Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editor: Victor R. Preedy

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Vinood B. Patel · Victor R. Preedy *Editors*

Biomarkers in Bone Disease

Springer

Biomarkers in Disease: Methods, Discoveries and Applications

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In the past decade there has been a sea change in the way disease is diagnosed and investigated due to the advent of high-throughput technologies, such as microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of Biomarkers in Disease.The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

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Biomarkers in Bone Disease

With 186 Figures and 134 Tables

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Preface

In the present volume, *Biomarkers in Bone Disease*, we have sections on

1. ***General Aspects and Introductory Material***
2. ***Body Fluids, Tissue, and Specific Biomarkers***
3. ***Genetic, Histological, Physical, and Imaging Methods***
4. ***Specific Diseases and Conditions***
5. ***Resources***

The editors recognize the difficulties in assigning particular chapters to particular sections, as some chapters can fit into more than one section. Nevertheless, the book has enormously wide coverage. Platforms and techniques include, for example, "omics," Raman spectroscopy, ultrasound, immunological, biochemical, histochemical methods, and imaging. Conditions and biomedical areas encompass bariatric surgery, bisphosphonate failure, bone quality, diabetes, effect of phytoestrogens, effects of statins, ethnicity, exercise, fracture risk, gestational hypertension, glucocorticoid treatments, HIV, intrauterine growth restriction, lumbar spine analysis, osteoarthritis, osteogenesis imperfecta, osteopetrosis type II, osteoporosis, Paget's disease, physiology, prematurity, remodeling, Rett syndrome, sclerosing disorders, sexual development, skeletal metastasis, spinal cord injury, and vascular remodeling. Analytes and measures include acid phosphatase, adiponectin, alkaline phosphatase, ameloblastin, calcium, carboxy-methyl lysine (CML), chitinases, creatine kinase, gene expression, geometry, integrin alpha2beta1, natural radionuclides, osteocalcin, parathyroid hormone, pentosidine, phosphorus, procollagen type 1 n-propeptide, PTX3, quality of life, radiomorphometric indices, relaxin, sclerostin, sirtuins, trabecular bone scores, ultrasound, uric acid, and vitamin D. There are also many other analytes and conditions described within this volume.

Finally, the last chapter is devoted to locating resource material for biomarker discovery and applications.

The chapters are written by national or international experts. This book is designed for clinical biochemists, orthopedic specialists. rheumatologists, those working within the wider field of skeletal disease, health scientists, epidemiologists,

doctors and nurses, from students to practioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and libraries as a reference guide.

The Editors

Series Preface

In the past decade, there has been major changes in the way diseases are diagnosed and investigated due to the advent of high-throughput technologies and advances in chemistry and physics. This has lead to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, and other new platforms. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these developments have gone hand in hand with analysis of biomarkers elucidated via traditional methods, such as histopathology, immunoassays, and clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is at the preclinical stage. There is thus a demand for a comprehensive and focused evidenced-based text that addresses these issues. Hence, the book series **Biomarkers in Disease: Methods, Discoveries and Applications**. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and treatments. It links conventional approaches with new platforms. The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Potential Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in ***Potential Applications to Prognosis, Other Diseases, or Conditions*** pertains to speculative or proposed areas of research, cross-transference to

other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The series is expected to prove useful for clinicians, scientists, epidemiologists, doctors and nurses, and also academicians and students at an advanced level.

The Editors

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About the Editors

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Dr. Vinood B. Patel is currently a Reader in Clinical Biochemistry at the University of Westminster and honorary fellow at King's College London. He presently directs studies on metabolic pathways involved in tissue pathology particularly related to mitochondrial energy regulation and cell death. Research is being undertaken to study the role of nutrients, antioxidants, phytochemicals, iron, alcohol, and fatty acids in tissue pathology. Other areas of interest include identifying new biomarkers that can be used for diagnosis and prognosis of liver disease, understanding mitochondrial oxidative stress in Alzheimers disease, and gastrointestinal dysfunction in autism. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his Ph.D. in Protein Metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. Dr. Patel is a nationally and internationally recognized liver researcher and was involved in several NIH-funded biomedical grants related to alcoholic liver disease. Dr. Patel has edited biomedical books in the area of nutrition and health, autism, and biomarkers and has published over 150 articles, and in 2014 he was elected as a Fellow to The Royal Society of Chemistry.

Victor R. Preedy is a senior member of King's College London. He is also Director of the Genomics Centre and a member of the Faculty of Life Sciences and Medicine.

Professor Preedy graduated in 1974 with an Honours Degree in Biology and Physiology with Pharmacology. He gained his University of London Ph.D. in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993 he gained his second doctoral degree for his outstanding contribution to protein metabolism in health and disease. Professor Preedy was elected as a Fellow to the Institute of Biology in 1995 and to the Royal College of Pathologists in 2000. Since then he has been elected as a Fellow to the Royal Society for the Promotion of Health (2004) and The Royal Institute of Public Health (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public Health, and in 2012 a Fellow of the Royal Society of Chemistry. In his career, Professor Preedy has carried out research at the National Heart Hospital (part of Imperial College London), The School of Pharmacy (now part of University College London), and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on the science of health and has a long-standing interest in biomarkers for over 30 years especially related to tissue pathology. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 600 articles, which include peer-reviewed manuscripts based on original research, abstracts and symposium presentations, reviews, and numerous books and volumes.

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Part I

General Aspects and Introductory Material

Overview of Biochemical Markers of Bone Metabolism

1

Pamela Maffioli and Giuseppe Derosa

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Abstract

The bone has the function of supporting the body; the bone is a tissue characterized by its rigidity, hardness, and power of regeneration and repair. The bone has several functions including protection of the vital organs, environment for marrow, mineral reservoir for calcium homeostasis, reservoir of growth factors and cytokines, and taking part in acid–base balance. Bone metabolism is a dynamic and continuous remodeling process that is normally maintained in a tightly coupled balance between resorption of old or injured bone and formation of new bone. Several hormones and factors are involved in bone metabolism, which regulation depends from the complex interaction among them. Considering the various phases of the bone cycle, markers of bone metabolism may be classified either as markers of bone formation, markers of bone resorption, and markers of bone metabolism regulation. The aim of this chapter will be to examine biochemical markers in bone metabolism in order to give readers a guide about the normal physiological process to better understand the mechanisms underlying bone diseases.

Keywords

Biomarkers • Bone diseases • Bone metabolism • Calcium • Regulation

List of Abbreviations

D-Pyr	Deoxypyridinoline
FGF	Fibroblast growth factors
IGF	Insulin-like growth factors
IL-1	Interleukin-1
IL-6	Interleukin-6

PG	Prostaglandins
PTH	Parathyroid hormone
Pyr	Pyridinoline
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α

Key Facts of Bone Metabolism

- The bone has several functions including protection of the vital organs, environment for marrow, mineral reservoir for calcium homeostasis, reservoir of growth factors and cytokines, and role in acid–base balance.
- Bone metabolism is a dynamic and continuous remodeling process that is normally maintained in a tightly coupled balance between resorption of old or injured bone and formation of new bone.
- Biomarkers relevant to bone metabolism include markers of bone formation, markers of bone resorption, and markers of bone metabolism regulation.
- The knowledge of biomarkers linked to bone metabolism is very useful to promptly identify bone abnormalities and to guide physicians in the right direction to better understand the mechanisms underlying bone diseases.

Definition of Word and Terms

Biomarker	The term refers to a measurable indicator of some biological state or condition that can be used for diagnosis or follow-up a particular disease.
Cytokines	The term refers to proteins released by cells with specific effect on the interactions among cells. Cytokines include interleukins, lymphokines, and cell signal molecules.
Osteoblast	The term refers to a cell producing the organic part of the bone matrix, an array of proteins collectively called "osteoid."
Osteoclast	The term refers to a cell with the function of resorption of existing bone and active early in the bone remodeling cycle.
Transforming growth factor type-β	The term refers to molecules implicated in the regulation of a variety of cellular events involved in the regulation of bone growth and turnover.

Introduction

The bone has the function of supporting the body; the bone is a tissue characterized by its rigidity, hardness, and power of regeneration and repair. The bone has several functions (Taichman 2005) including:

- Protection of the vital organs
- Environment for marrow (both blood forming and fat storage)
- Mineral reservoir for calcium homeostasis
- Reservoir of growth factors and cytokines
- Part in acid–base balance

Bone metabolism is a dynamic and continuous remodeling process that is normally maintained in a tightly coupled balance between resorption of old or injured bone and formation of new bone. On a microscopic level, bone metabolism always occurs on the surface of the bone at focused sites, called "bone metabolism unit." Global bone metabolism represents the cumulative behavior of many bone metabolism units such that defects in the organization of bone formation or any imbalance to the side of bone resorption can result in substantial changes in functional integrity over time. Changes can occur rapidly when the rate of turnover is increased. The bone has two components, the cortical and the trabecular bone. Cortical bone is dense and solid and surrounds the marrow space; on the other hand, the trabecular bone is composed of a network of trabecular plates and rods interspersed in the bone marrow compartment. The bone is composed of support cells, including osteoblasts and osteocytes, remodeling cells including osteoclasts, non-mineral matrix of collagen, and noncollagenous proteins called osteoid, with inorganic mineral salts deposited within the matrix (Fig. 1). The main characters in bone metabolism are osteoclasts and osteoblasts; they carry out bone metabolism at the fundamental bone metabolism unit site. Osteoblasts are involved in bone formation and differentiation from stromal marrow cells, in particular from precursor blood cells, and histologically have one nucleus and an extensive network of rough endoplasmic reticulum, responsible for synthesis of bone matrix proteins. Osteoblasts produce the

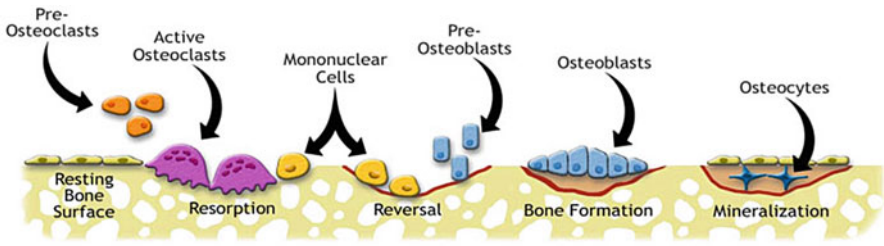

Fig. 1 Bone remodeling cycle

organic part of the bone matrix, an array of proteins collectively called "osteoid." Osteoid includes:

- **Collagen type I** which represents the bulk of osteoid. It consists of triple helix units containing two α1 chains and one α2 chain, which already form in the endoplasmic reticulum of the osteoblast after the individual chains have been posttranslationally hydroxylated on lysines and prolines. This procollagen unit is secreted, followed by proteolytic removal of C- and N-terminal peptides. The resulting collagen monomers spontaneously aggregate forming long fibrils that are subsequently covalently cross-linked via their hydroxylated lysines.
- **Osteocalcin** is a small protein that is carboxylated on glutamic acid residues with the help of vitamin K. Osteocalcin concentrated calcium in the bone, attracting Ca++ with the double-negative charges of glutamic acid residue after carboxylation. Osteocalcin, acting together with integrin-binding sialoprotein, nucleates crystals with phosphate ions to form hydroxyapatite $Ca_5(PO_4)_3(OH)$. Transcription of the gene of osteocalcin is induced by activated vitamin D. Osteocalcin itself, in non-carboxylated form, enters the bloodstream and enhances insulin activity. It stimulates proliferation of pancreatic β-cells and sensitizes fat cells to insulin by stimulating them to secrete adiponectin.
- **Osteonectin** is an osteoid component that makes contact to collagen type I as well as to hydroxyapatite, forming a link between organic and inorganic bone matrix.

On the other hand, osteoclasts are giant, multinucleated cells that derive from hematopoietic stem cells in the bone marrow, from the lineage leading to macrophages and neutrophils. A series of cytokines induces precursor cells to differentiate to osteoclasts, including interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and prostaglandin E. Osteoclasts reabsorb existing bone and are active early in the bone remodeling cycle. Osteoclasts use lysosomal chemistry, in addition to acidification and activation of acid hydrolases. Osteoclasts seal off a matrix area, which they acidify to dissolve hydroxyapatite, setting free Ca^{2+}. After the mineral has melted away, acid proteases like cathepsin K hydrolyze the remaining matrix proteins.

Several hormones and factors are involved in bone metabolism, which regulation depends from the complex interactions among them. Considering the various phases of the bone cycle, markers of bone metabolism may be classified either as:

- **Markers of bone formation**: osteoblasts, alkaline phosphatase, procollagen I extension peptides, and osteocalcin
- **Markers of bone resorption**: osteoclasts, urinary calcium, acid phosphatase, hydroxyproline, N-telopeptide, C-telopeptide, and pyridinoline (Pyr) and deoxypyridinoline (D-Pyr) cross-links
- **Markers of bone metabolism regulation**: parathyroid hormone (PTH) and calcitonin, thyroid hormones, estrogens, testosterone, vitamin D, cortisol, insulin, fibroblast growth factors (FGFs), insulin-like growth factors (IGFs, types I and II),

transforming growth factors (TGFs-β1 and TGFs-β2), prostaglandins, and interleukins.

Markers can be used in both generalized disorders of bone remodeling, such as osteoporosis or osteogenesis imperfecta, or in localized disorders of bone turnover, such as Paget's disease and cancer metastases. At this regard, the aim of this chapter will be to examine biochemical markers in bone metabolism in order to give readers a guide about the normal physiological process to better understand the mechanisms underlying bone diseases.

Markers of Bone Formation

Alkaline Phosphatase

Alkaline phosphatase (ALP) is present in mucosal epithelia of small intestine, proximal convoluted tubule of the kidney, bone, liver, and placenta. It is involved in lipid transportation in the intestine and calcification in the bone. Bone alkaline phosphatase (BAP) is the bone-specific isoform of alkaline phosphatase, a glycoprotein found on the surface of osteoblasts. Bone alkaline phosphatase is synthesized by the osteoblasts and is presumed to be involved in the calcification of bone matrix, though its precise role in the formation process is unknown; what is certain is that BAP reflects the biosynthetic activity of these bone-forming cells. Normal value of BAP ranges $\leq$20 mcg/L in males and $\leq$14 mcg/L in premenopausal women and $\leq$22 mcg/L in postmenopausal women (Nicoll 2007). Bone alkaline phosphatase has been shown to be a sensitive and reliable indicator of bone metabolism (Kress 1998). The highest amount of serum ALP activity is observed in Paget's disease, a metabolic bone disease caused by excessive rates of bone remodeling, resulting in local lesions of abnormal bone matrix. These lesions can result in fractures or neurological involvement. Under this condition, ALP activity is almost 10–25 times higher than the normal limit. The moderate increase in ALP activity is observed in osteomalacia, which is slowly decreased toward normal ranges in response to vitamin D therapy. The ALP activity rate is generally normal in osteoporosis, while in rickets disease, a 2–4 times increase is seen in the enzyme activity, which gradually moves toward the normal range following vitamin D therapy. Very high levels of ALP enzyme activity can be also found in patients with bone metastatic carcinoma and osteogenic sarcoma, while a transitory increase in the enzyme activity may be observed during the healing of bone fractures (Kubo et al. 2012; Hatayama et al. 2012; Corathers 2006; Simko 1991; Ross and Knowlton 1998).

Procollagen I Extension Peptides

Collagen is the major structural protein of the bone and comprises about 90% of the organic material. Collagen plays a central role in the integrity and strength of bone

matrix, and defects in its production lead to bone of poor quality, susceptible to fracture. Type 1 collagen is synthesized by osteoblasts as a larger precursor protein termed procollagen I (Risteli and Risteli 1993). The carboxy-terminal and amino-terminal ends of procollagen I are enzymatically "clipped" during extracellular processing and fibril formation prior to incorporation of type 1 collagen into the bone matrix. The amino-terminal ("N-terminal") and carboxy-terminal ("C-terminal") ends of the procollagen are released in the blood and can be quantified. Values of procollagen I extension peptides are increased during growth and in situations of increased bone formation, such as in Paget's disease, and in response to growth hormone.

Osteocalcin

Serum osteocalcin has been found to correlate with bone formation. Osteocalcin is a relatively small protein produced by osteoblasts during the matrix mineralization phase (Fottrell and Power 1991). Osteocalcin is both released into circulation and incorporated into the bone matrix, where it is the most abundant noncollagenous protein (Fottrell and Power 1991). In common with many factors involved in the coagulation cascade, osteocalcin synthesis is dependent on vitamin K, which posttranslationally modifies the gene product with gamma-carboxyglutamate (Gla) residues; due to this modification, osteocalcin is also known as bone γ-carboxyglutamate protein. The γ-carboxylated form binds hydroxyapatite and is abundant in bone extracellular matrix, while the undercarboxylated circulating form has been implicated as a novel hormone and positive regulator of glucose homeostasis. It is secreted into circulation and, in individuals having normal renal function, excreted in urine due to its low molecular weight (Kapustin and Shanahan 2011). Normal reference ranges for osteocalcin are 9–42 ng/mL for subjects of 18 years or older (Delmas et al. 2000). Elevated levels of osteocalcin can be found in conditions associated with increased bone formation, as happened in hyperparathyroidism, hyperthyroidism, and bone metastases. Reduced levels of osteocalcin can be found in condition with lower rates of bone formation, as seen in myeloma, or in patients taking glucocorticosteroids, or antiresorptive agents (bisphosphonates or hormone-replacement therapy), usually within 3–6 months after therapy begins. Decreasing osteocalcin levels indicate effective response to treatment. Within 3–6 months after surgical cure, osteocalcin levels in patients with primary hyperparathyroidism should return to the reference range (Harris et al. 2001).

Markers of Bone Resorption

Urinary Calcium

Calcium salts provide rigidity to the skeleton and calcium ions play a fundamental role. In the vertebrate skeleton, calcium is provided by a form of calcium phosphate, which approximates hydroxyapatite [$Ca_{10}(OH)_2(PO_4)_6$] and is embedded in

collagen fibrils. The average adult store of calcium is approximately 1–2 kg. The vast majority (99%) resides in the skeleton. Only a fraction of the stored calcium is present in extracellular fluid and available for the use in the form of ionized calcium. Ionized calcium is tightly regulated by PTH. Adult calcium plasma concentrations are normally between 8.5 and 10.5 mg/dL (2.2–2.6 mmol/L). Most of this circulating calcium is bound to albumin. Because of this, changes in serum protein concentrations can affect total blood calcium concentrations. Calcium enters the extracellular fluid through absorption from the gut and resorption from the bone. It is removed through secretion into the gastrointestinal tract and urine as well as losses in sweat and deposition in the bone. Urine calcium levels will reflect dietary intake. Normal values are collected on urine sample over 24 h, average between 100 and 250 mg of calcium (15–20 mmol). Moreover, calcium excretion is influenced by sodium excretion; patients following low-sodium diets tend to have a lower calcium excretion. High levels of urine calcium (>300 mg/24 h) are often a sign of an overactive parathyroid gland. Parathyroid hormone is produced in response to serum calcium levels: when serum calcium levels are low, parathyroid calcium-sensing receptors stimulate the PTH release. Parathyroid hormone works to increase serum calcium levels, increasing renal tubule resorption of calcium and simultaneously decreasing phosphorus resorption. Parathyroid hormone also causes resorption of calcium from the bone and increases synthesis of 1,25-dihydroxy vitamin D, which stimulates calcium absorption from the gut (Foley 2010). For the reasons reported above, hyperparathyroidism results in excessive uptake and increased concentrations of calcium in serum, leading to hypercalcemia, hypercalciuria, and hyperphosphaturia. Thus, urine calcium levels are often increased in the setting of hyperparathyroidism.

Acid Phosphatase

Acid phosphatase is an enzyme stored in lysosomes and localized in different organs. In particular, there are five isoenzymes of acid phosphatase in blood, localized in the bone, prostate, platelets, erythrocytes, and spleen (Moss and Henderson 1994). The bone isoenzyme is derived from osteoclasts, where it is present in high concentration and excreted into the microenvironment between the membrane sealing zone and the bone matrix. Acid phosphatase is a potent enzyme that plays an important role in the bone resorption process. It is released into circulation by "leakage," during resorption and after detachment of the osteoclast's sealing zone. Due to its molecular size, assays for acid phosphatase are serum or plasma based. Normal values of acid phosphatase range between 0 and 0.8 U/L. Abnormal levels of acid phosphatase in the blood may indicate Paget disease, hyperparathyroidism, and multiple myeloma.

Hydroxyproline

Proline and its metabolite hydroxyproline are unique amino acids, both chemically and biochemically (Hu et al. 2008; Kaul et al. 2008). Hydroxyproline is a modified

amino acid produced from the posttranslational hydroxylation of integral proline residues of type 1 collagen. It constitutes one-third of amino acids in the collagen proteins and has an essential role in collagen stability (Nelson and Cox 2005). It is also a major extracellular component in connective tissues (skin, tendon, cartilage, vessels of the vascular system, and bone). After collagen breakdown, hydroxyproline is not reutilized; 90% is degraded to the free amino acid form and passes through the glomerulus; moreover, hydroxyproline is almost completely resorbed and catabolized in the liver to urea and carbon dioxide. The remaining 10% of hydroxyproline is released in small polypeptide chains that pass through the glomerulus and are excreted in urine (Kivrikko 1983).

Testing hydroxyproline in the serum and in the urine is common. The reference range of hydroxyproline is different according to different age (Laitinen et al. 1966), in particular:

- Total hydroxyproline in the urine among subjects aged 18–21 years is 13–28 mg/24/m^2
- Total hydroxyproline in the urine among subjects aged 22–55 years is 8.5–23.5 mg/24/m^2
- Free hydroxyproline in the serum of males ranges between 0.7 and 1.55 μg/mL
- Free hydroxyproline in the serum of females ranges between 0.7 and 1.40 μg/mL

Elevated hydroxyproline levels can be found in Paget disease, hyperparathyroidism, osteomyelitis, hyperthyroidism, and skeletal metastases. However, also other diseases can give an increase of this marker, including rheumatoid arthritis, polyarteritis nodosa, Marfan syndrome, acromegaly, Turner syndrome, and pregnancy.

N-Telopeptide and C-Telopeptide

During bone resorption, amino- and carboxy-terminal fragments of collagen are released with cross-links attached. These fragments with attached cross-links are called telopeptides. N-Telopeptides and C-telopeptides are excreted in the urine. N-Telopeptides are measured by immunoassay using an antibody to the α2 chain of the N-telopeptide fragment. C-Telopeptides are measured by immunoassay (Watts 1999).

Pyridinoline (Pyr) and Deoxypyridinoline (D-Pyr) Cross-Links

Posttranslational modification of lysine and hydroxylysine produces the nonreducible pyridinium cross-links, pyridinoline (Pyr) and deoxypyridinoline (D-Pyr), that stabilize mature collagen. Both Pyr and D-Pyr are released from the bone in a ratio of approximately 3:1. Deoxypyridinoline is specific for the bone, while Pyr is also found in articular cartilage and in soft tissues such as ligaments and

tendons. Almost 60% of the cross-links released during resorption are bound to protein, with the remaining 40% being free. Pyridinium cross-links are not metabolized, and they can be measured in urine by HPLC or immunoassay either before or after hydrolysis.

Markers of Bone Metabolism Regulation

Parathyroid Hormone and Calcitonin

As already described above, PTH plays an important role in bone metabolism, regulating calcium levels in blood. When serum calcium levels are low, parathyroid calcium-sensing receptors stimulate the PTH release (Fig. 2). Parathyroid hormone works to increase serum calcium levels, increasing renal tubule resorption of calcium and simultaneously decreasing phosphorus resorption. Parathyroid hormone also causes resorption of calcium from the bone and increases synthesis of 1,25-dihydroxy vitamin D, which stimulates calcium absorption from the gut (Foley 2010). Parathyroid hormone is antagonized by calcitonin, a hormone produced by

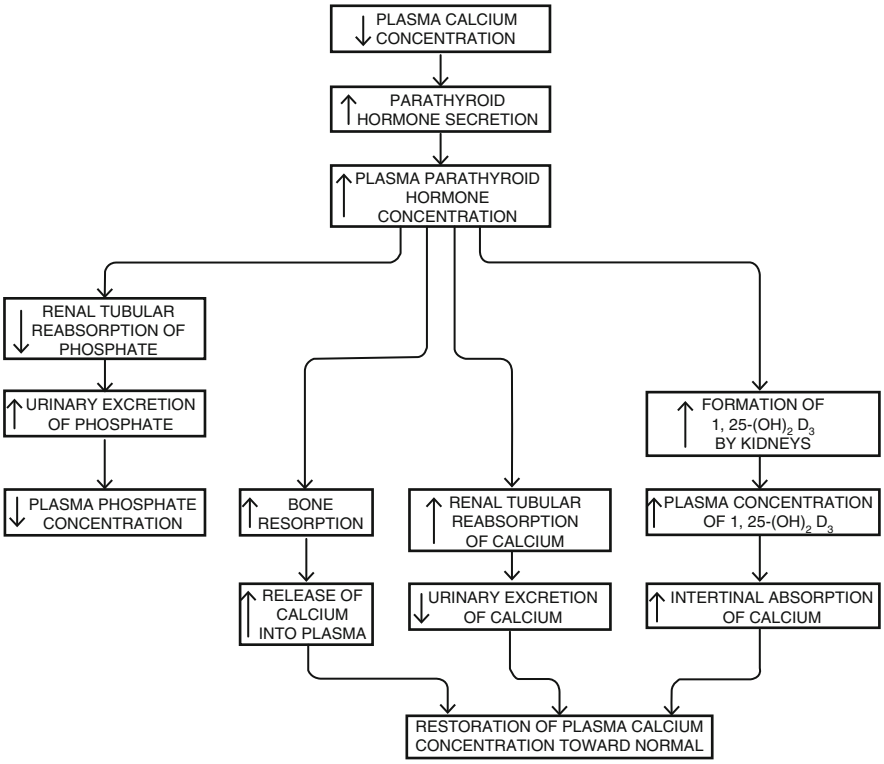

Fig. 2 Parathyroid, calcium, and phosphate metabolism

Table 1 Calcium and phosphate regulation

	Calcium deprivation	Calcium loading
Parathyroid hormone	Secretion stimulated	Secretion inhibited
Vitamin D	Production stimulated by increased parathyroid hormone secretion	Synthesis suppressed due to low parathyroid hormone secretion
Calcitonin	Very low-level secretion	Secretion stimulated by high blood calcium
Intestinal absorption of calcium	Enhanced due to activity of vitamin D on intestinal epithelial cells	Low basal uptake
Release of calcium and phosphate from the bone	Stimulated by increased parathyroid hormone and vitamin D	Decreased due to low parathyroid hormone and vitamin D
Renal excretion of calcium	Decreased due to enhanced tubular resorption stimulated by elevated parathyroid hormone and vitamin D; hypocalcemia also activates calcium sensors in loop of Henle to directly facilitate calcium resorption	Elevated due to decreased parathyroid hormone-stimulated resorption
Renal excretion of phosphate	Strongly stimulated by parathyroid hormone; this phosphaturic activity prevents adverse effects of elevated phosphate from bone resorption	Decreased due to hypoparathyroidism
General response	Typically see near-normal serum concentrations of calcium and phosphate due to compensatory mechanisms. Long-term deprivation leads to bone thinning (osteopenia)	Low intestinal absorption and enhanced renal excretion guard against development of hypercalcemia

C cells of the thyroid gland in response to high serum calcium levels. In order to decrease serum calcium levels, calcitonin prevents calcium loss from bones suppressing the activity of osteoclasts throughout receptors present on the surface of osteoclasts to stop them from breaking down the bone. Calcitonin also prevents the absorption of calcium from the intestine, maintaining normal blood levels of vitamin D. Calcitonin also regulates the level of calcium and other mineral levels in the kidneys. It reduces the kidney's reabsorption of calcium and magnesium, leading to increased calcium excretion via the urine (Stevenson 1982) (Table 1).

Thyroid Hormones

Thyroid diseases have systemic manifestations including effects on bone metabolism. Thyrotoxicosis is an important cause of secondary osteoporosis, while hypothyroidism has only a minimal effect on bone mineral metabolism (Donangelo and Braunstein 2011). Lower bone mass associated with hyperthyroidism may be caused by increased bone turnover as a result of imbalance between bone resorption and

formation. Thyroid hormones have direct catabolic effect on bone mineral homeostasis, leading to increased bone mineral resorption and calcium loss through kidneys. Histomorphometric studies demonstrate that thyroid hormones increase the activation of new remodeling cycles and stimulate osteoclastic and osteoblastic activity in trabecular and cortical bone, with an increase in number and activity of osteoclasts. The mechanisms of thyroid hormone-induced bone resorption include cAMP-mediated increased sensitivity of beta adrenergic receptors to catecholamines, increased sensitivity of bone cells to PTH, osteoclast activator factor, and IL-1-mediated increased bone resorption.

Estrogens

Estrogen actions on bone are complex. The major physiological effect of estrogen is to inhibit bone resorption. Bone cells have two kinds of intracellular steroid receptors for estrogen. When estrogen binds to the receptors, various genes become active. Estrogen also has effects that do not depend on activating the DNA. Estrogen effects may be mediated in part by growth factors and interleukins. For example, IL-6 is a potent stimulator of bone resorption, and estrogen blocks the osteoblast's synthesis of IL-6. Estrogen may also antagonize the IL-6 receptors (Väänänen and Härkönen 1996).

Testosterone

Men with hypogonadism are at increased risk of osteoporosis (Jackson et al. 1992). On the other hand, many observational studies have found an association between testosterone use in men and important gains in bone density, favorable changes in bone turnover biomarkers, and lower risk of osteoporotic fractures (Kenny et al. 2001). Androgen receptors have been identified in osteoblasts. Androgens likely stimulate longitudinal bone growth by their direct effects on growth plate chondrocytes. Androgen effects on the bone may also be indirectly mediated by regulation of cytokines and growth factors expressed locally in the bone. Androgens, in fact, upregulate TGF-β and IGFs, which stimulate bone formation, and downregulate IL-6, which stimulates osteoclastogenesis. Androgens inhibit PTH or IL-1-induced prostaglandin E2 (PGE2) production. Androgens stimulate IL-1β production and enhance the mitogenic effect of fibroblast growth factor (FGF) in cultured osteoblasts. Finally, dihydrotestosterone has been shown to reduce osteoprotegerin levels, which could potentially stimulate osteoclasts activity (Clarke and Khosla 2009).

Vitamin D

Vitamin D is important for normal development and maintenance of the skeleton. It is well known that vitamin D deficiency is related to rickets and osteomalacia.

Vitamin D is available either as ergocalciferol, or vitamin D2, derived from plants or as cholecalciferol, or vitamin D3, from animal sources. Both are converted by the liver to 25-hydroxyvitamin D, then by the kidneys to 1,25-dihydroxyvitamin D (Holick 2007). Ultraviolet B (UV-B) radiation (290–315 nm) converts 7-dehydrocholesterol in the deep epidermal layers to the provitamin cholecalciferol. Measurement of the active form 1,25-dihydroxyvitamin D is not useful in clinical practice. The serum 25-hydroxyvitamin D level, instead, reflects the vitamin D stores in the body. Normal serum 25-hydroxyvitamin D level values have been defined as >20 ng/mL (50 nmol/L). Deficiency is defined if serum values are <20 ng/mL (50 nmol/L); on the other hand, serum values of 25-hydroxyvitamin D level >200 ng/mL (500 nmol/L) are considered as toxic (von Domarus et al. 2011). As already said before, plasma calcium concentrations are maintained at a very constant level; if plasma becomes less than saturated with respect to calcium and phosphate, then mineralization fails, which results in rickets among children and osteomalacia among adults (Underwood and DeLuca 1984). The vitamin D hormone functions to increase serum calcium concentrations in three different ways:

1. Vitamin D is able to induce the proteins involved in active intestinal calcium absorption. Furthermore, it stimulates active intestinal absorption of phosphate.
2. Vitamin D is able to mobilize calcium from the bone when calcium is absent from the diet. Vitamin D, in fact, stimulates osteoblasts to produce receptor activator nuclear factor-kB ligand (RANKL). RANKL then stimulates osteoclastogenesis and activates resting osteoclasts for bone resorption (Suda et al. 2002). Both vitamin D and PTH are required for this mobilization event (Garabedian et al. 1974).
3. Vitamin D stimulates calcium absorption by the distal renal tubule, responsible for resorption of the last 1% of the filtered load of calcium (Yamamoto et al. 1984). Because 7 g of calcium are filtered every day, this represents a major contribution to the calcium pool. Again, both PTH and the vitamin D hormone are required.

Cortisol

The periosteum may affect bone formation by providing precursor cells, needed to achieve a normal osteoblastic cell population, or by providing bone growth factors, known to be released by cultured fibroblasts and intact bones. The long-term inhibitory effect of cortisol on bone collagen is secondary to a decrease in cell population, whereas the short-term stimulatory effect could be related to locally released growth factors (Canalis 1984). Data about cortisol effect on the bone come primarily from experience in patients with Cushing's syndrome, characterized by elevated levels of cortisol. In these patients, the prevalence of osteopenia and osteoporosis is usually estimated between 60–80% and 30–65%, respectively (Mancini et al. 2004). The end result of glucocorticoid excess is a loss of bone mineral content and increased bone fragility. The pathogenesis of glucocorticoid-induced

osteoporosis involves both skeletal and extraskeletal mechanisms. Glucocorticoids have extraskeletal effects including hypogonadotrophic hypogonadism, a decrease in intestinal calcium absorption and an increase in renal calcium excretion. Moreover, there is also a decreased secretion of adrenal androgens and estrogens and changes in the GH-IGF-1 axis and insulin. Glucocorticoids also have direct effects on skeleton, with a decrease in bone formation due to impaired osteoblastogenic differentiation, decreased osteoblast function, and increased osteoblastic apoptosis, resulting in decreased bone formation. The impairment of osteoblastic differentiation of bone marrow stromal cells parallels a shift toward the adipocytic lineage due to a decrease in bone morphogenetic protein-2, an increase in peroxisome proliferator-activated receptor-c-2 and CAAT enhancer-binding proteins, as well as inhibition of the Wnt/beta-catenin pathway (Tóth and Grossman 2013).

Insulin

The anabolic effects of insulin do not have to be confused with those of insulin-like growth factor (IGF-1), although the homology of molecular structure of both molecules may in fact account for some of the anabolic effects of insulin on the bone. A first difference between the two molecules is that insulin is produced in the pancreatic β-cells, while endocrine IGF-1 is synthesized in the liver. The release of insulin production is induced by glucose and osteocalcin, while IGF-1 is produced in response to growth hormone and the paracrine IGF-1 produced by bone cells, including pre-osteoblasts and osteoblasts, osteocytes, and osteoclasts (Klein 2014). Insulin proved to have an anabolic effect on the bone. Recent data suggest that, under normal conditions, insulin stimulates osteoblast differentiation to produce more osteocalcin, which would then stimulate more insulin production by the pancreas and greater insulin sensitivity of skeletal muscle. In insulin-resistant patients, such as in type 2 diabetes, osteocalcin levels are lower; moreover, insulin resistance is also caused by factors that cause bone resorption, such as IL-6-mediated chronic low-grade inflammation (Tarantino et al. 2010).

Fibroblast Growth Factors

Fibroblast growth factors (FGFs) are polypeptides originally isolated from the central nervous system but also found in a variety of tissues including the bone. Two forms of FGFs have been isolated: acidic (aFGF) and basic (bFGF); they have 55% homology in their amino acid sequence, have similar biological effects, and interact with the same cell receptors (Gimenex-Gallego et al. 1986). The skeletal tissue is a rich source of growth factors; both aFGF and bFGF are contained in bone matrix, suggesting that they are either trapped by the bone matrix or are synthesized by skeletal cells. They play a role in the local regulation of percent collagen synthesized. Fibroblast growth factors are important factors that promote osteoprogenitor cell proliferation and osteogenesis. They were found to exert

anabolic effects on bone formation in intact animals and to reduce bone loss in experimental models of osteoporosis (Fromigué et al. 2004).

Insulin-Like Growth Factors (IGFs, Types I and II)

IGF-I is the major mediator of growth hormone (GH) action, and it plays a central role in growth, development, and metabolism of skeletal tissue. GH stimulates skeletal growth indirectly by stimulating liver production of IGF-I to act in an endocrine manner to stimulate bone growth. However, subsequent studies showed that GH also has direct effects on the bone and that these effects are largely mediated via GH regulation of local IGF-I expression and its action in the bone. Osteoblasts contain GH receptors and GH treatment increases the production of IGF-I in these cell types (Mohan and Kesavan 2012). IGF-1 binds to IGF1-R, a type II tyrosine kinase, leading to autophosphorylation of Tyr residues 1131, 1135, and 1136 in the kinase domain, followed by phosphorylation of Tyr 950 in the juxtamembrane domain, which activates downstream substrates, insulin receptor substrate (IRS) proteins, and Shc by tyrosine phosphorylations. The IRS protein family consists of four isomers IRS1, 2, 3, and 4. Two of these proteins, IRS1 and IRS2, have been studied with respect to the bone; IRS1 is expressed in chondrocytes and osteoblasts; IRS2 is expressed in osteoblasts and osteoclasts but not in chondrocytes (Hernández-Sánchez et al. 1995).

Transforming Growth Factors (TGFs β1 and β2)

Transforming growth factors type-β (TGF-β1, TGF-β2, TGF-β3) have been implicated in the regulation of a variety of cellular events involved in the regulation of bone growth and turnover. They are produced by osteoblasts and chondrocytes and are highly concentrated in skeletal tissue. Autocrine and paracrine stimulation by TGF-β is important in the maintenance and expansion of the mesenchymal stem/progenitor cells, the progenitors of osteoblasts. The bone and cartilage contain large amounts of TGF-β and target cells for TGF-β activity. At earlier developmental stages, osteoblast-enriched populations from fetal bone are more sensitive to the mitogenic effect of TGF-β than similar populations from newborns. Furthermore, TGF-β signaling also promotes osteoprogenitor proliferation, early differentiation, and commitment to the osteoblastic lineage (Derynck and Akhurst 2007).

Prostaglandins

Prostaglandins are potent, multifunctional regulators of bone metabolism, which have both stimulatory and inhibitory effects. Prostaglandins stimulate bone resorption by increasing the number and activity of osteoclasts. Prostaglandin E2 is the most potent agonist, although other prostanoids, particularly prostacyclin (PGI2), are

potent stimulators. Most of the potent stimulators of bone resorption increased prostaglandin production in the bone by induction of COX-2, although they also stimulate resorption by prostaglandin-independent pathways. However, PG can stimulate bone formation by increasing replication and differentiation of osteoblasts. This effect is associated with an increase in the production of growth factors. There is indirect evidence that stimulation of bone formation is mediated by the EP2 receptor, which is expressed in osteoblast precursor cells.

Cytokines

Interleukin-1 is a prototypic pro-inflammatory cytokine that regulates a wide variety of cellular and tissue functions. There are two forms of IL-1, IL-1a and IL-1b, with similar biological activities but different functional roles. Both bind to IL-1 type I receptor (IL-1RI) with an equal affinity. In addition, IL-1 receptor antagonist (IL-1Ra) serves as a natural competitive inhibitor of IL-1a and IL-1b. The bone is very sensitive to IL-1, which regulates both bone formation and bone resorption. Interleukin-1 is an osteoclast-activating factor; it stimulates osteoclast formation indirectly by stimulating prostaglandin E2 synthesis in osteoblasts/stromal cells. Interleukin-1 also induces the fusion of mononuclear osteoclasts leading to multinucleation, it potentiates osteoclast function, and it is involved in the survival part of osteoclasts (Lee et al. 2010).

As already said above, IL-6 is an essential mediator of the bone loss. Interleukin-6 is a pleiotropic cytokine influencing many biological events in several organs including the bone marrow. In the bone, activation of the glycoprotein (gp)-130 signaling pathway by IL-6 and its soluble receptor (sIL-6R) is a key pathway for the regulation of osteoclastogenesis (Roodman 1992).

Another cytokine involved in bone resorption is tumor necrosis factor-α, a multifunctional cytokine mainly produced by activated macrophages, with numerous functions. Tumor necrosis factor-α is associated with several cell signaling systems via two types of cell surface receptors, namely, TNFR I and TNFR II. Both receptors are expressed on several cell types including bone marrow hematopoietic cells. Both TNFR I and II mediate biological properties of TNF-α. Osteoclast recruitment by TNF-α is probably essential in the pathogenesis of inflammatory osteolysis (Kwan Tat et al. 2004).

Potential Applications to Prognosis, Other Diseases, or Conditions

Bone metabolism is a dynamic and continuous remodeling process that is normally maintained in a tightly coupled balance between resorption of old or injured bone and formation of new bone. Several hormones and factors are involved in bone metabolism, which regulation depends from the complex interaction among them. The knowledge of biomarkers linked to bone metabolism is very useful to promptly

identify bone abnormalities and to guide physicians in the right direction to better understand the mechanisms underlying bone diseases.

Summary Points

- This chapter focuses on biomarkers relevant to bone metabolism.
- Biomarkers include measurable indicators of some biological state and are useful to diagnose or follow-up a specific condition or risk factor.
- Biomarkers relevant to bone metabolism include markers of bone formation, markers of bone resorption, and markers of bone metabolism regulation.
- The knowledge of biomarkers linked to bone metabolism is very useful to promptly identify bone abnormalities and to guide physicians in the right direction to better understand the mechanisms underlying bone diseases.

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Osteosarcoma Biomarkers Discovery Using "Omics" Approaches

2

Giulia Bernardini, Maurizio Orlandini, Federico Galvagni, and Annalisa Santucci

Contents

Abstract

Osteosarcoma is the most common malignant primary cancer of bone tissue affecting mostly children and young adults. Nowadays, reliable circulating or cellular/tissue biomarkers do not exist for early diagnosis, drug resistance, and relapses of osteosarcoma. Post-genomics represents an invaluable tool to disclose cancer complexity at a molecular as well as to discover novel diagnostic and prognostic biomarkers.

Although "omics" research on osteosarcoma has only been undertaken recently in respect to that on many other tumor types, these studies have brought

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to light several potential molecular biomarkers that represent the basis to develop novel and better strategies for early detection, outcome prediction, detection of disease recurrence, and therapeutic approach.

In this chapter, the discovery of such molecular markers through the emerging omics technologies, including miRNA-omics, transcriptomics, and proteomics, will be extensively reviewed.

Keywords

Osteosarcoma • Post-genomics • Omics approaches • miRNA • Transcriptomics • Proteomics • Biomarker

List of Abbreviations

1DE	Monodimensional polyacrylamide gel electrophoresis
2D-DIGE	Two-dimensional difference in gel electrophoresis
2DE	Two-dimensional polyacrylamide gel electrophoresis
CSC	Cancer stem cell
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FACS	Fluorescence-activated cell sorting
FT-ICR	Fourier transform ion cyclotron resonance
IHC	Immunohistochemistry
iTRAQ	Isobaric tags for relative and absolute quantitation
LC	Liquid chromatography
LTQ	Linear ion trap
miRNA	Micro RNA
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OB	Osteoblast
OC	Osteochondroma
OS	Osteosarcoma
PCA	Principal component analysis
PMF	Peptide mass spectrometry
q RT-PCR	Quantitative real time PCR
QToF	Quadrupole time of flight
SELDI-ToF/MS	Surface-enhanced laser desorption/ionization time of flight mass spectrometry
WB	Western blotting

Key Facts of Osteosarcoma

- Osteosarcoma is the most common type of bone cancer, which develops in growing bones and occurs more frequently in children and adolescents.

- The most common early signs of osteosarcoma are pain and swelling. The presence of a bone tumor has to be confirmed by a complete medical examination including blood test, since bone tumors can be associated with increased levels of certain enzymes in the blood; X-rays and other scans of the bone(s); and then a biopsy (removal of a sample of tissue) that will be examined by a pathologist to determine whether it is cancerous and if so what type of cancer it is.
- Osteosarcomas can be localized or metastasize to other parts of the body (mainly lungs). Microscopic spreads can occur even at the early phases of cancer progression, when the primary tumor has a very small size.
- Modern treatments of osteosarcoma require surgery (to remove all visible tumor tissue) and chemotherapy, given before (to shrink tumor size and to prevent metastasis) and after surgery (to kill cancer cells not completely removed by surgery). Many factors including site and location of the main tumor and other individual factors affect the type of surgery. When it is possible, limb-sparing procedures by an artificial device (endoprosthesis) or bones from other places in the body (bone graft) are preferred to amputation.
- Several factors affect the prognosis of osteosarcoma patients, including cancer spreading, size and location of tumor, type of osteosarcoma, surgery outcome, responsiveness to chemotherapy, and patient's general health.

Definitions of Words and Terms

Biological marker (Biomarker)	A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. (Biomarker Definitions Working Group – 1998).
miRNA	miRNAs are short noncoding RNA molecules that regulate gene expression at the posttranscriptional level by binding to the 3′ untranslated regions of target messenger RNAs. To date, nearly 2000 human miRNAs have been identified and any single miRNA can regulate dozens or hundreds of target genes.
Proteome	Large-scale inventory of the proteins expressed in cells, tissues, or organisms. Proteome reflects a specific developmental stage or physiological condition.
Proteomics	Comprehensive study of a specific proteome with the aim to catalog all protein species, to determine their structure and function, and to quantify the changing expression levels of each protein species during development and under different

	conditions. Proteomics approaches are conducted by means of high-throughput technologies.
Transcriptome	Large-scale inventory of the RNA transcripts (mRNAs, noncoding RNAs, and small RNAs) produced by the genome in cells, tissues, or organisms. Transcriptome reflects a specific developmental stage or physiological condition.
Transcriptomics	The study of a specific transcriptome with the aim to catalog all species of transcript, to determine the transcriptional structure of genes, and to quantify the changing expression levels of each transcript during development and under different conditions. Transcriptomics approaches are conducted by means of high-throughput technologies.

Introduction

Osteosarcoma (OS) is a rare neoplasm of bone that affects mainly young patients. Since OS have a high tendency to metastasize, they are classified among the most frequent sources of cancer-related death in childhood tumors (Botter et al. 2014). Despite the survival rate of OS patients has been improved as a result of refined surgical techniques and multiagent chemotherapy, the survival of patients that develop metastases still remains low (Anninga et al. 2011). Therefore, a more detailed understanding of the molecular mechanisms and specific identifying of biomarkers involved in tumor initiation, progression, and metastasis formation is of immediate importance to develop new and improved treatment strategies for OS.

Currently the diagnosis of OS occurs around 4 months from the onset of symptoms. Diagnosis of OS is based, after a first complete medical history of the patient, on imaging analysis, including radiographs, magnetic resonance imaging, bone scintigraphy, and biopsy which provide a definite diagnosis and grading/staging of the tumor. So far, reliable OS circulating markers do not exist. In fact, alkaline phosphatase (ALP) exhibits high plasmatic level only in 40% of cases, while lactate dehydrogenase (LDH) is elevated in around 30% of cases. These laboratory values also possess a moderate prognostic relevance: normal ALP and LDH levels in chemonaive patients have been associated with 5-year disease-free survival and a longer time to disease recurrence (Geller and Gorlick 2010). However, the most important prognostic factors in OS are represented by the presence of metastatic disease at the time of diagnosis and the histological response to preoperative chemotherapy.

Nowadays, the greatest challenge in OS management is the lack of reliable markers able to detect the tumor at an early stage, when there is a better chance for its treatment, or to predict the prognosis or the response to chemotherapy.

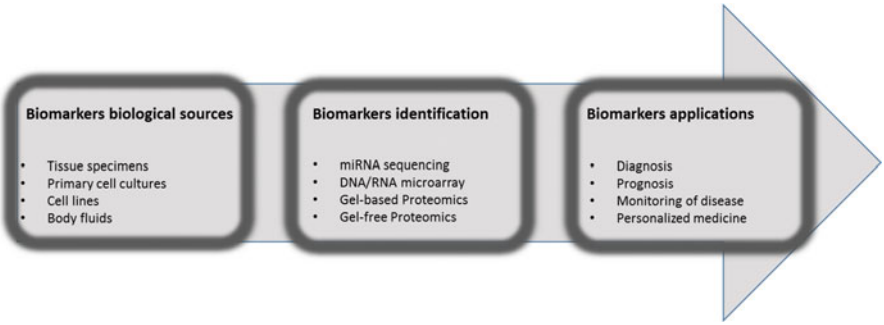

Fig. 1 Targeted "OMICS" approaches to biomarkers discovery. Different biological samples are collected from patients with OS and represent the source of molecular biomarker. Global profiles are obtained using high-throughput post-genomics technologies and then analyzed to identify candidate biomarkers

In the last few years, the significant progress in "omics" technologies (epigenomics, transcriptomics, and proteomics), allowing the simultaneous detection of thousands of molecular species in a large amount of biological samples, provided researchers with the opportunity to discover a variety of biomarkers with diagnostic and prognostic purposes. In this regard, the development of bioinformatics analytical tools suitable to mine the massive flood of data provided by high-throughput experiments is mandatory to integrate different "omics" approaches as well as to achieve robust and reliable finding with clinical relevance as well as to get novel clues for understanding cancer biology and pathophysiology (Bernardini et al. 2012, 2014).

Moreover, the evaluation of tumor-specific "omics" profiles may also allow the development of more efficient tools for cancer therapy through the identification of novel molecular targets and thus the development of personalized therapies (Fig. 1).

The main aim of the present chapter is to systematically summarize the most relevant post-genomic studies related to post-genomic biomarkers discovery in OS.

MicroRNAs

MicroRNAs (miRNAs) are a novel class of biomarkers, which could be helpful for OS diagnosis and determination of optimal treatment.

miRNAs are short noncoding RNA molecules ~22 nucleotides long that are synthesized by RNA polymerase II or III from endogenous transcription units. They regulate gene expression at the posttranscriptional level by binding to the 3′ untranslated regions (3′ UTRs) of target messenger RNAs (Ambros 2004). To date, nearly 2000 human miRNAs have been identified (miRBase, Homo sapiens miRNAs database, Manchester University), and any single miRNA can regulate dozens or hundreds of target genes (Rana 2007).

In the context of cancer cells, miRNAs can act as oncogenes (oncomiR) or tumor suppressor genes (anti-oncomiR) based on their inhibition of tumor-suppressive and oncogenic mRNAs, respectively, and expression deregulation of one or more miRNAs was demonstrated to be involved in development and progression of cancer (Calin et al. 2002; Sotiropoulou et al. 2009). The expression profiling of miRNAs is already used into cancer clinics as diagnostic and prognostic biomarkers to assess tumor initiation, progression, and response to treatment (Reddy 2015).

miRNAs Expression in OS

Since the recent discovery of the class of miRNAs in humans (Lagos-Quintana et al. 2001), the interest in the field has grown rapidly, and during the last 5 years the number of papers devoted to miRNAs in OS increased exponentially (Fig. 2).

In a recent study, comparison of 80 pairs of OS and corresponding noncancerous bone tissues revealed that miR-34a and miR-192 were downregulated in tumors, and OS patients with low miR-34a and miR-192 expression had shorter disease-free survival (Wang et al. 2015b). Thus, miR-34a and miR-192 are potential biomarkers associated with unfavorable prognosis. Interestingly, the expression of miR-34a is highly induced by p53 following DNA damage and oncogenic stress, and reduction of miR-34 function attenuates p53-mediated cell death (He et al. 2007). Moreover, in OS models, miR-34a inhibits proliferation, angiogenesis, and metastasis of tumor cells by targeting Notch-1, mTOR, c-Met, MDM4, and Eag1 (Li et al. 2013; Tian et al. 2014b; Wu et al. 2013; Yan et al. 2012).

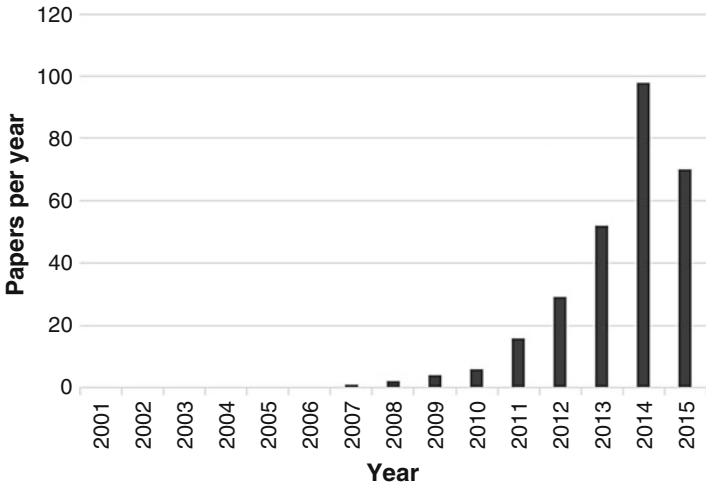

Fig. 2 Number of scientific publications related to miRNA investigation in OS. Annual number of peer-reviewed papers published with "osteosarcoma" and "miRNA" in their titles, keywords, or abstracts from 2001 (Year of discovery of the miRNA class in humans (Lagos-Quintana et al. 2001)) to May 2015 (Pubmed database)

In a sample of 52 patients, miRNA-22 was identified as a novel potential biomarker of unfavorable prognosis in OS (Wang et al. 2015a). In fact, miR-22 is downregulated in OS in comparison with noncancerous bone tissues, and its low expression level correlates with recurrence, metastasis, chemotherapy response, and poorer overall survival and DFS. miR-22 seems to act as tumor suppressor gene by targeting the 3′UTR of high-mobility group box 1 (HMGB1) and inhibiting its translation. In OS cells, high levels of HMGB1 (due to miR-22 downregulation) promote autophagy and consequent drug resistance (Guo et al. 2014).

As example of upregulated miRNAs in OS, miR-27a was found to be prognostic of metastatic disease in a sample of 18 patients (Jones et al. 2012). miR-27a is described to promote metastasis in OS, at least in part, through targeting the tumor suppressor CBFA2T3, which is downregulated in a majority of patients (Salah et al. 2015).

Up to now, several other miRNAs have been found to be implicated in OS (Zhang et al. 2015), and a tool was needed to manage the information regarding the expression patterns. To this aim, Korsching and coworkers constructed the Osteosarcoma Database, which provides a structured, annotated, and easy accessible overview of the protein-coding and miRNAs genes whose expression correlates with disease progression and that might be used as biomarkers (http://osteosarcoma-db.uni-muenster.de). At the time of the last update (October 2013), the Osteosarcoma Database contains 911 protein-coding genes and 81 microRNAs associated with OS according to 1,331 PubMed abstracts. The Osteosarcoma Database offers "the possibility to rank and sort the literature according to various parameters, including therapeutic and prognostic value of specific genes and microRNA and the type of sample used" (Poos et al. 2014).

miRNAs Detection Methods

Quantitative real-time PCR (qRT-PCR) technique is the most popular reference test to quantify miRNA expression, because of its speed, simplicity, low cost of exercise, and high sensitivity and specificity. The disadvantage is that this technique is time consuming and laborious if large number of miRNA has to be analyzed. Microarrays or microRNA sequencing (miRNA-seq), instead, are used when high throughput is desired, even if they need more complex steps of standardization and validation. Microarray platforms allow the analysis of thousands of miRNAs in a single experiment, and it is widely used in order to detect and quantify miRNAs. miRNA-seq uses next-generation sequencing technology to massively sequence miRNAs; it is relatively recent but is replacing microarrays. This miRNA-seq technology has the advantage of quantifying and identifying known miRNAs, as well as novel miRNAs.

A major difficulty in miRNA quantification from patient tissues is the availability of frozen samples. Recently, Spentzos and colleagues overcome this problem and published a large OS profiling study (Kelly et al. 2013). They used the Illumina cDNA-mediated annealing, selection, extension, and ligation (DASL) assay to analyze the expression of 1,146 miRNAs from the partially degraded RNAs

extracted from 91 formalin-fixed, paraffin-embedded (FFPE) OS diagnostic biopsy specimens and identified a cluster of miRNAs with predictive value for OS recurrence and survival. This cluster is located at the 14q32 locus, already linked to this type of cancer. Through this technology, they also identify nonoverlapping miRNA profiles predictive of chemoresponse.

Circulating miRNAs

miRNAs are not only regulators of gene expression in the same cell in which they are synthesized, but they can be secreted and transferred horizontally between cells, assuming also a role in intercellular communication and long-distance signaling, regulating target RNAs in recipient cells (Chen et al. 2012). Circulating miRNAs have been found in serum, plasma, and other body fluids and represent attractive biomarkers in noninvasive serological tests for the diagnosis or prognosis of cancer. This type of analysis presents the advantage of easier samples achievement and, consequently, it allows analyzing larger cohort of patients. Recent findings on circulating miRNA associated with OS are summarized in Table 1.

In some interesting example, such as miR-9 and miR-214, the differential expression of miRNAs and their prognostic value in OS were described for both plasma and tumoral tissue (Allen-Rhoades et al. 2015; Fei et al. 2014; Wang et al. 2014; Xu et al. 2014).

In conclusion, it is clear that the expression profiles of circulating miRNA are useful as biomarkers for OS diagnosis, prognosis, and chemoresponse. But despite the large progress in the field, a lot of work is still needed to identify, characterize, and validate the most predictive biomarkers, and several research findings have to be clarified, such as the opposite expression profile found in different studies for miR-199a-3p in plasma of patients (Lian et al. 2015; Ouyang et al. 2012).

Transcriptomics

Over the last few years, the analysis of differentially expressed genes by microarray combined with bioinformatics analysis has been used to identify key genes and cellular signaling pathways involved in OS progression and metastasis. However, OS genome-wide studies resulted extremely hard due to the rarity of the disease, the high genomically unstable OS cells, and the heterogeneity of tumor clinical samples (Kuijjer et al. 2013). In a recent review, the challenges of high-grade OS data analysis have been discussed (Kuijjer et al. 2013), giving an overview of the major findings on DNA/RNA microarray reports on OS. Therefore, here we will only review the most recent findings on OS obtained by microarray analyses.

Genetic regulation is pivotal for the occurrence and progression of tumors and the development of advanced technologies, such as serial analysis of gene expression

Table 1 Circulating miRNAs associated with OS

Circulating miRNAs	No. of patients	Expression	Correlation with	References
miR-9	118	Upregulated	Tumor size, metastasis, overall survival	Fei et al. 2014
miR-21	80 + 65	Upregulated	Metastasis, tumor subtype, Enneking stage, chemoresistance	Ouyang et al. 2012 Yuan et al. 2012
miR-34b	133	Downregulated	Metastasis	Tian et al. 2014a
miR-133b	100	Downregulated	Tumor grade, metastasis, recurrence, survival	Zhang et al. 2014b
miR-143	80	Downregulated	Metastasis, tumor subtype	Ouyang et al. 2012
miR-148a	89	Upregulated	Tumor size, metastasis, survival	Ma et al. 2014
miR-195-5p	90	Upregulated	Metastasis	Lian et al. 2015
miR-196a	100	Upregulated	Tumor grade, metastasis, and recurrence	Zhang et al. 2014a
miR-196b	100	Upregulated	Tumor grade, metastasis, and recurrence	Zhang et al. 2014a
miR-199a-3p	90	Upregulated	Metastasis, chondroblastic subtype	Lian et al. 2015
miR-199a-3p	80	Downregulated	Metastasis	Ouyang et al. 2012
miR-205-5p	40	Downregulated		Allen-Rhoades et al. 2015
miR-206	100	Downregulated	Tumor grade, metastasis, recurrence, survival	Zhang et al. 2014b
miR-214	40	Upregulated	Survival	Allen-Rhoades et al. 2015
miR-320a	90	Upregulated	Osteoblastic subtype	Lian et al. 2015
miR-335-5p	40	Upregulated		Allen-Rhoades et al. 2015
miR-374a-5p	90	Upregulated		Lian et al. 2015
miR-574-3p	40	Upregulated		Allen-Rhoades et al. 2015

provided the means to identify putative biomarkers for a large number of tumors, including OS. A summary of the most important genes and signaling pathways involved in the formation of OS and identified by genome-wide studies are provided

in Tables 2 and 3. By high-density oligonucleotide microarray, potential biomarkers of both prognostic and therapeutic significances were identified in OS cell lines (Zou et al. 2012). Interestingly, among them cancer testis antigens, such as melanoma antigen family A (MAGEA), were significantly increased and associated with a high risk of metastasis and poor survival. A meta-analysis study on different gene expression data of OS has allowed to detect differences between control tissues and OS, such as enrichment in focal adhesion pathway (Yang et al. 2014).

Recently, several microarray and meta-analysis studies have been carried out to unveil potential biomarkers for metastatic OS. In a screening using a DNA microarray, differentially expressed genes were identified and classified as upregulated, most significantly in cytoskeleton organization, and downregulated, mainly in wound healing (Diao et al. 2014). Seventeen differentially expressed genes were described to be metastasis related and considered as important players in tumor progression of osteoblastic OS, the predominant phenotype of the disease (Muff et al. 2012). New putative targets were supposed to be useful for the diagnosis and

Table 2 Major genes identified in genome-wide and microarray studies in OS

Gene	Function	References
IGFBP5	Tumor suppressor	Su et al. 2011
WIF1	Tumor suppressor	Kansara et al. 2009
LSAMP	Tumor suppressor	Kresse et al. 2009, Yen et al. 2009
Cyclin E3	Oncogene	Lockwood et al. 2011
RUNX2	Oncogene	Kresse et al. 2012, Sadikovic et al. 2009
DOCK5	Tmor suppressor	Sadikovic et al. 2009
TNFRSF10A	Tumor suppressor	Sadikovic et al. 2009
DLX5	Oncogene	Kresse et al. 2012
CXCL5	Tumor suppressor	Kresse et al. 2012
PRAME	Oncogene	Kresse et al. 2012, Zou et al. 2012
NKD2	Tumor suppressor	Zhao et al. 2015

IGFBP5 insulin-like growth factor binding protein 5, *WIF1* Wnt inhibitory factor 1, *LSAMP* limbic system-associated membrane protein, *VEGF* vascular endothelial growth factor, *RUNX2* runt-related transcription factor 2, *DOCK5* dedicator of cytokinesis 5, *TNFRSF10A* tumor necrosis factor receptor superfamily, member 10, *DLX5* distal-less homeobox 5, *CXCl5* chemokine (C-X-C motif) ligand 5, *PRAME* preferentially expressed antigen in melanoma, *NKD2* naked cuticle homolog 2

Table 3 Major signaling pathways identified in genome-wide and microarray studies in OS

Signaling pathway	References
Macrophage-associated genes correlated with better metastasis-free survival	Buddingh et al. 2011
DNA replication network	Cleton-Jansen et al. 2009, Sadikovic et al. 2009
Amplification of the VEGF pathway genes	Yang et al. 2011
Deregulation of the cell cycle	Kuijjer et al. 2012
Apoptosis, signal transduction	Kuijjer et al. 2012

treatment of metastatic OS, such as alpha-2-macroglobulin (A2M) and its interactive proteins (Niu et al. 2014), metalloproteinase 1 (MMP1), smoothened (SMO), Ewing sarcoma breakpoint region 1 (EWSR1), fasciculation and elongation protein 1 (FEZ1), brain-selective kinase 2 (BRSK2), aldo-keto reductase family 1 member B10 (AKR1B10) (Yao et al. 2015), brain-specific angiogenesis inhibitor 2 (BAI2), formin-like 1 (FMNL1), dual-specificity phosphatase 7 (DUSP7), transient receptor potential melastatin 2 (TRPM2) (Wang 2015), epiregulin (EREG), and carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2 (CHST2) (Chen et al. 2011). Moreover, by microarray analysis performed on a mouse model of localized and metastatic OS, it was demonstrated that downregulation of naked cuticle homolog 2 (NKD2) expression plays an important role in driving OS tumor growth and metastasis (Zhao et al. 2015).

Microarray analysis was also utilized to gain insights into the molecular mechanisms underlying signaling pathway or set of proteins known to be involved in OS progression and metastasis. Ribosomal protein S3 (RPS3) was identified as a downstream factor of GLI2 that mediates migration and invasion of OS (Nagao-Kitamoto et al. 2015); ΔNp63α, the predominant p63 isoform expressed in invasive OS cells, turned out to be necessary for the regulation of GLI2 expression to promote its oncogenic properties (Ram Kumar et al. 2014); and special AT-rich binding protein 2 (SATB2), highly expressed in OS, revealed the ability to regulate epithelial protein lost in neoplasma (EPLIN) and genes involved in cytoskeleton dynamics to increase OS migration and invasion (Seong et al. 2014).

OS is considered to be a differentiation disorder of mesenchymal stem cells, which produce defective, immature bone. Despite this simple definition, OS is highly heterogeneous and is subdivided into numerous different histological subtypes. Currently these subtypes are classified on the basis of morphological and histological criteria, but the identification of biomarkers that characterize each subtype would be of major importance to improve therapeutic and prognostic outcomes. Using microarray-based differential expression and gene set analysis, a different gene expression pattern was identified between osteoblastic and nonosteoblastic OS subgroups (Kubista et al. 2011), while gene expression analysis allowed to identify genes involved in plasticity of anoikis-resistant OS subgroups characterized by a rapid development of chemoresistance and altered growth rate, mimicking the early stages of latent metastasis (Foley et al. 2015).

OS is the most common primary bone malignancy in dogs. Canine OS shares several traits with human OS, making dogs a valuable comparative model that has strong potential applicability to the human disease (Sutter and Ostrander 2004). Indeed, using gene expression microarray analysis on canine OS samples, potential new biomarkers and novel pathways that may be targeted for therapeutic intervention were identified (O'Donoghue et al. 2010).

Gene expression profiling by microarray combined with other techniques resulted successful in several studies. Indeed, expression microarray analysis combined with the investigation of focal copy number aberrations has allowed identifying CKLF-like Marvel transmembrane domain containing 8 (CMTM8) as a new candidate tumor suppressor and G protein-coupled receptor 177 (GPR177) as a new putative

oncogene in OS (Both et al. 2014). Furthermore, combining proteomic analysis with previously obtained cDNA microarray results allowed detecting aldolase A fructose-bisphosphate (ALDOA) and sulfotransferase family cytosolic 1A phenol-preferring 3 (SULT1A3) as predictors of clinical outcomes for OS patients (Chen et al. 2014), while microarray-based comparative genomic hybridization (aCGH) allowed to gain a comprehensive understanding of the key driving pathways for OS, elucidating the contradictory role of Wnt signaling (Du et al. 2014), and identifying a functional crosstalk between vascular endothelial growth factor (VEGF) and runt-related transcription factor 2 (RUNX2) essential for the pathogenesis and angiogenesis of the disease (Yang et al. 2013).

Finally, although in some studies microarray failed to predict biomarkers for OS patients' outcome (Sabile et al. 2013), for researchers it certainly provides an useful tool to characterize the altered expression of genes involved in the development and behavior of OS subtypes and to identify the gene signature of an individual OS patient revealing distinct signaling events, which might account for the biological features specific for each tumor type.

Proteomics

Proteomic approaches to cancer research offer several advantages in respect to other high-throughput technologies such as genomics or transcriptomics. In addition to global protein profiling and protein identification, proteomics provides powerful tools to investigate the complexity of these highly dynamic macromolecules. In fact, disease-associated phenotypic alterations are consequences not only of deregulated (increasing/decreasing) expression of proteins but also of functional regulations by various processes such as proteins degradations, posttranslational modifications (e.g., phosphorylation, glycosylation, methylation), involvement in complex structures, and differential compartmentalization (e.g., nuclear localization).

Moreover, proteomic approaches can be applied to a variety of biospecimens ranging from biological models, such as cell lines, primary cell cultures, or animal models of disease, to clinical samples, including serum/plasma, urine, spinal fluid, synovial fluid, and tissue.

Comprehensive analysis of proteomic data from cancer patients' samples has notably improved our understanding of tumor pathogenesis and treatment, uncovering the different processes involved in cancer development and progression, along with the identification of novel target for cancer therapy.

The discovery of biomarkers with clinical relevance using proteomics is affected by several critical challenges, in particular the biological variability among patients' samples and the huge dynamic range of biomarkers concentration in biological fluids. In addition to these, another major obstacle to be taken into account is the thousands of cancer-associated proteins detected by high-throughput proteomic approaches that have to be properly validated.

Nevertheless, in the last decade proteomic approaches lead to the discovery of clinically relevant biomarkers for several types of cancers such as breast, esophageal, lung, liver, and colorectal cancer. All these biomarkers possess high values of specificity and sensitivity and represent unvaluable tool to be used for screening, early detection, and prediction of response to therapy in oncology (Sallam 2014).

Proteomics technologies include gel-based methods (1DE, 2DE, and 2D-DIGE), gel-free methods based on mass spectrometry (SELDI and MALDI ToF/MS, LC-MS/MS), or based on array (antibody array, reverse phase protein microarray (RPMA)) and bioinformatics.

Several proteomic approaches have been applied to OS research to elucidate the molecular mechanism underlining the development and progression of the diseases and also to identify new molecular markers for early diagnosis, prognosis, and chemotherapy responsiveness (Table 4).

To address these aims, different types of human biological samples have been used such as OS cell lines and primary cell cultures, OS bone tissue, or serum (Table 4).

Biomarkers from OS Cells

Comparative 2-DE was applied to total protein extract of OS cell lines (i.e., SaOS-2, U2OS, and IOR/OS9) and primary or SV-40 immortalized osteoblastic cells (i.e., hFOB1.19) (Spreafico et al. 2006; Guo et al. 2007; Liu et al. 2009). All three studies report a list of proteins whose expression was found altered in OS cell lines in respect to healthy counterparts. However, when comparing the results, a total absence of overlapping is noticeable. This inconsistency could rely on the type of control samples used by the authors, immortalized cells (Guo et al. 2007) or primary cells extracted from different anatomical sites (Spreafico et al. 2006; Liu et al. 2009), or on slight differences in the experimental procedures.

Subproteomic analyses of OS cell lines were also performed with particular attention to membrane (Zhang et al. 2010; Hua et al. 2011) and surface exposed proteins (Posthumadeboer et al. 2013). The group led by Cai applied a double approach to identify plasma proteins able to differentiate MG63 OS cells from hFOB1.19 cells (Zhang et al. 2010; Hua et al. 2011): CD151 was selected by a quantitative gel-free analysis (iTRAQ-LC/MS/MS) combined to bioinformatics (Zhang et al. 2010), while NDRG1 was identified by a gel-based approach (Hua et al. 2011). Both marker candidates were then validated by WB and IHC. Posthumadeboer et al. identified EPHA2 receptor as the most abundant surface proteins in several OS cell lines (SaOS-2, MG63, U2OS, and SaOS-2 LM7) and significantly overexpressed in OS cells and tissues in respect to normal samples (Posthumadeboer et al. 2013).

OS-specific proteins were also investigated by Folio in primary cells isolated from five paired samples of OS tumor and normal bone tissue (Folio et al. 2009). 2DE global protein profiling showed the upregulation of 56 protein spots in

Table 4 Protein biomarkers identified by proteomics in OS

Proteins	Application	Biological samples	Validation	Technique	References
Pyruvate kinase M1, L-lactate dehydrogenase B chain, triose phosphate isomerase 1, creatine kinase B chain, heat shock protein 90, 150 kDa oxygen-regulated protein, retinoblastoma-binding protein 4, alkaline phosphatase	Diagnosis	OS cell line (SaOS-2) and primary OB cells	No	2DE and PMF	Spreafico et al. 2006
Activator of 90 kDa heat shock protein, ATPase homolog 1 AHA1, and stomatin-like protein 2	Diagnosis	OS cell lines (SAOS-2, U2-OS and IOR/OS9) and OB cells (SV-immortalized hFOB1.19)	No	2DE and PMF	Guo et al. 2007
Cytochrome b-c1 complex subunit 1, ubiquitin carboxyl-terminal hydrolase isozyme L1, peroxiredoxin-4	Diagnosis	OS cell line (SaOS-2) and primary OB cells	Yes, WB	2DE and PMF	Liu et al. 2009
Ezrin, crystallin b chain	Diagnosis	Chemonaive primary OS and paired normal OB cells	Yes, RT-PCR and WB	2DE and nano-LC-ESI-QToF MS/MS	Folio et al. 2009
CD 151 (Membrane glycoprotein SFA-1)	Diagnosis	OS MG63 cells and OB cells (SV-immortalized hFOB1.19): plasma membrane proteins	Yes, IHC	iTRAQ-LC-MS/MS	Zhang et al. 2010
NDRG1	Diagnosis	OS MG63 cells and OB cells (SV-immortalized hFOB1.19): plasma membrane proteins	Yes, WB IHC	2DE and nano-LC-MS/MS	Hua et al. 2011
Ephrin type-A receptor 2 (EPHA2)	Diagnosis	OS cell lines (MG 63, U2OS, Cal-72, SaOS-2, SaOS-LM7) and primary OB cells (ORT-1, Hum31, Hum54, Hum63, Hum65: surface proteins)	Yes, FACS and IHC on tissue microarray	1DE and nano-LC-LTQ-FT MS/MS	Posthumadeboer et al. 2013

Aldolase A and sulfotransferase 1A3/1A4	Prognosis	OS cell lines with different metastatic potential (F5M2 and F4)	Yes, WB and ICH	2D-DIGE and PMF	Chen et al. 2014
Translationally controlled tumor protein, malate dehydrogenase, CBX3, dihydropyrimidinase-related protein 2, fructose-bisphosphate aldolase C	Diagnosis	OS cancer stem cells CHA59	CBX3: Yes, RT-PCR	2DE and LC-MS/MS	Saini et al. 2012
Activation of MAPKs pathway	Diagnosis	OS CSC (3-AB OS) and its parental OS cell line (MG-63)	No	Antibody array and knowledge-based analysis	Gemei et al. 2013
Protein signature of 10 protein spots	Therapy response	Chemonaive OS tissues classified as good/poor responders	No	2D-DIGE and PCA	Kawai et al. 2008
Vimentin, tubulin-a1c, lamin B2, coatomer protein complex, epsilon subunit, zinc finger protein 133, ferritin light polypeptide, myosin, light chain 6, ezrin, transferrin, a1-antitrypsin, chaperonin-containing TCP1	Diagnosis	OS and benign bone tumor tissues	Yes, WB and IHC	2DE and PMF	Li et al. 2010
Peroxiredoxin-2	Therapy response	Chemonaive OS tissues classified as good/poor responders	Yes, WB	2D-DIGE and LC-LTQ ion trap MS/MS	Kikuta et al. 2010, Kubota et al. 2013
Heat shock protein 90 and clusterin	Prognosis	OS tissues from older adults and desmoid tumor tissues	Yes, TMA	LC-MS/MS	Rao et al. 2013
Serum amyloid A	Diagnosis	Plasma form OS and OC	Yes, WB	SELDI-ToF/MS	Li et al. 2006

(*continued*)

Table 4 (continued)

Proteins	Application	Biological samples	Validation	Technique	References
Serum amyloid A	Diagnosis	Serum from OS patients and healthy subjects	Yes, WB and ELISA	2D-DIGE and PMF	Jin et al. 2007
Cytochrome-c1	Diagnosis	Serum from OS patients and healthy subjects	Yes, WB	SELDI-ToF/MS	Li et al. 2009
Serum amyloid A and transthyretin	Therapy response	Plasma from OS patients before and after preoperative chemotherapy and classified as good/poor responders	Yes, WB	SELDI-ToF/MS	Li et al. 2011
Gelsolin (decrease)	Diagnosis	Serum from OS patients and healthy subjects	Yes, WB and ELISA	2D-DIGE and PMF	Jin et al. 2012
Two protein peaks at *M/Z* of 3954 Da and 6438 Da (not identified)		Plasma from OS, OC, and healthy volunteers	No	SELDI-ToF/MS	Gu et al. 2014

transformed cells. The overexpression of two of these, namely, ezrin and alpha-crystallin B chain, were confirmed by immune histochemistry or real-time PCR.

Recently, researchers in the field of experimental and clinical oncology have focused their attention on cancer stem cells (CSC). Several lines of evidences indicate that CSCs posses an elevated genotypic and phenotypic plasticity responsible for the heterogenicity of tumors and are involved not only in carcinogenesis but also in the metastatic process, invasion, therapeutic poor responsiveness, and recurrence of cancer. Although our comprehension of OS CSCs has notably improved, their role in OS pathophysiology is still largely unknown (Bernardini et al. 2014). To strengthen our knowledge of OS CSCs, global protein profiling can be extremely useful in uncovering their complexity as well as in selecting novel putative biomarkers. Several phenotypic changes were detected in two OS CSCs when compared to their parental cell lines (Table 1; Saini et al. 2012; Gemei et al. 2013). However, since none of these potential markers have been validated, their use as diagnostic or prognostic factors is still to be demonstrated.

Biomarkers from OS Tissues

Proteomic studies to identify specific OS protein markers were also conducted on tissue samples obtained from patients' biopsies (Kawai et al. 2008; Kikuta et al. 2010; Li et al. 2010; Kubota et al. 2013; Rao et al. 2013).

Li et al. compared the protein expression profile of malignant (osteoblastic, chondroblastic, and fibroblastic OS) and several benign (chondroblastoma, osteoblastoma, and giant cell) bone tumors using 2DE combined to PMF (Li et al. 2010). The overexpression in OS of two (TUBA1C and ZNF133) out of 12 upregulated protein spots was validated by WB and IHC and thus selected as potential OS biomarkers. Although authors did not extend the validation phase to normal bone tissue, these two proteins represent a starting point for the development of important molecular tools for OS diagnostic.

Analogously, a gel-based proteomic approach was carried out by Kondo to identify prognostic markers of OS responsiveness to chemotherapy (Kawai et al. 2008; Kikuta et al. 2010; Kubota et al. 2013). Authors detected the overexpression of peroxiredoxin 2 in OS tissue samples from chemonaive patients who were afterwards classified as poor responder to different chemotherapy protocols: combination of IFO, DOX, and CDDP (Kikuta et al. 2010) or combination of MTX, DOX, and CDDP (Kubota et al. 2013). The overexpression of peroxiredoxin 2 was further validated in a larger cohort of OS patients by WB and ROC analysis (AUC = 0.90, sensitivity = 83.3%, specificity = 85.7%, $p = 0.015$) that demonstrated the reliability of such a prognostic marker (Kubota et al. 2013).

Heat shock protein 90 and clusterin were found by a gel-free proteomic approach to be able to differentiate OS tissues from benign desmoid tissues (Rao et al. 2013). In particular, OS tissues were isolated from older adult patients with different background (Paget's disease, OS associated to dedifferentiated liposarcoma,

extraosseus OS, dedifferentiated periosteal OS) with the aim to define the protein profile related to a highly metastatic cancer.

Circulating OS Biomarkers

Finally, the occurrence of specific OS protein biomarkers was also explored in plasma samples.

Serum amyloid protein A (SAA) was found to be present in higher amount in OS patients than in osteochondroma patients (Li et al. 2006) or in healthy controls (Jin et al. 2007). Moreover, several authors demonstrated that SAA levels in OS patients might be used as marker to monitor relapses or response to chemotherapy (Jin et al. 2007; Li et al. 2011). Other OS plasmatic biomarkers include high level of cytochrome C as an early diagnostic indicator, while high level of transthyretin suggests a poor response to therapy (Li et al. 2009, 2011).

Potential Applications to Prognosis, Other Diseases, or Conditions

Osteosarcoma is a heterogeneous tumor. This is due to the lack of characteristic chromosomal translocations or alterations, the occurrence in different anatomical sites, and the presence of different histologic subtypes. This heterogeneity, in addition to biospecimens variability (tissues, cells, body fluids, etc.), and to the low incidence of the pathology, is reflected in post-genomics studies leading to nonoverlapping or discordant results and to a very challenging validation phase of potential biomarkers. Therefore it is likely to be difficult to identify genes, miRNAs, or proteins that could have reliable diagnostic and/or prognostic value in osteosarcoma. To overcome the problem related to the scarcity of clinical cases, the scientific and medical community should promote networks of biobanks by means of national and international reference centers. These networks should be committed to harmonize procedures and set common standards for biospecimens and clinical data collection and storage and to facilitate access to biological samples. A similar approach should be used with high-throughput approaches and comprehensive and integrated post-genomic investigations of patients should be required in order to overcome the intrinsic limitation of each related technology.

Our chance to understand the relationships between the individual molecular asset and the pathogenesis of disease, as well as the diversity of clinical outcomes or responses to therapies, will only be guaranteed by the use of high-quality biological samples with accurately phenotyped clinical data. This will likely lead to personalized medicines for OS patients.

Summary Points

- Osteosarcoma is the most common primary bone cancer in adolescents and young adults.
- The presence of metastatic disease at the time of diagnosis and responsiveness to chemotherapy are the principal prognostic factors for OS.
- There is an urgency for reliable biomarkers for early detection of OS, prediction of chemoresponsiveness, monitoring of treatment or relapses.
- "Omics" approaches identified biomarkers for several types of cancers as diagnostic/prognostic indicators.
- Novel diagnostic and prognostic biomarkers can also represent novel target for more effective and personalized therapies.
- Rarity of OS hinders the large and proper validation of biomarkers selected by post-genomics approaches.

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Traditional Medicine and Use of Bone Biomarkers

3

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Contents

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Abstract

Traditional medicine gathers the knowledge, skills, and practices accumulated through years and transmitted generation to generation, aiming to maintain health and to treat illnesses. Although this folk medicine includes different techniques and practices, herbal medicine remains the most used by local populations in different parts of the world. In recent years, bone diseases and disorders become a major health problem. Although conventional drugs are effective, more and more studies are interested in herbal therapies. An increasing number of studies have shown promising antiosteoporotic activities of medicinal plants. Bone markers, considered as inexpensive and noninvasive tools, are used to manage the response to the herbal treatments both in vitro and in vivo. Bone turnover markers include formation markers (osteocalcin, alkaline phosphatase, osteoprotegerin, etc.) and resorption markers (CTX, NTX, Pyridinoline, Deoxypyridinoline, etc.). Clinical studies using bone markers demonstrated a preventive effect of different medicinal plants such as *Aristolochia longa* L., *Prunus domestica* L., or *Citrus unshiu* Marcow. regarding bone loss in menopausal women. This chapter focuses on herbal medicine and use of bone biomarkers.

Keywords

Traditional medicine • Medicinal plants • Bone • Osteoporosis • Markers

List of Abbreviations

ALP	Alkaline phosphatase
BALP	Bone-specific alkaline phosphatase
Ca	Calcium
CTX	Carboxy-terminal cross-linked telopeptides of type 1 collagen
DPD	Deoxypyridinoline
ICTP	Carboxy-terminal cross-linked telopeptide of type 1 collagen
NTX	Amino-terminal cross-linked telopeptide of type 1 collagen
OC	Osteocalcin
P	Phosphorus
PICP	Procollagen type 1 carboxy-terminal propeptide
PINP	Procollagen type 1 amino-terminal propeptide
PYD	Pyridinoline
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
TRAP	Tartrate-resistant acid phosphatase

Key Facts of Traditional Medicine

- Traditional medicine is linked to the local knowledge, skills, and practices used to treat ailments and/or maintain health.

- Traditional medicine is called folk medicine when used in its cultural context (acunpuncture used in China).
- Traditional medicine is called complementary medicine when used out of its cultural context, for example, when acupuncture is used for cancer pain in USA.
- Since medicinal plants are rich sources of chemical active compounds, antiosteoporotic effects, they are used by local populations and are studied regarding their antiosteoporotic effects.
- Several medicinal plants have shown promising antiosteoporotic activities through different molecular mechanisms.

Definitions of Words and Terms

Bone markers	Molecules released into the blood or urine during bone formation and/or resorption. Since bone formation and resorption are coupled, the bone turnover needs to be evaluated using both a formation marker and a resorption marker.
Bone turnover	Continuous process of formation and destruction of bone. The bone matrix is not static but is subject to permanent changes. To maintain it, every destroyed part (resorption) must be replaced (formation).
Flavonoids	Secondary metabolites produced by plants. They are about 6000 different molecules sharing the same chemical structure. Flavonoids are considered as bioactive compounds showing positive effects on health. They posses several activities including antioxidant, anticancer, anti-inflammatory, antiosteoporotic, antibacterial, etc.
Medicinal plants	Plants used to treat several ailments. Their activities are due to their richness in bioactive compounds, present in different parts of the plant.
Osteoporosis	A bone disease, often asymptomatic, in which bones become weak, porous, fragile, and more susceptible to fractures. In osteoporotic patients, it's often observed deterioration of bone tissue and a decrease in bone mass. Menopause and aging are the main causes of the disease.
Ovariectomy	A surgery performed to remove one or both ovaries. This surgery is practiced in preclinical studies to induce osteoporosis in rat model.
Phyto-estrogen	Plant phytochemicals considered as phyto-hormones that mimic natural estrogens. Their activity is weaker then the human estrogens.
Traditional medicine	The folk knowledge and practices transmitted from generation to generation, aiming to treat ailments and maintain health.

Introduction

Traditional medicine is defined by the WHO as "the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness."

The traditional medicine gathers the knowledge and practices accumulated over the years and transmitted from generation to generation with the aim to cure diseases and maintain health. Traditional medicine, called also indigenous medicine, becomes "alternative medicine" when practiced outside its cultural context. For example, acupuncture considered as traditional medicine in China is practiced in some occidental countries as "alternative medicine." We can distinguish some developed and documented traditional medicines such as the Chinese traditional medicine, the Indian ayurvedic medicine, the Islamic medicine, or the African folk medicine. Nowadays, about 70–90% of the populations living in developing countries use traditional medicines for primary care or for those living in developed countries as complementary and alternative medicine.

Although traditional medicine consists of different practices such as acupuncture and herbal medicine, the latter remains the most used by local population in different parts of the world. Herbal medicine use by ancient civilizations in Egypt, China, India, or Africa is well documented through historical references. In recent years, combining ethnobotanical studies, phytochemical investigations, and biological activities elucidation of medicinal plants gave a solid scientific support sometimes proving the virtues attributed to plants and/or correcting some errors.

Bone diseases such as osteoporosis or osteoarthritis are considered as major public health problems. Many studies (both in vitro and in vivo) have shown positive effects of traditional medicine(s) on bone health. Bone turnover markers are considered to be useful in the therapeutic management of bone diseases. Hence, this chapter focuses on herbal medicine and use of bone biomarkers.

Labisia pumila var. *alata*

Labisia pumila var. *alata* (Myrsinaceae) is a popular phytoestrogenic herb widely used in the Southeast Asia as an alternative to estrogens replacement therapy in the treatment of postmenopausal osteoporosis (Chua et al. 2012). Effects of *Labisia pumila* var. *alata* on bone markers were studied in ovariectomized rats. Serum osteocalcin and CTX levels were measured using ELISA. Supplementation of 17.5 mg/kg of *Labisia pumila* var. *alata* roots extract for 8 weeks maintained the levels of the bone formation marker osteocalcin decreased initially by ovariectomy. Furthermore, the plant was able to prevent the increase of the bone resorption marker CTX. Authors of the study suggested the use of *Labisia pumila* var. *alata* as an alternative treatment for postmenopausal osteoporosis (Shuid et al. 2011). Phenolic acids and flavonoids seem to be responsible of these effects. The total phenolic content in the leaves of the plant was

estimated to be 468 – 427 μg GAE/mg extract. Furthermore, nine phenolic acids (benzoic acid, gallic acid, protocatechuic acid derivative, vanillic acid, syringic acid, cinnamic acid, coumaric acid, salicylic acid, caffeic acid, and chlorogenic acid derivative), nine flavonols (quercetin, myricetin, kaempferol, etc.), and two flavanols (catechin and epigallocatechin) were identified (Chua et al. 2011).

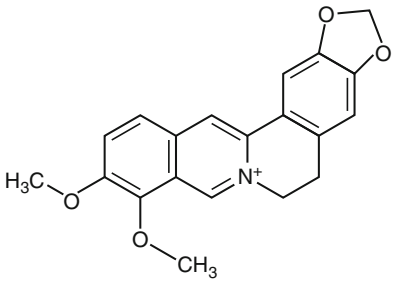
Aronia melanocarpa (Michx.) Elliott

Female rats treated with Cadmium (1 and 5 mg /kg) were supplemented with a polyphenol extract of *Aronia melanocarpa* (Michx.) Elliott (Rosaceae) for 3, 10, 17, and 24 months. The protective effects of the extract against cadmium-induced disorders in bone metabolism were investigated by measuring serum bone formation (osteocalcin, alkaline phosphatase, osteoprotegerin) and resorption markers (carboxy-terminal crosslinking telopeptides of type I collagen, soluble receptor activator of nuclear factor-kappaB ligand) in the serum. *Aronia melanocarpa* (Michx.) Elliott extract prevented the toxic metal-caused decrease in the serum activity of ALP and concentration of OC and the increase in sRANKL concentration and sRANKL/OPG in the serum (Brzóska et al. 2015). Phenolic acids characterized in the plant, especially p-Hydroxybenzoic acid, could explain the beneficial effects on bone function. Indeed, an estrogenic activity of p-Hydroxybenzoic acid has been demonstrated (Szopa et al. 2013).

Berberis aristata DC.

Berberis aristata DC. belongs to the Berberidaceae family. Yogesha et al. (2011) studied the antiosteoporotic activity of an aqueous-methanol extract of *Berberis aristata* DC. in ovariectomized Sprague–Dawley rats. Serum and urine calcium, inorganic phosphorus, and alkaline phosphatase activity served as bone loss markers. Ovariectomy-induced reduction of serum calcium and phosphorus was corrected by *Berberis aristata* DC. administered at 100, 300, and 500 mg/kg. Furthermore, the extract induced a significant dose-dependent decrease in serum ALP activity, urine calcium, creatinine, and phosphorous levels. This effect may be attributed to berberine, a yellow isoquinoline alkaloid, the major constituent of *Berberis aristata* DC. Berberine consumption produced a more favorable bone biomarker profile than lifestyle modification alone in healthy women. In fact, supplementation with 100 mg berberine (incorporated with hop rho iso-alpha acids, 500 IU vitamin D3, and 500 μg vitamin K1, twice daily) for 14 weeks in healthy postmenopausal women resulted in significant 34% mean reduction in serum osteocalcin at 10 weeks and 31% reduction at 14 weeks. Furthermore, treated women experienced a significant 8% increase in serum 25(OH)D concentration at 10 weeks and 13% at 14 weeks. On the other hand, supplementation resulted in a significant increase of serum IGF-I (Holick et al. 2010) (Fig. 1).

Fig. 1 Structure of berberine



Berberine has been reported to present antidiabetic, antihypertensive, anti-inflammatory, antioxidant, antidepressant, anticancer, antidiarrheal, hepatoprotective, and antiarrhythmic activities. Other bioactive compounds have also been extracted from different parts of the plant (Karachine, taxilamine, berbamine, aromoline, palmatine, oxyberberine, hydrazlin, malic acid, etc.) (Tamilselvi et al. 2014).

Drynaria fortunei (Kunze ex Mett.) J. Sm.

Drynaria fortunei (Kunze ex Mett.) J. Sm. is a traditional Chinese medicine widely used to manage osteoporosis and other musculoskeletal disorders. It has been demonstrated that an aqueous extract of *Drynaria fortunei* (Kunze ex Mett.) J. Sm. at 0.5 and 1 g/kg body weight/day for 1, 2, 3, and 6 months increased osteocalcin biosynthesis in bone tissues of ovariectomized rats. In this study, immunohistochemistry and immunoblotting were used to measure levels of osteocalcin (Lee et al. 2014). The osteogenic effects of the plant may be attributed to its richness in flavonoid compounds. Indeed, five flavonoid aglycones, naringenin, kurarinone, kushennol F, xanthogalenol, and sophoraflavanone G, were characterized in rhizomes of *Drynaria fortune*. Although, these compounds have not enhanced proliferation of osteoblastic cells, but significantly increased the ALP activity of the cells at most of the concentrations from 10 nm to 1000 nM (Wang et al. 2011). Naringenin, the metabolized form of orally administered naringin, has been reported to have promising osteogenic effects both in vitro and in vivo (Fig. 2) (Li et al. 2010).

Davallia formosana Hayata

Davallia formosana Hayata belongs to Davalliaceae family and used to manage bone disorders and diseases such as osteoporosis and arthritis. The 75% ethanolic extract of *Davallia formosana* Hayata rhizomes prevented bone loss in ovariectomized rats. Administered at 200 and 500 mg/kg daily for 12 weeks, the extract did not affect the activity of serum alkaline phosphatase (used as bone formation marker). Interestingly, it resulted in a significant decrease of urinary deoxypyridinoline levels from 214.2 $\pm$ 55.4 (nmol/nmol creatinine) in ovariectomized rats to 170.6 $\pm$ 36.1 and 122.4 $\pm$ 16.4 (nmol/nmol creatinine) in treated rats with 200 and

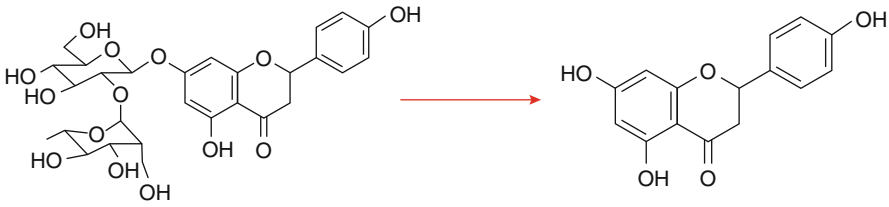
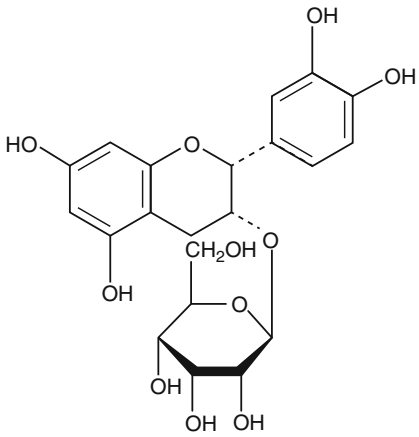


Fig. 2 Structure of naringin and naringenin

Fig. 3 Structure of (−)-epicatechin 3-*O*-β-D-allopyranoside



500 mg/kg, respectively. Similarly, elevated urinary calcium contents have been significantly decreased. The active compound of *Davallia formosana* Hayata extract was found to be (−)-epicatechin 3-*O*-β-D-allopyranoside (Ko et al. 2012) (Fig. 3).

Oral administration of ethanol extract of fresh *Davallia formosana* Hayata (50 or 200 mg/kg/day for 33 days) to ovariectomized rats resulted in a significant decrease of serum CTX levels. On the other hand, the extract inhibited osteoclasts differentiation via the inhibition of NF-kB activation (Lin et al. 2013).

Angelica sinensis (Oliv.) Diels

A. sinensis (Apiaceae), a perennial plant native to southwest China, is one of the most commonly used medicinal plants in China, Europe, and North America. Dried roots of the plant are used to treat ailments including hypertension, osteoarthritis, cardiovascular problems, and anemia. The antiosteoporotic effects of the plant have been demonstrated in ovariectomized rats. The 70% ethanol extract from roots of *A. Sinensis* at 300 mg/kg/ 4 weeks resulted in significant decrease in serum ALP, CTX, and OC concentrations compared to the ovariectomized rats. In addition estrogen concentrations were not affected by the treatment (Lim and Kim 2014) (Fig. 4).

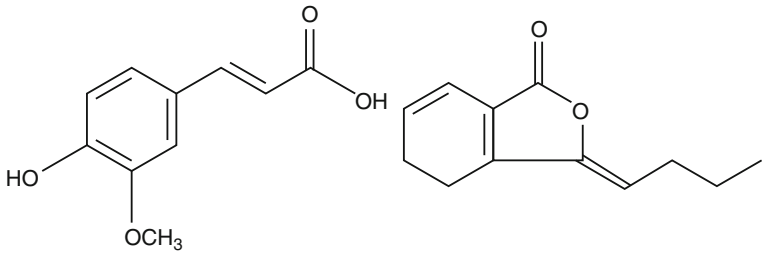


Fig. 4 Chemical structure of ferrulic acid (left) and Z-ligustilide (*right*)

It has been demonstrated that an aqueous extract of *Angelica sinensis* stimulated in vitro the proliferation of human osteoprecursor cells, alkaline phosphatase activity, and type I collagen synthesis in a dose-dependent fashion (Yang et al. 2002). In line with these results, similar osteogenic effects have been reported in osteosarcoma MG-63 cells (common cell line used in studying osteoblast differentiation) with enhancing alkaline phosphatase activity (Zhang et al. 2013). Positive effects of *Angelica sinensis* on bone metabolism have been attributed to its major compounds: ferulic acid or Z-ligustilide. In fact, preventive effect of ferrulic acid in ovariectomized rats has been demonstrated after 8 weeks of treatment (Sassa et al. 2003).

Prunus domestica L.

Dried plum or prunes (*Prunus domestica* L. – Rosaceae) is one of the most effective edible fruits in preventing bone loss. Dried plums intake (100 g/day/3 months) by postmenopausal women was associated with increased serum levels of insulin-like growth factor-I (IGF-I) and bone-specific alkaline phosphatase activity (markers of bone formation), without affecting serum and urinary markers of bone resorption (Arjmandi et al. 2004). Moreover, consumption of 100 g dried plum per day resulted in a time-dependent significant decrease in bone turnover markers (bone-specific alkaline phosphatase, osteocalcin, and tartrate-resistant acid phosphatase-5b) of healthy postmenopausal women at 3-, 6-, and 12-month time intervals (Hooshmand et al. 2011).

Dried plum reversed bone loss in osteopenic rats by significantly decreasing bone resorption markers (urinary deoxypyridinoline and serum TRAP) and increasing insulin-like growth factor (IGF)-I (Bu et al. 2007). Similarly, dried plum supplementation has been shown to restore PINP and IGF-1 increase induced by ovariectomy in female mice (Rendina et al. 2012). Recently, Smith et al. (2014) demonstrated that dried plum supplementation to ovariectomized female Sprague Dawley rats significantly suppressed the increased levels of both urinary DPD and serum P1NP. These findings support that dried plum suppresses the increase in bone turnover caused by ovariectomy. Dried plum supplementation to male orchidectomized rats has been demonstrated to result in a significant decrease (by 57%) in total forms of DPD and a significant increase (by 15.7%) in serum insulin-like growth factor (IGF)-I.

In contrast, serum alkaline phosphatase and osteocalcin remained unaffected. These effects were mediated through downregulation of gene expression for receptor activator of NFk-B ligand and osteoprotegerin in the bone (Franklin et al. 2006).

Panax ginseng C.A. Mey.

Panax ginseng C.A. Mey. belonging to Araliaceae family is called Korean ginseng and used to treat several ailments. Lee et al. (2015) reported that ginseng extract inhibited bone loss associated with estrogen deficiency. Indeed, 300 and 500 mg/kg of the extract for 8 weeks protected female rats against ovariectomy-induced rise in serum ALP activity, serum osteocalcin levels, and urinary DPD/creatinine ratio. Ginseng has been also shown to prevent radiation-induced bone loss in C3H/HeN mice. Treatment (250 mg/kg of body weight/d) for 12 weeks reduced serum ALP and TRAP concentrations (Lee et al. 2013). The major active compounds in the plant extract were identified as about 30 ginsenosides, triterpenoidal saponins (polycyclic aglycones attached to one or more sugar side chains, derivatives of the triterpene dammarane) (Sunwoo et al. 2013). It has been suggested that the ginsenosides may increase the osteogenesis of bone marrow stromal cells and preosteoblast cells (Siddiqi et al. 2013).

Cibotium barometz (L.) J. Sm.

Cibotium barometz (L.) J. Sm. belongs to *Dicksoniaceae* family and is widely used for its anti-inflammatory activity. Ovariectomized virgin female Sprague–Dawley rats treated with 100, 300, and 500 mg/kg/day of 70% ethanol extract *Cibotium barometz* (L.) J. Sm. for 16 weeks resulted in a significant suppression of the ovariectomy-induced increases in urinary DPD/creatinine levels and serum OC levels (Zhao et al. 2011). Xu et al. (2014a) demonstrated similar positive effects of different extracts of *Cibotium barometz* (L.) J. Sm. rhizomes on retinoic acid-induced male rats osteoporosis by using s-TRAP and total scores of OPG, Ca, P, IL-6, TNF-alpha, and IL-1 as bone metabolism markers. RW-Cb, one of the processed products of *Cibotium barometz* (L.) J. Sm., and its major compounds *p-acid* and *p-aldehyde* promoted proliferation and differentiation of primary rat osteoblasts, which can explain the antiosteoporotic effects of the plant (Xu et al. 2014b). Rhizomes contain several compounds that could explain their antiosteoporotic activity such as β-sitosterol, phenolic acids, and tannins (Fig. 5).

Citrus unshiu Marcow.

The treatment of ovariectomized rats with dried *C. unshiu* Marcow. peel 300 mg/kg for 8 weeks significantly decreased ALP, CTX, and OC serum levels, reflecting a significant decrease of bone resorption (Lim et al. 2014). Moreover, oral intake of

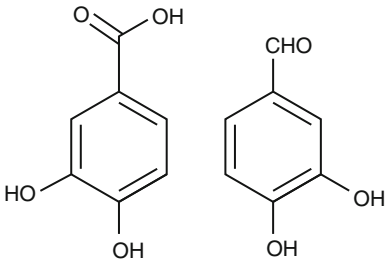


Fig. 5 Chemical structure of *p-acid* (*left*) and *p-aldehyde* (*right*)

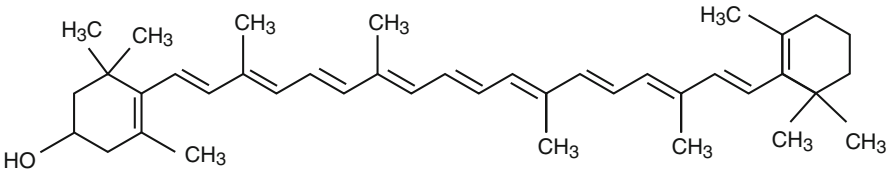


Fig. 6 Chemical structure of β-cryptoxanthin

C. unshiu Marcow. juice for 28 or 56 days resulted in significant increase of bone-specific alkaline phosphatase activity and γ carboxylated osteocalcin levels and decreased serum TRACP and type I collagen *N*-telopeptide. In contrast, serum calcium, inorganic phosphorous, and parathyroid hormone (intact) were not affected by the treatment (Yamaguchi et al. 2006).

These effects on bone metabolism may be explained by the presence of several active compounds such as β-cryptoxanthin, a carotenoid showing anabolic effects on bone, both in vitro and in vivo. It has been demonstrated that oral administration of β-cryptoxanthin (5 or 10 μg/100 g body weight) once daily for 3 months to ovariectomized (OVX) rats significantly prevented the decrease of calcium content and alkaline phosphatase activity in the femoral diaphyseal and femoral metaphyseal tissues and deoxyribonucleic acid (DNA) content in the metaphyseal tissues (Uchiyama and Yamaguchi 2006). Recently, β-cryptoxanthin has been shown to prevent bone loss via inhibition of differentiation and maturation of osteoclasts through repression of the nuclear factor-kB-dependent transcriptional pathway (Ozaki et al. 2015) (Fig. 6).

It has been found that hesperidin (monomethoxylated flavanone) was one of the most abundant major active compounds in *Citrus unshiu* Marcow. extract, and it is considered as the most studied flavonone regarding bone health. It's suggested that dietary flavonones could have a good bioavailability and may reach target cells in sufficient concentrations to exert their biological activities. Hesperidin intake (500 mg hesperidin/day in two biscuits) by healthy postmenopausal women (50–65 years old) for 24 months was studied, and its effect on bone loss was assessed using PINP and CTX-1 as bone metabolism markers. Hesperidin intake resulted in better balance in bone metabolism, as reflected by the bone turnover index (procollagen I N-terminal propeptide: carboxy-terminal collagen crosslinks type I ratio) (Habauzit et al. 2011).

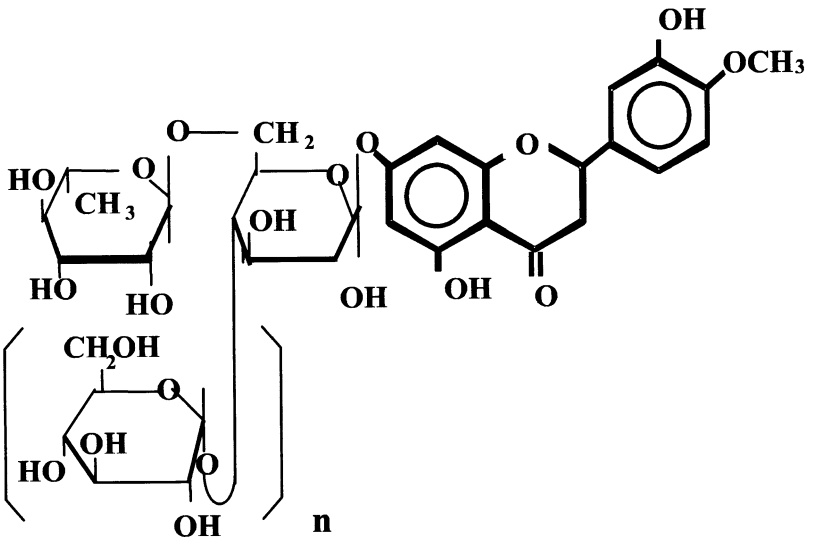


Fig. 7 Chemical structure of α-glucosylhesperidin

Chiba et al. (2003) studied the effect of supplementation of hesperidin on bone loss. Ovariectomized rats were fed a diet containing 5.0 g/kg hesperidin or 7.0 g/kg for 4 weeks.

They found that hesperidin prevented bone loss in ovariectomized mice by a decrease in the osteoclast number without affecting the uterus (Fig. 7).

Hesperidin consumption (a casein-based diet supplemented with 0.5% hesperidin) for 90 days caused total inhibition of the ovariectomy-induced increase in DPD excretion in 6-months-old rats and a partial inhibition in 9-months-old animals. However, heperidin did not affect serum osteocalcin concentrations. It has been suggested that hesperedin suppressed NF-kB signaling through NIK/IKK, ERK, p38, and JNK signaling pathways (Horcajada et al. 2008).

Solanum virginianum L.

Solanum virginianum L. belongs to Solanaceae; it is a green perennial herb, woody at the base, and 2–3 m height. The fruits are berry 1–3 cm diameter, yellow or with white green strips, edible and considered as important herbal therapy. The plant is widely used for its antifertility, antihyperlipidemic, antiasthmatic, hypoglycemic, anti-inflammatory, and anticancer activities. Since the plant is rich in phytosterols, its use as antiosteoporotic was expected. Aswar et al. (2014) studied the effect of an aqueous extract of *Solanum virginianum* L. administered at 200 and 400 mg/kg/day per os for 90 days to ovariectomized rats. They demonstrated that *Solanum virginianum* L. extract (200 mg/kg) significantly increased serum calcium and decreased ALP level. The major active compounds were found (by thin layer chromatography) to be

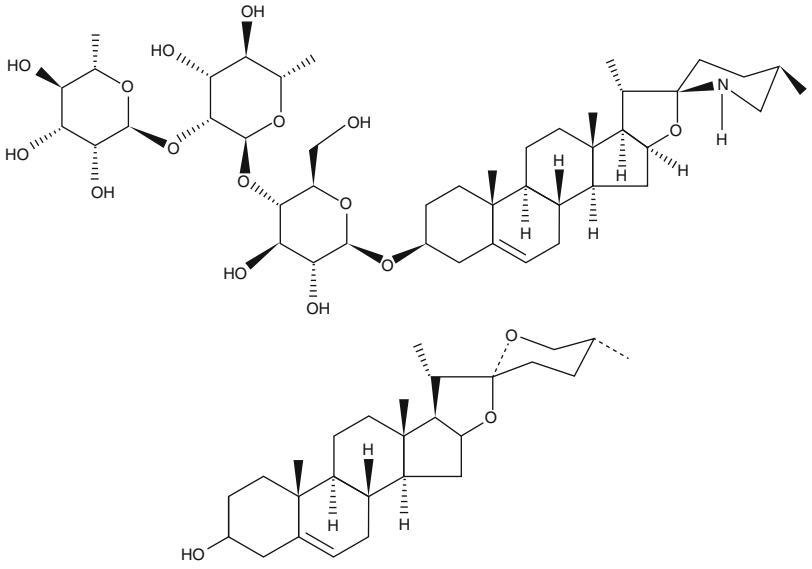


Fig. 8 Chemical structure of α-solamargine (*left*) and diosgenin (*right*)

steroids. Indeed, the plant contains several steroidal compounds such as lupeol, sitosterol, disogenin, solasodine, tomatidenol, and α solamargine.

Recently, Zhao et al. (2016) have demonstrated that diosgenin prevented bone loss on retinoic acid-induced osteoporosis in rats. Diosgenin (30 and 90 mg/kg) significantly increased estradiol and osteocalcin levels and decreased those of ALP and TRAP (Fig. 8).

Asparagus racemosus Willd.

Asparagus racemosus Willd. is a perennial herbaceous plant belonging to Liliaceae. The plant is widely used in India and other neighboring countries. The plant has been shown to possess phytoestrogenic, anticancer, galactogogue, antilithiatic, antibacterial, antidiabetic, immunomodulatory, antioxidant, antiulcer, adaptogenic, antiosteoporotic, aphrodisiac, neurprotective, and hepatoprotective effects.

Ovariectomized rats were treated with aqueous and methanolic extracts of *Asparagus racemosus* Willd. (50, 250 mg/kg) for 40 days. The aqueous extract at 250 mg/kg significantly decreased alkaline phosphatase levels, reflecting ameliorative effect on osteoblastic activity. Moreover, the methanolic extract treatment resulted in significant rise in serum calcium concentrations (Chitme et al. 2009). Recently, Di Pompo et al. (2014) demonstrated that *Asparagus racemosus* Willd. decoction was effective and inhibited osteoclasts formation (osteoclastogenesis) without inducing osteoclasts apoptosis. It has been found that shatavarins (steroidal saponins) and asparagamine

(alkaloid) were the major active compounds in *Asparagus racemosus* Willd. Quercetin and rutin were also reported as major constituents of the plant (Fig. 9).

Rubia cordifolia L.

Rubia cordifolia L. (Rubiaceae) is a flowering plant, used for its roots and stems. Ethanolic extract of *Rubia cordifolia* L. roots at doses of 200 and 400 mg/kg body weight was administered to ovariectomized rats for 90 days. Its effects on bone metabolism were assessed using serum tartrate-resistant acid phosphatase (TRAP), alkaline phosphatase activity, and serum calcium. The ovariectomy-induced increase of ALP activity was prevented by *Rubia cordifolia* L. (200 and 400 mg/kg). Similarly, the extract administered at 400 mg/kg significantly decreased TRAP levels. On the other hand, *R. cordifolia* L. extract treatment resulted in a strong rise of serum calcium levels, decreased by ovariectomy. These findings could be attributed to improved calcium absorption, enhanced by *R. cordifolia* L. roots extract (Shivakumar et al. 2012). The antiosteoporotic effect may be attributed to mollugin, one of the major compounds of *R. cordifolia* L. (Fig. 10).

Recently, Baek et al. (2015) elucidated the molecular mechanism of antiresorptive activity of mollugin. The latter suppressed RANKL-induced osteoclast differentiation of bone marrow macrophages and bone resorbing activity of mature osteoclasts. Mollugin suppressed RANKL-induced c-Fos and NFATc1 expression, inhibited RANKL-induced phosphorylation of ERK, p38, JNK, Akt,

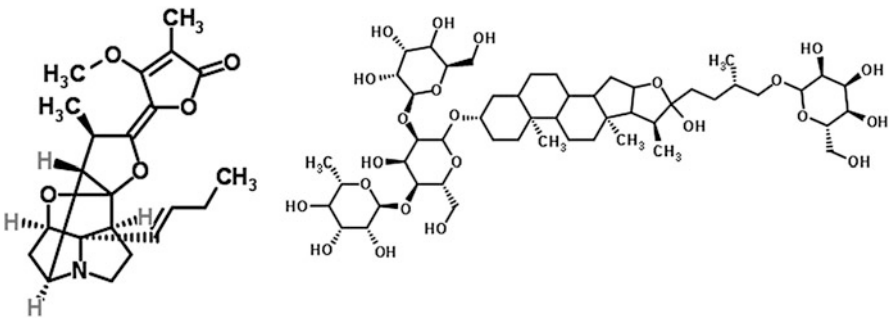


Fig. 9 Chemical structure of asparagamine (*left*) and shatavarin I (*right*)

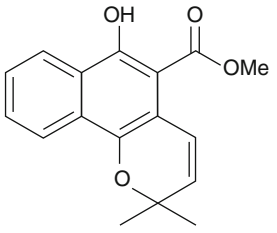


Fig. 10 Chemical structure of mollugin

and GSK3β, and downregulated the expression of different genes involved in osteoclast differentiation and function (OSCAR, TRAP, DC-STAMP, OC-STAMP, integrin αν, integrin β3, CtsK, and ICAM-1). Furthermore, mollugin restored bone mass density in mice with lipopolysaccharide-induced bone loss and resulted in the suppression of osteoclast activity in bone tissue.

Emblica officinalis Gaertn.

Emblica officinalis Gaertn. (Euphorbiaceae) is native of India and grows in Pakistan, Uzbekistan, Srilanka, Southeast Asia, China, and Malaysia. Fruits, seeds, leaves, and flowers *E. officinalis* Gaertn. possess several biological activities such as anticancer, antidiabetic, and hepatoprotective. Ovariectomized rats were treated with 200 mg/kg/day of an aqueous extract of *E. officinalis* Gaertn. for 8 weeks. The *E. officinalis* Gaertn. significantly increased the serum ALP levels and prevented ovariectomy-induced elevation of serum TRAP and hydroxyproline (Sirasanagandla et al. 2013). Gallic acid, ellagic acid, chebulinic acid, quercetin, corilagin, and other bioactive compounds were isolated and characterized in different parts of *E. officinalis* Gaertn.

Magnolia biondii Pamp.

Magnolia biondii Pamp. belongs to Magnoliaceae family, and it is widely used in Chinese medicine to treat several illnesses such as rhinitis, sinusitis, and inflammatory diseases. Jun et al. (2012) demonstrated that *Magnolia biondii* Pamp. showed promising antiosteoporotic activity. In fact, *Magnolia biondii* Pamp. markedly inhibited the ovariectomy-induced increase of serum levels of bone turnover marker such: alkaline phosphatase, tartrate-resistant acid phosphatase 5b, calcium, and osteocalcin. Furthermore, *Magnolia biondii* Pamp. has been shown to reduce the RANKL-mediated osteoclast differentiation and bone resorption by inhibiting the activities of matrix metalloproteinases and cathepsin K in mouse bone marrow macrophages. The major compounds were found to be magnolin, eudesmin, and lirioresinol B dimethyl ether.

Aristolochia longa L.

Aristolochia longa L. (Aristolochiaceae), called “berrostom” by local populations, is widely used in Algerian phytotherapy. Roots of the plant are used to manage several illnesses but mainly to treat different types of cancers. The aqueous extract of the roots was shown to be highly cytotoxic to Burkitt’s lymphoma (BL41) cells in a dose-dependent manner. The cytotoxic effect was mediated through apoptosis induction by triggering the mitochondrial (intrinsic) pathway: loss of mitochondrial membrane potential and the activation of caspases-9 and -3 followed by poly(adenosine diphosphate-ribose) polymerase cleavage (Benarba et al. 2012). Moreover, we have

demonstrated (data under publication) that *A. longa* L. was effective against multiple myeloma (LP1 cell line) and breast cancer (HBL-100 and MDA-MB 231 cell line).

Breast cancer patients use *A. longa* L. roots mixed with honey or milk to manage their disease. We have studied the effect of intake of 1 g of *A. longa* L. roots per day on bone resorption markers in Algerian breast cancer postmenopausal women. A standardized questionnaire was used to assess retrospectively the *A. longa* L. intake. Subjects were interviewed after diagnosis and before initiating treatment. We have compared two groups of patients: Breast cancer *A. longa* L. group (Al) (n = 54) and non-*A. longa* L. group (non-Al) (n = 24). Since *A. longa* L. contains arsitolochic acids, well-known high nephrotoxic agents, kidney function markers (creatinine, urea, uric acid) were measured. Furthermore, bone resorption was assessed by measuring urinary excretion of pyridinoline (PYD) and deoxypyridinoline (DPD) by HPLC. Results of our study demonstrated that the intake of 1 g of *A. longa* L. by breast cancer patients resulted in a pronounced significant increase of mean concentrations of bone resorption markers. *A. longa* L. intake resulted in a marked increase of median levels of DPD (free, conjugated, and total form), increased by 71.55%, 20.85%, and 54.08%, respectively. On the other hand, median concentrations of PYD (free, conjugated, and total form) were significantly higher by 83.26%, 34.22%, and 19.06%, respectively. We have concluded that "the intake of *A. longa* roots is detrimental for kidney function and resulted in high bone resorption, maybe due to the reduction in renal function caused by the aristolochic acids contained in the roots." (Benarba et al. 2014).

Herbs Mixtures

Wang et al. (2012) evaluated the ICTP and BAP concentrations in a bone cancer pain rat model after topical treatment with Chinese TLSJ gel. The latter composed of extracts prepared from seven herbs: *Piper kadsura*, *Trachelospermum jasminoides*, *Dioscorea nipponica*, *Corydalis yanhuso*, *Melia toosendan* Sieb. et Zucc, *Boswellia carterii*, *Commiphora myrrha*) significantly and progressively decreased serum ICTP and BAP levels.

Serum osteocalcin, as marker of bone formation, and serum osteoclast derived tartrate-resistant acid phosphatase 5b (TRACP5b), as marker of bone resorption, were used to investigate the effects of the traditional Chinese medicine Hachimi-jio-gan (mixture of eight plants, widely used in Japan and Korea to treat osteoporosis) on bone microstructure in ovariectomized rats. Hachimi-jio-gan treatment resulted in a significant decrease of serum TRACP5b concentrations. On the other hand, serum osteocalcin levels remained unchanged. These results were confirmed by micro-computed tomography (Chen et al. 2012).

A herbal formula containing *Epimedium sagittatum* (Sieb. et Zucc) (Berberidaceae, whole herb), *Curculigo orchioides* (Hypoxidaceae, rhizome), *Morinda officinalis* (Rubiaceae, root), *Angelica sinensis* (Oliv.) (Umbelliferae,

root), *Phellodendron chinense* (Rutaceae, bark), and *Anemarrhena asphodeloides* (Anthericaceae, rhizome) (300, 600 mg/kg, daily) for 12 weeks has showed an antiosteoporotic activity via reduction of serum osteocalcin and ALP in ovariectomized rats (Nian et al. 2006).

Recently, Elkomy and Elsaid (2015) studied the effect of a three herbs mixture of the Lamiaceae family (*Salvia officinalis* L., *Rosmarinus officinalis* L., and *Thymus vulgaris* L.) in ovariectomized rats. Herbs mixture at 30 g/kg for 1 month (from day 30 to day 60, after ovariectomy) restored the decreased levels of serum Ca and P to normal values and improved bone metabolic markers ALP and ACP. Furthermore, herbs mixture administration resulted in a significant decrease of osteocalcin levels from 4.54 $\pm$ 0.06 to 3.82 $\pm$ 0.05 ng/ml.

Lai et al. (2015) studied the effect of the traditional Chinese medicinal formula Zuo-Gui-Wan (ZGW) on bone loss in mice with bone loss induced by estrogen deficiency using CTX-I as bone resorption marker. The traditional formula (ZGW) is a mixture of *Rehmannia glutinosa* Libosch. (Scrophulariaceae), *Lycium barbarum* L. (Solanaceae), *Dioscorea opposita* Thunb. (Dioscoreaceae), *Cornus officinalis* Sieb.et Zucc. (Cornaceae), *Cuscuta chinensis* Lam (Convolvulaceae), *Cervus elaphus* Linnaeus (Cervidae), *Chinemys reevesii* (Gray) (Emydidae), and *Cyathula officinalis* Kuan (Amaranthaceae). Results of this study showed that ZGW administration at 8, 16, and 32 g/kg/day for 12 weeks significantly decreased the serum CTX-I levels in a dose-dependent manner.

BHH10, a herbal formulation consisting of the root of *Astragalus membranaceus*, the bark of *Cinnamomum cassia*, and the bark of *Phellodendron amurense*, has been studied for its bone protective effects in ovariectomized Sprague–Dawley rats. BHH10 administered in a single oral dose of 0, 500, 1000, or 2000 mg/kg BHH10 significantly decreased the serum levels of ALP, osteocalcin, BMP-2, and CTX. These results gave evidence that BHH10 mixture reversed bone metabolism in ovariectomized rats and confirmed its ethnomedicinal use as antiosteoporotic herbal therapy (Huh et al. 2015). The antiresorptive effect of BHH10 herbal formulation was attributed to its major compounds such as cinnamic acid, formononetin, and berberine.

Koo et al. (2014) reported that a combinatory mixture of *Rubus coreanus* Miquel and *Astragalus membranaceus* Bunge extracts had an antiosteoporotic effect in ovariectomized mice. They found that the mixture (50, 100, and 200 mg/kg body weight/day, three times per week for 12 weeks) significantly decreased the level of RANKL and increased the level of OPG in a dose-dependent manner. Therefore, the RANKL/OPG ratio marked rise induced by ovariectomy was reversed following the treatment. Similarly, *Rubus coreanus* Miquel and *Astragalus membranaceus* Bunge mixture significantly decreased the levels of osteocalcin and TNF-α. It has been suggested that the antiosteoporotic effect demonstrated in this study was mediated through inhibition of the RANK-signaling pathway in osteoclast cells by downregulating the expression of TRAF6 and NFATc1 activated in RANK– RANKL binding (Koo et al. 2014) (Tables 1 and 2).

Table 1 Some ethnomedicinal plants used in bone healing/health

Plant	Use	Region
Glycine soja Sieb. et Zucc	Bone strength	Algeria
Sorghum annum Trab		
Marribium vulgare	Arthritis	
Rosmarinus officinalis L.		
Aloe vera (L.) Burm. f.	Bone fracture	Brazil
Byrsonima crispa A. Juss.		
Camarea ericoides A. St.-Hil		
Coronopus didymus (L.) Sm.		
Myracrodruon urundeuva		
Scoparia dulcis L.		
Solidago microglossa DC.		
Stryphnodendron adstringens (Mart.)		
Chenopodium ambrosioides L.	Bone regeneration	
Ainsliaea pertyoides Franch. var. *albo-tomentosa*	Bone fracture	China
Erythrina arborescens Roxb		
Eucommia ulmoides Oliver		
Gynura japonica (Thunb.) Juel		
Helwingia chinensis Batalin		
Kalanchoe pinnata (Lam.) Pers.		
Lobelia seguinii H. Lev. and Vaniot		
Lycoris aurea Herb		
Peperomia heyneana Miq		
Peperomia tetraphylla (G. Forst.) W.R.B. Oliv.		
Plumbago zeylanica L.		
Sambucus williamsii Hance		
Thunia alba Rchb.f		
Tinospora sinensis (Lour.) Merr		
Toricellia angulata var. *intermedia*		
Cyclea barbata Miers	Bone sticking	
Brugmansia candida Pers.	Bone fracture	Ecuador
Ficus subandina Dugand		
Siparuna eggersii Hieron		
Boerhavia diffusa L.	Arthritis	India
Calotropis gigantea Linn.	Bone dislocation	
Cissampelos pareira L.	Bone fracture	
Cyclea peltata Hook. F and Thoms		
Cyperus rotundus		
Mimusops elengi Linn.		
Saraca asoca (Roxb.) De Wilde		
Tinospora cordifolia Miers		
Cissus quadrangularis. Linn.	Bone healing	
Senna occidentalis	Bone setting	

(*continued*)

Table 1 (continued)

Plant	Use	Region
Allium iranicum	Osteoporosis	Iran
Elaeagnus angustifolia L.		
Lonicera nummulariifolia Jaub. and Spach		
Glycyrrhiza glabra L.	Bone fracture	
Scrophularia striata Boiss	Bone healing	
Origanum compactum Benth	Bone fracture	Morocco
Nigella sativa L.		
Tetraclinis articulata Benth.		
Epipremnum pinnatum (L.) Engl.	Bone fractures, dislocated joints, and sprains	New Guinea
Cyclandrophora laurina (A. Gray) Kosterm	Bone fracture	
Ficus hispidioides L.f.		
Gnetum gnemon L.		
Pometia pinnata J.R. Forst. and G. Forst		
Cissus repens	Bone fracture	Nepal
Diospyros lanceifolia	Bone dislocation	
Rosa canina L.	Bone pain	North-Western Ligurian Alps
Malva neglecta Wallr.	Bone fracture	Pakistan
Sellaria media L.	Bone fracture	
Symphytum officinale L.	Bone fracture	Serbia
Allium sativum L.	Bone pain	Spain
Dittrichia viscosa (L.) Greuter	Bone fracture	
Equisetum telmateia Ehrh		
Retama sphaerocarpa		
Symphytum tuberosum L.		
Urtica dioica L.	Arthritis	

Potential Applications to Prognosis, Other Diseases, or Conditions

Traditional medicine considered as alternative and complementary when practiced with conventional medicine is widely used to treat or manage different ailments. Bone diseases such as osteoporosis or metastases became a major health issue both in developed and developing countries. Management of these diseases includes use of a variety of drugs and molecules. Their use has been correlated to an increasing incidence of some cancers and serious adverse effects (ulcers, irritations, cancers). Therefore, the use of alternative medicines has gained much interest, and several studies have been carried out to assess the effectiveness of herbal medicines use in the prevention and/or treatment of bone diseases and disorders. An increased number

Table 2 Medicinal plants studied with regard to bone markers

The plant	Family	Possible active compound (s)	Bone markers studied	Reference(s)
Labisia pumila var. *alata*	Myrsinaceae	Phenolic acids Flavonoids	Osteocalcin, CTX.	Shuid et al. (2011)
Aronia melanocarpa (Michx.) Elliott	Rosaceae	Phenolic acids (*p*-Hydroxybenzoic acid)	Osteocalcin, alkaline phosphatase, osteoprotegerin, CTX.	Brzóska et al. (2015)
Berberis aristata DC.	Berberidaceae	Berberine	Serum and urine calcium, inorganic phosphorus, alkaline phosphatase	Yogesha et al. (2011)
Drynaria fortunei (Kunze ex Mett.) J. Sm.	Polypodiaceae	Naringenin, Kurarinone,	Osteocalcin	Lee et al. (2014)
Davallia formosana Hayata	Davalliaceae	(−)-epicatechin 3-*O*-β-D-allopyranoside	Alkaline phosphatase, deoxypyridinoline, CTX, urinary calcium	Ko et al. (2012), Lin et al. (2013)
Angelica sinensis (Oliv.) Diels.	Apiaceae	Ferulic acid Z-ligustilide	Alkaline phosphatase, CTX, osteocalcin	Lim and Kim (2014)
Prunus domestica L.	Rosaceae	Chlorogenic acids Proanthocyanidins 3-caffeoylquinic acid	IGF-I, bone-specific alkaline phosphatase, osteocalcin, tartrate-resistant acid phosphatase-5b, deoxypyridinoline, PINP	Bahram et al. (2004), Hooshmand et al. (2011), Bu et al. (2007), Rendina et al. (2012), Smith et al. (2014)
Panax ginseng C.A. Mey.	Araliaceae	Ginsenosides	Alkaline phosphatase, serum osteocalcin, deoxypyridinoline, tartrate-resistant acid phosphatase-5b	Lee et al. (2015), Lee et al. (2013)
Cibotium barometz (L.) J. Sm.	*Dicksoniaceae*	β-sitosterol, Phenolic acids, Tannins	Deoxypyridinoline, osteocalcin, s- tartrate-resistant acid phosphatase-5b, OPG, Ca, P, IL-6, TNF-alpha, IL-1	Zhao et al. (2011), Xu et al. (2014a)

(*continued*)

Table 2 (continued)

The plant	Family	Possible active compound (s)	Bone markers studied	Reference(s)
***Citrus unshiu* Marcow.**	*Rutaceae*	β-cryptoxanthin Hesperidin	Alkaline phosphatase, bone-specific alkaline phosphatise, serum osteocalcin, CTX, type I collagen *N*-telopeptide	Lim et al. (2014), Yamaguchi et al. (2006)
Solanum virginianum L.	*Solanaceae*	Lupeol Sitosterol Disogenin α solamargine	Alkaline phosphatase, serum calcium	Aswar et al. (2014)
Asparagus racemosus Wild.	*Liliaceae*	Shatavarins Asparagamine Quercetin Rutin	Alkaline phosphatase, serum calcium	Chitme et al. (2009)
Rubia cordifolia L.	Rubiaceae	Mollugin	Serum tartrate-resistant acid phosphatase, alkaline phosphatase, serum calcium	Shivakumar et al. (2012)
Emblica officinalis Gaertn.	Euphorbiaceae	Gallic acid Ellagic acid Quercetin Corilagin	Serum tartrate-resistant acid phosphatase, hydroxyproline, alkaline phosphatase	Sirasanagandla et al. (2013)
Magnolia biondii Pamp.	Magnoliaceae	Magnolin, eudesmin, lirioresinol B dimethyl ether	Alkaline phosphatase, tartrate-resistant acid phosphatase 5b, calcium, osteocalcin	Jun et al. (2012)
Aristolochia longa L.	Aristolochiaceae	Aristolochic acids	Deoxypyridinoline, pyridinoline	Benarba et al. (2014)

of plant species has been shown to prevent bone loss and to be effective in the treatment of osteoporosis.

Bone markers are useful in clinical practice to evaluate the bone turnover either in pathologic cases or response to treatments. Interestingly, bone markers reflect changes of bone formation and/or resorption rates as early as some months compared to bone mineral density measurements. Bone markers are useful in diagnosis of bone diseases and evaluation of bone loss level and relative fracture risk. Both formation and resorption markers are used in monitoring response to antiresorptive therapies. Hence, bone markers may be useful to evaluate the response to herbal medicines treatments. Use of bone markers provide an inexpensive and noninvasive tool for detecting populations at high risk of osteoporosis-related fractures such as breast cancer postmenopausal women or postmenopausal women under corticoid treatment.

Summary Points

- This chapter focuses on bone turnover markers use with traditional medicine practices.
- The traditional medicine consists of the folk knowledge and practices accumulated through history to cure diseases and maintain health.
- Herbal medicine is the most widely used traditional medicine by different populations throughout the world.
- Bone diseases such as osteoporosis are considered as a major public health problem, and several studies have shown promising positive effects of traditional medicine(s) on bone health. These effects were assessed using different bone turnover markers.
- Bone turnover markers include formation markers (osteocalcin, alkaline phosphatase, osteoprotegerin, etc.) and resorption markers (CTX, NTX, Pyridinoline, Deoxypyridinoline, etc.).
- Preclinical studies have shown that different medicinal plants prevented bone loss induced by ovariectomy or radiation in mice and rats. This effect reflected by changes in bone turnover markers may be attributed to the major compounds such as flavonoids, alkaloids, essential oils, etc.
- Clinical studies demonstrated a preventive effect of different medicinal plants regarding bone loss in menopausal women. Bone turnover markers have been shown to be useful in managing the effect of herbal treatment in patients.

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Bone Markers Throughout Sexual Development: Epidemiological Significance and Population-Based Findings

4

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Abstract

Bone markers are used in adults to predict fracture risk and to monitor anabolic or catabolic therapies. Much less is known about their usefulness during childhood and adolescence when, besides remodeling, bone markers also reflect modeling and linear growth of the skeleton. Adolescence is a sensitive period for bone health during which a substantial proportion of bone mass is accrued. Therefore, it is important to understand the significance of bone markers at this stage, mainly in identifying individuals at increased risk of bone fragility. Sections of this chapter focus on the trajectories of bone markers during sexual development, as well as their normative values, determinants, and clinical significance. In general, both resorption and formation markers peak at puberty, decreasing from this point onward. In girls, peak metabolism rate occurs earlier and decreases faster than in boys. In both genders, markers are weakly associated with bone physical properties. Few studies have addressed modifiable determinants of bone markers, and the effect of behaviors on bone metabolism is far from consensual. Some successful attempts have been made to use bone markers in the clinical setting to diagnose and monitor pediatric diseases. Today, even though the measurement of bone markers in children and adolescents can be useful in the clinical setting, lack of standardized methods for determination still limits their widespread use.

Keywords

Bone markers • Remodeling • Modeling • Growth • Sexual development • Adolescent • Bone density • Fracture • Cohort studies

List of Abbreviations

BALP	Bone-specific alkaline phosphatase
BMC	Bone mineral content
BMD	Bone mineral density
CTX-I	Cross-linked C-terminal telopeptide of type I collagen
DXA	Dual-energy X-ray absorptiometry
ECLIA	Electrochemiluminescence immunoassay
ELISA	Enzyme-linked immunosorbent assay
EPITeen study	Epidemiological Health Investigation of Teenagers in Porto
ICMA	Immunochemiluminometric assay
ICTP	C-Terminal cross-linking telopeptide of type I collagen
IRMA	Immunoradiometric assay
NTX	Cross-linked N-terminal telopeptide of type I collagen

OC	Osteocalcin
OPG	Osteoprotegerin
PBM	Peak bone mass
PICP	Procollagen type I carboxy terminal propeptide
PINP	Procollagen type I amino terminal propeptide
RANK	Receptor activator of nuclear factor kB
RANKL	Receptor activator of nuclear factor kB ligand
RIA	Radioimmunoassay
TRACP	Tartrate-resistant acid phosphatase

Key Facts of Bone Marker Interpretation

- Bone markers have high inter- and intraindividual variability.
- The use of different methods for the determination of bone markers contributes to their analytical variability.
- Even when the same method is used, interlaboratory variation is still a problem.
- Biological variability of bone markers arises from factors such as age, sex, and puberty.
- There is sexual dimorphism in bone markers trajectories during puberty: levels peak earlier and decline faster in girls than in boys.
- Bone markers have limited individual clinical utility in a single point in time, but are valid at a group level (by comparison with reference samples or over time).
- So far, no standardized methods have been proposed to measure bone markers.

Key Facts of the EPITeen Cohort

- EPITeen stands for Epidemiological Health Investigation of Teenagers in Porto.
- This is a population-based cohort of teenagers born in 1990 and living in Porto, Portugal.
- The cohort was assembled during the school year 2003/2004 in public and private schools when teenagers were 13 years old (78% of 2787 eligible adolescents agreed to participate).
- The sample initially recruited was reevaluated during the 2007/2008 school year, at 17 years of age (21% attrition from baseline).
- Data regarding anthropometrics, clinical history, and lifestyles as well as serum bone markers PINP and CTX-I, osteoclast-regulating cytokines osteoprotegerin and RANK-ligand, and forearm bone mineral density were collected.
- Follow-up of this cohort continued at 21 and 24 years old.
- This project is funded through FEDER–COMPETE, through the Foundation for Science and Technology – FCT (Portuguese Ministry of Education and Science) – within the projects PTDC/SAU-EPI/115254/2009 (FCOMP-01-0124-FEDER-

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Definition of Words and Terms

Bone biomechanical properties	Set of behaviors of a bone that depend on its structural dimension (including size, shape, and architecture) and on its material dimension (including apparent density and microstructural arrangement).
Bone formation	Process that results from the deposition of the bone by osteoblasts and that results in bone gain.
Bone growth	Skeletal development in length that lasts until late adolescence.
Bone markers	Molecules that result from bone metabolism, as a result of osteoclast or osteoblast activity (bone resorption and formation markers, respectively).
Bone modeling	Change in bone size and shape up to peak bone mass, through uncoupled processes of bone formation in the periosteal surface and bone resorption within the endosteal surface.
Bone remodeling	Continuous replacement of old or damaged bone through coupled resorption and formation, to maintain the integrity of bone structure throughout life.
Bone resorption	Breakdown of mineralized bone tissue conducted by osteoclasts, which results in bone loss.
Bone strength	Bone resistance to mechanical stress.
Fragility fracture	Fracture resulting from a low-energy trauma or that occurs at an anatomical site with decreased bone strength; its incidence increases after 50 years of age.
Peak bone mass	Maximum amount of bone mass acquired during life, believed to be an important determinant of fragility fracture risk.

Introduction

Bone is composed by cells and an extracellular matrix, mineralized by the deposition of calcium hydroxyapatite. Bone has three distinct cell types: osteoblasts, or bone-forming cells; osteoclasts, or bone-resorbing cells; and osteocytes, which are osteoblasts entrapped within lacunae. Bone is constantly being resorbed by osteoclasts and then replaced by osteoblasts in a process called bone remodeling (Kini and Nandeesh 2012).

Bone markers are molecules that result from the synthesis or degradation of the bone matrix and can be grouped into resorption and formation markers, as they result from the activity of osteoclasts or osteoblasts, respectively. Bone markers can be used to estimate bone turnover in adults. They have been tested as short-term proxies of the turnover process, as surrogate markers for osteoporosis and fracture risk, and tested in the context of monitoring drug effects (Nishizawa et al. 2013; Vasikaran et al. 2011). Nevertheless, important challenges to their widespread use remain to a large extent due to substantial inter- and intraindividual variability, as well as to differences between laboratory methods used for their quantification (Hannon and Eastell 2000).

Little is known about the usefulness of bone turnover markers in pediatric ages mainly due to the relatively low prevalence of metabolic bone disease in this age and to the effect of the growing process on these markers. Thus, there is a lack of evidence about their clinical significance, namely regarding clinical meaning and possible relation with bone physical properties. Bone markers in healthy children are the net result of normal bone growth and modeling, as well as homeostatic remodeling, at each stage of skeletal maturity. Deviations from reference levels or individual trajectories over time may be used not only to detect pediatric metabolic bone disease (interindividual variation) but also to monitor prognosis in children with established disease (intraindividual variation). In both cases, clinical application of bone markers requires that reference trajectories are obtained from healthy aged-matched populations (Eapen et al. 2008).

The importance of studying bone turnover in the pediatric population may extend beyond the first decades of life. Even in healthy children, subclinical disturbances to the process of bone mineralization may impair the acquisition of optimal peak bone mass, which may predispose to an increased risk of age-related fragility fractures in adulthood (Heaney et al. 2000). Adolescence is a particularly critical phase for bone development, since a substantial proportion of bone mineral mass is acquired during this stage. Understanding how bone turnover markers vary in this period can contribute to better model bone health throughout life and ultimately to inform on how to prevent fragility fractures.

The main focus of this chapter is on the variation of bone markers during sexual development in the context of bone fragility/osteoporosis. A review of selected bone markers in the general adolescent population is presented, including their trajectories in this age range, as well as their associations with osteoclast-regulating cytokines, bone physical properties, and potential modifiable determinants. The Epidemiological Health Investigation of Teenagers in Porto (EPITeen study) prospective cohort is used as a case study (Ramos and Barros 2007).

A Focus on Bone Mechanical Fragility

Bone is a metabolically active organ with key functions in mineral homeostasis as well as glucose metabolism (Kini and Nandeesh 2012; Lee and Karsenty 2008). In terms of its physical role, bone maintains the mechanical integrity of the organism, providing leverage for locomotion (Raisz 1999).

Whole **bone strength** is defined as the load-bearing capacity of a bone – its ability to resist fracture – and depends on its **biomechanical properties**. These include structural properties, which are influenced by the spatial distribution of bone tissue (size, shape, and architecture) and material properties, which reflect the intrinsic characteristics of bone tissue (apparent density and microstructural arrangement of trabeculae) (Bouxsein 2005). **Bone fragility** is said to occur when the biomechanical properties of the organ as a whole are compromised, leading to increased fracture risk (Jordan and Cooper 2002). Therefore, the concept of bone fragility is closely related to that of osteoporosis – a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture (NIH Consensus Development Panel on Osteoporosis Prevention Diagnosis and Therapy 2001).

Fragility fractures can be defined as those that result from a low-energy trauma, such as a fall from a standing height or collision with an object during daily routine (Warriner et al. 2011). Alternatively, they can also be operationalized as those that occur at a skeletal site with decreased bone strength (e.g., femoral neck, vertebra, or distal forearm) and whose incidence increases after the age of 50 (Johnell and Kanis 2005). Age-related fragility fractures are frequent events that are associated with high case fatality (Bliuc et al. 2009) as well as important short- and long-term morbidity (Paul 2003). It is expected that, due to population aging, the prevalence of osteoporosis will increase by 23% from 2010 to 2025, which corresponds to an estimate of 33.9 million fractures in 2025 (Hernlund et al. 2013). Thus, fractures will continue to pose an important financial burden on individuals, health systems, and ultimately on the society.

Bone Health as a Life Course Investment

Bone strength in adulthood is seen as the result of several influences acting throughout the entire life span. In this framework, two main factors are thought to determine the ultimate risk of sustaining a fragility fracture: the amount of bone lost with age and the maximum bone strength acquired during the first decades of life (Heaney et al. 2000). While most preventive efforts have been directed toward the reduction of bone loss with advancing age, the promotion of optimal bone strength accrual during the first decades of life is also contributive to fracture risk reduction (Seeman 2008). Since bone strength is a broad and complex construct, and as body size changes substantially along the life course, total body bone mineral mass is used to represent individual trajectories since birth. It has even been suggested that bone accrual before **peak bone mass** (PBM) – the maximum amount of bone mass an individual has during the life course – may be more important than unbalanced resorption in adulthood in determining fracture risk (Hernandez et al. 2003).

The second decade of life is a key period for mineral accrual, as it features a rapid increase in bone mass. Therefore, it is likely to present greater scope for plasticity than other life periods. Common endocrine control of body composition determines

that maximum linear growth drives other tissue velocities: peak height velocity was documented to occur at a mean (standard deviation) 11.8 (0.9) years in girls, while peak lean mass velocity was observed at 12.1 (1.0) years, followed by peak bone mineral content (BMC) velocity (12.5 (0.9) years) and by peak fat mass velocity (12.6 (2.0) years). Among boys, peak height velocity was reported to take place at 13.4 (1.0) years, whereas peak lean mass velocity, peak BMC velocity, and peak fat mass velocity occur at 13.7 (0.9), 14.0 (1.0), and 14.0 (1.3) years, respectively (Iuliano-Burns et al. 2001).

After the linear growth spurt, sexual hormones, in addition to growth hormones, also regulate bone metabolism, and these influences make adolescence a particularly sensitive period for determining PBM (Heaney et al. 2000). In a study that followed children from 8 to 16 years of age, BMC tripled at all sites (hip, spine, and total body) and areal bone mineral density (BMD) increased 60% at the spine, 56% at the hip, and 35% in the whole body (Foley et al. 2009). Compartment volumetric BMD does not change substantially in the phase of fastest linear growth, but there is evidence of a 30% increase during late puberty (Neu et al. 2001). Therefore, understanding bone mineralization during adolescence and identifying factors that contribute to the attainment of the genetically programmed PBM can help forestall the impact of bone loss, preventing fragility fractures.

A useful functional model was proposed – the **mechanostat** – that provides an integrative framework for the major factors influencing a healthy bone development (Fig. 1) (Rauch and Schoenau 2001). The model builds on the premise that one-way relations of molecular, cellular, and behavioral factors with bone accrual are overly simplistic. Bone accrual during growth requires constant regulation due to the need for the skeletal framework to adapt to changing external circumstances. The implication is that bone physiology is a controlled effector-sensor system where the

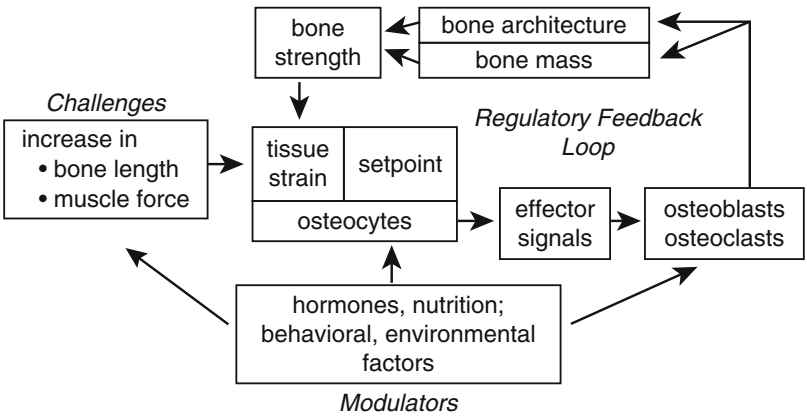

Fig. 1 The mechanostat – a functional model of the bone. The mechanostat model applied to the developing bone. Tissue strain is detected by osteocytes, resulting in the feedback loop so that bone strength can be adapted to the new circumstances (Reprinted by permission from Macmillan Publishers Ltd: Pediatric Research (Rauch and Schoenau 2001), copyright (2001))

purpose of homeostatic mechanisms is to maintain the strain (i.e., the mechanically induced deformation of bone) to a certain predetermined set point. The mechanostat model assumes that, during growth, bone stability is continuously challenged by increases in bone length and muscle mass, and the resulting bone deformation is sensed by osteocytes, which in turn signal for **bone formation**. According to this model, hormonal, nutritional, or behavioral influences on bone homeostasis act by altering the mechanostat set point or the response to a disturbance to that point, i.e., by modifying the effect of mechanical strain on bone. The most relevant aspect of the model is that it places the maintenance of mechanical properties at the center of the bone development process.

The mechanostat is particularly well suited to model bone accrual in adolescence, a phase of profound anthropometric challenges and endocrine adaptations. It is also a stage of behavioral changes, which may modify the physiologic effects of those well-known influences. In a life course paradigm, such a period of high mineral accrual before PBM may be conceptualized as critical or sensitive, i.e., a time frame during which beneficial or harmful exposures have lasting and irreversible effects on bone health (Rauch and Schoenau 2001).

Understanding Bone Metabolism

The outline of the human skeleton, known as patterning, is attained during fetal life and includes shaping and positioning of long bones (Davies et al. 2005). Subsequent mineral accrual up to PBM features a continuous process of **bone growth** in length and diameter, which occurs over the first decades of life. Longitudinal growth of long bones occurs through endochondral ossification, where new trabecular bone is formed by using a cartilaginous template generated by the growth plate (Gafni and Baron 2007; Kini and Nandeesh 2012). During linear growth, prechondrocytes in growth plates differentiate into proliferative and then hypertrophic chondrocytes that lay a cartilaginous matrix that is then replaced with mineralized tissue, thereby increasing the size of the metaphyseal compartment. Linear growth ceases when chondrocytes at growth plates exhaust their proliferative capacity and the closure of epiphyses occurs (Nilsson and Baron 2004). Additionally, in order to achieve substantial changes in diameter and shape in the diaphysis, bone undergoes **modeling**, a process characterized by the uncoupling between bone formation, in the periosteal surface, and **bone resorption**, in the endosteal surface. The net balance of this cellular activity is in favor of formation and leads to an increase in diameter and cortical thickness. As a result of modeling drifts, substantial changes are made to the size and shape of bone, optimizing its geometry with respect to mechanical properties (Robling et al. 2006). The end result is an altered spatial disposition of bone tissue that happens in coordination with linear growth.

Apart from the major changes that occur through bone modeling, maintenance of the shape, mass, and size of the skeleton depends on a different, continuous process of turnover named **remodeling**. Through remodeling, bone tissue is able to repair microfractures and to modify its structure in response to biomechanical stress

(Hadjidakis and Androulakis 2006). Mechanical properties are maintained or modified through constant metabolic activity in four different bone surfaces: periosteal, endocortical, trabecular, and intracortical (or Haversian). Remodeling is characterized by the coupled actions of matrix formation and resorption in each of these envelopes and is achieved by basic multicellular units that comprise osteoblasts and osteoclasts that act in a coordinated manner, following an activation-resorption-formation sequence (Robling et al. 2006; Sims and Martin 2014). Within each envelope, the balance of bone formation and resorption allows bone to adapt structure to function, optimizing resistance to stress. Once the skeleton reaches maturity, modeling rates reduce substantially but homeostatic remodeling remains active throughout the life course. Remodeling is thus essential for the maintenance of overall bone strength and for the repairing of damaged tissue.

Both major structural changes to bone organization (linear growth and modeling) and homeostatic replacement of bone tissue (remodeling) result from the interplay between formation and resorption conducted by osteoblasts and osteoclasts, respectively (Kini and Nandeesh 2012). Osteoblasts derive from the mesenchymal cell lineage and secrete osteoid, which is composed of type I collagen as well as noncollagenous proteins (e.g., osteocalcin). The osteoid creates a template for the mineralization and production of mature, mineralized bone. In addition to bone formation, osteoblasts stimulate osteoclasts to initiate bone resorption (Morgan et al. 2010). Bone resorption is carried out by osteoclasts, which are multinucleated cells derived from the hematopoietic lineage. Osteoclasts secrete hydrochloric acid and proteases, which degrade mineralized tissue, contributing to the regulation of calcium homeostasis. Thus, monitoring bone metabolic activity at a given point in time requires the measurement of products generated by the activity of osteoblasts and osteoclast, i.e., **bone markers**.

Bone Metabolism Markers

Bone markers in adults reflect bone remodeling and have been studied as predictors of fracture risk and to monitor both anabolic and catabolic therapies (Vasikaran et al. 2011). In children and adolescents, bone markers represent not only remodeling but also modeling and linear growth of the skeleton, given that all these processes occur simultaneously. Bone markers in children and adolescents have been studied as contributors for the diagnosis of metabolic bone diseases and also as prognostic tools by using intraindividual variation. Additionally, during adolescence, the study of bone markers in healthy populations may also provide an important dynamic dimension of skeletal maturity that may not be captured by examining physical properties alone. Indeed, low cumulative exposure periods to external influences and compensatory "mechanostat"-type mechanisms may preclude the observation of the effects of certain bone health determinants on mechanical properties. Short-term effects might be primarily observable at a physiological dimension, i.e., through levels of bone metabolism markers (Rauch and Schoenau 2001). A summary of commonly used markers is presented below.

Bone formation markers are the result of the cellular activity of osteoblasts and the most commonly used are:

- Bone-specific alkaline phosphatase (BALP): This is an osteoblastic ectoenzyme that plays a role in bone mineralization (Jürimäe 2010). Alkaline phosphatase has four isoforms – produced in the bone, liver, kidney, and intestine – but in growing children, the bone isoform is the most abundant, contributing up to 90% of the total circulating alkaline phosphatase (van der Sluis et al. 2001). BALP is measured in serum through immunoradiometric assay (IRMA), enzyme immunoassay, electrophoresis, lectin precipitation, and calorimetry (Eapen et al. 2008; Čepelak and Čvorišćec 2009). The main sources of undesired variability of this marker relate to assay characteristics and cross-reactivity with the other ALP isoforms. BALP has a relatively long half-life (1–2 days) and consequently shows negligible circadian variation (Eapen et al. 2008).
- Osteocalcin (OC): It is the most abundant noncollagenous protein within the bone (Eapen et al. 2008). This protein not only plays a role in mineralization by acting as a nucleator protein for hydroxyapatite crystals but is also involved in glucose metabolism by regulating insulin secretion and sensitivity. There are two forms of OC, carboxylated and undercarboxylated. Mature OC undergoes a posttranslational carboxylation, a process after which it becomes biologically active (Shao et al. 2015). Osteocalcin is measured in serum through competitive radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), electrochemiluminescence immunoassay (ECLIA), and IRMA (Čepelak and Čvorišćec 2009). Osteocalcin can also be measured in urine (Vasikaran et al. 2011), by RIA and immunofluorometric or immunoassays, even though no commercial assays are available at the moment (Ivaska et al. 2005). Regarding pre-analytical variability, OC presents a circadian variation, with higher levels in the morning, which determines variability related to the timing of sample collection (Eapen et al. 2008; Jürimäe 2010).
- Procollagen type I amino terminal propeptide (PINP) and procollagen type I carboxy terminal propeptide (PICP): PINP and PICP are both derived from cleavage of collagen type I, and while the first is the result of cleavage at the amino terminal end (N-terminal), PICP is cleaved at the carboxy end (C-terminal). These propeptides are released during the processing of type I procollagen to collagen and are released in their trimeric form, being rapidly converted to the monomeric form (Jürimäe 2010). Similarly to the previous bone markers, PINP and PICP are measured in serum using RIA or ELISA, and assays are also available for plasma (Čepelak and Čvorišćec 2009; Vasikaran et al. 2011); there are assays that allow measuring both its trimeric and monomeric form (Dreyer and Vieira 2010; Yang and Grey 2006). Since PINP measurements have some practical advantages, such as lower diurnal variation and clearance unaffected by renal dysfunction (metabolism occurs mainly in the liver), it is more frequently used than PICP (Dreyer and Vieira 2010).

Bone resorption markers are the dynamic reflex of osteoclast activity and the most commonly used are:

- Cross-linked C-terminal telopeptide of type I collagen (CTX-I) and cross-linked N-terminal telopeptide of type I collagen (NTX): These are cross-linked telopeptides, which result from the degradation of type I collagen at the amino or carboxy terminals, respectively. Both markers can be measured in serum or urine. Serum CTX-I is analyzed through ELISA or by ECLIA, and these assays are also available for plasma (Vasikaran et al. 2011); when measured in urine, it can be quantified by ELISA or RIA (Chubb 2012). As for NTX, both urine and blood samples are measured by ELISA, RIA, or immunochemiluminometric assay (ICMA) (Čepelak and Čvorišćec 2009). Serum assays are preferred to urinary ones because the former are less affected by analytic and biologic variability. Also, CTX-I is more frequently used than NTX, due to better assay availability, higher bone specificity, and well-documented variability (Vasikaran et al. 2011).
- C-terminal cross-linking telopeptide of type I collagen (ICTP): This telopeptide is generated by cleavage of type I collagen by cathepsin K, an osteoclast enzyme (Eapen et al. 2008). This bone marker is measured only in serum by radioimmunoassay (Eapen et al. 2008; Čepelak and Čvorišćec 2009), but it is not commonly used due to lack of sensitivity (Garnero et al. 2002).
- Tartrate-resistant acid phosphatase type 5b isoform (TRACP5b): This marker is an isoform of the enzyme TRACP that is secreted by bone-resorbing osteoclasts. This enzyme is measured in serum through colorimetry, RIA, or ELISA (Čepelak and Čvorišćec 2009). It has been demonstrated that TRACP5b has low diurnal variation. In addition, this enzyme is cleared by the liver, meaning that renal function does not affect its excretion (Halleen et al. 2001).

Besides the abovementioned sources of pre-analytical variability – including the type of specimen (urine or blood) and circadian variation – there are important technical sources of variability common to all markers, such as sampling, handling, and storage of the primary biological sample. An additional source of variability is the use of different commercial determination kits or methods (interlaboratory variation): in a study that aimed to quantify this variation, samples were submitted to 73 different laboratories and, even with identical assays, results differed markedly (Seibel et al. 2001).

As for biological variability – the actual target of bone marker measurements – several factors are known to interact to determine measured concentrations: sex, pubertal stage, age, ethnicity, recent fracture, menstrual cycle phase, pregnancy and postpartum, underlying medical conditions (such as metabolic bone disease and liver or renal failure), drugs (antiresorptive agents, anticonvulsants, or oral contraceptives), smoking, and physical exercise (Eapen et al. 2008). Even though all the markers mentioned above have been used in research, serum CTX-I and PINP have been recommended as reference analytes for bone metabolism in clinical studies. This is not only due to the availability of commercial assays but also because sources

of both analytical and biological variations, though considerable, have been well documented (Vasikaran et al. 2011).

The following sections present the most widely used bone markers in adolescents with regard to (a) reference or normative values; (b) variation with sex, age, and sexual development; (c) relation with physical properties of bone tissue; and (d) associations with modifiable risk factors.

Reference Values

Reference or normative values are fundamental to interpret the clinical meaning of bone markers as diagnostic and/or prognostic tools. Thus, attempts have been made in order to establish these for children and adolescents. In Table 1, results from different studies including different markers are shown, comprising children in a wide range of ages and sexual maturity stages (Bayer 2014; Rauchenzauner et al. 2007; Fischer et al. 2012; Huang et al. 2011). Since the focus of this chapter is on the postpubertal period, reference values are presented only for the ages of 13 and 17 years old, spanning most of adolescence.

Even though absolute values differ between studies, similar patterns are observed for all markers: levels decrease from 13 to 17 years old, in both boys and girls, which are consistent with the convergence toward skeletal maturity, featuring a deceleration of skeletal growth and modeling and the takeover of remodeling as the main phenomenon responsible for bone dynamics in adulthood. In the case study – the EPITeen cohort – the same decreasing trend with age was observed both for girls and boys with regard to resorption and formation markers. Additionally, for CTX-I, the mean values determined were within the 3rd and the 97th percentiles obtained by Huang et al. and by Rauchenzauner et al. (Fig. 2).

Attempts to establish reference values are limited, on the one hand, by the large analytical variability observed between studies and, on the other hand, by the small number of children and adolescents included in most investigations. Also, most studies present data for a limited number of bone markers, hampering comparisons between populations. Additionally, most of the research has been conducted in Europe and Canada and included only White children and adolescents. Therefore, reference values are only applicable to this population, since ethnicity is one of the factors that contribute to biological variability. Indeed, a study conducted among Black and White children found that Black children had significantly higher urinary NTX and serum OC values. Serum BALP value was also increased in Black children but not significantly (Hill et al. 2012).

Influence of Sex, Age, and Sexual Development on Bone Markers

Growth and sexual hormones regulate bone metabolism and thus it is expected that levels of bone markers are strongly influenced by sex, age, and sexual development.

Table 1 Normative values of bone markers in 13- and 17-year-old subjects. Formation and resorption marker values are presented as median, 3rd and 97th percentile; when these were not available, the 2.5th and 97.5th percentiles are shown Girls, Boys. Generically, a decrease in bone formation and bone resorption markers is observed throughout adolescence, both in girls and in boys

Girls			
		Age (years)	
		13	17
Markers	First author, year	P50th (P3rd;P97th)	P50th (P3rd;P97th)
Bone formation			
OC (ng/mL)	Rauchenzauner, 2007	20 (8;60)	18 (8;42)
	Huang, 2011[a,b]	(24.1;232.1)	(21.1;76.7)
	Bayer, 2014[a]	75 (13;240)	30 (25;40)
BALP (U/L)	Rauchenzauner, 2007	42 (20;103)	19 (8;45)
	Huang, 2011[a,c]	(20.8;172.3)	(8.1;43.9)
	Fischer, 2012	74 (40;163)	27 (12;58)
PINP (ng/mL)	Bayer, 2014[a]	300 (50;1200)	75 (50;100)
	Huang, 2011[a,c]	(109;1346)	(49;277)
Bone resorption			
ICTP (μg/L)	Rauchenzauner, 2007	17 (7;38)	9 (4;20)
CTX-I (ng/L)	Rauchenzauner, 2007	1.800 (0.500;3.800)	1.000 (0.250;2.200)
	Huang, 2011[a,b]	(0.49;2.76)	(0.00;1.59)
TRACP 5b (U/L)	Rauchenzauner, 2007	4.6 (1.9;7.5)	2.9 (0.1;5)
	Fischer, 2012	8.9 (4.8;18.7)	3.3 (1,8;6.8)
Boys			
		Age (years)	
		13	17
Markers	First author, year	P50th (P3rd; P97th)	P50th (P3rd; P97th)
Bone formation			
OC (ng/mL)	Rauchenzauner, 2007	21 (9;70)	28 (8;71)
	Huang, 2011[d,e]	(48.2;226.4)	(22.5;151.3)
	Bayer, 2014[d]	120 (56;237)	50 (12;163)
BALP (U/L)	Rauchenzauner, 2007	60 (29;130)	38 (16;89)
	Huang, 2011[d,f]	(45.5;208.4)	(13.1;80.0)
	Fischer, 2012	48 (69;197)	69 (26;134)
PINP (ng/mL)	Bayer, 2014[d]	600 (250;1500)	100 (50;500)
	Huang, 2011[d,g]	(339;1399)	(61;718)
Bone resorption			
ICTP (μg/L)	Rauchenzauner, 2007	18 (8;39)	15 (7;28)
CTX-I (ng/mL)	Rauchenzauner, 2007	2.000 (0.500;4.500)	1.400 (0.200;3.900)
	Huang, 2011[d,e]	(1.00;2.90)	(0.50;2.43)
TRACP 5b (U/L)	Rauchenzauner, 2007	6 (3.2;8.8)	4 (0;8.0)
	Fischer, 2012	14.3 (6.8:27.7)	6.8 (2.5;15.0)

[a]Values for percentiles 2.5th, 50th, and 97.5th are presented
[b]Data from age ranges of >10 to 14 and >15 were considered
[c]Data from age ranges of 11 to 14 and >15 were considered
[d]Values for percentiles 2.5th, 50th, and 97.5th are presented
[e]Data from age ranges of >9 to 15 and >15 were considered
[f]Data from age ranges of >11 to 15 and >15 were considered
[g]Data from age ranges of >11 to 14 and >15 were considered

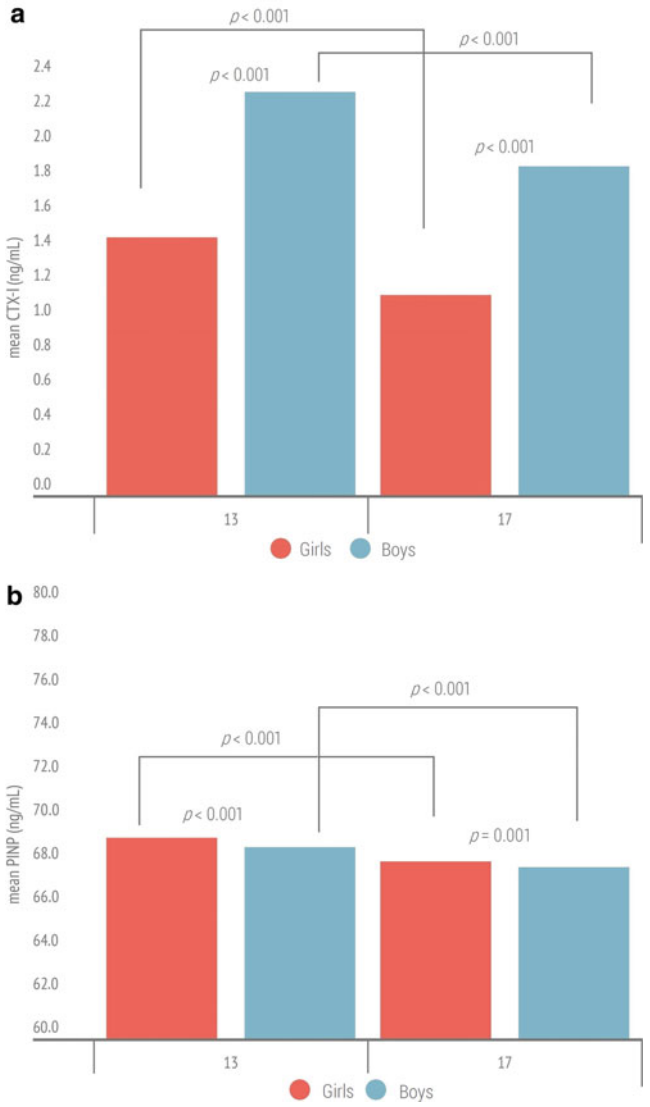

Fig. 2 Bone marker mean values in EPITeen cohort. Cross-linked C-terminal telopeptide of type I collagen (CTX-I) and procollagen type I amino terminal propeptide (PINP) mean values at 13 and 17 years old for girls and boys. **A**, CTX-I; **B**, PINP. For both markers, a decrease throughout adolescence is observed in girls and in boys. Boys present significantly higher values of both markers both at 13 and 17 years old. Girls, $n = 300$; boys, $n = 300$. Unpublished data

Adolescence is characterized by biological changes that accelerate skeletal development, leading to a rapid increase in bone formation, which then decreases progressively; this is reflected by an increase followed by a decrease in bone markers levels. During this life stage, chronological age and sexual development coevolve, but their

relation is not linear and different trajectories are expected for different children. The evolution of bone markers with sexual development, usually defined using the Tanner staging system (Tanner and Whitehouse 1976), reflects skeletal maturation even more closely than that with chronological age, but the standardization of sexual development assessment outside the clinical setting presents several practical challenges. Thus, chronological age remains a universal indicator of growth used to describe most phenotypes.

Formation Markers

Serum bone-specific alkaline phosphatase levels are higher in boys than in girls, either when stratified by age (Rauchenzauner et al. 2007; Fischer et al. 2012; Huang et al. 2011; Mora et al. 2009) or sexual development (Mora et al. 2009). Among girls, BALP levels peak earlier and also start to decrease at an earlier age. The age group of peak BALP in girls varied across studies as follows: 13–17 years old in a Chinese population (Tsai et al. 1999), 6–11 in a Canadian study (Huang et al. 2011), 9–11 in an Italian (Mora et al. 2009) and in a German sample (Fischer et al. 2012), around 9–13 in an Austrian study (Rauchenzauner et al. 2007), and 10–12 in Brazilian adolescents (Fortes et al. 2014). In boys peak BALP was found at 10–14 (Tsai et al. 1999), 12–15 (Huang et al. 2011), 11–13 (Mora et al. 2009), 12–14 (Fischer et al. 2012), and around 10–15 years of age (Rauchenzauner et al. 2007), depending on the population studied.

Regarding BALP variation according to sexual development, Mora et al. reported that, in girls, levels increased from Tanner stage I to II, but from there to stage III, a large decrease was verified. Progressive decrease was observed up to stages IV and V. Overall, BALP levels were significantly higher in Tanner I and II, compared to subsequent stages. As for boys, BALP levels presented the same trend, but the decrease from Tanner stage II to III was much attenuated when compared to girls and was more pronounced in stages IV and V. In addition, BALP levels at stage II were significantly higher than BALP levels at stages I, IV, and V (Mora et al. 2009). Fortes et al. found that BALP levels at Tanner I, II, and III were significantly higher than at stages IV and V (Fortes et al. 2014). In the Italian study (Mora et al. 2009), the decrease in BALP levels occurred earlier (Tanner II to Tanner III), a difference that might arise from different criteria for sexual development classification between studies.

Osteocalcin is also increased in boys comparing to girls. Magnusson et al. described an increase of OC levels from age 7 up to 13 in both sexes, but in girls, the decrease started at 13–14 years old, while in boys was after 15–16 years old (Magnusson et al. 1995). Similarly to BALP, peak OC age is not consistent among studies, ranging from 9 to 12 in an Austrian study (Rauchenzauner et al. 2007), 10–14 in a Canadian sample (Huang et al. 2011), 11–12 in a sample from the Czech Republic (Bayer 2014) to 12 years old in a study from the Netherlands (van der Sluis et al. 2001), for girls. In boys these values varied between 12–15 (Rauchenzauner et al. 2007), 9–15 (Huang et al. 2011), 13–14 (Bayer 2014), and 15 years old (van der Sluis et al. 2001). In some studies it is not possible to observe a reversal age, since all participants were in postpubertal ages, and only a decrease is observed (Gracia-Marco et al. 2010; Fortes et al. 2014).

Focusing on sexual development, van der Sluis et al. reported higher OC levels throughout Tanner stages in boys, with exception of Tanner stage I, in which girls presented a slightly higher value, but not statistically significant. Furthermore, boys at Tanner II–IV had significantly higher values than boys at stage I, but levels decreased in Tanner stage V (van der Sluis et al. 2001). Girls at Tanner V presented significantly lower OC values than those at stages II–IV. These results are in line with those presented by a Spanish study: a statistically significant decrease in OC levels throughout Tanner stages, both for boys and girls, was reported (Gracia-Marco et al. 2010). The study conducted in Brazilian female adolescents showed an increase of OC levels until Tanner III, decreasing from this stage onward (Fortes et al. 2014). The same trend was reported by Bayer et al. for both boys and girls – an increase of OC levels until Tanner III and a decrease thereafter (Bayer 2014).

PINP, another formation marker, shows higher levels in boys than in girls, either when bone markers are analyzed by Tanner stage (van der Sluis et al. 2001; Gracia-Marco et al. 2010) or by age (Gracia-Marco et al. 2010; Huang et al. 2011; Bayer 2014). Again, the age at which PINP peaks is not consistent throughout studies. A Canadian study reported that, for girls, the trend reverses at 10–11 years old, while for boys it occurred at 11–14 years old (Huang et al. 2011). On the other hand, a study from the Czech Republic reported peak PINP levels at 11–12 years old for girls and 13–14 years old for boys (Bayer 2014). Gracia-Marco et al. reported a significant decrease of PINP levels, in both sexes, from age group 12.50–14.99 to 15.00–17.50 (Gracia-Marco et al. 2010).

With regard to sexual development, van der Sluis et al. reported a decrease throughout Tanner stages for boys and girls, even though only significant from Tanner II–IV to V. As noted above PINP levels were higher for boys, with exception of Tanner I, in which girls presented slightly higher values (van der Sluis et al. 2001). Bayer et al. reported that for boys in Tanner I and II, PINP levels were similar, increasing on to stage III and decreasing progressively to Tanner IV and V. As for girls, PINP levels were quite alike in Tanner I, II, and III, gradually decreasing afterward (Bayer 2014).

Regarding PICP, levels are generically higher in boys than girls, when stratified either by sexual development or age (van der Sluis et al. 2001; Tsai et al. 1999). Like the other markers, PICP also presents an increase followed by a decrease, when analyzed by age. A study conducted in Japanese children showed peak PICP at 12 years old both for boys and girls (Kikuchi et al. 1998), while a study conducted in a Chinese population reported the peak at 9–12 and 10–14 years old for girls and boys, respectively (Tsai et al. 1999). Another study estimated this peak at 12 years old for girls and 15 for boys (van der Sluis et al. 2001).

Relatively to sexual development, Rotteveel et al. reported that both for boys and girls, PICP levels increased from Tanner I to II and remained similar through Tanner III. Up to Tanner IV, PICP levels increased again and decreased after Tanner V (Rotteveel et al. 1997). The same trend was obtained by van der Sluis et al., who reported an increase from Tanner I to Tanner II–IV and a decrease after Tanner V, both in boys and girls. However, none of those changes were statistically significant.

PICP levels were higher for girls in Tanner I than for boys at the same stage; for the remaining stages, boys presented higher PICP levels (van der Sluis et al. 2001).

Resorption Markers

Serum CTX-I levels are reported to be higher in boys than in girls (Rauchenzauner et al. 2007; Huang et al. 2011; Gracia-Marco et al. 2010), and in both sexes an increase followed by a subsequent decrease is verified during the second decade of life (Rauchenzauner et al. 2007; Huang et al. 2011; Fortes et al. 2014). Rauchenzauner et al. reported that for boys, peak CTX-I was observed at the age range 13–15 years old and for girls at 10–13 years old (Rauchenzauner et al. 2007). Huang et al. reported superimposable, though wider age ranges >9–15 for boys and >10–14 for girls (Huang et al. 2011). Gracia-Marco et al. reported that boys had significantly higher levels of serum CTX-I than girls, both at age 12.50–14.99 and 15.00–17.50. In addition, in girls, a statistically significant decrease of this marker's levels was observed, while in boys the observed decrease was not significant (Gracia-Marco et al. 2010). Also Fortes et al., in Brazil, observed that serum CTX-I levels were similar from 10 to 12 years old and decreased progressively in the subsequent groups, being significantly lower at 17–19 years old (Fortes et al. 2014).

Regarding the variation of serum CTX-I levels with sexual development, Gracia-Marco et al. observed significant higher values in boys than in girls, both in Tanner ≤ IV and V. Also, a significant decrease from the earlier to the later sexual development group was verified in both sexes (Gracia-Marco et al. 2010). Fortes et al. described a slight increase of serum CTX-I levels from Tanner I to III, decreasing abruptly after Tanner IV and more smoothly after Tanner V (Fortes et al. 2014).

The only study assessing both urinary and serum CTX-I values reported similar trajectories, but no significant differences were found for Tanner stage ≤ IV among boys and girls. Also, the variability in the measurements obtained by urinary CTX-I was broader (Gracia-Marco et al. 2010), due to analytical constrains, namely, the need for adjustment for excretion rate.

NTX is also characterized by an age-related increase followed by a decrease. Serum NTX was shown to peak around 12 years old for girls and around 15 years old for boys. When considering its variation according to sexual development, van der Sluis et al. observed, in boys, an increase from Tanner I to II–IV, even though not significant, followed by a significant decrease to Tanner V. In girls, serum NTX levels were similar in Tanner I and II–IV, decreasing significantly to stage V. Also, boys presented higher levels of serum NTX in Tanner II–IV and Tanner V than girls, but not in Tanner I (van der Sluis et al. 2001).

Urinary NTX also shows an increase followed by a decrease in both genders, with higher levels observed in younger girls and in earlier Tanner stages, when compared to boys. In fact, boys only presented higher values than girls in later pubertal stages and after peak concentration is attained (Mora et al. 1998). Peak urinary NTX levels were observed at 8–12 years old for girls and 10–14 years old

for boys. In a more recent study of Japanese children aged 6–11 years old, a decrease in urinary NTX levels was reported for boys, with levels significantly higher in 6- than in 11-year-old boys. For girls, a decrease between 6 and 9 years old was reported, followed by an increase after this age (Sato et al. 2010). Regarding sexual development, girls in Tanner stages I, II, and III presented higher levels of this marker than boys in the same developmental stage. However, boys at Tanner stages IV and V presented higher urinary NTX levels than girls (Mora et al. 1998).

ICTP presents a similar trend to the previous markers. The age at which ICTP peaked was 12 years old for Japanese girls and boys (Kikuchi et al. 1998), 13 years old for girls and 14 years old for boys in a Dutch study (van der Sluis et al. 2001), and between 13–15 and 11–13 years old in Austria for boys and girls, respectively (Rauchenzauner et al. 2007). In all studies, values reported for boys were slightly higher than those described for girls.

When ICTP levels were evaluated as a function of sexual development, a statistically significant increase from Tanner I to II–IV was reported in boys, followed by a significant decrease from there to Tanner V. Regarding girls, in Tanner I and II–IV, ICTP levels remained quite similar, decreasing significantly to Tanner V. ICTP levels were higher in boys than in girls throughout all Tanner stages (van der Sluis et al. 2001).

Finally, TRACP5b presents higher values in boys than in girls. Rauchenzauner et al. reported significant higher levels for boys than girls in the 13–17 age group. In girls TRACP5b seemed to decrease since the first years of life, until late adolescence. In boys, TRACP5b remained fairly constant until 13 years old, decreasing from this point on (Rauchenzauner et al. 2007). In a more recent study, boys also presented higher levels of TRACP5b than girls, and the peak level was observed at 8–10 years old for girls and 11–14 years old for boys (Fischer et al. 2012). Regarding sexual development, results resemble to those observed by age, with concentrations decreasing at Tanner IV and V (Rauchenzauner et al. 2007).

Case Study: Bone Markers in the EPITeen Cohort A frequently referred limitation of published evidence on normative levels is that most studies are cross-sectional and use different individuals in different development stages to infer on the effects of age or sexual maturity. In the EPITeen cohort, it was possible to measure serum concentrations of one formation (PINP) and one resorption marker (CTX-I) in the same individuals at two different time points: 13 and 17 years of age. The results obtained are generally in line with those presented above for cross-sectional studies: both CTX-I and PINP levels decreased from early to late adolescence, and boys had significantly higher levels of both markers, at both ages (Fig. 2). Regarding sexual development – measured only in girls using menarche age – earlier menarche (i.e., higher sexual maturity for the same age) was associated with lower levels of CTX-I, both in early and late adolescence. However, for PINP, no such differences were observed (Fig. 3).

Overall, it is clear from the published literature that both formation and resorption markers increase in the first decade of life, peaking at puberty and decreasing

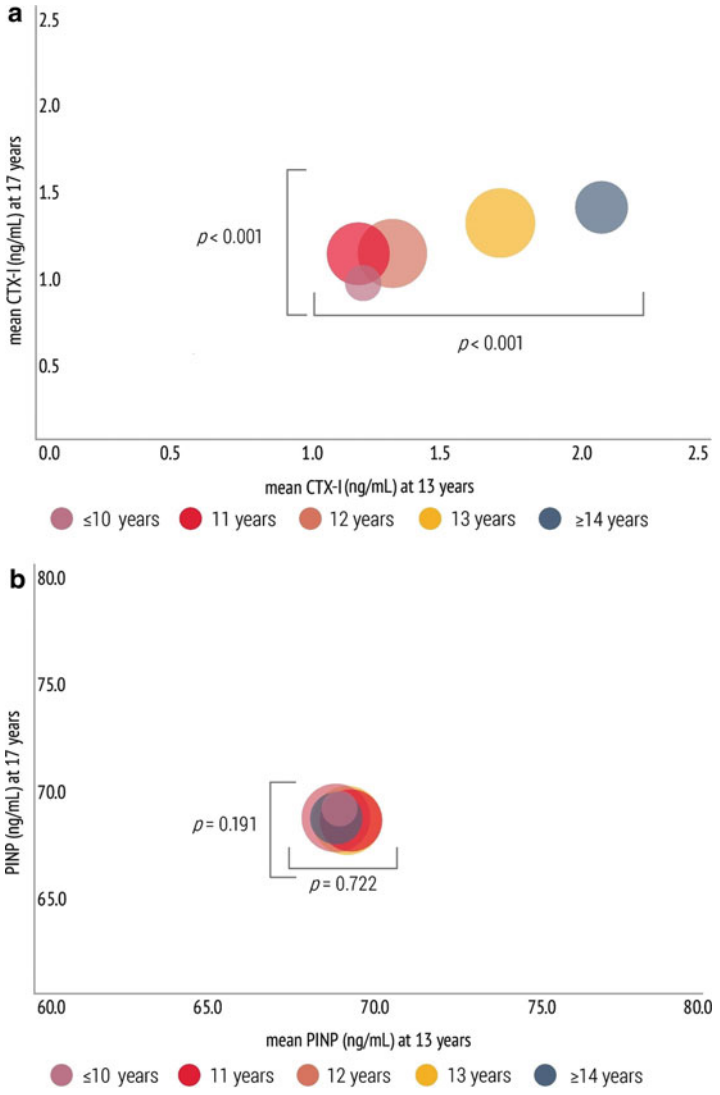

Fig. 3 Bone marker mean values for girls according to sexual maturity (EPITeen). Cross-linked C-terminal telopeptide of type I collagen (CTX-I) and procollagen type I amino terminal propeptide (PINP) mean values at 13 (X axis) and 17 (Y axis) years old for girls according to menarche age. **A**, CTX-I; **B**, PINP. Girls that had menarche before 12 years old present lower levels of the CTX-I than girls that had the menarche later on. Girls $n = 300$. The *circle* area is proportional to the number of subjects within the group. Unpublished data

from this point onward. This is consistent with the attainment of skeletal maturity, which is accompanied by a clear deceleration of linear growth and modeling rates toward peak bone mass. Since pubertal development trajectories differ between children and populations, the chronological age at which bone markers peak is not

consistent between studies, but the overall trajectories are remarkably similar. It is also clear that boys present higher levels of bone markers than girls, especially at later pubertal stages, which is consistent with their later timing of skeletal maturation.

Clinical Significance of Bone Markers

To clarify the potential usefulness of biomarkers in the clinical setting, it is necessary to understand how they correlate with circulating levels of bone bioactive molecules and with the physical properties of bone tissue. To illustrate this, the following section presents evidence on the relation of markers with osteoclast-regulating cytokines and with bone mineral content and density.

Example 1: Bone Markers and Osteoclast-Regulating Cytokines The system osteoprotegerin (OPG)/receptor activator of nuclear factor kB (RANK)/RANK-ligand (RANKL) is a major regulator of metabolism at the bone tissue level. The system contributes greatly to regulate bone modeling and remodeling by mediating the local effects of systemic factors, such as hormones and cytokines (Vega et al. 2007). This system plays a fundamental role in bone resorption by regulating osteoclast formation and activation (Boyce and Xing 2008). Osteoclast differentiation is dependent on the binding of RANK, a transmembrane protein expressed by mature osteoclasts and osteoclast precursors, and RANKL, a cytokine expressed by osteoblasts. OPG, secreted by osteoblasts, is an inhibitor of osteoclastogenesis by acting as a decoy receptor to RANKL (Martin and Sims 2015). In an effort to relate these two signaling molecules with the established bone markers, it was found, in the EPITeen cohort, that RANKL was directly associated with CTX-I, both in early (13 years old) and late (17 years old) adolescence, whereas the OPG/RANKL ratio was inversely correlated with CTX-I (Lucas et al. 2014). For PINP, no significant correlation was observed with either RANKL or OPG. In boys, OPG and OPG/RANKL were directly correlated with CTX-I only at age 13 (Fig. 4). So, even though serum RANKL and OPG levels varied markedly throughout adolescence, correlations with bone markers were not robust: in girls RANKL was associated with bone resorption only in late adolescence, and in boys, OPG correlated with the bone resorption only in early adolescence.

In general there is little evidence on how bone markers correlate with bone active molecules in the general population, but associations seem to be generally weak. There are several likely reasons for that, such as the pleiotropic effects of bone active molecules, which reflect a multitude of processes other than bone metabolism; the instantaneous nature of serum determinations of these parameters, which might reflect only very short-term homeostatic processes; or the technical variability of methods for quantification.

Example 2: Bone Markers and Bone Physical Properties As a result of linear growth, modeling, and remodeling, bone physical properties change substantially

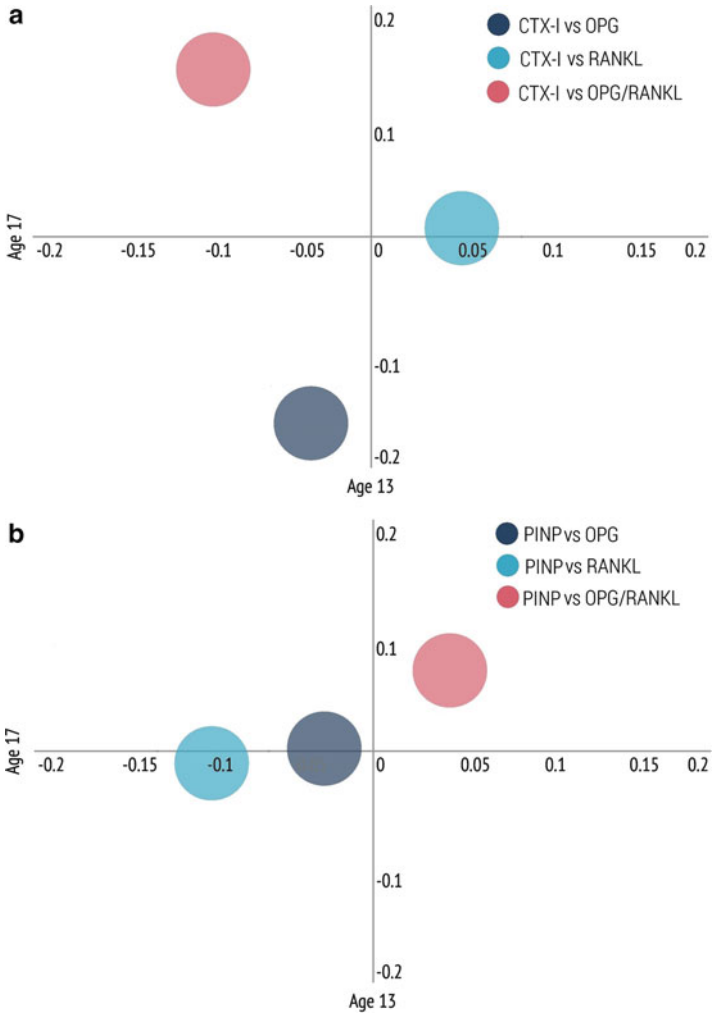

Fig. 4 Correlations between bone markers and osteoclast regulators in boys (EPITeen). Pearson correlations for 13 (Y axis) and 17 years old (X axis). **A**, Correlation for cross-linked C-terminal telopeptide of type I collagen (CTX-I) and OPG (osteoprotegerin), CTX-I and RANKL (receptor activator of nuclear factor kB ligand), and CTX-I and OPG/RANKL at 13 and 17 years old. **B**, Correlation for procollagen type I amino terminal propeptide (PINP) and OPG, PINP and RANKL, and PINP and OPG/RANKL at 13 and 17 years old. Significant correlation for CTX-I and OPG/RANKL was observed at 13 years old. Boys $n = 300$. Unpublished data

throughout the first decades of life. Since physical properties in early life are a main determinant of fracture risk (Johansson et al. 2009), it would be useful if bone markers could predict properties such as bone mineral density (BMD) or bone mineral content (BMC). Especially in large-scale epidemiological studies of healthy

children, this would be an interesting alternative to the gold standard dual-energy X-ray absorptiometry (DXA), which implies some radiation exposure.

As previously noted, levels of bone markers decrease after puberty with evolving sexual development and aging. As linear growth ceases and skeletal maturity is attained, increases in BMD and BMC are seen up to peak bone mass (Arabi et al. 2004; Gracia-Marco et al. 2010; Guo et al. 2013; Jeddi et al. 2013; Kardinaal et al. 2000). Thus, inverse associations between bone markers and bone physical properties are expected in the healthy adolescent population. Studies that have explored these associations generally report weakening correlations with growing sexual development, as seen in the examples below.

A study of Japanese females showed that serum BALP and NTX correlated negatively with lumbar spine BMC at 12 ($r = -0.489$ and $r = -0.494$, respectively) and 14 years of age ($r = -0.293$ and $r = -0.274$). Regarding total hip BMC, both BALP and serum NTX presented a negative statistically significant correlation at 12 years old ($r = -0.236$ and $r = -0.260$). The same study reported significant negative correlations between BMD (lumbar spine, LS, and total hip, TH) and both BALP and serum NTX, for ages 12 (LS, $r = -0.454$ and $r = -0.455$; TH, $r = -0.327$ and $r = -0.329$), 14 (LS, $r = -0.293$ and $r = -0.274$; TH, $r_{BALP} = -0.297$), and 16 (LS, $r_{NTX} = -0.203$) (Orito et al. 2009).

Additionally, a study conducted in Estonian boys showed a negative correlation between BMC and both PINP and ICTP in Tanner stages I and II, even though only significant at Tanner I ($r = -0.84$ and $r = -0.66$, respectively). The same study also reported significant negative correlations of total BMD ($r = -0.87$) and lumbar spine BMD ($r = -0.67$) with PINP at Tanner stage I. With regard to ICTP, a negative significant correlation was observed with lumbar spine BMD ($r = -0.59$) and total BMD ($r = -0.75$). For lumbar spine BMD, the correlation with ICTP was positive for Tanner II and Tanner III, while for total body BMD, the correlation was negative for Tanner II, but positive at Tanner III (Jürimäe et al. 2009).

In Brazilian girls, an inverse significant correlation between BMD and all markers was found – BALP, OC, and serum CTX-I – at the lumbar spine ($r = -0.696$, $r = -0.367$, and $r = -0.627$, respectively), femur ($r = -0.519$, $r = -0.334$, and $r = -0.644$), and total body ($r = -0.655$, $r = -0.425$, and $r = -0.695$) (Fortes et al. 2014).

Another study conducted in American girls showed statistically significant inverse correlations for BALP, OC, and urinary NTX with lumbar spine BMD ($r = -0.363$, $r = -0.129$, and $r = -0.202$, respectively) (Harel et al. 2007).

Nevertheless, in Dutch children and young adults, van der Sluis et al. did not find significant correlations between BMD and the bone markers OC, NTX, ICTP, PICP, or PINP (van der Sluis et al. 2001).

Case Study: Bone Markers and Bone Mineral Density in the EPITeen Cohort In the EPITeen cohort, inverse significant correlations between serum CTX-I and forearm BMD were found for boys and girls, both in early and late adolescence. For PINP, negative correlations were found, but did not achieve statistical significance. It is worthy of note that all correlations found were generally weak (Fig. 5).

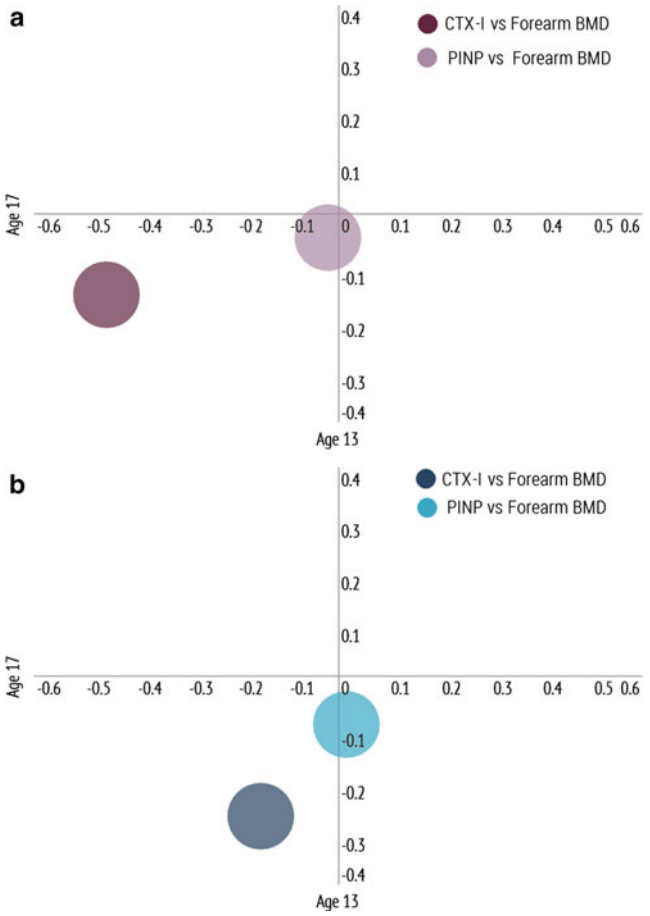

Fig. 5 Correlations between bone markers and bone mineral density (BMD) (EPITeen). Pearson correlations are presented. **A**, Girls. **B**, Boys. A statistically significant correlation was found between cross-linked C-terminal telopeptide of type I collagen (CTX-I) and forearm BMD both at 13 and 17 years old, for girls and boys. Girls: $n = 300$. Boys: $n = 300$. Unpublished data

In conclusion, a relatively consistent inverse correlation has been reported between bone physical properties and bone markers, which is consistent with the attainment of skeletal maturity. This correlation is observed at different skeletal sites and with varying bone resorption and formation markers. However, in general, such correlations have been weak, which argues for caution when using instantaneous measures of metabolic activity to translate dimensions of bone tissue that are acquired cumulatively throughout several decades of life, as are physical properties. Therefore, the use of these markers in population-based samples might be primarily to rank individuals with regard to skeletal maturity within their source distribution rather than to predict overall bone quality.

Modifiable Determinants of Bone Markers

Unlike bone physical properties, for which the role of modifiable determinants has been widely studied, determinants of bone markers have been much less evaluated and, as a consequence, knowledge on their determinants is quite limited. So far, published studies regarding modifiable determinants of bone markers in adolescents are restricted to vitamin D status (Jones et al. 2005; Ginty et al. 2004), physical activity (Karlsson et al. 2003; Bennell et al. 1997; Chahla et al. 2015), and weight (Dimitri et al. 2011; Mora et al. 2009).

Regarding vitamin D, a study conducted in Swiss adolescents reported that intake was not a significant determinant of serum CTX-I or PINP, in boys or girls, either in pooled analysis or stratified by Tanner stage (Ginty et al. 2004). However, in a study performed in Australian boys aged 16–18 years old, a serum vitamin D threshold analysis showed that those with vitamin D levels below 55 nmol/L had significant higher BALP levels (Jones et al. 2005).

Studies regarding physical activity usually compare athletes with controls. In a study conducted in Australia (Bennell et al. 1997) comparing power athletes, endurance athletes, and controls, no significant differences were observed for OC between groups. Another study conducted in active Swedish premier young male soccer players reported significantly higher levels of OC and ICTP, compared to age- and gender-matched controls. PICP also presented higher levels in active athletes comparatively to controls, though not significant (Karlsson et al. 2003). Reduction in athletes' physical activity was associated with increased bone resorption, i.e., after 2 weeks of rest, ICTP was significantly higher in athletes than in controls, but no significant differences in OC and PICP levels were observed. Additionally, a recent study conducted in normal weight and obese children reported that less physically active children had significantly lower levels of OC, but this was not observed for BALP (Chahla et al. 2015).

Weight is also a possible modifiable determinant of bone markers, due to its fundamental role in defining bone physical properties. In a study conducted in children aged 5–16 years in the United Kingdom, obese children had significantly higher serum CTX-I levels than normal weight children, but this was not observed for PINP (Dimitri et al. 2011). Also, in a study conducted in Italian children, a significant direct correlation was shown between BALP and weight (Mora et al. 2009).

Case Study: Modifiable Determinants of Bone Markers in the EPITeen Cohort In the EPITeen cohort, associations between modifiable factors – smoking, drinking, weight, physical activity, and calcium and vitamin D intake – and the bone markers CTX-I and PINP, for boys and girls separately, were studied (Figs. 6 and 7).

In general, no significant associations were found between CTX-I and the modifiable factors tested in girls. The exception was body size at 13 and 17 years old, where normal weight girls had higher CTX-I than overweight girls (Fig. 6AIII). A marginally

significant result was found between physical activity and CTX-I levels at 17 years old, where girls that did not practice physical exercise had lower levels compared to those that practiced physical exercise more than three times per week (Fig. 6AIV). As for PINP, no statistically significant differences were found in girls (Fig. 7A).

Regarding boys, a similar inverse association was found between CTX-I and weight at ages 13 and 17 years old (Fig. 6BIII). For PINP, a significant difference between normal and overweight boys was found at 17 years old (Fig. 7BIII). Also

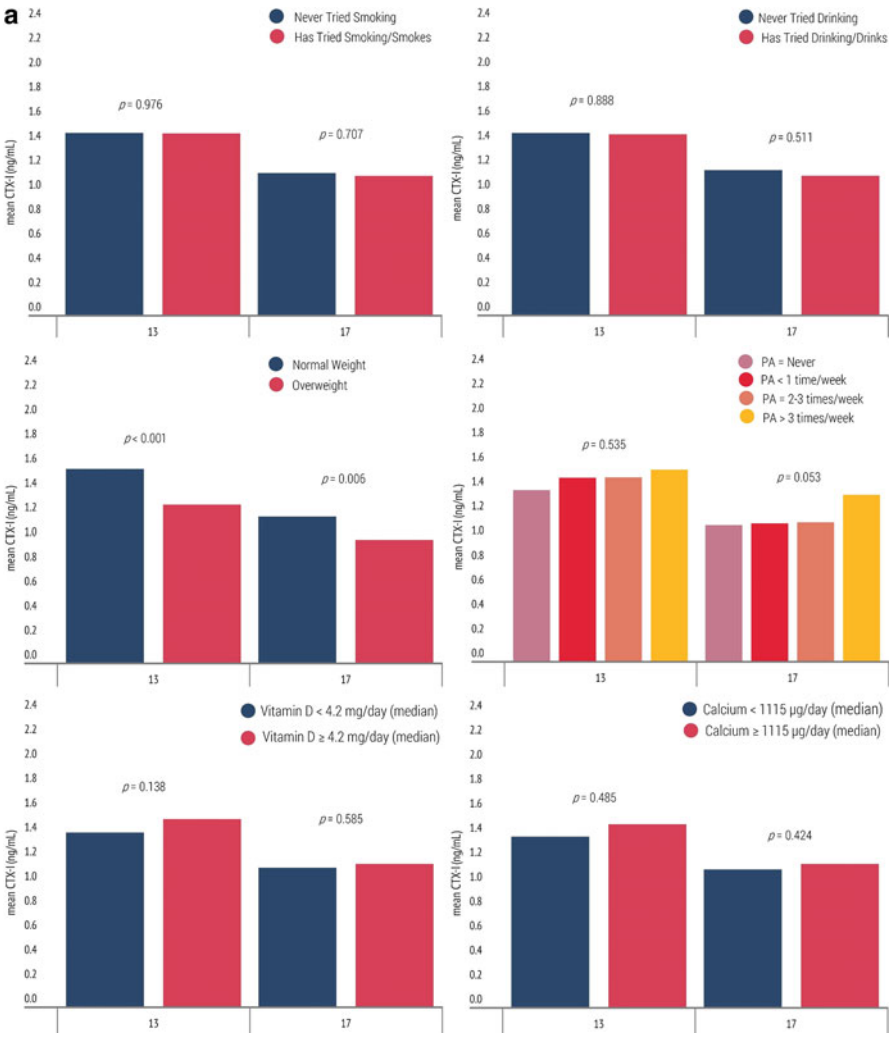

Fig. 6 (continued)

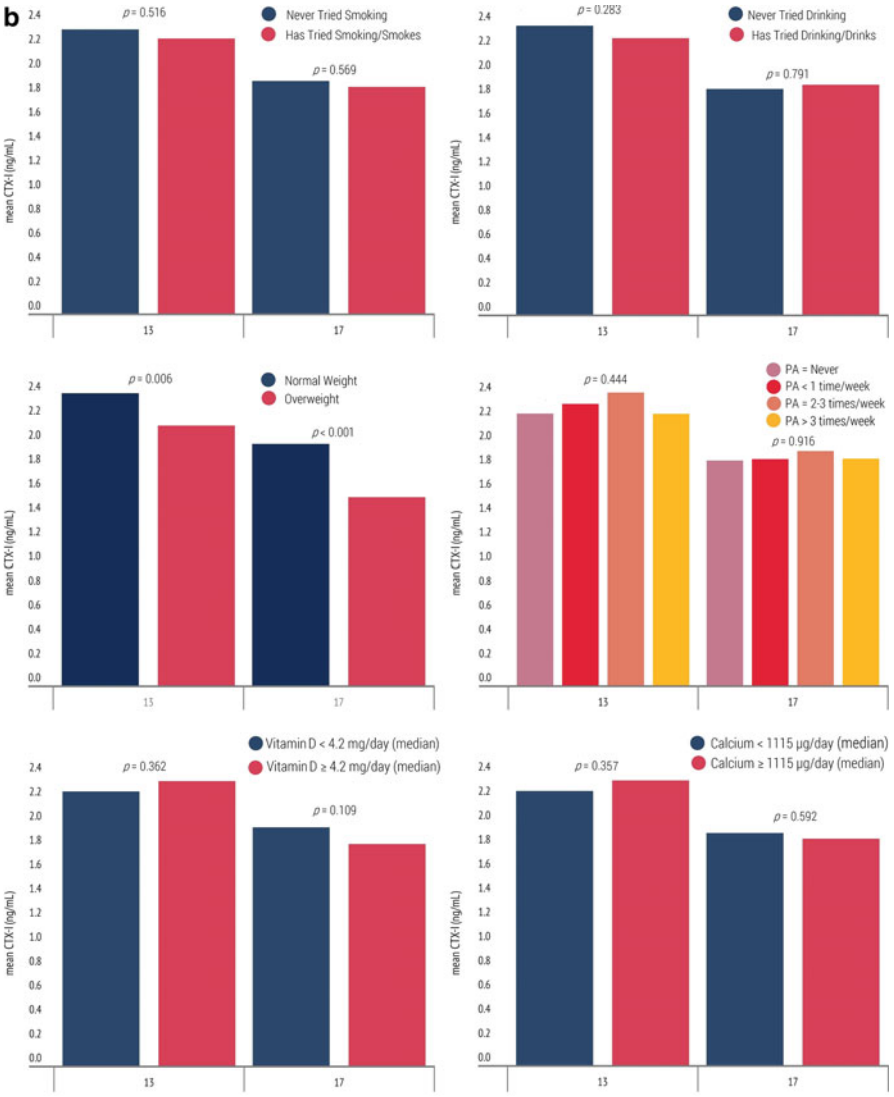

Fig. 6 Cross-linked C-terminal telopeptide of type I collagen (CTX-I) according to modifiable determinants (EPITeen). CTX-I mean values at 13 and 17 years old according to levels of exposure to modifiable determinants. **A**, Girls. **B**, Boys. I, smoking; II, drinking; III, weight; IV, physical activity; V, vitamin D intake at age 13; VI, valcium intake at age 13. Normal weight girls present significantly higher CTX-I levels than overweight girls both at 13 and 17 years old; the same was observed for boys. Girls, $n = 300$; boys, $n = 300$. Unpublished data

for PINP, boys who never smoked or drank presented significantly higher PINP levels (Fig. 7BI, BII). A marginally significant result was found between physical activity and PINP levels at age 17 (Fig. 7BIV).

Potential Applications to Prognosis and to Other Diseases or Conditions

Although bone markers are potentially valuable and useful in clinical practice, they have not been widely used in children. So far, in adults, markers have been used to help in the diagnosis of metabolic bone disorders and to monitor their progression, as well as to monitor the effectiveness of antiresorptive therapies (Nishizawa

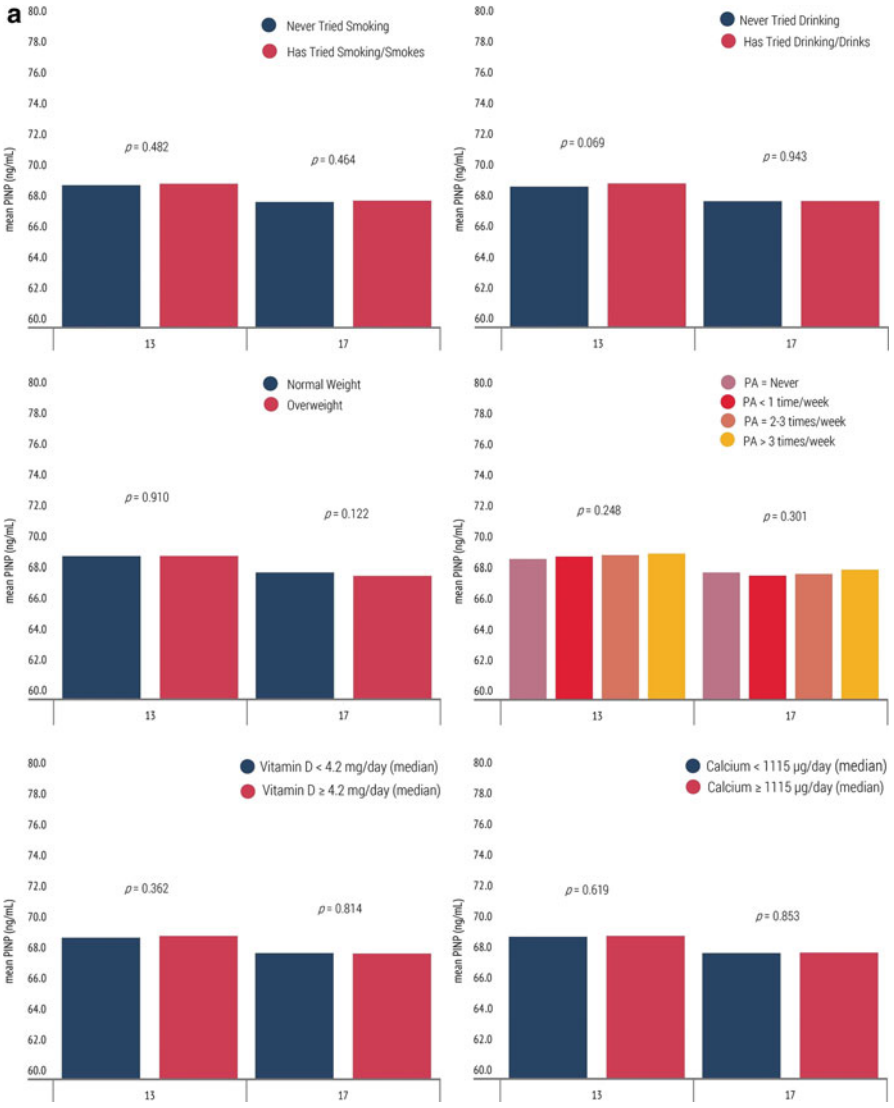

Fig. 7 (continued)

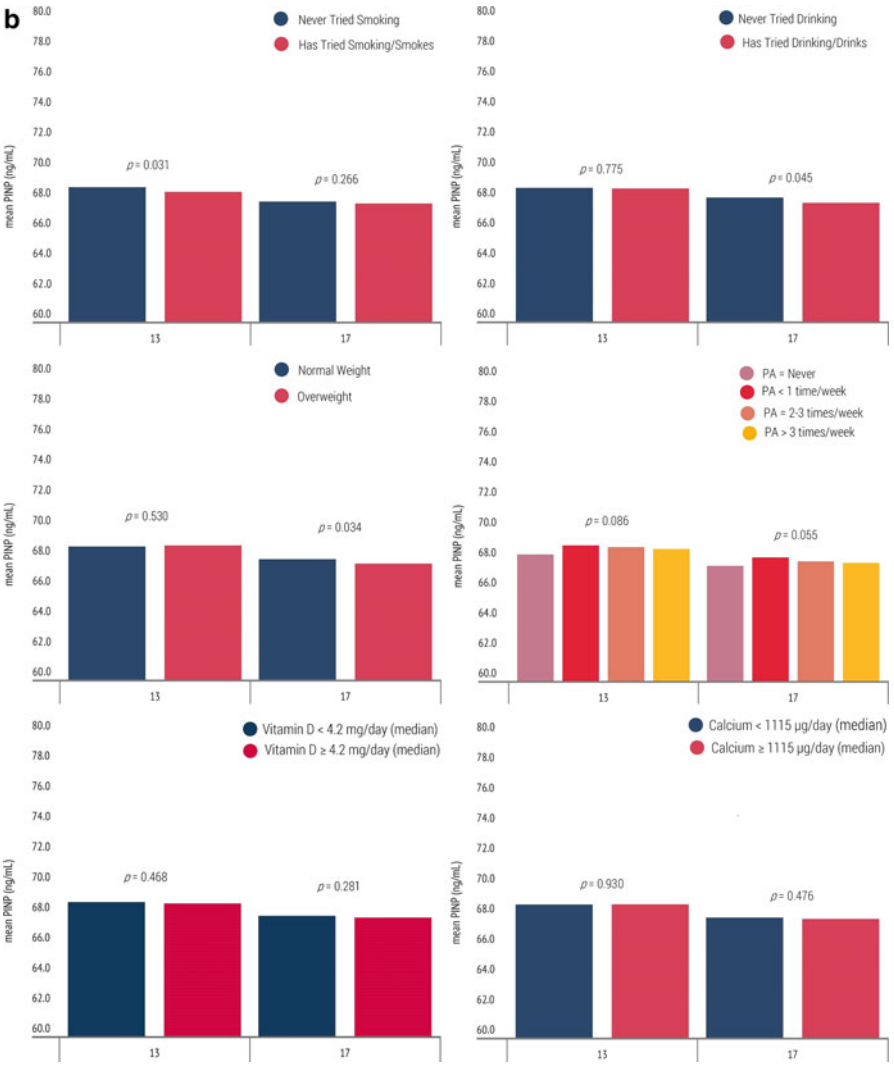

Fig. 7 Procollagen type I amino terminal propeptide (PINP) according to modifiable determinants (EPITeen). PINP mean values at 13 and 17 years old according to levels of exposure to modifiable determinants. **A**, Girls. **B**, Boys. I, smoking; II, drinking; III, weight; IV, physical activity; V, vitamin D intake at age 13; VI, calcium intake at age 13. At 13, boys who never smoked have significant higher PINP values than those that tried smoking or smoke. At 17, for drinking status, the same results are observed. Also at 17 years of age, normal weight boys have higher PINP values than overweight ones. Girls, $n = 300$; boys, $n = 300$. Unpublished data

et al. 2013; Civitelli et al. 2009; Singer and Eyre 2008). Additionally, bone markers have been proposed to predict fracture risk, independently of BMD measured by DXA (Glendenning 2011).

Bone markers have prognostic applications in children, including the assessment of bone metabolism disturbances secondary to established conditions, for instance, osteogenesis imperfecta, juvenile idiopathic arthritis, and chronic kidney conditions. In these conditions, bone markers are also used to monitor the effectiveness of antiresorptive, growth hormone, or corticosteroid therapies on bone metabolism (Rooney et al. 2000; Simm et al. 2011; Boyce et al. 2014; Rijks et al. 2015; Kandemir et al. 2002; Doyon et al. 2015). For this purpose, assessment of bone markers as intermediate endpoints for treatment is advantageous when compared to DXA measures, because BMD or BMC changes take much longer to occur when compared to changes in bone markers (Civitelli et al. 2009).

The use of bone markers in cancer is also a growing area, particularly to detect bone metastases. The applicability of these markers in adults has been widely studied in breast, prostate, multiple myeloma, and lung cancer, since these have higher incidence of bone metastases (Chao et al. 2010). At younger ages, attempts to evaluate the usefulness of bone markers for the diagnosis and prognosis of primary bone tumors have been made. However, the variability associated with age, pubertal stage, and growth velocity of bone markers represents an obstacle to their use in differential diagnosis (Wang et al. 2007). Nevertheless, evidence suggests that these markers may be useful, for instance, to monitor clinical treatment of osteosarcoma in pediatric patients (Ambroszkiewicz et al. 2010).

Bone markers have considerable usefulness in clinical practice both for diagnosis and prognosis purposes. However, until now, they have not been widely used due to their substantial pre-analytical variability, such as individual biological variation. Additionally, variability in laboratory methods and commercial assays also pose a problem. At present, lack of standardized methodologies still precludes the widespread application of bone markers to routine clinical practice (Singer and Eyre 2008).

Summary Points

- Bone fragility results from an accumulation of influences throughout the life course.
- Adolescence is a key period for mineral accrual, through linear growth, modeling, and remodeling of bone tissue.
- Bone metabolism features two fundamental processes: bone formation and resorption.
- Formation and resorption markers are molecules that reflect the rate of bone metabolism. They have been evaluated in the context of drug monitoring, in fracture risk prediction, and in the diagnosis of bone metastasis and to monitor complications of renal disease.
- Markers have been extensively used in adults but much less is known on how they evolve throughout growth and sexual development.

- Knowledge on normative values and trajectories of bone markers in healthy children and adolescents is key for their use as diagnostic and prognostic tools.
- After an increase in bone marker levels in the first decade of life, a gradual decrease is seen in adolescence, with the progression of sexual development and chronological age.
- Sexual dimorphism in bone metabolism is clear from puberty: marker levels peak earlier and decline faster in girls than in boys.
- Bone markers correlate negatively with bone mineral density and content, which translates a deceleration of linear growth and modeling as peak bone mass approaches. However, reported correlations between bone markers and physical properties vary substantially.
- Few studies have addressed modifiable determinants of bone markers, and those that did are far from conclusive in terms of practical meaning.

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Biomarkers of Natural Radionuclides in the Bone and Teeth

5

Biomarkers in Disease: Methods, Discoveries, and Applications

Basim A. Almayahi

Contents

Abstract

This study aimed to estimate the radioactive accumulation of the radionuclides (^{40}K, ^{137}Cs, ^{210}Pb, ^{226}Ra, ^{228}Ra, and ^{228}Th) and the radiobiological analysis of natural alpha emitters in extracted human teeth and animal bones. The natural radionuclides were measured by high-purity germanium spectroscopy and CR-39 alpha-particle track detector in extracted human teeth and animal bones, from people and animals living in different states in the Northern Malaysian Peninsula. The average ^{40}K, ^{137}Cs, ^{210}Pb, ^{226}Ra, ^{228}Ra, and ^{228}Th concentrations in teeth were found to be 12.31 ± 7.27 Bq g^{-1}, 0.48 ± 0.21 Bq g^{-1}, 0.56 ± 0.21 Bq g^{-1}, 0.55 ± 0.23 Bq g^{-1}, 1.82 ± 1.28 Bq g^{-1}, and 0.50 ± 0.14 Bq g^{-1}, respectively. The corresponding concentrations on bones were found to be 3.79 ± 0.81 Bq g^{-1}, 0.07 ± 0.02 Bq g^{-1}, 0.08 ± 0.02 Bq g^{-1}, 0.16 ± 0.04 Bq g^{-1}, 0.51 ± 1.08 Bq g^{-1}, and 0.06 ± 0.02 Bq g^{-1}, respectively. The corresponding radionuclide concentrations on teeth from smokers were higher than those in nonsmokers. The corresponding radionuclide concentrations were higher in female teeth than in

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male teeth. The values of radionuclides in teeth decreased in order $^{40}K > ^{228}Ra > ^{226}Ra$ and $^{210}Pb > ^{228}Th > ^{137}Cs$. The corresponding radionuclide concentrations were higher in teeth than in bones. Significant positive correlations between $p < 0.01$ were found between the radionuclides in bones and teeth.

The lowest and highest alpha emission rates in teeth in the Kedah and Perak states were 0.0080 ± 0.0005 mBq cm^{-2} and 0.061 ± 0.008 mBq cm^{-2}, whereas those of bones in the Perlis and Kedah states were 0.0140 ± 0.0001 mBq cm^{-2} and 0.7700 ± 0.0282 mBq cm^{-2}, respectively. The alpha emission rate for male teeth was 0.0209 ± 0.0008 mBq cm^{-2}, whereas that of female teeth was 0.0199 ± 0.0010 mBq cm^{-2}. The alpha emission rate for teeth is higher in smokers (0.0228 ± 0.0008 mBq cm^{-2}) than in nonsmokers (0.0179 ± 0.0008 mBq cm^{-2}). Such difference was found statistically significant ($p < 0.01$).

Keywords

Biomarkers • Environmental radionuclides • Natural alpha radioactivity • CR-39 detector • Gamma-ray • Bones • Teeth • Smokers

List of Abbreviations

EPR	Electron paramagnetic resonance
HPGe	High-purity germanium
IAEA	International Atomic Energy Agency
ICRP	International Commission on Radiological Protection
MDA	Minimum detectable activity
NMP	Northern Malaysian Peninsular
SSNTD	Solid-state nuclear track detector
UNSCEAR	United Nations Scientific Committee on the Effects of Atomic Radiation
WHO	World Health Organization

Key Facts About Radionuclides

- The gamma radionuclides increase in age possibly because they can be directly inhaled from soil dust, by contrast, ^{40}K and ^{137}Cs decrease with the donor age possibly because of the biological half-time of ^{40}K and ^{137}Cs in the teeth of donors aged $\geq$53 years.
- The corresponding radionuclide concentrations are higher in the male teeth of smokers than in nonsmokers, the radionuclide concentrations on teeth are higher in females than in males, and the overall highest radionuclide concentrations on female teeth can be transferred to the fetus and be dangerous to the latter's health because the fetus mainly receives nutrients from the mother.
- The degree of accumulations of radionuclides in animal bones and human teeth was as follows: human teeth > mice > chicken > fish > cow for ^{228}Th, ^{210}Pb, ^{137}Cs, and ^{228}Ra; human teeth > mice > fish > chicken > cow for ^{40}K and ^{226}Ra.

- The alpha emission rate values are slightly higher in the teeth of smokers than in nonsmokers, and alpha activities of tooth samples are low and no cause dangerous effects on human health.
- These results can be a marker for the transfer rate of radionuclides from soil, water, food, and air to teeth and bones, and this study is useful in determining the alpha and gamma exposure of the population within the study sites for radiological protection and prevention from extreme exposure.
- Teeth and bones are good biological markers for exposure to environmental contamination.

Definition of Words and Terms

(OHAp) and $Ca_{10}(PO_4)_6(OH)_2$	Hydroxyapatite (OHAp) and $Ca_{10}(PO_4)_6(OH)_2$ are used as a model of the inorganic components and is the most important component in bones and dental enamel. The main components of the apatite family (a group of phosphate minerals, usually referring to hydroxylapatite, fluorapatite, and chlorapatite, with high concentrations of OH^-, F^-, and Cl^- ions, respectively, in the crystal) are hydroxyapatite (OHAp, $Ca_{10}(PO_4)_6(OH)_2$), chlorapatite (ClAp, $Ca_{10}(PO_4)_6C_{l2}$), and carbonated apatites and fluorapatite (FAp, $Ca_{10}(PO_4)_6F_2$).
^{137}Cs	Cesium-137, or radiocaesium, is a radioactive isotope of caesium which is formed as one into the more common fission products by the nuclear fission of uranium-235 and other fissionable isotopes in nuclear reactors and nuclear weapons. It is among the most problematic of the short-to-medium-lifetime fission products because it easily moves and spreads in nature due to the high water solubility of caesium's most common chemical compounds, which are salts.
^{210}Pb	The longest lived radioisotopes are ^{205}Pb with a $T_{1/2} = 15.3$ million years and ^{202}Pb with a $T_{1/2} = 53{,}000$ years. Of naturally occurring radioisotopes, the shortest $T_{1/2}$ is ^{210}Pb with a $T_{1/2}$ of 22.20 years.
^{226}Ra	Radium (Ra) has no stable or nearly stable isotopes, and thus a standard atomic mass cannot be given. The longest lived, and most common, isotope of radium is ^{226}Ra with a $T_{1/2}$ of 1600 years. ^{226}Ra occurs in the decay chain of ^{238}U

	(the radium series.) Radium has 33 known isotopes from ^{202}Ra to ^{234}Ra.
^{228}Ra	Radium has 33 known isotopes, with mass numbers from 202 to 234: all of them are radioactive. Four of these – ^{223}Ra ($T_{1/2}$ = 11.4 days), 224Ra (3.64 days), ^{226}Ra (1600 years), and ^{228}Ra (5.75 years) – occur naturally to the decay chains of primordial thorium-232, uranium-235, and uranium-238 (^{223}Ra from uranium-235, ^{226}Ra from uranium-238, and the other two from thorium-232).
^{228}Th	^{228}Th is an isotope of thorium with 138 neutrons. It was once named radiothorium, due to its occurrence in the disintegration chain of thorium-232. It has a $T_{1/2}$ of 1.9116 years.
^{40}K	Potassium-40 is a radioactive isotope of potassium which has a very long half-life of 1.251×10^9 years. It makes up 0.012% (120 ppm) of the total amount of potassium found in nature.
Biological half-time	The biological half-life time is the time an organism takes to eliminate one-half the amount of a compound or chemical on a strictly biological basis.
DNA	DNA, or deoxyribonucleic acid, is the hereditary material in humans and all organisms. Nearly every cell in a person's body has the same DNA. DNA is located in the cell nucleus, but a small amount of DNA can be found in the mitochondria (mitochondrial DNA or mtDNA).
Half-life	$\mathbf{T_{1/2}}$ is the amount of time required for the amount of something to fall to half its initial value. The term is commonly used in nuclear physics to describe how quickly unstable atoms undergo radioactive decay, but it is used for discussing any type of exponential decay.
NaOH	The CR-39 detectors were then etched under controlled conditions in NaOH etchant solution. Etching to expose radiation damages is typically performed using solutions to caustic alkalis such as sodium hydroxide, regularly at high temperatures for a number of hours.
PADC-TASTRACK CR-39	A substance frequently used in SSNTDs is polyallyl diglycol carbonate (PADC), also known as Tastrak, CR-39 and CR39.

	$C_{12}H_{18}O_7$. CR-39 is a transparent, colorless, and inflexible plastic with the chemical formula $C_{12}H_{18}O_7$ (polycarbonate of allyl diglycol). The monomer contains two of the following functional groups: $CH_2 = CH\text{-}CH_2$.
SSNTDs	A solid-state nuclear track detectors (etched track detector or a dielectric track detector, DTD) is a section of a solid material (photographic emulsion, crystal, glass, or plastic) uncovered to nuclear radiation, etched, and inspected microscopically. SSNTDs are frequently used to learn more about cosmic rays, long-standing radioactive elements, radon concentration in houses, and the age of geological samples.

Introduction

Experimental studies on naturally occurring environmental radionuclides contribute to the current knowledge of geological processes and atmospheric phenomena. Environmental radionuclides can be divided into three groups: radionuclides of primordial origin, decay products of primordial radionuclides, and radionuclides generated by human activities. Radionuclides are commonly found in rocks, soil, and water that make up the planet, buildings, and homes. Soil is an important environmental material used as raw material and product for buildings, roads, playgrounds, and land filling. The functioning of terrestrial Earth systems continuously affects man and induced global changes. These effects are reflected by changes in the ecological functions of terrestrial systems such as surface water bodies (flood prevention), soils (fertility for food production), and groundwater (drinking water supply) (Froehlich 2010). Contamination of land and water can result from deposition of waste material originally introduced into the atmosphere, discharge directly released into surface or subsurface waters, and wastes in or on the ground. The primary reason for this concern is that radioactive contamination of the environment may result in exposure to humans. Groundwater or surface water erosion coupled to subsurface aquifers, soils, and the atmosphere may eventually mobilize ground contaminants. Atmospheric pollutants become deposited on soils or surface waters. The mechanisms for removing contaminants from soil involve transportation by water through a sequence of processes, including surface runoff and leaching into soil water that seeps into streams. Humans are surrounded by natural radioactivity. Radioactive isotopes are present in human bodies, houses, air, water, and the ground (Eisenbud and Gesell 1997; Henriksen and Maillie 2003). ^{226}Ra and ^{228}Ra isotopes are considered important natural radionuclides of the ^{238}U and ^{232}Th series, respectively. The distributions of ^{238}U and ^{230}Th in bones are similar and somewhat lower in concentrations than ^{232}Th.

These radioisotopes chemically and physiologically behave like calcium and are inclined to concentrate on bones and teeth (Dewit et al. 2001). In the physical environment, radioisotopes form soluble compounds and can contaminate underground reservoirs, soils, plantations, food sources and, consequently, the human food chain. ^{226}Ra is a well-known "bone-seeking" radionuclide that accumulates in calcareous tissues because of its chemical similarity to calcium (Whicker and Schultz 1982). The movement toward ^{226}Ra through food chains is considered "moderate," and 99% of the ^{226}Ra body content is in human bones (ICRP Committee II 1959). ^{226}Ra is expected to be present in bone tissues because this radionuclide tends to be moderately transferable in the physical environment.

^{226}Ra is taken up by vegetation from the soil and efficiently assimilated from the gut when ingested by animals (NCRP 1999). The retention of ^{226}Ra in bones is high and accumulates over time for conditions of chronic intake. The levels measured in human bones from several urban locations have ranged from 0.03 Bq kg^{-1} to 0.37 Bq kg^{-1} (Eisenbud and Gesell 1997). Among the radionuclides derived from anthropogenic sources, ^{137}Cs is the major source but represents only between 2% and 3% of the total gamma-ray dose rate. ^{137}Cs is derived from land mammal consumption. Studies have shown that the distribution of ^{137}Cs in surface soils is attributed to differences in climate and topographical situations pertinent to a location (Tang et al. 2002). The International Commission on Radiological Protection assumes that ^{137}Cs and ^{40}K are homogenously distributed throughout the body of an organism. ^{137}Cs and ^{40}K are generally not considered bone seekers; however, the accumulation of their radionuclides causes internal radiation exposure in humans and animals (Hong et al. 2011). The population's primary source of gamma exposure is naturally occurring radionuclides, particularly ^{40}K, which is found in soil, water, and food. The largest doses and risks of radionuclides in the physical environment are from naturally occurring radionuclides, mostly ^{210}Po, ^{210}Pb, ^{228}Th, and ^{232}Th. When lead to enter the circulatory system, it is distributed among the body, accumulates in bones, and lingers from several years to decades (Brito et al. 2005). Gamma-emitting radionuclides such as ^{137}Cs, located in a source organ, can irradiate target organs long distances away within the body. The Chernobyl accident in 1986, along with other localized releases from various sources, contaminated the terrestrial and marine environment (Strand et al. 2002). Indigenous populations that are basically dependent on wildlife are vulnerable to radioactive exposure from ingestion of terrestrial and marine food (Cooper et al. 2000; Macdonald et al. 2007; Hamilton et al. 2008). ^{210}Pb is a bone-seeking element with a long physical and biological half-life of 20 years. ^{210}Pb is a beta emitter, but 4.25% of the decays are accompanied by the emission of a gamma photon with an energy of 47 keV (Wahl 1998), which can be measured in HPGe detector. The uptake routes of ^{210}Pb and ^{226}Ra are through plants and from the soil, respectively (Pietrzak-Flis and Skowronska-Smolak 1995). ^{238}U, ^{232}Th series, ^{40}K, and their daughters are the main contributors to internal radiological doses received by humans from the natural radioactive sources.

The internal radiation doses vary from the radionuclide concentration in the air, soil, water, foodstuff, and rate of intake. This concentration differs from one human group to another. Therefore, radionuclides accumulated in teeth depend on the transfer rate of radionuclides from air, soil, and water to the teeth. WHO (2001) showed that the human body incorporates 90 g of uranium by ingestion or inhalation of air and that 66% of this element is found in bones (59.4 mg), 16% in the liver (14.4 mg), 8% in the kidney (7.2 mg), and 10% in other tissues. UNSCEAR (2008) showed that the concentration of ^{232}Th in the human body ranges from 6 mBq kg^{-1} to 24 mBq kg^{-1}.

As part of evaluating a problem of radiological health, the ^{226}Ra uptake from public water supplies to teeth was studied as part of an evaluation of a radiological health problem (Samuels 1964). The results of the study by Samuels (1966) focused on radium metabolism as it relates to teeth. The bone is providing a socket to surround and support the roots of the teeth. The main core of each tooth is composed of dentine and a highly sensitive calcified tissue. Dentine is covered with enamel at the crown portion and cementum at the roots. Teeth are an extension of the skeleton and accumulate contaminating stable and radioactive bone-seeking metals that enter the human body. The alpha particle levels in teeth are 10–15 times greater than elsewhere in the body, and the distribution of activity shows considerable structure. Studies on autopsy tissues have mainly aimed at determining the levels for the principal alpha emitters present, namely, ^{210}Po, ^{226}Ra, and ^{238}U. These nuclei are found in human tissues with typical activity values of 1 mBq g^{-1} to 3 mBq g^{-1}, 0.2 mBq g^{-1} to 0.3 mBq g^{-1}, and 0.05 mBq g^{-1}, respectively. Considering ^{210}Po occurs to the end of the ^{222}Rn decay chain, ^{222}Rn exposure is considered another potential source of increased ^{210}Po and ^{210}Pb of teeth. Clemente et al. (1982, 1984) found a relationship between the ^{210}Pb content in teeth and the exposure to radon and radon daughters and determined that the incremental ^{210}Pb teeth content is due to excessive exposure to radon daughters, especially in people living near the Bad Gastein Spa in Austria. Henshaw et al. (1994) studied the uptake and microdistribution of the natural alpha radioactivity in human teeth using a CR-39 detector and found the transference of ^{226}Ra in the pulp and dentine teeth through systemic blood circulation. Yamamoto et al. (1994) measured the activity of ^{226}Ra in the human teeth and bones of the inhabitants at several locations in Japan. They reported the mean ^{226}Ra activity values of 0.23 and 0.41 mBq g^{-1} in the teeth and bones, respectively. The mean ^{226}Ra concentration (0.51 mBq g^{-1}) in teeth samples obtained from Tokyo is less than the concentration (1.11 mBq g^{-1}) reported on vertebral bone samples of this city. The radionuclide concentrations on teeth and bones are good indicators of the levels of radioactive contamination in the human body. Teeth have been widely used as markers for biological exposure to environmental pollution (Budd et al. 1998; Appleton et al. 2000; Carvalho et al. 2000; Gomes et al. 2004). CR-39 detectors are used in radon detection and alpha-particle spectroscopy to measure the natural alpha radioactivity in human and animal tissues (Henshaw 1989; Henshaw et al. 1994). Aghamiri et al. (2006) showed that the teeth

of the inhabitants of Iran have more ^{226}Ra radioactivity concentration than those of the inhabitants of low-radiation areas because of higher ^{226}Ra content of soil and water. Radionuclide concentrations on teeth are good indicators of radiation contamination in the human body. However, studies on this subject are limited. A possible reason is that earlier investigators have assumed that the uptake and distribution of radionuclides in teeth are similar to those in bones. As such, these investigators have neglected the study of the distribution of radionuclides and the histopathologic changes in dental tissues. In addition, analysis of teeth and bones may aid in the investigation into low doses and low dose-rate exposure of radionuclides potentially emitted throughout the process of nuclear energy production (Culot et al. 1997) because the roots of teeth exhibit a bone-like structure (Gulson and Gillings 1997). The radionuclide concentrations of bones samples of other countries were measured by HPGe spectroscopy. Navarro et al. (1998) measured ^{137}Cs concentration on bird bones in Spain. The mean activity concentration of ^{137}Cs was found to be 64 Bq kg^{-1} (47–590 Bq kg^{-1}). Chibowski and Gładysz (1999) calculated the activity concentrations of ^{137}Cs (1.23 Bq kg^{-1}) and ^{40}K (56 Bq kg^{-1}) in poultry bones in Poland using a germanium cylindric detector (JGC-13). Mietelski et al. (2000) measured ^{137}Cs, ^{226}Ra, and ^{40}K concentrations in the bones of wild, herbivorous animals in Poland. The gamma activities ranged from 1.9 Bq kg^{-1} (deer) to 6 Bq kg^{-1} (boar) for ^{137}Cs, just below 10 Bq kg^{-1} for ^{226}Ra and from 18 Bq kg^{-1} (elk) to 30 Bq kg^{-1} (boar) for ^{40}K. Mietelski et al. (2001) measured ^{228}Th concentration on the bones of wild herbivorous animals (deer, roe-deer, boars, and elks). The activity of ^{137}Cs ranged from <0.01 Bq kg^{-1} to 8 Bq kg^{-1}. Gaca et al. (2005) measured the ^{137}Cs and ^{40}K concentrations on mammals (rodents, insectivores, and owls) bones in Poland.

The ^{137}Cs activities ranged from <1.2 Bq kg^{-1} to 10 Bq kg^{-1} (rodents), <0.9 Bq kg^{-1} to 5 Bq kg^{-1} (insectivores), and <1.3 Bq kg^{-1} to <7 Bq kg^{-1} (owls). The ^{40}K activities ranged from <10 Bq kg^{-1} to 75 Bq kg^{-1} (rodents), <14 Bq kg^{-1} to 50 Bq kg^{-1} (insectivores), and 39 Bq kg^{-1} to 93 Bq kg^{-1} (owls). Mietelski et al. (2006) calculated the activity concentrations of ^{137}Cs (0.8 Bq kg^{-1} to 51 Bq kg^{-1}) and ^{40}K (11 Bq kg^{-1} to 482 Bq kg^{-1}) in the bones of eagles in Poland. Rabitsch and Pichl (2008) investigated the ^{137}Cs and ^{40}K activity levels of the ribs and sternum of a cow in Austria. The results showed a nonuniform distribution of ^{137}Cs and ^{40}K in different skeletal bones and their adherent tissues in cattle. They found that the ^{137}Cs and ^{40}K concentrations varied up to a factor of 2.5 in the ribs. The minimum and maximum values of ^{137}Cs were 30 and 332 Bq kg^{-1}, respectively, whereas the corresponding ^{40}K values were 21 and 132 Bq kg^{-1}, respectively. Komosa et al. (2009) measured ^{137}Cs, ^{226}Ra, ^{210}Pb, and ^{40}K concentrations in the bones of prey birds in Poland. ^{137}Cs, ^{226}Ra, ^{210}Pb, and ^{40}K concentrations ranged within 1.1–27, 12–74, 11–260, and 100–350 Bq kg^{-1}, respectively. Hong et al. (2011) analyzed the anthropogenic ^{137}Cs and natural radionuclides (^{40}K, ^{210}Pb, and ^{226}Ra) of caribou bone in Canada. The mean activity of ^{137}Cs, ^{210}Pb, ^{226}Ra, and ^{40}K was found to be less than the detection limits, 246, 7, and 78 Bq kg^{-1}, respectively.

The alpha particle activities in the tooth and bone samples of other countries were measured using a CR-39 detector. Henshaw et al. (1988) measured the average ^{210}Po and ^{226}Ra activity values in human bones in the UK, which were 1.46 Bq kg^{-1} and 0.003 Bq kg^{-1}, respectively. Henshaw (1989) found that the average ^{226}Ra activity values in human teeth in the UK were found to be 9 Bq kg^{-1}. James et al. (2004) showed that ^{210}Pb-supported ^{210}Po accumulates on the outer enamel of teeth. Furthermore, the activity concentration on this surface best harbors a measure of cumulative exposure. The mean alpha activity concentration measured in human teeth obtained in the UK was 5 Bq kg^{-1}. O'Donnell et al. (1997) measured the levels of plutonium and the total alpha emitters in human teeth in the UK and obtained 5 mBq kg^{-1} and 7 Bq kg^{-1}, respectively. Bunzl and Kracke (1983) measured the plutonium level in the human bone collected in Germany and obtained 4 mBq kg^{-1}. Henshaw et al. (1994) studied the uptake and distribution of natural alpha radionuclides and found that the ^{226}Ra activity concentration on fetal teeth in the UK was 2.05 Bq kg^{-1}. All natural elements that have atomic numbers $Z > 82$ are emitters of radiation; ^{232}Th, ^{235}U, and ^{238}U series are the natural radioactive series.

The ^{232}Th series begin with ^{232}Th nuclei and end with ^{208}Pb. The ^{235}U series begin with ^{235}U nuclei and end with ^{207}Pb, which is a stable element. The ^{238}U series begin with ^{238}U nuclei and end with ^{206}Pb, which is a stable element. Experience with the dangers of radioactive materials preceded by discovery of the phenomenon by many years (Eisenbud and Gesell 1997). The discovery of the radioactivity phenomenon led to testing for soil and water, which were then shown to contain high concentrations of natural radioactive elements. Radiation exposure varies from the environment depending on radioisotope concentrations (e.g., ^{210}Pb, ^{137}Cs, ^{228}Th, ^{226}Ra, ^{228}Ra, ^{234}Th, ^{214}Pb, ^{214}Bi, ^{212}Pb, ^{212}Bi, ^{208}Ti, ^{235}U, and ^{40}K) in soil. Contamination in land and water is caused by deposition of waste materials originally introduced into the atmosphere, discharge of waste placed on the ground into surface or subsurface water waste placed on the ground, with ground contamination eventually mobilized by groundwater or erosion. Elements that are easily absorbed on sediments and suspended matter include Cs, Mn, Fe, Co, and the actinide elements. Artificial radionuclides behave in a similar manner, and pollution of the food chain all over the world by radioactive elements produced during atmospheric nuclear weapon testing may have occurred during the past half-century. For example, ^{90}Sr and ^{137}Cs fallout from nuclear weapon testing in the upper 10 cm of soil was found to occur for the first few years after deposition (Eisenbud and Gesell 1997). Erosion by rainfall or runoff is one mechanism to transport of radionuclides inserted into the soil surface. Erosion by wind causes resuspension of pollutants that has settled on the surface. The suspended radionuclides settle on plants, water, and soil and eventually enter the food chain. These radionuclides may be deposited on skin and clothing, allowing them accessed to the human body by inhalation and ingestion, or directly affect the human body by external exposure, as shown in Fig. 1.

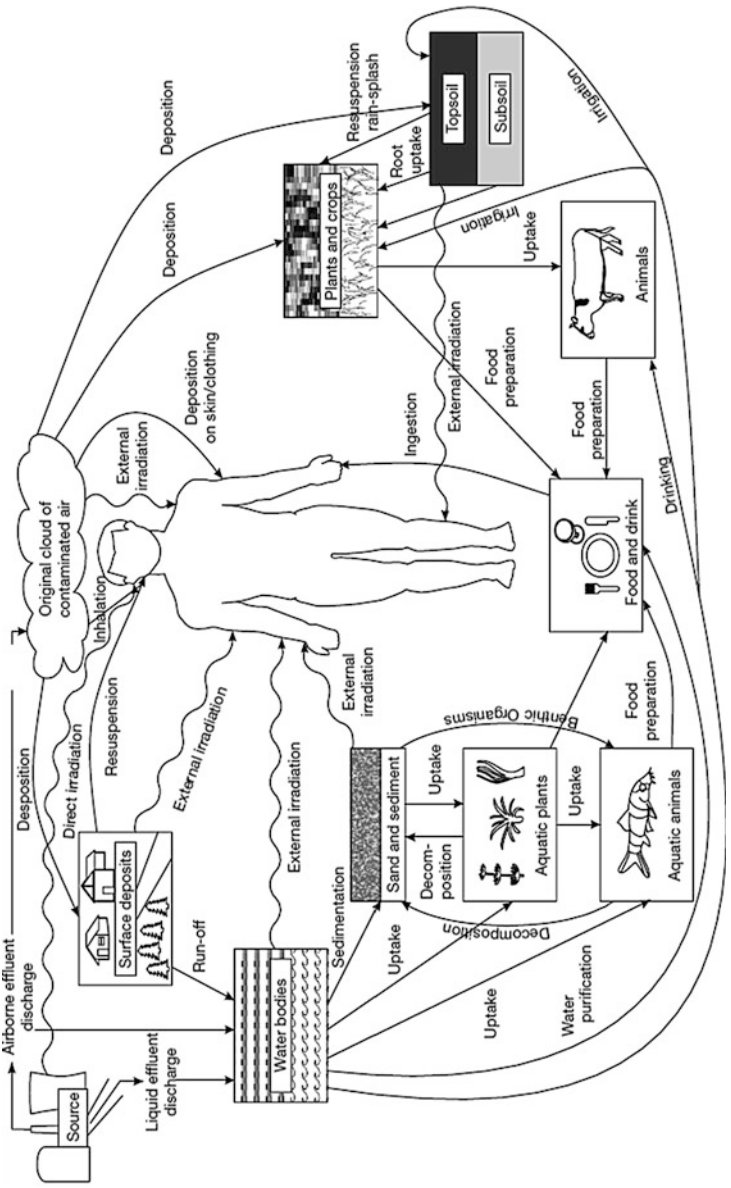

Fig. 1 The possible pathways to exposure to members of the public as a result of discharges of radioactive material to the environment. Main environment pathways to human radiation exposure are shown the suspended radionuclides settle on plants, water, soil, and eventually enter the food chain. These radionuclides may be deposited on skin and clothing, allowing them access to the human body by inhalation and ingestion (Chart is from IAEA (2005), with permission from the Publisher)

Potential Applications to Prognosis, Other Diseases, or Conditions

Natural radionuclides accumulate in the human body after intake of food, water, and air. The two main exposure pathways are inhalation and ingestion. Inhalation may be an important exposure pathway to the public. Ingestion of contaminated soil, water, and food can also lead to internal exposure. Radiation damages to the lung caused by inhalation of radon in the air may increase the risk of cancer. Exposure to increased levels of radium for long periods can lead to death and severe health problems such as cancer (especially bone, liver, and breast cancer), anemia, fractured teeth and cavities, as well as cataracts. Radiation exposure to radionuclides within the body depends on the radionuclide type, inhaled or ingested, and duration of stay in the body (biological half-time). The biological half-life time is the time an organism takes to eliminate one-half the amount of a compound or chemical on a strictly biological basis. Hydroxyapatite (OHAp) and $Ca_{10}(PO_4)_6(OH)_2$ are used as a model of the inorganic components in bones and teeth. Radium can replace calcium and enter the hydroxyapatite structure (Yamamoto et al. 1994). Lead may be stored for long periods in mineralizing tissue (teeth and bones). The stored lead could be released again into the bloodstream, especially in times of calcium stress. Bone-to-blood lead mobilization increases in periods of pregnancy, lactation, menopause, physiologic stress, chronic disease, hyperthyroidism, kidney disease, broken bones, and advanced age, all which are exacerbated by calcium deficiency. The bones and teeth of adults contain about 94% of their total lead body. Lead in mineralizing tissues is not uniformly distributed, and it tends to accumulate in bone regions undergoing the most active calcification at the time of exposure. Biomarker represents the event or changes into human biological systems as results of exposure or diseases. Exposures can be estimated by measuring pollutant levels in various body tissues (hair, blood, urine, nails, bone, and teeth) as biomarkers (WHO 2008). Biomarker represents a means to monitor environmental exposure by characterizing an individual's dose (mass of contaminant over some time) of a contaminant from sources of exposure. Biomarker is believed to be predictive of health effect than external measures of exposure. DNA can itself be altered or its expression can change as results of exposure to a genotoxic agent, and then white blood cell DNA may be used for biomarkers of exposure to genotoxic agents (Carrano and Natarajan 1988). Interpretation of genotoxic response is complicated because DNA damages can result in cell death or removal of the marker by DNA repair or may alter cell functions (Perera 1987). A DNA adduct is an abnormal part of DNA which is bonded with contaminants. DNA adducts are used as biomarker of exposures. In cancer risk assessment, biology markers can utilize for hazard identification, characterization, and for exposure assessment (Figs. 2, 3, 4, and 5).

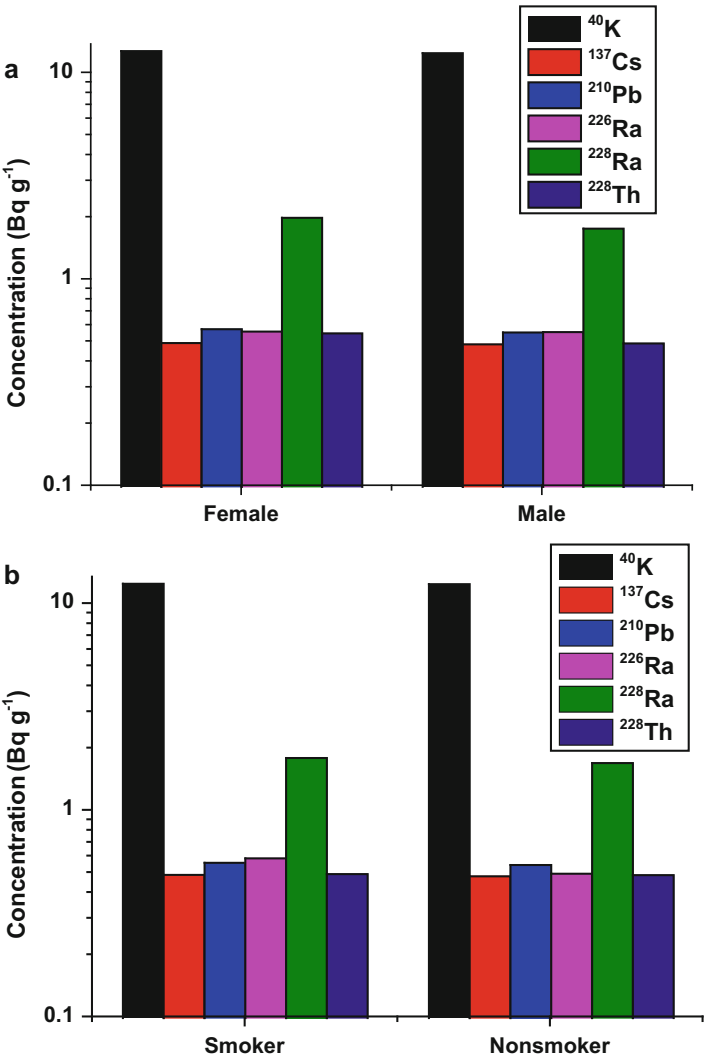

Fig. 2 Average radionuclide concentrations on teeth samples as a function of gender (**a**) and males in relation to smoking (**b**). The concentrations of ^{40}K, ^{137}Cs, ^{210}Pb, ^{226}Ra, ^{228}Ra, and ^{228}Th in male teeth were found to be 12.62, 0.48, 0.56, 0.55, 1.76, and 0.49 Bq g^{-1}, respectively, whereas female teeth resulted in slightly higher concentrations on 12.71, 0.49, 0.58, 0.56, 1.99, and 0.55 Bq g^{-1}, respectively (**a**). This difference may be due to biological retention of radionuclides was greater in females than in males. Besides, physiological differences between females and males have some sort of effect, may be excreted estrogens hormones in females and the fertility period of females. With regard to smoking, the comparison between smokers and nonsmokers was plotted for males only because most females in Malaysia do not smoke. Women who smoke are also not likely to admit that they do because smoking among females is socially rejected by most Malaysians. With the exception of ^{40}K, the radionuclide concentrations on teeth were higher in smokers (12.43, 0.49, 0.56, 0.58, 1.79, and 0.49 Bq g^{-1} for ^{40}K, ^{137}Cs, ^{210}Pb, ^{226}Ra, ^{228}Ra, and ^{228}Th, respectively) than in nonsmokers (13.01, 0.48, 0.54, 0.49, 1.69, and 0.48 Bq g^{-1}, respectively) (**b**) (Data are from Almayahi et al. (2014a), with permission from the Publisher)

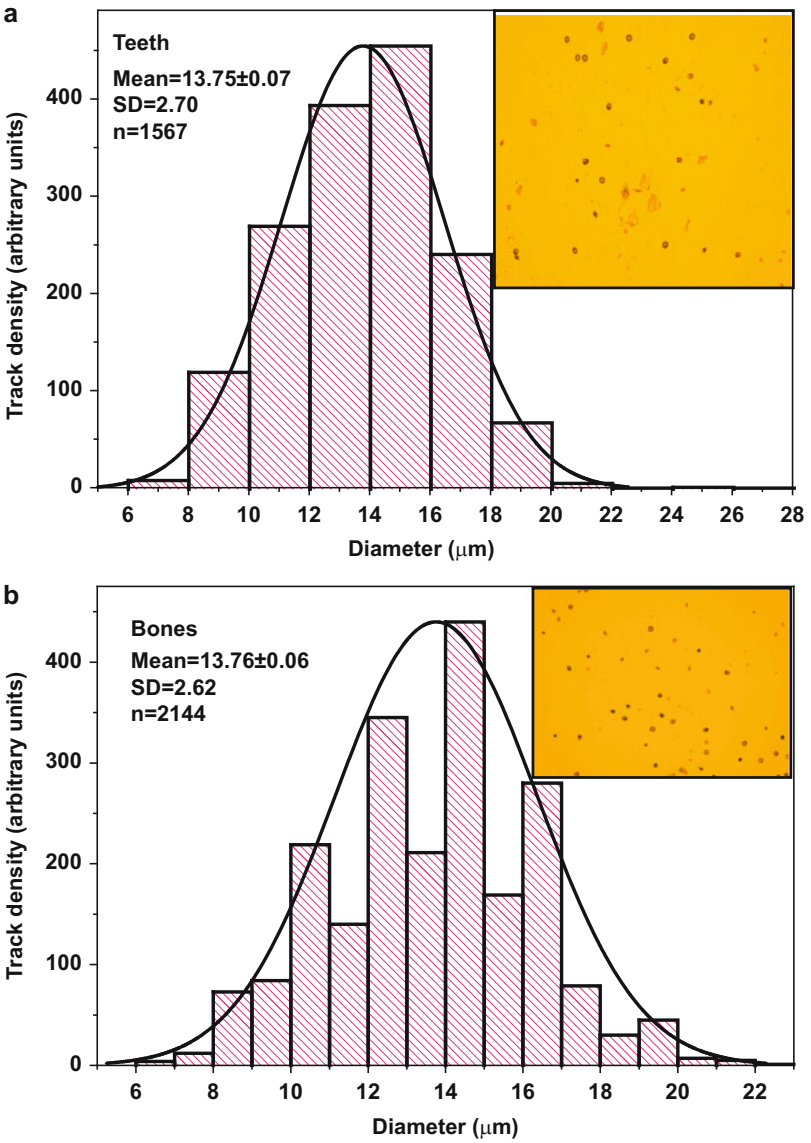

Fig. 3 Track distribution and Gaussian fit for (**a**) teeth sample and (**b**) bones sample. Track diameter histogram distribution of teeth and bone samples of etching time (10 h). The mean value of track diameters in the teeth samples (13.75 ± 0.07 μm) is similar to that in the bone samples (13.76 ± 0.06 μm) (Data are from Almayahi et al. (2014b), with permission from the Publisher)

The relationship between the biomarkers and the carcinogenic response should be established (Committee on Carcinogenicity 2013). Teeth constitute a unique medium for assessment of past exposure. Depending on the tooth type and part of

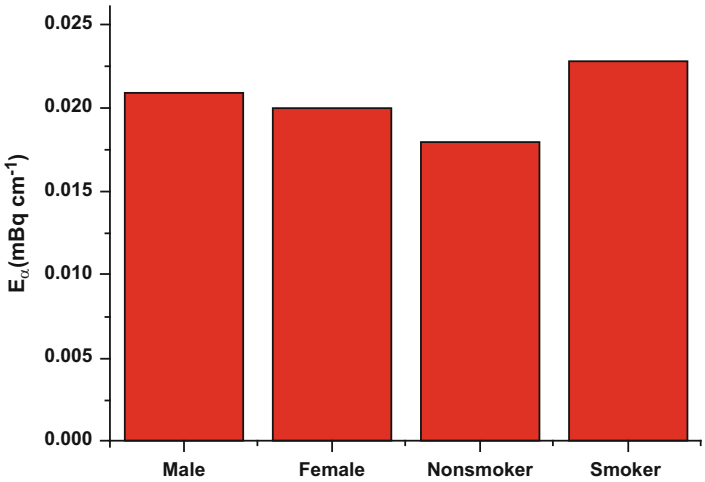

Fig. 4 Alpha emission rates in teeth samples as a function of gender and males in relation to smoking. The alpha emission rate of teeth was found slightly higher in smokers (0.0228 ± 0.0008 mBq cm^{-2}) than in nonsmokers (0.0179 ± 0.0008 mBq cm^{-2}). The average alpha emission rate of male teeth was 0.0209 ± 0.0008 mBq cm^{-2}, whereas that in female teeth was 0.0199 ± 0.0010 mBq cm^{-2} (Data are from Almayahi et al. (2014b), with permission from the Publisher)

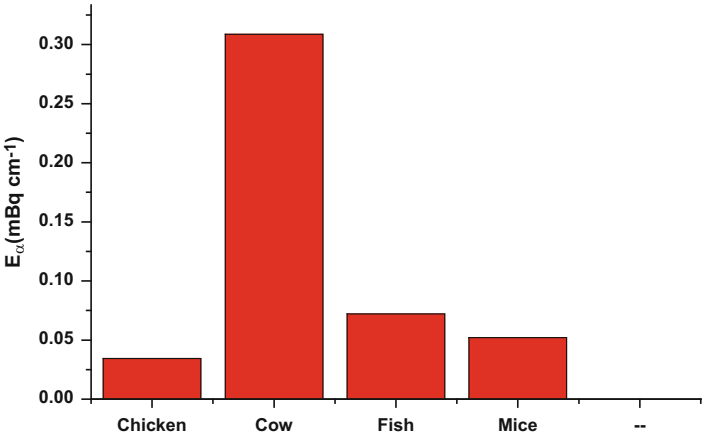

Fig. 5 Average alpha emission rates in bones (cow, fish, chicken, and mice). The average alpha emission rates in cows, fish, chicken, and mice bones, obtained using the CR-39 detector. The degrees of accumulation of alpha emitters in the animal bones (cow, fish, chicken, and mice) are ranked as follows: cow > fish > mice > chicken. These differences were statistically significant at the 0.001 level (Data are from Almayahi et al. (2014b), with permission from the Publisher)

Table 1 Analysis of variance results of radionuclides concentrations with donor age. Indicate there are no statistically significant differences in correlations between radionuclides concentrations (^{210}Pb, ^{137}Cs, ^{226}Ra, ^{228}Ra, and ^{40}K), except for ^{228}Th which is statistical significant at the 0.01 level. Then, ^{210}Pb, ^{137}Cs, ^{226}Ra, ^{228}Ra, and ^{40}K have the same effect at all ages, except ^{228}Th (Data are from Almayahi et al. (2014a), with permission from the Publisher)

	Source of variation	Sum of squares	Degrees of freedom	Mean square	*F* ratio	*F* probability
^{137}Cs	Between groups	1.193	39	0.030	0.945	0.570
	Within groups	1.360	42	0.032		
	Total	2.554	81			
^{40}K	Between groups	1285.376	39	32.958	1.120	0.358
	Within groups	1235.444	42	29.415		
	Total	2520.820	81			
^{210}Pb	Between groups	1.709	39	0.04382	0.648	0.913
	Within groups	2.839	42	0.06758		
	Total	4.548	81			
^{226}Ra	Between groups	1.977	39	0.050	0.868	0.671
	Within groups	2.454	42	0.058		
	Total	4.431	81			
^{228}Ra	Between groups	27.680	39	0.710	0.939	0.577
	Within groups	31.736	42	0.756		
	Total	59.417	81			
^{228}Th	Between groups	7.578	39	0.194	2.474	0.002
	Within groups	3.298	42	0.078		
	Total	10.876	81			

the tooth, one can reconstruct early childhood exposures to bone-seeking elements, such as lead (Rabinowitz et al. 1989). Electron paramagnetic resonance (EPR) tooth dosimetry has been used to validate dose models of acute and chronic radiation exposure (Kleinerman et al. 2006). The bone represents both past exposure to bone-seeking elements and source for future internal exposure to these elements. The element concentrations on bones represent long-term exposure and storage of contaminants. Teeth are very good indicators of bone-seeking radionuclides that are transferred from the mother's blood plasma to the fetus. An analysis of milk teeth allows a retrospective measurement of prenatal and early postnatal trace-element uptake during a critical period of child development, as the deciduous enamel forms from week 13 in utero up to 9 months postnatal (Dolphin et al. 2005) (Tables 1 and 2).

Table 2 Summary statistics and 95% confidence intervals of mean radionuclides concentration in teeth for each age interval. The average value of activity concentration and 95% confidence intervals for the mean radionuclides concentrations for each age interval (Data are from Almayahi et al. (2014a), with permission from the Publisher)

							95% confidence interval for mean	
Radionuclides	Age interval	Mean	St. deviation	Min.	Max.	St. error	Lower bound	Upper bound
^{137}Cs	15–28	0.5075	0.1168	0.33	0.76	0.0291	0.4453	0.5697
	29–43	0.4724	0.2020	0.22	0.92	0.0404	0.3890	0.5558
	44–53	0.5320	0.1893	0.19	0.90	0.0423	0.4434	0.6206
	54–68	0.4590	0.1762	0.16	0.86	0.0384	0.3788	0.5393
	Total	0.4904	0.1776	0.16	0.92	0.0196	0.4514	0.5294
^{40}K	15–28	12.9844	3.7852	4.57	17.91	0.9463	10.9674	15.0013
	29–43	12.7500	6.8414	4.87	33.71	1.3683	9.9260	15.5740
	44–53	12.4810	5.4966	3.06	21.98	1.2291	9.9085	15.0535
	54–68	11.9914	5.4676	3.84	22.28	1.1931	9.5026	14.4803
	Total	12.5359	5.5786	3.06	33.71	0.6161	11.3101	13.7616
^{210}Pb	15–28	0.4706	0.1512	0.27	0.75	0.0378	0.3901	0.5512
	29–43	0.5552	0.2631	0.20	0.96	0.0526	0.4466	0.6638
	44–53	0.6410	0.2221	0.30	0.97	0.0496	0.5370	0.7450
	54–68	0.5733	0.2592	0.10	0.99	0.0565	0.4553	0.6913
	Total	0.5643	0.2369	0.10	0.99	0.0261	0.5122	0.6163

^{226}Ra	15–28	0.4606	0.2292	0.15	0.95	0.0572	0.3385	0.5827
	29–43	0.6456	0.2122	0.30	1.17	0.0424	0.5580	0.7332
	44–53	0.5830	0.2055	0.29	0.89	0.0459	0.4868	0.6792
	54–68	0.5119	0.2607	0.14	0.94	0.0569	0.3932	0.6306
	Total	0.5600	0.2339	0.14	1.17	0.0258	0.5086	0.6114
^{228}Ra	15–28	1.5731	0.5634	0.64	2.40	0.1409	1.2729	1.8733
	29–43	2.0256	1.0723	0.83	5.65	0.2145	1.5830	2.4682
	44–53	2.0310	0.8173	0.80	3.74	0.1827	1.6485	2.4135
	54–68	1.7005	0.7500	0.72	3.42	0.1637	1.3591	2.0419
	Total	1.8554	0.8565	0.64	5.65	0.0945	1.6672	2.0436
^{228}Th	15–28	0.4688	0.2070	0.18	0.92	0.0517	0.3584	0.5791
	29–43	0.5720	0.5250	0.16	2.48	0.1050	0.3553	0.7887
	44–53	0.4975	0.2614	0.22	1.21	0.0584	0.3752	0.6198
	54–68	0.5029	0.3314	0.11	1.12	0.0723	0.3520	0.6537
	Total	0.5160	0.3664	0.11	2.48	0.0407	0.4355	0.5965

Summary Points

- The uptake of natural radioactivity in teeth and bones is estimated; the teeth and bone samples are analyzed using a high-purity semiplanar germanium (HPGe) detector manufactured by DSG detector systems GmbH.
- The alpha emission rate in bone and individual teeth was determined using SSNTD, α-sensitive plastic track detector (PADC-TASTRACK CR-39, Track Analysis Systems Ltd, Bristol, UK) with the chemical composition ($C_{12}H_{18}O_7$), density 1.32 g cm^{-3}, and 2 cm × 2 cm dimensions.
- A total of 157 extracted human tooth samples were collected from 14 clinics and hospitals distributed across four states (Penang, Perlis, Kedah, and Perak) in Malaysia, and a total of 73 animal bones (cow, fish, chicken, and mice bones) were collected from Penang, Perlis, Kedah, and Perak in Malaysia (17 sites).
- Gamma-ray peaks of 47 keV (^{210}Pb), 352 keV (^{226}Ra), 583 keV (^{208}Tl peaks indicating ^{228}Th), 609 keV (^{226}Ra), 662 keV (^{137}Cs), 911 keV, 969 keV (^{228}Ac peaks indicating ^{228}Ra), and 1462 keV (^{40}K) were found in the tooth and bone samples.
- The variation in the gamma-ray of ^{210}Pb, ^{137}Cs, ^{228}Th, ^{226}Ra, ^{228}Ra, and ^{40}K concentrations and alpha emission rates in the teeth and bones collected from Penang, Perlis, Kedah, and Perak may depend on the transfer rate of radionuclides from soil, water, food, and air to the teeth and bones.
- The results demonstrate the considerable influence of smoking, age, and gender on the ^{210}Pb, ^{137}Cs, ^{228}Th, ^{226}Ra, ^{228}Ra, and ^{40}K contents in human teeth.
- The correlation between the donor age and the observed radioactivity in teeth was performed and linearly fitted according to Eq.

$$y = \alpha x + \beta$$

where y is the radionuclides concentrations, x is the donor age, and α and β are coefficients.

- It is concluded that ^{210}Pb, ^{137}Cs, ^{228}Th, ^{226}Ra, ^{228}Ra, and ^{40}K concentrations in teeth higher than in bones and significant positive correlations at $p < 0.01$ were found between the radionuclides in bones and teeth, and statistical analysis of variance results of the radionuclide concentrations for bones from Perak, Perlis, and Kedah states are not significantly different at the 0.05 level. The results demonstrated no considerable effect of smoking, age, and gender on the alpha emission rates in human teeth, no statistically significant difference exists between the alpha emission rate and age intervals, and the alpha particles have the same effect at any age.
- The degree of accumulation of alpha particles of bones is ranked as follows: cow > fish > mice > chicken, and these differences were statistically significant at the 0.001 level. The degree of accumulation of the alpha particles of tooth samples is ranked as follows: Perak > Perlis > Kedah; the differences were statistically significant at the 0.001 level; by contrast, the alpha emission rate in bone samples

is ranked as follows: Perlis < Perak < Kedah, and these differences were statistically significant at the 0.05 level.

- The mean activity concentrations of ^{40}K, ^{137}Cs, ^{210}Pb, ^{226}Ra, ^{228}Ra, and ^{228}Th in teeth samples from NMP were found to be 12.31 Bq g^{-1}, 0.48 Bq g^{-1}, 0.56 Bq g^{-1}, 0.55 Bq g^{-1}, 1.82 Bq g^{-1}, and 0.50 Bq g^{-1}, respectively, and the highest concentrations of radionuclides were above the detection limits, except for ^{137}Cs and ^{226}Ra were ≤MDA.

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Parathyroid Hormone (PTH) Assays and Applications to Bone Disease: Overview on Methodology

6

Ashutosh Kumar Arya and Naresh Sachdeva

Contents

Abstract

Biologically active parathyroid hormone (PTH) is an 84-amino acid-long hormone that mediates calcium homeostasis and is responsible for normal functions of bone and kidney. Accurate assessment of circulating PTH is essential for the diagnosis of hyperparathyroidism, bone diseases, and chronic kidney disease (CKD). Immunoassays have been extensively used for the measurement of PTH in biological fluids for more than 60 years. Besides several pre-analytical

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factors, differences in types of immunoassays influence estimation of PTH in clinical samples. First-generation PTH radioimmunoassays use a single antibody which detects fragmented PTH and does not entirely reflect levels of biologically active PTH. Second-generation assays use two antibodies directed against distinct N-terminal (12–20/26–32) and C-terminal (39–84) epitopes, respectively, to detect intact PTH (iPTH), but these antibodies also cross-react with N-terminally truncated PTH fragments. To avoid such cross-reactivity, the third-generation assays came which use an N-terminal antibody directed against the first 4 amino acids of PTH with identical C-terminal antibody, as used in second-generation assays. Both second- and third-generation assays are equally good in diagnosis of primary hyperparathyroidism (PHPT) and CKD patients. Third-generation assays are superior in performing intraoperative PTH measurement for predicting successful parathyroidectomy in PHPT patients. The ratio of PTH levels determined by the third-generation over the second-generation assay is another useful tool in detecting parathyroid carcinoma and severe PHPT. Relative measurements of PTH (1–84) and PTH (7–84) in clinical samples may provide insights in their biological roles in CKD. Recently, developed liquid chromatography-assisted mass spectrometry-based PTH assays are more accurate in quantitation of PTH, but require sophisticated instrumentation and expertise. The utility of such advanced assays to differentiate various modified forms of PTH (phosphorylation, oxidation, etc.) needs to be further explored in bone-related pathologies.

Keywords

Parathyroid hormone • Intact PTH • Amino-PTH • Immunoassay • Primary hyperparathyroidism • Bone • Renal osteodystrophy • Chronic kidney disease

List of Abbreviations

CAP	Cyclase-activating PTH
CIP	Cyclase-inactivating PTH
CKD	Chronic kidney disease
CLIA	Chemiluminescence immunoassay
C-PTHR	C-terminal PTH receptor
ECLIA	Electrochemiluminescence immunoassay
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IO-PTH	Intraoperative PTH
IRMA	Immunoradiometric assay
LC-MS/MS	Liquid chromatography-assisted mass spectrometry
n-oxPTH	Non-oxidized PTH
N-PTH	Amino-PTH
oxPTH	Oxidized PTH

PHP	Pseudohypoparathyroidism
PHPT	Primary hyperparathyroidism
PTH	Parathyroid hormone
PTH1R	PTH/PTHrP 1 receptor
RIA	Radioimmunoassay
SHPT	Secondary hyperparathyroidism

Key Facts

Parathyroid Hormone

- Secretory product of chief cells of parathyroid glands.
- JB Collip (1925) prepared the parathyroid gland extract and was the first to demonstrate physiological role of parathyroid glands in calcium regulation.
- In 1963, Berson et al. developed the first RIA-based immunoassay for estimation of human and bovine PTH in biological fluids.
- Complete amino acid sequence of human PTH was identified in the year 1978 by Keutmann et al.
- PTH (1–34) is biologically equivalent to the intact PTH (1–84) molecule (Potts et al. 1971).
- PTH is very fragile and gets degraded rapidly in the liver and kidney.
- In circulation, PTH is present as intact as well as different fragments like N-terminal PTH fragments (4–84, 7–84, 10–84, and 15–84), mid-region fragments, and C-terminal fragments (34–84, 35–84, 37–84 and 45–84).

Monoclonal Antibodies

- Polyclonal antibodies are those antibodies that originate from different B-cell clones that recognize diverse epitopes of a specific antigen.
- The major disadvantages of polyclonal antibodies are batch-to-batch variation in production and multiple specificities.
- Monoclonal antibodies are antibodies that are produced from a single clone of cells and have specificity for a single epitope of an antigen.
- The technique for synthesis of monoclonal antibodies is first devised by Köhler and Milstein in the year 1975.
- Monoclonal antibodies are widely used in diagnostic and research assays as well as in immunotherapy for diseases.

Biological Modifications of Parathyroid Hormone

- Posttranslational modifications are mainly limited to N-terminal region of PTH; only two amino acids methionine and serine are known to be posttranscriptionally modified.
- Methionine presents at the 8th and 18th position in PTH and can be single or double oxidized, but methionine 8 oxidizes more slowly than methionine 18.
- Oxidized PTH variations are most common modifications and but are biologically less active than non-oxidized PTH variants.

- Serine at the 17th position can get phosphorylated and form another N-terminal molecular form, amino-PTH.

Primary Hyperparathyroidism

- A major pathological condition of parathyroid glands and the third most common endocrine disease.
- PHPT is defined as hypercalcemia in association with inappropriately elevated concentrations of PTH.
- PHPT is most commonly observed in postmenopausal women and men over the age of 50 years, with women predominating by approximately 3:1.
- Classical clinical features of PHPT were first described by Fuller Albright (1948) as a pentad of bone pains, kidney stones, abdominal groans, psychic moans, and fatigue overtones.
- With recent advances in screening programs and assays, it is now possible to diagnose PHPT much earlier before it develops into symptomatic disease without obvious signs of hypercalcemia or PTH excess.
- In many developing countries such as India, Brazil, and China, most of the PHPT cases are still presented with classical clinical symptoms.

Teriparatide

- Teriparatide is a recombinant PTH (1–34) molecule.
- It is an anabolic agent for the treatment of osteoporosis in postmenopausal women and men with primary or hypogonadal osteoporosis who are at high risk of fracture.
- Once daily administration of teriparatide preferentially stimulates bone formation in trabecular and cortical bone.
- Teriparatide activates the osteoblasts (bone-forming cells) more than osteoclasts (bone-resorbing cells).

Definition of Words and Terms

Amino-PTH	A newly described molecular form of PTH with intact first 5-amino acids at N-terminal and phosphorylation of serine residue at 17th position.
Chronic kidney disease	Chronic kidney disease is a pathological condition of kidney characterized by progressive loss of kidney function and can lead to renal failure, which can be fatal without dialysis or kidney transplant.
Electrochemiluminescence immunoassay	It is in vitro chemiluminescent assay that is initiated electrically and generates highly reactive species from stable precursors on the surface of the electrode. The highly reactive species reacts with a chemiluminescent substrate to produce light.

Epitope	The epitope is a part of a molecule or an antigen that is being recognized by a specific antibody or T-cell receptor, also known as antigenic determinant.
Intraoperative PTH	Intraoperative PTH refers to the PTH levels measured after minimum invasive parathyroidectomy to predict the success of surgery which is indicated by at least 50% decrease in PTH concentration within 10 min. from pre-incision PTH value.
Mass spectrometry	Mass spectrometry is an analytical technique to quantify the amount or type of molecules in a sample on the basis of the mass-to-charge ratio. The function of the mass-to-charge ratio for ion signal is plotted and known as mass spectrum.
Parathyroid hormone/parathyroid hormone-related peptide 1 receptor	PTH1R is a G-protein-coupled transmembrane receptor. Its activity is mediated by Gsα and activates adenylate cyclase and phosphatidylinositol–calcium second messenger system.
PTH (7–84)	It is the biologically active fragment of PTH (1–84) which starts from the 7th amino acid (lysine); binds to a different receptor, the C-terminal PTH receptor; and performs antagonistic function to PTH.
PTH IS 95/646	It is the recombinant human PTH (1–84) considered as the first international standard of human PTH (1–84) recognized by the World Health Organization.
Radioimmunoassay	It is an analytical technique to quantify the amount of a molecule using radiolabeled or radiotracer antibody directed against a specific region (epitope) of that molecule.
Renal osteodystrophy	Renal osteodystrophy is a metabolic bone disorder of kidney which is mainly due to failure of kidney to maintain normal levels of calcium and phosphorus in circulation. Renal osteodystrophy includes osteitis fibrosa cystica (OFC), adynamic bone disease, and osteomalacia.

Introduction

Parathyroid hormone (PTH), a major regulator of mineral homeostasis, is produced by chief epithelial cells of the parathyroid glands. It is synthesized as a 115 amino acid pre-pro-PTH precursor molecule which undergoes two successive proteolytic

cleavages leading to the formation of pro-PTH and its biologically active form, intact PTH (iPTH) (84 amino acids), respectively. PTH molecules are stored in secretory granules. In response to external stimuli, especially, decreased extracellular ionized calcium concentration, these secretory granules fuse with cell membrane to release PTH in the bloodstream (Brown 2001). Circulatory PTH increases plasma calcium concentration by stimulating bone resorption and calcium absorption from the kidney. These actions are mediated through the PTH/PTHrP receptor or PTH1 receptor (PTH1R), a G-protein- coupled receptor that is ubiquitously expressed and abundantly found in the bone and kidney. PTH also increases the activation of 1-alpha hydroxylase enzyme in the kidney that converts inactive vitamin D to active vitamin D (1,25-dihydroxyvitamin D) to increase calcium absorption in the small intestine. PTH also decreases the phosphorus concentration in circulation by decreasing phosphorus absorption in the proximal tubule of the kidney. Overall, PTH has a positive role in bone formation, and recombinant form of PTH is used as a therapeutic anabolic agent in the treatment of bone diseases.

Along with iPTH, different carboxyl (C)-terminal fragments are also released into circulation. These fragments are formed within parathyroid glands as well as in the liver and are eliminated by glomerular filtration and subsequent degradation in the kidney. In circulation, PTH undergoes proteolytic cleavage into C-terminal fragments, mid-region fragments, and N-terminal fragments. Being a small polypeptide, PTH is highly fragile in nature. The circulating half-life of PTH (1–84) in blood is only 2–4 min; however, C-terminal fragments, especially PTH (7–84) known as "N-terminal truncated PTH," have five to ten times longer half-life and make approximately 80% of circulating immunoreactive PTH in normal individuals. Recent discoveries such as identification of PTH (7–84) as another biologically active form of PTH, identification of C-terminal PTH receptor (C-PTHR), and an amino molecular form of PTH, amino-PTH (N-PTH), which is distinct from PTH (1–84), have made the understanding of PTH physiology more complex. These findings suggest multifaceted roles of PTH and its forms and their possible involvement in pathophysiological conditions.

Altered synthesis and secretion of PTH is involved in various diseases of the parathyroid glands, bone, and kidney, like hyperparathyroidism, hypoparathyroidism, and metabolic bone diseases. Therefore, accurate and reproducible measurement of PTH in clinical samples is an important prerequisite for diagnosis and treatment of these diseases.

Evolution of PTH Assays

Immunoassays are being used for the measurement of PTH in biological fluids for more than 60 years. An immunoassay is a technique to detect or quantify any analyte in blood or body fluids employing the inherent property of an antibody to bind to a specific region of the molecule, termed as epitope. Estimation of PTH in biological fluids has witnessed a constant evolution and has become much easier following the development of antibodies directed against specific epitopes on the hormone

together with a better understanding of the physicochemical properties of PTH. The first PTH immunoassay was developed in the 1960s based on the radioimmunoassay (RIA), generally referred as first-generation PTH immunoassays (Berson et al. 1963). Initial RIA for PTH used single polyclonal antibody directed against the PTH extracted from bovine (porcine) glands and later from human parathyroid adenoma glands. Identification of amino acid sequences of PTH in year 1978 demonstrated that initial PTH RIA detected only the mid-region or C-terminal fragments of PTH. However, quantification of PTH by one RIA assay differed from another assay under similar experimental/clinical conditions. In the early 1980s, N-terminal PTH RIA was developed that could quantify PTH (1–34) which is biologically equivalent to PTH (1–84). The N-terminal assays had better predictive values in disease setup compared to mid-region and C-terminal assays since it could detect biologically active part of PTH. C-terminal assays had limited clinical value as C-terminal fragments have little biologically activity and low clearance rate from the kidney. Major limitations of these assays were low affinity and sensitivity of antibodies, availability of the antisera, and limited applicability to bone disease in CKD patients. These assays were in use till the 1980s.

In the late 1980s, immunometric assays were introduced for most of the hormonal estimations. These assays use two different antibodies directed against two distinct epitopes, one against the N-terminal and another against C-terminal region of the PTH (Nussbaum et al. 1987). One antibody is known as capture antibody bound to solid support and second antibody bound to isotope labeled and used as signal antibody. Thus, these assays measure only "intact (1–84) PTH." By definition, iPTH assay means measurement of a PTH molecule that binds and activates PTH1R. Later, radioisotopes were replaced by enzyme-based chemiluminescent substrates (luminol/peroxidase system) or electrochemiluminescent compounds (ruthenium/tripropylamine). These second-generation assays proved to be superior to RIA and also had better reproducibility in CKD patients and demonstrated good correlation with bone histomorphometric findings from renal osteodystrophy patients. However, these assays overestimate PTH levels in secondary hyperparathyroidism (SHPT) and CKD patients as C-terminal fragments, especially PTH (7–84) are cleared from kidney and form major part of total circulating PTH (90–95%). To overcome this problem and to measure only iPTH, in the year 1999, an immunoassay was developed using signaling antibody directed against the first 4 amino acids of the PTH molecule. These assays are known as "whole" or "bioactive" or "bio-intact" PTH assays. These assays detect only those PTH molecules which have these N-terminal 4-amino acids and are thought to theoretically measure biologically active PTH. In the year 2005, D'Amour et al. demonstrated that an amino-terminal form of PTH (N-PTH) cross-reacted with the antibodies in the third-generation assays. By using HPLC purification, it was identified that N-PTH has a phosphorylated serine amino acid at 17th position. Third-generation PTH assays are expensive and not widely used in diagnostic laboratories. Further, to quantify only PTH (1–84), liquid chromatography-assisted mass spectrometry (LC-MS/MS)-based PTH estimation methods have been developed (Kumar et al. 2010; Lopez et al. 2010). MS-based PTH measurement utilizes immunoaffinity

extraction with a C-terminal anti-PTH capture antibody, followed by digestion with trypsin, and finally quantification by LC-MS/MS method. These mass spectrometry-based PTH assays have high sensitivity and provide accurate estimation of iPTH molecule.

Earlier studies have shown that PTH can be oxidized at methionine residues present at position 8 and 18. The oxidized forms of PTH are biologically inactive but cross-react in third-generation assays. The oxidized PTH can be separated from non-oxidized PTH (active form) by using a specific antibody directed against oxidized PTH and further quantification by LC-MS/MS methods (Hocher et al. 2013). A timeline showing the development of different PTH assays is shown in Table 1.

With increasing knowledge of the heterogeneous forms of PTH, an optimized assay which is highly specific, sensitive, accurate, and inexpensive for the measurement of iPTH and its different molecular forms in routine clinical samples is thus yet to be achieved, and the field is still open for more research.

Pre-Analytical Factors Affecting Estimation of PTH

The prime requirement for any successful biochemical estimation is the provision of optimal pre-analytical conditions as there are several factors that may influence the analysis of samples as well as the final interpretation of results in context of the disease. Intact PTH is a relatively unstable molecule with varying secretory amounts and very low levels in circulation. PTH levels in circulation also vary during different pathophysiological conditions. In order to evolve consistency in PTH estimation, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) has established a PTH-working group (Hanon et al. 2013) with following aims:

(a) To prepare good practice recommendations for the optimal pre-analytical handling of patients and samples
(b) To encourage worldwide implementation of recombinant international standard for PTH (IS 95/646) by manufacturers of diagnostic kits
(c) To establish inter-assay limits for harmonization among the different platforms
(d) To develop a standard reference measurement procedure for PTH
(e) To prepare a panel of reference plasma samples to establish standard reference intervals

Sample Type, Storage, and Stability

Ever since the inception of the PTH assays, measurements have been performed in whole blood, plasma, or serum separated in different blood collection tubes like plain tubes, tubes containing anticoagulants (potassium-ethylenediaminetetraacetic acid (EDTA), citrate, or lithium heparin), and/or gel-separating tubes. Studies have demonstrated that PTH is stable at room temperature in EDTA, whole blood for up to

Table 1 History of development of PTH assays

	Year	Group	Description
First-generation immunoassays Radioimmunoassay (RIA)			
1	1963	Berson et al.	Developed polyclonal antibody directed against PTH extracted from bovine (porcine) glands
2	1970	Fischer et al.	Developed antibodies against synthetic PTH peptides used for iodine labeling
3	1971	Patts et al.	Discovered that synthetic PTH (1–34) (amino-terminal fragment) is biologically active and functionally equivalent to PTH (1–84)
4	1977	Desplan et al.	Developed amino-terminal specific RIA PTH assay
5	1978	Keutmann et al.	Described complete amino acid sequence of human PTH
6	1980	Manning et al.	Developed first antisera against peptide extracted from human parathyroid adenoma glands
Second-generation immunoassays "Two-site" or "sandwich" intact PTH assays Two antibodies directed against distinct amino- and carboxyl-terminal regions			
7	1987	Nussbaum et al.	Developed first two-site immunoradiometric assay (IRMA) for PTH measurement
8	1996	Brossard et al.	Described a non-PTH (1–84) circulatory form, PTH (7–84) that interferes with intact PTH assays
9	2001	Divieti et al.	Identified carboxyl-terminal specific receptor (C-PTHR) that binds to PTH (7–84)
Third-generation immunoassays "Whole" or "bioactive" or "bio-intact" PTH immunoassay Signal antibody specifically directed against epitope of 1–4 amino acids of PTH			
10	1999	Scantibodies Clinical Laboratories	Developed the first third-generation assay, cyclase-activating PTH (CAP) assay
11	2005	D'Amour et al.	Identified amino-PTH (N-PTH) by HPLC that showed reactivity to third-generation assays
12	2006	Zhang et al.	Immunoextracted PTH fragments and analyzed by LC fractionation and then by MALDI-TOF MS in normal and diseased individuals sera
13	2010	Kumar et al.	Developed PTH LC-MS/MS assay using isotopically labeled recombinant PTH (1–84) as internal reference standard
14	2010	Lopez et al.	Developed quantitative PTH LC-MS/MS assay using individual isotopically labeled tryptic peptides as standard reference
15	2012	Hocher et al.	Generated antibody directed against oxidized PTH (inactive PTH) and measured oxidized PTH by nano-LC-MS/MS

24 h, and in plasma up to 48 h, but in case of clotted blood and serum, PTH suffers significant decline in immunoreactivity within 2–3 h after venipuncture (Stokes et al. 2011; Oddoze et al. 2012; Glendenning et al. 2002). We and others have also reported that PTH starts degrading at room temperature but remains within

acceptable range until 24 h (3.5–7.3% degradation), whereas after 48 h, samples show 9–14.8% degradation (Omar et al. 2001; Arya and Sachdeva 2014). Very few studies have directly compared the use of lithium heparin whole blood and EDTA whole blood and showed similar stability of PTH (Levin and Nisbet 1994; Teal et al. 2003; Stokes et al. 2011).

Stability of PTH has also been analyzed at lower temperatures such as, 4 °C, −20 °C, and −80 °C. Most studies compared the EDTA plasma and serum for PTH stability at 4 °C and showed that PTH is more stable in EDTA plasma (Parent et al. 2009; Oddoze et al. 2012; Evans et al. 2001). Combining all the available data, it can be concluded that at 4 °C PTH is stable in EDTA plasma for at least 72 h, serum for at least 24 h, and EDTA whole blood for at least 18 h. Few studies have assessed the stability of PTH in plasma or serum kept at −20 °C and −80 °C. In one contrasting report, Cavalier et al. (2007) have shown that when samples are kept at −20 °C, stability of PTH in serum and EDTA plasma is similar, but after 5 days, degradation of PTH was higher in EDTA plasma. Brinc et al. (2012) reported that PTH is unstable in serum and degraded up to 16% in 2 months when kept at −80 °C. Studies involving long-term storage have shown that PTH is stable in serum for 14 days and EDTA plasma for 12 months when kept at −20 °C and −80 °C, respectively, as analyzed by third-generation PTH assays (Inaba et al. 2004; Cavalier et al. 2012). Different commercial assays also influence data on PTH stability. At −80 °C, PTH was stable till 9 months in serum and only 2 months in EDTA plasma when analyzed by the DiaSorin Liaison method, but when assessed by the Roche Elecsys system, PTH was found to be stable until 2 years in EDTA plasma (Cavalier et al. 2007, 2009).

Therefore, based on the previous data and PTH working group of IFCC, blood samples for routine diagnostic PTH measurements should be collected in EDTA tubes; after venipuncture, plasma should be separated immediately and measured as soon as possible or should be stored at 4 °C and analyzed within 72 h (Hanon et al. 2013; Arya and Sachdeva 2014; Cavalier et al. 2007).

Sampling Site

The PTH working group strongly recommends that blood samples should be collected from the same site (central or peripheral) for comparison, both within and between individuals as central venous PTH concentrations were higher than peripheral venous concentrations.

Sampling Time

PTH has a circadian rhythm with higher secretion in the night (12–6 am) and nadir (8–10 am) in the morning with another slight higher secretion in the afternoon (Fig. 1). Studies have shown the variations in secretory nadir, but none have compared the effect of sampling time on the diagnostic accuracy of PTH measurement. Most of the studies suggest pre-noon time as the best suitable period for

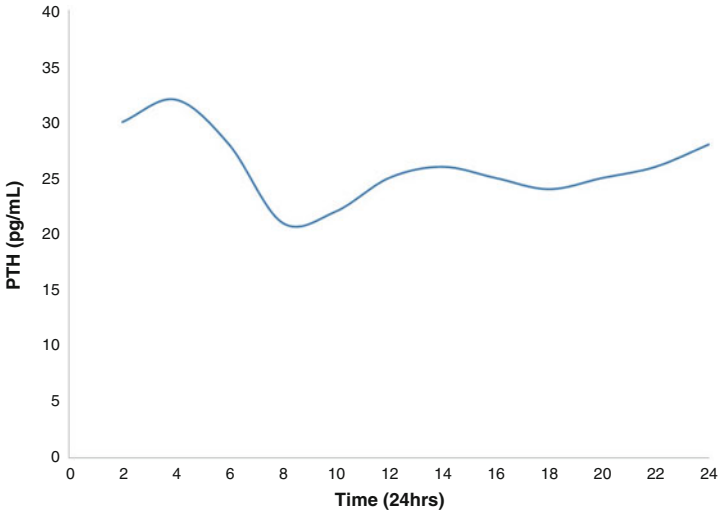

Fig. 1 Schematic representation of diurnal circadian rhythm of PTH secretion. Secretion of PTH is highest during sleep (12–6 AM), then decreases in morning (8–10 AM) and then again slightly increases in the afternoon (12–4 PM)

collecting blood samples for PTH measurements (Herfarth et al. 1992; Ahmad et al. 2003; Logue et al. 1989). Recently, Hanon et al. (2013) have suggested a broader time period between 10 am and 4 pm for collecting blood for PTH estimations. Calcium and phosphorus concentrations are influenced by meals (Statland et al. 1973) and that may finally alter the PTH concentration in the circulation; so, for practical purpose, blood should be collected in fasting condition and preferably before 10 am.

Other Factors

Few studies have tried to assess the effect of other factors like seasonal variation, fasting, diet and sleep, etc. PTH concentrations are influenced by season variability and are higher in the winter season than summer (Woitge et al. 2000). Continuous 2 days or more fasting also leads to loss of circadian rhythm (Schlemmer and Hassager 1999), whereas PTH levels are not affected by sleep (Chapotot et al. 1996). Intake of vegetarian diet also increases the PTH concentrations in blood (Moe et al. 2011).

Several areas associated to affect the PTH stability are still open for research like comparison of the third-generation with second-generation assays to check stability of PTH in EDTA samples, requirement of standard reference range data, and further comparison related to the effect of freezing temperature on the PTH stability by both second and third-generation assays.

Table 2 Optimum pre-analytical factors for estimation of PTH from blood specimens (Arya and Sachdeva 2014; Hanon et al. 2013; Cavalier et al. 2007; Woitge et al. 2000; Stokes et al. 2011; Oddoze et al. 2012)

Recommendation	Factor	Guidelines
# 1	**Type of sample tube**	Blood samples for PTH measurement should be taken in EDTA tubes, and plasma should be separated as soon as possible after venipuncture
# 2	**Sample storage**	PTH should be measured as soon as possible or should be stored at 4 °C and analyzed within 72 h
# 3	**Sampling site**	Blood for PTH measurements should be drawn from same (central or peripheral) site every time
# 4	**Time of collection**	Blood samples should be collected preferably before 10 AM, and results should be interpreted against a reference interval derived for this sampling time
# 5	**Season variations**	Consider season, vitamin D status, and latitude while reporting PTH levels, and interpret the results accordingly in individuals
# 6	**Transfer of samples**	Use pre-chilled EDTA tube after drawing blood transport on ice (without freezing), and use a cold centrifuge to separate plasma
# 7	**Protease inhibitors**	If available, addition of protease inhibitors (e.g., aprotinin) in sample tube before blood collection improves stability of PTH

Based on the data available till date, optimum pre-analytical conditions for PTH measurement have been summarized in Table 2.

PTH Immunoassays: Methods

First-Generation Immunoassays

The first-generation PTH immunoassays were based on competitive radioimmunoassay (RIA). In PTH RIA, radiolabeled hormone (^{125}I-PTH) and unlabeled PTH compete for binding to an immobilized antibody or antisera generated against PTH. Antiserum is prepared from immunized rabbits or guinea pigs with affinity-purified bovine PTH. Purified PTH is also used as standard as well as for the radioiodination of PTH.

Procedure: Antisera against PTH and a fixed amount of radiolabeled hormone (^{125}I-PTH) are incubated in a test tube followed by addition of a specific quantity of unlabeled PTH in samples. The unlabeled PTH competes with the ^{125}I-PTH for binding to the anti-PTH antibody and displaces a proportional amount of ^{125}I-PTH from the antibody. Following washing and precipitation of antigen antibody complexes, the amount of radioactivity in the tube is measured in a gamma counter. This process is continually repeated, using progressively higher concentrations of unlabeled PTH. Radioactivity remained or bound for each standard (**B**) is

compared to the zero standard having no unlabeled PTH ($\mathbf{B_0}$) using the following formula:

$$\%\mathbf{B}/\mathbf{B_0} = \frac{\textbf{Net cpm of standard or sample} \times \textbf{100}}{\textbf{Net cpm of zero standard}}$$

Then a standard-binding curve is plotted between concentration of the unlabeled PTH and the radioactivity (counts per minute, cpm) remaining in each tube (standard) ($\mathbf{\%B/B_0}$), with $\%B/B_0$ on y-axis and log of concentration of PTH on x-axis. By comparing the resulting radioactivity of unknown sample with the standard-binding curve, concentration of the PTH in the sample can be calculated.

Types: On the basis of epitopes used, PTH RIA can be categorized into three types – C-terminal (58–69, 76–84), mid-region (44–53), and N-terminal (1–34) PTH assay. No single assay estimates the complete PTH molecule. The N-terminal assay detects the biologically active form of PTH, as well as the intact PTH, and has a better correlation with the clinical conditions mainly diagnosis of metabolic bone disease in CKD patients than the other two types of assays (Solal et al. 1991; Coen et al. 1993).

Limitations: Generation of antibody or antisera, use of polyclonal antibody, availability of purified PTH, availability of the radiolabeled tracer molecule, radiolysis of radiolabeled PTH, and exposure to radioactive compounds are the major limitations of RIA for PTH determination.

Second-Generation Immunoassays

Second-generation immunoassays are two-site sandwich immunoassays that use two antibodies, the first N-terminal antibody as the signal antibody and second C-terminal antibody as a capture antibody. The capture antibody is immobilized on the solid surface to bind any PTH molecule that has a C-terminal sequence, and the signal antibody is labeled with a detection molecule to bind the N-terminal region of the PTH molecule. Different commercial assays are available that use the signal antibody directed against the proximal (12–20) or distal (26–32) N-terminal region of PTH. These assays are easy to perform and widely used for diagnosis of PHPT and renal osteodystrophy in CKD patients.

Limitations: Second-generation assays also detect PTH (7–84), which is a major C-terminal component of bioactive PTH. Initially, it was thought that PTH (7–84) is biologically inactive, but in vivo studies have shown that PTH (7–84) binds to uncharacterized C-PTHR and increases the bone resorption (Brossard et al. 1993; D'Amour and Brossard 2005).

Third-Generation Immunoassays

Third-generation immunoassays, also known as "whole" or "bio-intact" or "bioactive intact" PTH assays, detect only full-length biologically active PTH and use

signal antibody directed against first 4-amino acids of N-terminal PTH and a capture antibody, identical to second-generation assays. Thus, the third-generation assays have an upper hand over second-generation assays in diagnosis of primary hyperparathyroidism and bone diseases.

Limitations: A non-1–84 PTH fragment, amino-PTH, is overexpressed in parathyroid carcinoma and severe primary hyperparathyroidism and shows cross-reactivity with third-generation assays. It was suggested that amino-PTH is phosphorylated at 17 serine residue that explains no cross-reactivity with second-generation immunoassays having signal antibody in distal N-terminal region (D'Amour et al. 2005; Rubin et al. 2007).

Types: Second-generation and third-generation PTH assays use similar detection systems. Different types of labels and label detection systems are available which are used in the second and third-generation PTH assays. The main aim of developing different detection systems was to increase the sensitivity and reduce background noise. Currently, almost all PTH estimations are performed using automated platforms. The major variants of second and third-generation immunoassays based on the detection systems are as follows:

1. Immunoradiometric assay (IRMA)
2. Chemiluminescence immunoassay (CLIA)
3. Electrochemiluminescence immunoassay (ECLIA)
4. Enzyme-linked immunosorbent assay (ELISA)

Immunoradiometric Assay (IRMA)

The immunoradiometric assay is a noncompetitive assay, which has higher sensitivity and specificity than classical RIA. In PTH IRMA, first, polyclonal/monoclonal N-terminal antibody is labeled with radioiodine (^{125}I) (signal antibody) rather than using radioiodine-labeled PTH as a tracer. Second, C-terminal antibody (capture antibody) is linked to a solid-phase support such as magnetic cellulose particles, glutaraldehyde-BSA, or polystyrene beads. First, plasma is incubated with the solid-phase antibody alone, effectively extracting the analyte from the sample. After washing, the secondary antibody labeled with ^{125}I is added. This antibody reacts with an alternative antibody binding sites on the solid-phase complex to form a solid-phase antibody-analyte-labeled antibody complex. A further washing step separates the complex from the unreacted/free labeled antibody. Then complex is analyzed on a gamma counter. Finally, a standard curve is constructed, and concentration of an unknown sample is calculated, which is directly proportional to the measured radioactivity, unlike classical RIA.

Chemiluminescence Immunoassay (CLIA)

In a chemiluminescence immunoassay (CLIA), the signal antibody is directed against the N-terminal region of the PTH and is conjugated to an isoluminol derivative (DiaSorin Liaison) or acridinium ester (Advia Centaur), while the capture antibody is directed against the C-terminal PTH and is immobilized on magnetic particles or biotinylated (immobilized on streptavidin-coated paramagnetic

particles). During incubation, PTH first binds to the solid-phase and then to N-terminal antibody. After incubation, the unbound/free material is washed, the starter reagent is added, and a flash chemiluminescence reaction is initiated. The light signal is measured by a photomultiplier as relative light units (RLUs) and is proportional to the concentration of PTH in sample.

Electrochemiluminescence Immunoassay

Electrochemiluminescence immunoassay (ECLIA) is based on a process in which highly reactive species are generated from stable precursors at the surface of an electrode. ECL immunoassays use biotinylated monoclonal C-terminal PTH-specific antibody and a monoclonal N-terminal PTH-specific antibody labeled with a ruthenium complex (tris (2, 2′-bipyridyl) ruthenium (II) complex ($Ru(bpy)_3^{2+}$). These antibodies after treatment with analyte samples attach to streptavidin-coated paramagnetic microparticles. The application of voltage to the immunological complexes leads to excitation of ruthenium complex that emits light which is detected by a photomultiplier. The amount of light produced is directly proportional to the amount of PTH in the sample. In the third-generation ECLIA assay, the signal antibody is directed against 1–5 amino acids of PTH, while in the second-generation assay, signal antibody is directed against a distal region (26–32) of N-terminal PTH. This makes amino-PTH (N-PTH) cross-reactive in both second- and third-generation PTH ECLIA.

Cyclase-Activating PTH (CAP) Immunoassay

Cyclase-activating PTH (CAP) refers to intact PTH molecules featuring the first 34-amino acids that bind to PTH1R and activate adenylate cyclase stimulating the synthesis of cyclic adenosine monophosphate (cAMP). On the contrary, cyclase-inactivating PTH (CIP) is another form of PTH that does not bind to PTH1R; therefore, cAMP doesn't get synthesized. Studies have shown that CIP is basically PTH (7–84) that binds to C-PTHR and lowers bone turnover through inhibition of osteoclast formation and differentiation resulting in an overall inhibition of bone resorption (D'Amour 2006; Nguyen-Yamamoto et al. 2001).

CAP immunoassay is the first third-generation IRMA assay developed by Scantibodies Clinical Laboratories, USA. CAP assay only quantifies PTH (1–84) without any cross-reactivity of PTH (7–84). Then CIP level can be calculated by subtracting the PTH levels determined by the CAP assay from the PTH levels determined by the iPTH assay. Finally, CAP/CIP ratio [PTH (1–84) /PTH (7–84) ratio)] can be calculated that has both theoretical and practical advantages over iPTH assay for CKD patients since they usually have large amounts of PTH (7–84) in circulation (Grzegorzewska and Mlot 2004).

Enzyme-Linked Immunosorbent Assay (ELISA)

Current available ELISA assays for determination of PTH are mainly limited to research applications and are available for both intact and total PTH measurement. Principally, in this assay, standards, controls, or samples are simultaneously incubated with an enzyme-labeled antibody and a biotinylated antibody in the well of a streptavidin-coated microtiter plate. Following the incubation period, each

Table 3 Comparison of first-, second-, and third-generation PTH immunoassays

	First-generation immunoassays	Second-generation immunoassays	Third-generation immunoassays
Methodology	RIA	IRMA, CLIA, ECLIA	IRMA, CLIA, ECLIA
Antibody	Single antibody directed against C-terminal, mid-region, or N-terminal PTH	Two antibodies for two distinct epitopes **Capture antibody:** against C-terminal PTH **Signal Antibody**: against N-terminal PTH	Two antibodies for two distinct epitopes **Capture antibody**: against C-terminal PTH **Signal Antibody**: against first 4 amino acids at N-terminal PTH
Detection	C-terminal, mid-region, or N-terminal at a time	Intact PTH and C-terminal fragment (7–84) PTH	Intact PTH and amino-PTH
Advantages	–	Excellent analytical quality and significant correlation with bone biopsy parameters	Measure only biologically active PTH
Limitations	Measures mainly fragments, low-analytical sensitivity, does not distinguish active and inactive PTH	Cross-reactivity with 7–84 and overestimation in CKD patients	No major superiority over second-generation assays

microwell is washed to remove any unbound components followed by the addition of a chromogenic substrate, such as tetramethylbenzidine (TMB). Finally, the reaction is terminated by the addition of stop solution, and the intensity of color formed is read at 450 nm on an ELISA reader. The intensity of the color is directly proportional to the concentration of PTH in the standard or the sample. In most of these assays, antibodies used are directed against distinct epitopes of PTH just as in second and third-generation immunoassays.

A comparison of first-, second-, and third-generation PTH immunoassays has been shown in Table 3.

Mass Spectrometry

In the last 10–15 years, liquid chromatography-assisted mass spectrometric (LC-MS/MS) detection has been widely applied for quantifying small molecules (<1 kDa), such as steroids, hormones (testosterone, vitamin D), drugs, and their metabolites from clinical samples (Rauh 2009). This technique is a combination of chromatographic separation with a detection system that determines the mass-to-charge (m/z) ratio of the analyte and provides far superior specificity over immunoassay-based detection. There have been published reports on quantitative LC-MS/MS methods

for the analysis of PTH (1–84) in clinical samples that have led to their application in diagnostics (Kumar et al. 2010; Lopez et al. 2010; Krastins et al. 2013).

Quantitative LC-MS/MS Assay: Albumin and IgG make 90% of the total proteins in plasma and mask the expression of proteins present in minimal concentrations. Therefore, quantification of low-concentration peptides like PTH in plasma by MS requires extensive sample enrichment. Immunoaffinity extraction (also referred as immunoextraction) is increasingly being used for sample enrichment. Immunoaffinity extraction of PTH is performed by using either C-terminal PTH capture antibody immobilized on polystyrene beads (Kumar et al. 2010) or by immobilizing the capture antibody (polyclonal goat antihuman PTH39-84) on micro-column embedded in pipette tips for automation (Lopez et al. 2010). After washing steps to remove nonspecific binding, the captured PTH (1–84) and related fragments are digested using trypsin. Selected tryptic peptides are then quantified on LC-MS/MS. Quantification of the first 1–13 amino acid tryptic peptide (SVSEIQLMHNLGK) is used as a surrogate for the measurement of PTH (1–84). The LC-MS/MS method has been calibrated using standard recombinant human PTH WHO IS 95/646. For internal reference control, isotopically labeled intact (^{15}N) PTH (1–84) or isotopically labeled tryptic peptides are used. LC-MS/MS PTH quantification methods have good analytical performance, linearity, and sensitivity. MS analysis has nonsignificant interference with any of the other PTH fragments [PTH (1–44), PTH (7–84), PTH (43–68), PTH (52–84), and PTH (64–84)] or other PTH-related forms like PTHrP, amino-PTH even in hemolyzed, lipemic, and icteric samples.

MS-based PTH assays are still in developmental stages. The major limitations of MS are the complexity of the procedure right from sample preparation, enzymatic digestion, and further quantification of PTH fragments and sophisticated instrumentation and costs.

PTH Modifications and Immunoassays

Studies have revealed that posttranslational modifications can occur at different amino acids in PTH. Phosphorylation of serine 17-residue has been reported in human and bovine parathyroid glands. Another possible modification is oxidation of methionine present at positions 8 and 18 in PTH. In vitro studies using high-resolution MS have indeed identified that both methionine residues can be single or double oxidized to form sulfoxides and sulfones, respectively, (Pan et al. 2010; Chu et al. 2004). These oxidation products are biologically inactive because they lose their ability to bind to PTH1R. The oxidized PTH forms are identified in excess in CKD patients as these patients suffer with high oxidative stress (Nabuchi et al. 1995; Tepel et al. 2013). Classical PTH immunoassays do not differentiate between biologically active non-oxidized PTH (n-oxPTH) and non-active oxidized PTH (oxPTH). Hocher et al. (2013) recently generated monoclonal antibody against the oxPTH immobilized on CNBr-activated sepharose-4B beads and separated all oxidized forms of PTH (1–84) from plasma samples. These samples containing purified n-oxPTH can be further measured by high-resolution MS after tryptic

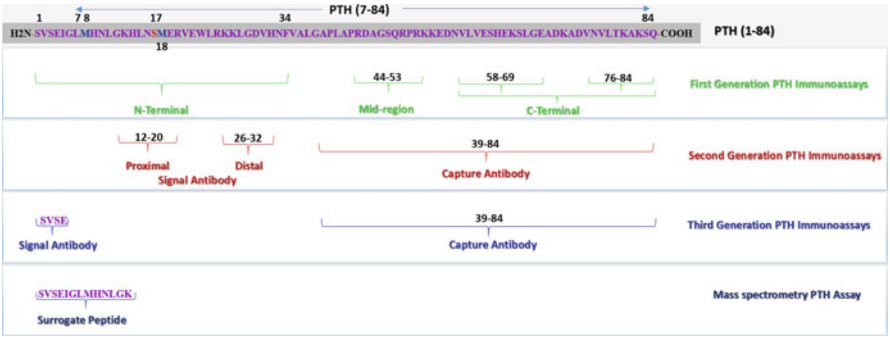

Fig. 2 PTH structure, modification sites, and epitopes selected for antibodies in different generation of PTH assays. Sequence (1–34) represents the amino-terminal, and sequence (35–84) represents carboxyl-terminal of PTH molecule. Sequence of PTH (7–84) is also depicted which starts from lysine amino acids at 7th position. Amino acid methionine, at 8th and 18th position, is a possible site for PTH oxidation. Oxidized PTH is biologically inactive. A newly described molecular form of PTH, amino-PTH, can be generated by phosphorylation of serine present at the 17th position. Different generations of PTH immunoassays were developed using antibodies directed against the specific epitopes on the PTH molecules. First-generation assays used single antibody, but second and third-generation assays used two antibodies directed against N-terminal region (signal antibody) and C-terminal region (capture antibody). Mass spectrometry-based methods are the latest to estimate PTH values using 13 amino acid tryptic peptide (SVSEIQLMHNLGK) as surrogate peptide to estimate only intact PTH (1–84) from the samples

digestion or by immunoassays using targeted antibodies. The sequence of PTH with its modification sites and epitopes used in different generations of assays are highlighted in Fig. 2. With growing evidences on various posttranslational and post-secretion modifications in PTH, it is imperative that immunoassays be constantly improved to enable detection of biologically active PTH in clinical samples. Recent literature suggests the requirement of a fourth-generation PTH assay for improving detection of bioactive and intact PTH with simultaneous detection of other fragments and forms of PTH.

Tissue-Specific PTH Expression

Ultrasound-assisted fine-needle aspiration (FNA) can be utilized to obtain a tissue biopsy from the potential tumor sites and can be used as localizing and diagnosis technique for severe and misdiagnosed primary hyperparathyroidism (Stephen et al. 2005). Cytological examination as well as PTH measurement can both be performed, simultaneously in the aspirates to establish the correct diagnosis of primary hyperparathyroidism. Biopsy can also be utilized for confirming tumor by analyzing the expression of PTH by immunohistochemistry. Gene and protein expression analysis of PTH by real-time PCR and western blotting, respectively, can also serve as adjunct tools in the diagnosis or prognosis of PTH-associated diseases.

Potential Applications to Prognosis, Other Diseases, or Conditions

Primary Hyperparathyroidism: PHPT is mainly characterized by hypercalcemia and inappropriately elevated PTH levels. Therefore, measurement of PTH is a primary diagnostic criterion for PHPT patients. Second-generation PTH assays are widely in use and have good sensitivity and reproducibility in PHPT patients. These assays can also detect subnormal levels of PTH and discriminate hypoparathyroidism from a mild form of PHPT or hypercalcemia due to malignancy. However, the second-generation PTH assays also detect the (7–84) PTH fragment, which was later proved to have antagonist functions to PTH. Hence, third-generation assays were developed that detect only the active iPTH. Several studies have compared the sensitivity and specificity of second and third-generation assays in PTH measurement. Direct comparison between second- and third-generation PTH assays showed that both assays provide good sensitivity with minimal differences in PTH levels of PHPT patients (Boudou et al. 2005; Silverberg et al. 2003). The recently held Fourth International Workshop on Asymptomatic PHPT also concluded that diagnostic sensitivity of both the assays was similar (Eastell et al. 2014). However, more studies are required to assess the superiority of third-generation assays over second-generation assays in the diagnosis of PHPT.

Intraoperative PTH Measurement: Rapid measurement of intraoperative PTH (IO-PTH) is a procedure to confirm the successful removal of a parathyroid adenoma during minimally invasive parathyroidectomy after preoperative localization of putative adenoma. Assessment of IO-PTH is becoming a useful tool in guiding operative decisions in PHPT (Irvin et al. 2004; Chen et al. 2005). According to the well-accepted *Miami criterion* (Carneiro and Irvin 2002), $\geq$ 50% decrease in PTH levels compared to the highest pre-incision or pre-excision PTH levels at 10 min. after removal of hyperfunctioning gland represents successful parathyroidectomy. Since iPTH is degraded immediately after release, third-generation PTH assays show faster decrease in PTH levels than second-generation PTH assays, when PTH is measured at different time intervals of 5, 10, and 15 min post-excision of parathyroid adenoma. At 5 min., PTH levels dropped more than 50% in all the patients after parathyroidectomy as determined by third-generation assays but only in 92% of patients when second-generation assays were used (Yamashita et al. 2002). Therefore, third-generation assays appear to be better in assessing IO-PTH levels. Sohn et al. (2015) have demonstrated that in PHPT patients with CKD, decrease in IO-PTH is slower as after 10 min only 89% of patients with chronic renal insufficiency (CRI) met intraoperative cure criteria, and at 15 min only 95% met the criteria. The site of blood collection (peripheral or central) doesn't influence the success of parathyroidectomy and predictive accuracy of IO-PTH levels (Abdel-Misih et al. 2011).

Parathyroid Carcinoma: With differences in the size of PTH fragments analyzed, the ratio of PTH levels detected in third-generation assays over the second-generation assays can be utilized for differentiating parathyroid carcinoma from benign parathyroid tumors. The third-/second-generation PTH ratio is normally less than 1.0 which increases in parathyroid carcinoma (Cavalier et al. 2010; Caron et al. 2011). In

parathyroid carcinoma and severe PHPT, amino-PTH is secreted which is only detected by second-generation assays with a signal antibody in the distal N-terminal region and all third-generation PTH assays (Rakel et al. 2005; Rubin et al. 2007). In healthy individuals, amino-PTH makes 2–4% of total PTH in circulation, but in severe PHPT and CKD patients, it increases up to 15–20%. The third-/second-generation PTH ratio has the sensitivity of 81.8% and specificity of 97.3% in detecting parathyroid carcinoma among the PHPT patients (Cavalier et al. 2014).

In context of the recent observations, these assays also need to be standardized and validated in symptomatic PHPT, where levels of PTH remain very high in parathyroid carcinoma as well as in benign tumors.

Secondary Hyperparathyroidism: Secondary hyperparathyroidism (SHPT) is a complex entity comprising of the bone and mineral metabolism-related complications that have a direct effect on the kidney, such as in CKD. SHPT is associated with hyperphosphatemia, vitamin D deficiency, and skeletal resistance to PTH. CKD patients at stage 3, stage 4, and stage 5 may develop or already have developed SHPT. Metabolic bone disorders associated with CKD are referred to as renal osteodystrophy, two major types being osteitis fibrosa cystica and adynamic bone disease. Osteitis fibrosa cystica is a high-turnover bone disease with elevated PTH levels that stimulate osteoclast activity and bone resorption. Adynamic bone disease is a low-turnover disease with normal mineralization and may result from low PTH levels. The use of vitamin D agents and/or calcimimetics increases the incidence of adynamic bone diseases due to misdiagnosis of PTH levels and further suppression of PTH secretion in CKD patients. Iliac crest bone biopsy with double tetracycline labeling and bone histomorphometry analysis is a gold standard for the diagnosis of skeletal complications in the CKD patients although it is difficult to obtain bone biopsies from the patients.

PTH measurement with bone turnover markers (alkaline phosphatase, tartrate-resistant acid phosphatase) is essential for the diagnosis of CKD patients as well as for monitoring the patient's response to therapy. According to Kidney Disease Outcomes and Quality Initiative (KDOQI) clinical practice guidelines for metabolic bone diseases in CKD patients, PTH levels should be maintained between 150 and 300 pg/ml in CKD patients. PTH measurement also acts as a screening tool for differentiating osteitis fibrosa cystica from adynamic bone disease. Usually, second-generation PTH assays provide good sensitivity and a high correlation with bone histomorphometry studies and biochemical bone turnover markers in CKD patients. However, PTH fragments mainly PTH (7–84) accumulate in the kidney and further released into circulation after glomerular filtration and make up to 45–50% of total PTH in CKD patients. This leads to the overestimation of active PTH by second-generation assays that finally influence the diagnosis and management in CKD patients (D'Amour et al. 2006; D'Amour and Brossard 2005). Several studies had also found a strong correlation of PTH levels between second- and third-generation assays in CKD patients. However, the PTH levels measured by third-generation assays were 30–50% lower than measured by second-generation assays (John et al. 1999; Savoca et al. 2004; Souberbielle et al. 2006). Monier-Faugere et al. (2001) have suggested that PTH (1–84)/PTH (7–84) ratio can be helpful in

differentiating normal or high bone turnover disease (osteitis fibrosa cystica) with low bone turnover disease (adynamic bone disease) in CKD patients. They have reported that PTH (1–84) /PTH (7–84) ratio can be less than 1 in adynamic bone disease and more than 1 in normal or high bone turnover diseases. However, other studies were unable to confirm these findings (Coen et al. 2002; Salusky et al. 2003). Therefore, more focused studies are required to evaluate the third- generation assays and relevance of PTH (7–84) in the diagnosis of CKD.

Pseudohypoparathyroidism: Pseudohypoparathyroidism (PHP) is a group of rare diseases where resistance to various hormones that activates G-protein coupled receptors, primarily PTH is observed. PHP is mainly of two types, PHP-I and PHP-II. PHP-I is a major type and further subdivided into two major subtypes – PHP-Ia and PHP-Ib. PHP-Ia is caused by inactivating mutations in exons of GNAS gene which encode G stimulatory protein (Gsα) that affect the maternal allele (Aldred and Trembath 2000; Ahmed et al. 1998). PHP-Ib is mainly caused by loss of genomic imprinting at the exon A/B in the GNAS gene (Bastepe and Juppner 2005). Clinically, PHP-I patients show resistance to PTH in renal proximal tubules that lead to hypocalcemia and hyperphosphatemia and increase circulatory PTH levels (Weinstein et al. 2001; Bastepe and Juppner 2000). It has been shown that besides elevated PTH, PTH (7–84)/PTH (1–84) ratios were also higher in PHP-Ia and Ib patients (Hatakeyama et al. 2003).

Osteoporosis: PTH is essential for normal bone function, and elevated PTH levels increase the risk of low bone mineral density, bone resorption, osteoporosis, and fractures in elderly persons, whereas intermittent doses of PTH increase bone formation and bone mass and is used as an anabolic agent [teriparatide (PTH (1–34)), recombinant PTH (1–84)] for the treatment of osteoporosis (Canalis et al. 2007). PTH treatment also has some adverse effects like mild hypercalcemia, hypercalciuria, and possibly increased uric acid concentrations (Canalis et al. 2007; Greenspan et al. 2007). Currently, PTH is the only approved anabolic agent available for treatment of osteoporosis treatments. Hence, measurement of accurate PTH levels in osteoporotic patients is required for deciding the correct dose of PTH. As the third-generation assays only detect the biologically active PTH (1–84), so, they have an advantage over the second-generation assays in the accurate assessment of PTH levels in osteoporosis patients.

Other Diseases: Elevated PTH levels as in PHPT patients also affect the cardiovascular and gastrointestinal systems, and these patients show improvement in symptoms after successful parathyroidectomy (Agarwal et al. 2013; Abboud et al. 2011; Shah et al. 2014; Barletta et al. 2000; Walker et al. 2013). In case of the cardiovascular system, recent reviews suggest that chronic PTH level increases the risk of hypertension, cardiac hypertrophy, and myocardial dysfunction. PTH receptor is also present in myocardium and leads the hypertrophic function. Recent report from Atherosclerosis Risk in Communities (ARIC) group demonstrated that PTH is elevated, but not an independent risk factor for cardiovascular diseases (Folsom et al. 2014). It is suggested that hypocalcemia may induce inappropriate rise in PTH levels in pancreatic patients. Levels of PTH are variable in acute to a chronic form of pancreatitis, but higher levels were observed in patients with GI complications (McKay et al. 1994). The European

Table 4 Circulating levels of PTH and its molecular forms in different disease conditions

Disease	Levels of PTH and its molecular forms
Primary hyperparathyroidism	Increased or inappropriate normal levels of **PTH (1–84),** Increased percentage of **PTH (7–84)** Increased percentage of **amino-PTH**, particularly in parathyroid carcinoma and severe PHPT
Secondary hyperparathyroidism	Increased **PTH (1–84)** levels Increased percentage of **PTH (7–84)** in high bone turnover disease, which decreases in adynamic bone disease Increased percentage of **amino-PTH**, particularly in CKD.
Hypoparathyroidism	Decreased **PTH (1–84)** levels
Pseudohypoparathyroidism	Increased **PTH (1–84)** levels with increased percentage of **PTH (7–84)**
Coronary heart disease	Increased **PTH (1–84)** levels
Acute pancreatitis	Increased **PTH (1–84)** levels in severe acute pancreatitis
Anemia	Increased **PTH (1–84)** levels
Colorectal cancer	Increased **PTH (1–84)** levels particularly in males

Prospective Investigation into Cancer and Nutrition (EPIC) group reported that high PTH levels may be associated with the incidence of sporadic colorectal cancer, particularly in males (Fedirko et al. 2011). High levels of PTH also inhibit the erythropoiesis that leads to the genesis of anemia. This pathway is also associated with patients of PHPT and SHPT (Bhadada et al. 2009; Brancaccio et al. 2004; Chutia et al. 2013). Therefore, with growing knowledge about the extra-osseous roles of PTH, the utility of accurate assessment of PTH particularly iPTH becomes important. Variations in circulating levels of PTH and its molecular forms in different disease conditions have been summarized in Table 4.

Current Challenges and Future Prospectives

PTH measurements from currently available second- and third-generation assays correlate well, but differ sometimes. Besides, the difference in the N-terminal antibody, another reason for this inter-assay variability, is calibration of the assays using synthetic PTH (1–84) from different origins and measurement of different PTH forms. Souberbielle et al. (2006) had suggested the use of correction factors for each assay considering allegro intact PTH assay as a reference to overcome this problem. However, the use of correction factors cannot be the final solution. For this, all manufacturers need to calibrate the assays against the currently available WHO International Standard IS PTH 95/646 for uniformity. Heterogeneity of the PTH molecules and the potential physiological functions of the different molecular forms of PTH have made our understanding of calcium homeostasis complex. The scientific community needs to work on structure–function relationship of PTH and its molecular forms in different pathophysiological conditions and to develop more sensitive assays. The MS-based PTH assay is an accurate method for quantification

of iPTH as it identifies the molecules on the basis of the mass-to-charge ratio and fragmentation property and is not interfered by different forms of PTH cross-reacting with the antibody. But as discussed earlier, MS is technically challenging, is costly, and requires sophisticated instrumentation. Also, currently used assays still do not entirely differentiate iPTH from its modified forms [phosphorylation at serine (17) and oxidation at methionines (8 & 18)].

In a clinical set up, PTH is highly associated with vitamin D status of the individuals and has an inverse relationship with PTH secretion. Populations targeted for PTH measurements mostly have vitamin D insufficiency and should be avoided in generation of reference data. Nonetheless, PTH still has a long way to go before it is used as a surrogate marker for bone turnover. Extensive studies are thus required to address the pathophysiology of PTH and its different molecular forms and their diagnostic applicability.

Summary Points

- Accurate assessment of PTH in clinical samples is essential for diagnosis of diseases associated with parathyroid glands, bone, and kidney.
- First-generation radioimmunoassays, based on the use of a single antibody, were the first to quantify PTH in vitro and were used till the late 1980s.
- Second-generation PTH assays measure intact PTH (1–84), but also detect PTH (7–84) fragment and are widely used in clinical laboratories worldwide.
- PTH measured by second-generation assays correlates well with bone histomorphometric studies of metabolic bone disorders (diagnostic gold standard) in CKD patients.
- Third-generation PTH assays detect bioactive PTH (1–84) and do not show cross-reactivity with PTH (7–84) fragment.
- The ratio of PTH levels obtained from the third-generation over the second-generation assays can be used for reliable diagnosis of parathyroid carcinoma and severe primary hyperparathyroidism.
- Liquid chromatography-assisted mass spectrometry (LC-MS/MS)-based assays are recent addition to the PTH assays, provide accurate assessment of PTH, and can distinguish different fragments of PTH.
- Commercially available second- and third-generation assays have a lot of variability in measured PTH values and need to be calibrated against WHO International Standard PTH IS 95/646.

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Part II

Body Fluids, Tissue, and Specific Biomarkers

Sirtuins as Markers of Bone Disease: A Focus on Osteoarthritis and Osteoporosis

7

Élie Abed, Pascal Reboul, and Daniel Lajeunesse

Contents

Abstract

Sirtuins are widely distributed class III NAD^+-dependent histone deacetylases (HDAC) involved in epigenetic regulation. There are seven protein members of the sirtuins, SIRT1-7, each of which specific sites of action linked with the intracellular localization, and acting on selective target proteins. Sirtuins are involved in a number of processes ranging from cell cycle regulation,

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apoptosis/proliferation, DNA repair, tumor suppression, energy metabolism, mitochondrial homeostasis, metabolism, cellular senescence/aging, and inflammation. Sirtuins also play a key role on the recruitment and differentiation of mesenchymal stem cells and via this pathway play an important role in the maintenance and function of bone tissue. Hence, sirtuins are involved in bone pathologies such as osteoarthritis and osteoporosis and can serve as biomarkers for these pathologies.

Keywords
Sirtuins • Osteoarthritis • Osteoporosis • Mesenchymal stem cells • Wnt/β-catenin

List of abbreviations

ADP	Adenosine diphosphate
AMPK	5' Adenosine monophosphate-activated protein kinase
AP1	Activator protein-1 complex IkBα and NF-kB
BMI	Body mass index
BMP2	Bone morphogenetic protein-2
Cbfa1/Runx2	Core-binding factor alpha 1/ Runt-related transcription factor 2
cWnt	Canonical Wnt/β-catenin
DAN	Cysteine-knot protein abberative in neuroblastoma
DKK1	Dickkoft-1
EP300	E1A binding protein p300
FoxO	Forkhead box O
Fz	Frizzled
GSK3β	Glycogen synthase kinase 3 beta
H3K9	Histone 3 is deacetylated at lysine
HATs	Histone acetyl transferases
hBMSC	Human bone marrow stromal cells
	Mmp13 matrix metalloproteinase-13
HDAC	NAD + -dependent histone deacetylases
HGF	Hepatocyte growth factor
IL	Interleukin
JNK	c-Jun N-terminal kinase
Lef	Lymphoid enhancer-binding factor
LRP5/6	Low-density lipoprotein receptor-related protein 5/6
LXR	Liver X receptor
MSC	Mesenchymal stem cell
NAD	Nicotinamide adenine dinucleotide
NANOG	Transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells
OA	Osteoarthritis
OCT4	Octamer-binding transcription factor 4
OP	Osteoporosis
OPG	Osteoprotegerin

PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor γ
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor kappa-B ligand
Rho GTPase	Rho family of guanosine triphosphate hydrolase
sFRP	Secreted frizzled-related proteins
SIRT1	Sirtuin 1
SIRT6	Sirtuin 6
SOST	Sclerostin
SOX2	SRY (sex determining region Y)-box 2
T2D	Type 2 diabetes
TCF	T cell factor
TGF-β1	Transforming growth factor beta-1
TNF	Tumor necrosis factor
Wif1	Wnt inhibitory factor 1
Wnt	Wingless

Introduction

The acetylation and deacetylation of histones are regulated by two enzyme systems, the histone acetyl transferases (HATs) and histone deacetylases (HDACs). HADCs remove acetyl groups from a lysine residue on a histone, which in turn regulates gene expression. Involved in epigenetic regulation, especially the deacetylation of histones, 18 members of the HDAC family have been identified. HDACs are classified into four groups of which the sirtuins form an important group of class III NAD^+-dependent histone deacetylases. In addition to their HDAC activity, sirtuins also deacetylate other target proteins besides histones (Schemies et al. 2010). Indeed, targets of SIRT1 activity include important transcription factors and cofactors. Members of the forkhead box O (FoxO) family and the Wnt-canonical signaling target β-catenin figure among those factors (Michan and Sinclair 2007).

Seven members of the sirtuin family have been identified in mammals, SIRT1–7. Sirtuins share structural similarities with their catalytic core domain of around 250 amino acids exhibiting between 25% and 60% sequence identified across organisms and variable N- and C-terminal domains flanking the catalytic core (Sauve et al. 2006; Schemies et al. 2010). Classical HDACs (classes I, II, and IV) possess a zinc ion in the catalytic site, whereas, sirtuins (class III HDACs) require nicotinamide adenine dinucleotide (NAD) as a cofactor for catalysis and show no sequence similarity to classical zinc-dependent HDACs. Structural studies of sirtuins have revealed; they possess an elongated core domain which adopts the classic NAD^+/NADH or $NADP^+$/NADPH pyridine dinucleotide-binding fold, referred as the Rossmann fold, with a structurally more variable zinc-binding motif opposite to

it and a series of loop connecting the two protein folds (Avalos et al. 2005; Schemies et al. 2010).

Sirtuins can be divided into four classes linked with specific enzymatic behavior. Class I, comprising SIRT1, SIRT2, and SIRT3, class II with SIRT4 as the sole representative sirtuin, class III with only SIRT5, and class IV comprising SIRT6 and SIRT7 (D'Onofrio et al. 2015). Two primarily different NAD^+-consuming activities of sirtuins have been identified. Sirtuins ADP ribosylate the amide oxygen of the acetyl group of acetylated lysines resulting in a transient and labile intermediate, which finally leads to deacetylation. In line with this, SIRT1–3 show strong deacetylase activities, whereas, SIRT5–7 are weaker deacetylases yet they all use NAD^+ to cleave acetyl groups from lysine residues on target proteins. The biochemical reaction of sirtuins also shows a reaction similar to ADP ribosyltransferases or ADP cyclases, which also depend in their catalysis on NAD^+. This is particularly true for SIRT4 and 6. To date, no deacetylase activity has been recognized for SIRT4. Hence, SIRT6 is the only sirtuin with a double enzymatic role. In addition, other weaker enzymatic roles have been associated with SIRT2, 4, and 5. The cellular localization of sirtuins corresponds to their substrate specificity. Whereas SIRT1 is found both in the cytosol and nucleus, SIRT2 is mainly located in the cytosol and sometimes in the nucleus linked with the cell cycle (Vaquero et al. 2006). SIRT3, SIRT4, and SIRT5 are located in the mitochondria, whereas SIRT6 is nuclear while SIRT7 is nucleolar (D'Onofrio et al. 2015).

Sirtuins are involved in a number of processes ranging from cell cycle regulation, apoptosis/proliferation, DNA repair, tumor suppression, energy metabolism, mitochondrial homeostasis, metabolism, cellular senescence/aging, and inflammation. Among the seven mammalian sirtuins family members, SIRT1 is the best characterized in human studies (Michan and Sinclair 2007; Finkel et al. 2009), while recent articles now describe a key role for SIRT6 in a number of processes. SIRT1 is an important regulator of life span extension during caloric restriction and affects cell survival, differentiation, and proliferation (Blander and Guarente 2004; Sauve et al. 2006). The exact role of sirtuins on life span extension, however, still remains to be demonstrated. Indeed, whereas SIRT1 removal from the mice leads to a reduction of their life span, a life span extension in transgenic mice overexpressing SIRT1 is not observed compared to control. In contrast, amounting studies suggest a key role of SIRT1 in energy metabolism (Imai et al. 2000; Landry et al. 2000). SIRT1-null mice do not utilize ingested calories appropriately nor can they adapt to caloric restriction or to fasting (Boily et al. 2008). SIRT1 directly affects PGC-1α, the peroxisome proliferator-activated receptor γ (PPARγ), and liver X receptor (LXR), metabolic enzymes that regulate glucose, fatty acids, and cholesterol metabolism (Picard et al. 2004). SIRT1 affects mesenchymal stem cell (MSC) recruitment and proliferations and is reduced during aging (Yuan et al. 2012; Chen et al. 2014). Likewise, SIRT6 also affects MSC recruitment and differentiation (Sun et al. 2014).

The regulation of sirtuin activity can be modulated via a number of mechanisms and/or ligands. Indeed, the polyphenol resveratrol can activate both SIRT1 and

AMPK, hence the same molecular pathways that are triggered by caloric restriction (Lam et al. 2013; Ramis et al. 2015). The targets of resveratrol are numerous, with a direct action on fat-producing cells via its effect on SIRT1 that regulates PPARγ, the regulation of cellular autophagy, which reduces the activity of the mammalian target of rapamycin responsible for inflammatory and oxidative processes (Ghosh et al. 2010) involved in both osteoarthritis (OA) and osteoporosis, while the effect of resveratrol on autophagy may also be SIRT1 independent (Liu et al. 2010).

Role of Sirtuins in Mesenchymal Stem Cell Recruitment and Differentiation

In adults, MSCs from the bone marrow possess the capacity to differentiate into a number of different cell types, namely, osteoblasts, chondrocytes, adipocytes, myoblasts, and tenocytes (Abdallah and Kassem 2008). The maintenance of bone tissue homeostasis is based on a permanent replacement of osteoblasts which are short lived and terminally differentiated cells responsible for the formation and mineralization of the bone matrix. MSC progenitors are responsible for the continuous replacement and maintenance of bone tissue osteoblasts (Komori 2006). This renewal capacity, or stemness, is based on a number of cellular controls including pluripotent transcription factors such as SOX2, OCT4, and NANOG in both embryonic stem cells and adult stem cells including bone marrow stem cells (Tsai et al. 2012; Seo et al. 2013). Inasmuch as all these factors are included in the regulation of stemness, the role of SOX2 is considered as crucial for maintaining the self-renewal and multipotency of bone marrow-derived stem cells (Basu-Roy et al. 2010). Interestingly, recent studies aimed at understanding the role of SOX2 revealed that its activity is under the regulation of SIRT1 (Yoon et al. 2014). Indeed, the acetylation of SOX2 drives its exit from the nucleus and leads to its proteasomal degradation.

SIRT1 induces the deacetylation of SOX2 in the nucleus to promote its activity and triggers SOX2 target genes. Via this regulation in adult stem cells, the SIRT1-SOX2 axis regulates two opposite functions of stem cells, renewal and differentiation. Of note, the regulation of SOX2 can control the osteogenic capacity of bone marrow stem cells and the mineralization capacity of osteoblast progenitors via the regulation of dickkoft-1 (DDK1), a known antagonist of Wnt/β-catenin signaling that is important for osteogenesis (Park et al. 2012). This is interesting since SIRT1 can also directly deacetylate β-catenin which in turn also regulates the differentiation of mesenchymal stem cells (Simic et al. 2013). In addition, SIRT1 acts upon the FoxO transcription factor which sequesters β-catenin in the cytosol therefore interfering with its activity. Via this regulation, SIRT1 can stimulate cortical bone formation and promotes osteoblast progenitors in mice (Simic et al. 2013; Iyer et al. 2014). Hence, SIRT1 activity leads to bone tissue homeostasis via different routes and therefore is crucial for bone tissue regulation.

Role of the Wnt Signaling Pathway in MSC Lineage Commitment

Besides the mechanisms discussed above, Wnt signaling plays a crucial role in MSC self-renewal in adult tissues (Reya and Clevers 2005) and tissue renewal following trauma, disease, and aging (Ling et al. 2009). Via its interaction with frizzled (Fz) and LRP5/6 coreceptors, Wnts inactivate the axin-GSK3β complex. This complex phosphorylates and drives the degradation of β-catenin, while Wnt ligands inhibit this activity. Nonphosphorylated β-catenin then translocates into the nucleus to form a complex with T cell factor (TCF)/lymphoid enhancer-binding factor (Lef) transcription factors to activate Wnt target genes (Reya and Clevers 2005). Whereas the phosphorylated state of β-catenin is a master regulator of its activity, it remains that other regulatory mechanism(s) such as the level of acetylation of β-catenin also drives its transport toward the nucleus (Simic et al. 2013). Some Wnt ligands do not activate this canonical pathway but act via other effectors including JNK, Rho GTPase, or Ca^{2+}/PKC (Veeman et al. 2003). Wnt3a and Wnt7b are among the most potent Wnt agonists in bone tissue (Gordon and Nusse 2006). Five families of extracellular Wnt antagonists are identified: secreted frizzled-related proteins (sFRP), Wnt inhibitory factor 1 (Wif1), Cerberus, Wise, and DKK. Genetic studies have identified that Wnt/β-catenin activity is essential for the commitment of MSC into osteoblasts in normal osteogenesis (Day et al. 2005). In mice and humans, targeted overexpression of Wnts or deficiency of Wnt antagonists is associated with increased bone formation. Although some in vitro studies showed that Wnts stimulate the differentiation of murine MSC toward the osteoblastic lineage (Gaur et al. 2005), both stimulatory or inhibitory effects have been reported for human MSC (de Boer et al. 2004; Gregory et al. 2005), possibly linked with culture conditions. Under conditions permissive for in vitro binary lineage differentiation of adipocytes and osteoblasts, reminiscent of the in vivo situation, differences in sensitivity for Wnt ligands alter this equilibrium, where Wnt ligands at low concentrations potently block adipogenesis while stimulating the recruitement of osteoprogenitors, and shift the commitment of normal MSC from adipocytes toward osteoblasts (Liu et al. 2009). Hence, the balance of Wnt ligands and Wnt antagonists, leading to the resulting Wnt/β-catenin signaling capacity of MSC, triggers adipogenesis and osteogenesis. As osteoblast progenitors then progress through their differentiation toward osteoblasts, they progressively express more Wnt ligands and Wnt antagonists that locally control this balance to further promote either adipogenesis or osteogenesis of neighboring cells. In mouse, the local triggering of Wnt/β-catenin stimulates the differentiation of MSC into osteoblasts which increases the expression of Wnt ligands, namely, Wnt7b and Wnt10b, and antagonists, namely, DKKs and sFRPs (Taipaleenmaki et al. 2011).

Role of Sirtuins in Bone Homeostasis

Inasmuch as sirtuins are involved with age-related diseases, and that SIRT1 activity declines with age, it is likely that sirtuins could play a role in bone homeostasis. Indeed, aging and senescence directly affect the cells involved in both bone

formation, i.e., osteoblasts, and in bone resorption, i.e., osteoclasts. Likewise, articular cartilage, composed mainly of chondrocytes, is also affected by aging, and chondrocytes also express sirtuins. This prompted the research of an association between SIRT1 activity and bone loss and/or bone deterioration.

SIRT1 has been shown to regulate bone accrual via different mechanisms. On one hand, SIRT1 promotes bone formation (Haigis and Guarente 2006) and on the other hand it reduces osteoclastogenesis (Shakibaei et al. 2011). SIRT1 is expressed in bone cells, and not surprisingly the targeted osteoblast deletion of SIRT1 in mice leads to delayed bone mineralization (Cohen-Kfir et al. 2011). Targeted disruption of SIRT1 in either osteoblasts or mesenchymal stem cells also leads to bone mass loss (Edwards et al. 2013). Conversely, overexpression does not lead to a net increase in bone mass but rather protects against age-dependent bone loss especially in male mice (Herranz et al. 2010) and from ovariectomy-induced bone loss in female mice (Artsi et al. 2014). Cortical bone formation is promoted by SIRT1 in mice by preventing β-catenin sequestration via FoxO transcription factors in osteoblast progenitors expressing osterix1 (Iyer et al. 2014). Moreover, the deacetylation of β-catenin also regulates the differentiation of mesenchymal stem cells (Simic et al. 2013).

As SIRT1 deletion leads to major bone effects, the regulation of SIRT1 activity also impacts on bone tissue. Indeed, modulating the diet of rodents can lead to impressive modifications of bone density. Rodents fed a high fat, high cholesterol, and casein protein diet, reminiscent of our modern Western world diet, showed reduced bone mineral density and strength linked with osteoblast senescence. However, replacing casein by soy protein isolate without modifying the lipid portion of the diet reversed these effects. This was linked with alterations of SIRT1 expression, with the soy protein isolate stimulating SIRT1 expression whereas the Western-like diet reducing it, in addition to an increase in caveolin-1 production (Chen et al. 2015). In turn, SIRT1 levels regulated both peroxisome proliferator-activated receptor γ (PPARγ) and p53/p21 signaling pathways in these animals. Hence, a combination of factors can regulate SIRT1 expression and activity at different levels yet all leading to bone changes.

Role of Sirtuins in Osteoarthritis (OA)

Osteoarthritis (OA) is the most common form of arthritis. OA is characterized by progressive articular cartilage loss, appositional new bone formation and sclerosis of the subchondral trabeculae and growth plate, and formation of osteophytes (Lohmander 1994). Synovitis is often observed and is considered to be secondary to the changes in hard tissues within the joint. The widely held belief that OA is a disease of articular cartilage with cartilage erosion being the main identifying feature has, particularly in recent years, been a subject for debate, and several investigations (Lajeunesse and Reboul 2003; Burr 2005) have led to the postulate that bone changes may account for subsequent joint deterioration and development of OA. Risks factors for this disease in humans include age, gender, genetic

predisposition, mechanical stress and/or joint trauma, obesity (Felson et al. 1988), and type 2 diabetes (T2D) (Ehnert et al. 2014; Magnusson et al. 2015). A better preserved bone mass than healthy individuals (Foss and Byers 1972; Carlsson et al. 1979), independently of body weight (Vandermeersch et al. 1990; Hordon et al. 1993), is observed in OA patients, leading to higher than average body mass index (BMI), and this altered bone mass is noted for both affected sites, such as the knee and hip, as well as non-synovial sites, such as the lumbar spine. Genetic studies point toward the expression of a factor involved in a metabolic pathway rather than a structural skeletal protein to explain OA. Studies examining a metabolic link between obesity and OA have reported conflicting findings. Some studies were able to show significant association between hypertension, uric acid, cholesterol, leptin, TD2, and OA, whereas other studies failed to substantiate these relationships (Ehnert et al. 2014; Magnusson et al. 2015).

As tissue homeostasis is disturbed and joint integrity impaired, normal wear and tear could then lead to cartilage damage, the hallmark of OA. The loss of cartilage may then be the result of mechanical forces, but this would not be the cause of the disease itself. Furthermore, the OA process may modify the formation and biosynthetic activity of MSC (Aspden et al. 2001). Inasmuch as adipocytes share a common mesenchymal stem cell precursor with osteoblasts, chondrocytes, tenocytes, and myoblasts, all cells affected by OA, this suggests a link between lipid metabolism and connective tissues. Such a link may be related to leptin, a known factor involved in body weight regulation and obesity and in lipid metabolism. Leptin favors within the bone marrow the local differentiation of mesenchymal stromal cells into osteoblasts while impeding the maturation of adipocytes (Thomas et al. 1999). Of note, the production of osteoblasts is increased while that of adipocytes, and chondrocytes are blunted in bone marrow stromal cells from OA patients (Murphy et al. 2002), therefore implying a key role for leptin in this process. In bone tissue, leptin can alter the production of TGF-β1 (Dumond et al. 2003), and recent studies indicate a key role of TGF-β1 in the development of OA (Zhen et al. 2013). In particular, a targeted increased activity of TGF-β1 in the subchondral bone tissue and alteration of MSC recruitment thereof can lead to OA (Zhen et al. 2013). However, the mechanisms that would lead to this increased TGF-β1 activity in the subchondral bone tissue remain unknown. The activity of the canonical Wnt/β-catenin signaling pathway is also crucial for the recruitment and differentiation of MSC into osteoblasts and adipocytes. Indeed, β-catenin levels must vary within a narrow limit at specific time points during MSC, pre-osteoblast, and osteoblast development, and recent studies indicated that the Wnt/β-catenin signaling is reduced in human OA osteoblasts compared to normal due to an elevated expression of TGF-β1. Therefore, a unifying hypothesis taking into account all features of the pathology could be that OA is a metabolic disease that induces changes in skeletal tissues which alter the formation and biosynthetic activity of MSC.

Recent studies indicated a potential role for SIRT1 in mouse models of OA (Gabay et al. 2012; Matsuzaki et al. 2014). In addition, in human chondrocytes SIRT1 affects cartilage-specific gene expression (Fujita et al. 2011). Indeed, previous studies have described the potential of SIRT1 in cartilage biology

(Dvir-Ginzberg et al. 2008). Reduced SIRT1 production in the heterozygous SIRT1 knock-out mouse model leads to increased apoptosis in chondrocytes and increased OA-like phenotype indices (Gabay et al. 2012), with decreased collagen type II and glycosaminoglycan release by isolated chondrocytes and increased metalloproteinases (MMPs). Likewise, conditional SIRT1-KO in cartilage leads to early OA and increases surgery-induced OA in mice (Matsuzaki et al. 2014). SIRT1 also plays a key role on osteoblast regulation (Kwon and Ott 2008; Canto et al. 2010), represses SOST expression in bone (Fulco and Sartorelli 2008; Cohen-Kfir et al. 2011), and interestingly affects MSC self-renewal and differentiation (Yoon et al. 2014). Diet and nutrient reduction for OA patients has been considered to be beneficial via body weight reduction (Christensen et al. 2007) whereas, a high fat diet reduces SIRT1 expression and levels (Coste et al. 2008) while nutrient starvation increases SIRT1 expression (Nemoto et al. 2004) in animal models. Of note, obesity is a risk factor for OA patients (Felson et al. 1988, 1997) and a high fat diet enhances the OA burden. However, recent studies indicate that nutrient reduction could be linked with an increase in muscle strength in OA patients (Henriksen et al. 2012) whereas, SIRT1 levels increase in muscle of starved animals (Canto et al. 2010). Hence, promoting SIRT1 expression in affected joint tissues of OA patients could potentially restore normal cell physiology in OA tissues.

Role of Mesenchymal Stem Cells in OA

The potential role of bone tissue in OA initiation or progression, leading to cartilage loss, the gold standard in OA, may be due to its capacity to serve as a reservoir for MSC and to provide nutrition of the hyaline cartilage. The role of MSC in the appearance and/or progression of OA is a key issue that received recent attention. Because there are increasing data on the abnormal behavior and phenotypic features of osteoblasts, chondrocytes, adipocytes, myoblasts, and tenocytes in OA joints, MSC development and differentiation are likely to be altered in affected individuals. MSC numbers, proliferation rate, population-doubling time, and the capacity to differentiate into different lineage cells may be altered in OA (Muschler et al. 2001; Murphy et al. 2002), yet no mechanism(s) directly responsible for this situation have been identified. The response to cytokines and growth factors by MSC in OA individuals may also be altered (Lisignoli et al. 2004). These results indicate that as their differentiation into target cells could be altered in vivo, this could lead to abnormal tissue homeostasis (Scharstuhl et al. 2003; Luyten 2004). It also suggests that cells not presently residing in the affected tissue may profoundly affect behavior and homeostasis of this tissue. Because the chondrogenic and adipogenic capacity of OA MSC is impaired (Murphy et al. 2002), OA MSC could either remain undifferentiated or differentiate into limited lineage cell, such as the osteogenic line. This could explain why all joint tissues except bone are impaired in OA individuals. Indeed, although muscle strength is reduced beyond the normal age-related loss (Hurley 1999), possibly due to muscle cell dysfunction (Hurley 1998), alterations in the differentiation capacity of MSC to form myocytes may also be altered in these individuals. However, no data are available to date to substantiate this hypothesis. In contrast, an increased osteogenic capacity of OA MSC may explain the increased

proliferation capacity of OA osteoblasts and an increased response to growth factors (Mutabaruka et al. 2010). The alteration in OA MSC may also affect the important immunoregulatory role played by MSC. This is modulated by specific signaling molecules such as TGF-β1 and hepatocyte growth factor (HGF). Indeed, the elevation of TGF-β1 and HGF levels in OA cartilage could be linked with osteophyte formation, whereas it has been shown that OA osteoblasts express more mRNA and protein levels of both TGF-β1 and HGF while chondrocytes do not express HGF (Guévremont et al. 2003; Abed et al. 2015). Osteophyte formation may be considered a repair response to stabilize the damaged joints, and it requires the local recruitment of specific MSC. In addition, Abed et al. recently demonstrated that elevated HGF levels in OA osteoblasts are responsible for their altered phenotype and abnormal canonical Wnt/β-catenin (cWnt) signaling whereby HGF increased TGF-β1 expression and reduced bone morphogenetic protein-2 (BMP-2) and cWnt signaling (Abed et al. 2015).

Regulation of SIRT1 Expression in OA Tissues

In animal studies, a high fat diet reduces SIRT1 expression and levels (Coste et al. 2008), whereas nutrient starvation increases SIRT1 expression (Nemoto et al. 2004). Of note, obesity is a risk factor for OA patients (Felson et al. 1988, 1997; Powell et al. 2005), and a high fat diet enhances the OA burden (Davies-Tuck et al. 2009). Diet and nutrient reduction for OA patients has been considered to be beneficial via body weight reduction (Christensen et al. 2007). However, recent studies indicate that this could also be linked with an increase in muscle strength in OA patients (Henriksen et al. 2012), whereas SIRT1 levels increase in muscle of starved animals (Canto et al. 2010). These data suggest that promoting *SIRT1* expression in affected joint tissues of OA patients, namely, the cartilage, bone, and muscle, could potentially restore normal cell physiology in OA tissues (Fig. 1). Previous studies have described the potential of *SIRT1* in cartilage biology (Dvir-Ginzberg et al. 2008). Reduced SIRT1 production in the heterozygous SIRT1 knock-out mouse model leads to increased apoptosis in chondrocytes and increased OA indices in these animals (Gabay et al. 2012). Moreover, a reduced SIRT1 activity in mice leads to a decreased collagen type II and glycosaminoglycan release by chondrocytes isolated from these animals, whereas it also leads to an increased release of MMPs, indices of an OA-like phenotype. Therefore a key role for SIRT1 in OA pathophysiology is now suggested and may represent a potential target to treat OA. However, a direct assessment of the role of SIRT1 in either muscle and bone tissues of OA patients has not been reported. In contrast, Abed et al. recently showed for the first time that SIRT1 expression is reduced in human OA osteoblasts and leads to an alteration of osteoblast functions (Abed et al. 2014). Indeed, reduced SIRT1 expression increases the expression of TGF-β1 and SOST which can both alter the phenotype of human OA osteoblasts. This study also confirmed that SOST production is increased in human OA cartilage, as previously reported (Chan et al. 2011), and in human OA subchondral bone tissue (Abed et al. 2014).

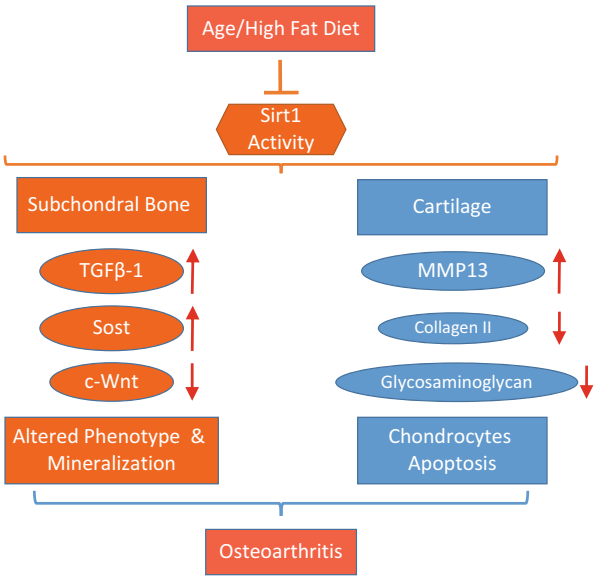

Fig. 1 Schematic representation of the potential effect of SIRT1 activity on the development of osteoarthritis

Role of Sirtuins in Osteoporosis

The World Health Organization considers osteoporosis (OP) as the most common metabolic bone disorder (Watts et al. 2008; Rizzoli et al. 2009). The gold feature of osteoporosis is the presence of a low bone mass resulting from an imbalance between bone resorption over bone formation. Bone tissue in osteoporotic patients shows microarchitectural deterioration leading to reduced bone strength and increased low-energy fractures (Lupsa and Insogna 2015). Bone remodeling is altered and leads to poor bone tissue quality. Moreover, the presence of microfractures at multiple bone sites leading to overt fractures characterizes osteoporotic patients. The balance between bone formation and bone resorption is also not the sole responsible for osteoporosis as evidence indicates that bone mass accrual to reach a peak bone mass during growth also contributes to the burden of the disease. In addition to changes in bone structure and bone mass, alterations in bone tissue quality are involved in the pathology of osteoporosis. Hence, multiple mechanisms are altered leading ultimately to fractures, a key feature in osteoporosis.

Risks factors for this disease in humans include being a female, ethnic group where Causasians are more at risks than any other group, loss of estrogen, fractures incidence during adulthood, low BMI or low body weight, smoking, and prolonged corticosteroid therapy (Leslie and Morin 2014; Lupsa and Insogna 2015). Bone tissue in osteoporotic patients is thinner and may show overt loss of bony trabeculae and perforation. Bone loss can be explained by a decrease in bone formation, i.e., the activity of osteoblasts that synthesize the organic extracellular matrix which becomes mineralized, an increase in bone resorption, whereby osteoclasts degrade the extracellular matrix, or both. Bone tissue homeostasis is maintained by the

delicate equilibrium of these activities. Hence, the osteoblasts form the organic matrix called osteoid, whose thickness depends on the time interval between matrix formation and its subsequent calcification. Mononuclear/macrophage lineage stem cells give rise to giant multinucleated osteoclasts that are responsible for bone resorption. A number of key factors prevail the formation of osteoclasts, and among these, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 (calcitriol), sex hormones, and cytokines such as tumor necrosis factors (TNFs) and interleukins (ILs) are the most important.

Data on the role of sirtuins in human osteoporosis per se is rather limited, whereas studies of animal model with osteopenia or ovariectomy-induced osteoporosis have provided insightful information on potential processes. Most importantly, studies have revealed that two members of the sirtuin family are key players controlling bone accrual or preventing bone loss, namely, SIRT6 and SIRT1, via direct deletion of these genes in mouse models. Indeed, both SIRT1-KO or SIRT6-KO mouse models lead to alterations of bone mineral density and bone tissue quality.

Role of SIRT6 in Osteoporosis

SIRT-6 KO mice present low-turnover osteopenia characterized by both impaired bone formation and bone resorption leading to altered bone remodeling. In particular, histone 3 is deacetylated at lysine 9 (H3K9) which leads to abnormal expression of target genes Runx2 and osterix in $SIRT6^{-/-}$ osteoblasts that contribute to alter osteoblastogenesis. In addition, the same alteration in H3K9 also leads to up-regulation of DKK1 and osteoprotegerin (OPG). DKK1 alters the function of osteoblasts while OPG impairs osteoclasts function. Together, triggering these mechanisms leads to an alteration of bone remodeling characterized by reduced bone formation and reduced differentiation of osteoclasts (Sugatani et al. 2015). In human bone marrow stromal cells (hBMSC), SIRT-6 inhibits the hypoxia-induced glycolysis pathway and prevents the hypoxia-induced apoptosis of these cells. Whereas, SIRT-6 activity inhibits inflammation, it is also a direct target of inflammatory mediators in hBMSC and is downregulated by hypoxia (Kok et al. 2015).

Role of SIRT1 in Osteoporosis

The involvement of SIRT1 in osteoporosis has been studied in a number of animal models, namely, mice, rats, and dogs. The regulation of H3K9 by SIRT1, hence via the same mechanism shown for SIRT6 (Sugatani et al. 2015), leads to an alteration of the expression of sclerostin (SOST). Indeed, SIRT1 inhibits SOST, a bone formation inhibitor. Indeed, in $SIRT1^{+/-}$ females, but not in males, a reduction in bone mass was observed linked with reduced bone formation indices and an increased marrow adipogenesis, implying that SIRT1 regulates bone formation and that SIRT1 could be an anabolic approach for the treatment of osteoporosis (Cohen-Kfir et al. 2011). The key role of SOST was demonstrated in bone marrow-derived cells from $SIRT1^{+/-}$ mice, since reducing SOST expression via a siRNA technique or blocking SOST with a neutralizing antibody restored the expression of osteocalcin and bone sialoprotein, two markers of bone formation, in these cells. SIRT1 also regulates the production of PTH-dependent matrix metalloproteinase-13 (Mmp13)

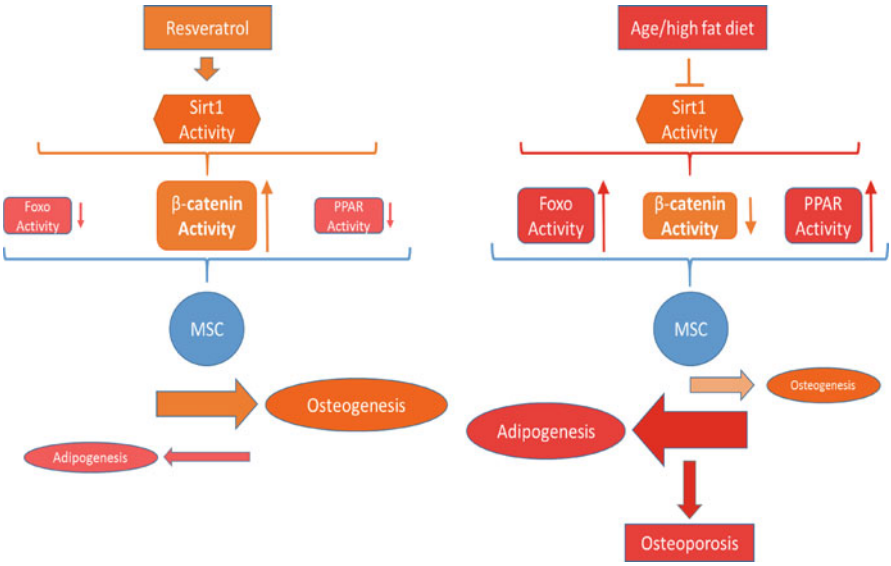

Fig. 2 Schematic representation of the potential effects of resveratrol or age/high fat diet on the development of osteoporosis via the regulation of SIRT1 activity

in mouse osteoblasts (Fei et al. 2015). Whereas, osteoblast specific SIRT1$^{-/-}$-animals showed increased Mmp13 mRNA expression and protein levels, the addition of resveratrol to the rat osteoblast-like cell model UMR 106–01 blocked the PTH-induced Mmp13 expression while using the SIRT1 inhibitor, EX527, enhanced this pathway. In contrast, acute treatment with PTH increased SIRT1 association with c-jun, a component of the activator protein-1 complex (AP1) promoting SIRT1 interaction with the AP-1 site on the Mmp13 promoter. Resveratrol further promoted this interaction, which implies SIRT1 as a feedback inhibitor regulating Mmp13 transcription (Fig. 2).

Up-regulation of SIRT1 activity using resveratrol also contributes to regulate the NF-kB pathway leading to alterations in osteoclast function. Using a rat osteoporosis model of ovariectomized female rats, Feng et al. (Feng et al. 2014) showed that medium and high doses, 25 and 45 mg/kg/d, respectively, resveratrol reversed the loss of bone mineral density observed in the osteoporosis model. Serum osteocalcin and alkaline phosphatase levels were also partly restored in these animals. Moreover, hematoxylin eosin staining and micro-computed tomography revealed alterations in bone structure in ovariectomized rats that were corrected by resveratrol. Resveratrol promoted the activity of alkaline phosphatase and the expression of type 1 collagen in rat bone marrow-derived MSC, while, blocking the expression of SIRT1 via a siRNA technique prevented this effect of resveratrol (Feng et al. 2014). Further, resveratrol reduced the activity of NF-kB via a decreased expression of phosphorylated IkBα and NF-kB p65. A similar link between resveratrol, SIRT1, and NF-kB was observed in canine primary osteoblasts. Indeed, using in vitro high-density bone

cultures derived from dog femoral heads during total hip replacement surgery, Shakibaei et al. (2011) showed that RANKL induced the formation of tartrate-resistant acid phosphatase-positive multinucleated cells similar to osteoclasts, whereas, high-density bone cultures showed well organized bone-like structures and extracellular matrix. RANKL promoted the expression of EP300, and this histone acetyltransferase prompted the acetylation of NF-kB and its nuclear translocation which triggered the osteoclastogenesis in this in vitro model. Resveratrol treatment prevented this RANKL-induced osteoclastogenesis and further promoted the transcription of Cbfa1/Runx2 and the formation of SIRT1-Cbfa1 complexes.

Potential Applications to Prognosis, Other Diseases, or Conditions

Inasmuch as sirtuins are involved in multiple age-related processes and the regulation of intracellular cell signaling pathways in mesenchymal stem cells of the osteogenic, chondrogenic, and osteoclastogenic lineages and act upon different steps of their differentiation pathways, they are key proteins that can modulate osteogenesis, chondrogenesis, and osteoclastogenesis. In particular, SIRT1 and SIRT6 are known to play key roles in bone tissue homeostasis. The targeted disruption of these two deacetylases leads to reduced bone tissue homeostasis, whereas activation of sirtuins such as SIRT1 via resveratrol leads to bone tissue accrual. Since sirtuins act at different cellular sites yet can be released into the circulation, serum sirtuin levels and activities can be readily evaluated such as in frailty (Kumar et al. 2014). Hence, sirtuin levels could also be evaluated in OA and OP as indicators of bone tissue homeostasis.

Summary Points

- This chapter focuses on the role of sirtuins in bone cell metabolism.
- SIRT1 and SIRT6 are key members of the sirtuin family involved in bone cell metabolism.
- Sirtuins play key roles in mesenchymal stem cell lineage fate and recruitment.
- Sirtuins play key roles in the canonical Wnt/β-catenin signaling pathway.
- Sirtuins are key players in osteoarthritis and osteoporosis.
- Sirtuins regulate key functions in osteoblasts.

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Creatine Kinase as Biomarker in Osteogenesis Imperfecta

8

Patrizia D'Eufemia, Mauro Celli, Anna Zambrano, and Roberto Finocchiaro

Contents

Abstract

Creatine kinase (CK) plays a storage and distribution role in cellular energetics. There are two mammalian CK cytosolic isoforms, the muscle type (CKm) and the brain type (CKb) that form homodimers or heterodimers as CKmm, CKmb, and CKbb. CKbb is present in a range of tissue, including the bone. Osteogenesis

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imperfecta (OI) is a heterogeneous group of heritable connective disorder causing bone fragility of varying severity. In most cases, the diagnosis is based on clinical and radiological data, but serum determination of marker bone formation and resorption may help for therapy decision. Actually, N-BPs are considered the current standard of care for treating OI since they potently inhibit bone resorption by suppressing the activity of osteoclasts.

In the past, clinical studies reported an increased serum CKbb in patients with genetic osteopetrosis (OPT) and in a patient with acquired OPT due to prolonged N-BP therapy. A recent report evidenced an increase of serum CKbb in children with OI type I during therapy with N-BPs probably reflecting suppression of osteoclast function. The hypothesis that osteoclasts could represent an important source of CKbb has been confirmed in a recent in vitro study, performed on rabbit-stimulated osteoclasts incubated in medium containing various N-BPs. This study confirmed that osteoclasts are the source of CK release from the bone and that this is an osteoclast apoptosis-related event.

Although until now no significant correlation has been found between serum CKbb and parameter of clinical outcome, it is likely that serum CKbb determination could help to evaluate risk condition due to oversuppression of osteoclast activity before the occurring of clinical evidence of pathological changes of bone density.

Keywords

Creatine kinase • Creatine kinase isoforms • Osteogenesis imperfecta • Collagen type I • Osteoclast • Osteoblast • Bisphosphonates

List of Abbreviation

ANT	Adenine nucleotide translocator
Apppl	Analog of ATP
BMD	Bone mineral density
BPs	Bisphosphonates
CK	Creatine kinase
CKbb	Brain-type creatine kinase
CKmb	Muscle/brain-type creatine kinase
CKmm	Muscle-type creatine kinase
Cr	Creatine
CRT	Cr transporter
CTx	C-Terminal cross-linked telopeptide of collagen
FPPS	Farnesyl diphosphate synthase
G	Glycolytic enzymes
IPP	Isopentenyl diphosphate
N-BPs	Nitrogen-containing bisphosphonates
NTx	C-Terminal cross-linked telopeptide of collagen I
OI	Osteogenesis imperfecta
OP	Oxidative phosphorylation
OPG	Osteoprotegerin

OPT	Osteopetrosis
PARP-1	Poly(ADP-ribose) polymerase-1
PCr	Phosphocreatine
PICP	C-Terminal propeptides of procollagen I
PINP	N-Terminal propeptides of procollagen I
RANK	Receptor activator of nuclear factor kB
RANKL	Receptor activator of nuclear factor kB ligand
TNF	Tumor necrosis factor
V-ATPase	Vacuolar-ATPase

Key Facts of Osteogenesis Imperfecta

The following are the key facts of OI regarding notes of historical background and differential diagnosis:

- The first report of osteogenesis imperfecta (OI) comes from ancient Egypt dating to 1000 BCE and consists of a painted coffin protecting a skeleton of a small child. In general the bones show clinical and radiological characteristics of OI associated with dentinogenesis imperfecta (Gray 1970).
- The first case suggestive of OI is relating to a mythical Danish prince, Ivan Benloss (boneless, legless) who led the Scandinavian invasion of England. He had to be carried into battle on the back of a shield as he was unable to walk on his soft legs.
- Interestingly, in 1788 it has been described in a doctoral thesis by Ekman, a Swedish military surgeon, the presence of four generations residing in a mining district in Uppland who were severely handicapped by their softness or fragility of bones and bony malformations.
- Since OI is characterized by fractures with a minimal history of injury, mild phenotypes of this disease can be misdiagnosed as child abuse because fractures are the second most common presentation in physical abuse after skin lesions (McMahon et al. 1995).
- As child abuse is a pervasive disorder and OI is rare, OI may not necessarily be considered in suspected cases of abuse. Nevertheless the misdiagnosis of OI as a child abuse is devastating to the family and health-care providers.

Definition of Words and Terms

Bone matrix	The intercellular substance of bone tissue consisting of collagen fibers, ground substance, and inorganic bone salts.
Bone remodeling	Bone remodeling is defined as an active process of resorption and apposition of bone matrix,

	essential for calcium homeostasis and preserving the integrity of the skeleton, through the coupled activity of osteoclasts and osteoblasts.
Bone turnover	Refers to the total volume of the bone that is both resorbed and formed over a period of time, usually expressed as percent/year that can be estimated by measuring relevant bone biomarkers.
Cross-linking of collagen type I	The formation of covalent cross-links between specific peptidyl residues of lysine and hydroxylysine in the non-helical portion of collagen type I l molecules that stabilizes the fibers.
Mevalonic pathway	An important metabolic pathway which plays a key role in multiple cellular processes by synthesizing sterol isoprenoids, such as cholesterol, and non-sterol isoprenoids, molecules that are essential for cell growth and differentiation and appear to be potential interesting therapeutic targets for many areas of ongoing research.
OPG/RANK/RANKL axis	An intercellular signaling pathway that regulates the differentiation of precursors into multinucleated osteoclasts and osteoclast activation and survival both normally and in most pathological conditions associated with increased bone resorption.
Osteopetrosis	A rare disorder characterized by a generalized increase in bone density, probably caused by faulty bone resorption resulting from a deficiency of osteoclasts.
Protein prenylation	Involves the transfer of either a farnesyl or a geranyl-geranyl moiety to C-terminal cysteine (s) of the target protein that promotes their membrane interactions given the hydrophobicity of the lipids involved.
Proteomics	A large-scale comprehensive study of the entire complement of proteins produced by an organism or a cellular system in order to understand cellular processes.
V-ATPase	V-ATPase is a macromolecular complex proton pump located on the ruffled border plasma membrane of bone-resorbing osteoclasts, mediating extracellular acidification for bone demineralization during bone resorption.

Introduction

New technologies in molecular biology, like proteomics approaches, provide new insight on mechanisms underlying pathophysiology of several diseases. In the field of bone diseases, they contributed to the comprehension of the molecular processes that regulate bone remodeling in physiological and pathological conditions. These knowledges allow us to better understand the role of known biomarkers in cellular metabolism, opening further perspectives for the discovering of new biomarkers. These biomarkers could be useful in the monitoring of clinical management and could represent potential targets for new pharmacological strategies.

An example elucidating these concepts is creatine kinase (CK), an enzyme involved in energy metabolism and employed as a serum biomarker in the last decades.

CK had been isolated, purified, and partially crystallized from rabbit skeletal muscle in 1954 (Kuby et al. 1954), and successively this enzyme started to became an important marker for the detection of carriers for Duchenne muscular dystrophy. Later it has been observed that serum CK could be high both in health and diseases. A serum CK increase has been suggested occurring after low- to moderate-intensity exercise (Baird et al. 2012), and elevated value CK has been related to high intracellular CK such as in healthy black people (Brewster et al. 2012). Regarding clinical diseases an elevation of serum CK was found in conditions characterized by muscular damage like rhabdomyolysis and myositis, as well as in disorders including the use of medication as statins (Stroes et al. 2015) and endocrine disorder like hypothyroidism (Hekimsoy and Oktem 2005), malignant hyperthermia (Johannsen et al. 2013), and neuroleptic malignant syndrome (O'Dwyer and Sheppard 1993). Therefore serum CK was investigated as a routinely biochemical parameter in emergency and in clinical practice. When CK isoforms (CKmm, CKmb, and CKbb) have been identified, many studies increased widely in this field enhancing the significance of the serum determination of these parameters. In 1970s CKmb was noted to be elevated and highly specific for acute myocardial infarction so that it has been considered for many years the most useful marker in differential diagnosis and clinical monitoring. Likewise elevated serum CKbb was found in neurological diseases producing acute neuronal damage (Castro-Gago et al. 2001). Successively the expression of CKbb has been demonstrated in non-neuronal cells. Recently this isoform was evidenced highly expressed in osteoclast, and its crucial role in bone remodeling was postulated (Chang et al. 2008). This knowledge opens the way for the use of this parameter as a biomarker in bone diseases.

Creatine Kinase

Found in all vertebrates, CK is a member of the phosphagen kinase family of guanidino kinases (ATP-guanidino phosphotransferases) that, as shown in Fig. 1, catalyzes the reversible transfer of a phosphoryl group from MgATP to creatine (Cr), producing phosphocreatine (PCr) and MgADP (McLeish and Kenion 2005).

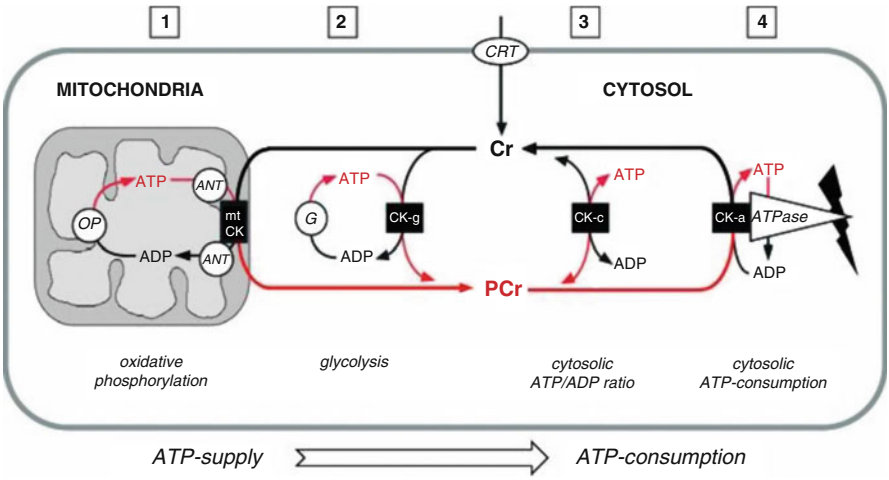

Fig. 1 The creatine kinase/phosphocreatine system for temporal and spatial energy buffering in cells of high and fluctuating energy requirements. Creatine (Cr) enters the target cells via Cr transporter (CRT). Inside the cells, PCr/Cr and ATP/ADP equilibria are adjusted by a soluble fraction of cytosolic CK isoforms (CK-c, see (3)). Another fraction of cytosolic CK (CK-g, see (2)) is specifically coupled to glycolytic enzymes (G), accepting glycolytic ATP, while mitochondrial CK isoforms (mtCK, see (1)) are coupled to adenine nucleotide translocator (ANT), thus accepting ATP exported from the matrix and generated by oxidative phosphorylation (OP). The contribution of both of these so-called microcompartments to total PCr generation depends on the cell type. The PCr thus generated is fed into the large PCr pool that is available as a temporal or spatial energy buffer. Another fraction of cytosolic CK (CK-a, see (4)) specifically associated with subcellular sites of ATP utilization also forms tightly coupled microcompartments regenerating the ATP utilized by the ATPase reaction in situ on the expense of PCr. The proposed CK/PCr energy shuttle or circuit connects, via highly diffusible PCr and CR, subcellular sites of ATP production (glycolysis and mitochondrial OP) with subcellular sites of ATP utilization (ATPases). This model is based on functionally coupled, subcellular CK microcompartments, where ATP production and ATP consumption are tightly connected to CK/PCr action (Data are from Wallimann et al. (2011), with the permission from the publisher)

The so-called phosphagens are "metabolically inert" compounds and as such do not interfere with primary metabolism (Wallimann et al. 2011). CK is crucially involved in several bioenergetic processes and plays a significant role in the energy homeostasis of various tissue cells. It is highly expressed in tissues that require large energy fluxes such as skeletal, cardiac, and smooth muscle, kidney, brain and neuronal cells, retina photoreceptor cells, spermatozoa, and sensory hair cells of the inner ear (Wallimann et al. 1992, 2007). As reported in Table 1, four different isoforms of CK are known to exist in the cells of mammals; two are cytosolic (CKb and CKm) and two are mitochondrial (CK_umt and CK_smt) which are named according to their tissue distribution or subcellular localization. All CK isoforms are encoded by separate nuclear genes, and in most tissues, a single cytosolic CK isoform is co-expressed together with a single mitochondrial CK isoform (Wallimann et al. 2011). Therefore, cytosolic CKs exist as homodimers (CKmm and CKbb)

Table 1 Gene, protein, cellular isoform, and distribution of creatine kinase. Five separate nuclear genes are known to encode for two cytosolic and two mitochondrial subunits of CK. The brain-type is present as homodimer (CKbb) in a large variety of cells and tissue where is co-expressed with ubiquitous mitochondrial isoform (CK_umt). The muscle-type exists as homodimer (CKmm) in sarcomeric muscle where the only cytosolic isoform is co-expressed with sarcomeric mitochondrial isoform (CK_smt). In cardiac muscle both CKb and CKm chains are expressed resulting in the presence of heterodimeric isoform (CKmb)

Gene	Protein	Cellular Isoform	Distribution
CKB	Creatine kinase, cytosolic, brain, CKb	Homodimer, CKbb	Ubiquitous
		Heterodimer, CKmb	Cardiac muscle
CKM	Creatine kinase, cytosolic, muscle, CKm	Homodimer, CKmm	Sarcomeric muscle
CKMT1A, CKMT1B	Creatine kinase, mitochondrial 1, ubiquitous, CK_umt	Octamer	ubiquitous
CKMT2	Creatine kinase, mitochondrial 2, sarcomeric CK_smt	Octamer	Sarcomeric muscle

under physiological conditions. The exception is the cardiac muscle in which both CKb and CKm proteins are expressed generating heterodimers CKmb that represent 25% of cytosolic CK isoforms.

Structure

CK cytoplasmic isoforms exist as a dimer and each subunit is composed of two domains: a smaller N-terminal domain containing only a-helices and a larger C-terminal domain with both b-sheets and a-helix secondary structures (Bong et al. 2008). The enzyme active site is located at the cleft of the two domains and is thought to facilitate the entry of substrates as well as inhibitors (McLeish and Kenion 2005). The two mitochondrial isoforms usually exist as octamers but can be dissociated into dimers (Bong et al. 2008).

Function

Creatine kinase isoforms regulate ATP homeostasis by the transfer of phosphates between creatine and adenine nucleotides. The main physiological role ascribed to CK was the maintenance of energy homeostasis at sites of high-energy requirement. The discovery that cytosolic and mitochondrial isoforms are located in different subcellular compartments where they are associated with sites of ATP production and consumption sustained the concept of a *creatine/phosphocreatine shuttle*: "the mitochondrial isoform generates PCr, which is shuttled to cytosolic isoforms

localized to specific subcellular regions to provide ATP at sites where high energy fluxes are required" (Wallimann et al. 1992). The shuttle hypothesis takes into account the dual localization and the different equilibrium constants of CK isoforms that favor the formation of PCr in mitochondria and the consumption of PCr in cytosol. This system, also referred to as a *spatial energy buffer*, functions to maintain constant levels of ATP and ADP, acting as a transport mechanism for high-energy phosphates between the local production of ATP (glycolysis in cytosol and oxidative phosphorylation in mitochondria) and the place of ATP consumption (Wallimann et al. 2011). In Fig. 1 it is evident how CK reactions may run in different (forward or backward) directions, but on the global cellular or organ level, the CK system appears to be as if in equilibrium.

The observation that cells expressing CK are characterized by intermittently high and fluctuating energy requirements suggested the concept that creatine/phosphocreatine system could act as a *temporal energy buffer*. In these cells a large cytosolic phosphocreatine pool serves as a local fuel for rapid re-phosphorylation of ADP, thereby ensuring rapid regeneration of ATP in condition of changing demand (Wallimann et al. 2011).

Cell and Tissue Distribution of CK Isoforms

The most important feature for the cellular functions of CK is the presence of tissue- and cell-specific isoforms with defined subcellular locations (Wallimann et al. 2011).

The muscle-type CK (CKmm) is the predominant isoform in highly differentiated skeletal muscle tissue. Only the CKmm is able to interact with the M-band region of a myofibrillar sarcomere, thus suggesting a functional importance for this specific CK isoform in maintaining high ATP requirements during muscle contraction (Wallimann et al. 1984).

The heterodimeric isoform CKmb is the predominant form in adult cardiac muscle (Clark et al. 1996).

The ubiquitous brain-type cytosolic isoform (CKbb) is found primarily in the brain and retina where it is associated with ion transport pumps in the brain (Kaldis et al. 1996). This isoform is also widely distributed in other cells, such as smooth muscle cells, spermatozoa, kidney, salt glands, myometrium, placenta, pancreas, thymus, thyroid, intestinal brush-border epithelial cells, endothelial cells, cartilage and *bone cells*, macrophages, blood platelets, tumor, and cancer cells (Wallimann and Hemmer 1994).

The mitochondrial isoforms are concentrated at sites where the inner and outer membranes are in close opposition (Wallimann et al. 1992). The sarcomeric mitochondrial isoform is expressed in skeletal muscle together with the cytosolic CKmm isoform, while in non-muscle cells, the ubiquitous mitochondrial isoform is expressed together with the cytosolic CKbb. In cardiac muscle, all five isoforms are found and the ratio between them is developmentally regulated (Clark et al. 1996).

CKbb in Bone Metabolism

Animal studies on CKbb knockout have demonstrated the importance of CK for the energetics of bone metabolism and osteoclast function for bone resorption (Chang et al. 2008).

Osteoclasts are multinucleated bone-resorbing cells that play a crucial role in physiological bone remodeling, as well as in pathological bone resorption such as osteoporosis and periodontal disease (Teitelbaum 2000; Tanaka et al. 2005).

To decalcify bone osteoclasts, degrade the organic extracellular matrix using a large number of plasma membrane and intracellular transport systems, including the vacuolar-ATPase (V-ATPase), a highly conductive chloride channel, chloride bicarbonate exchangers, and accessory pumps (Francis et al. 2002). Mature osteoclasts exhibit high citric acid cycle activity and active mitochondrial respiration to generate a large amount of ATP that is used by V-ATPase to pump protons into an extracellular resorption area (Francis et al. 2002), which is critical for the bone resorption function (Frattini et al. 2000). Expression of CKbb is considered to exert a crucial role for ensuring high-energy fluxes in condition of high requirement necessary for osteoclast function and bone resorption (Chang et al. 2008).

Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a heterogeneous group of heritable connective disorder causing bone fragility and several other connective tissue abnormalities with a prevalence of 1 in 15,000–20,000 births. Other than bone fragility, OI is characterized by low bone mineral density (BMD), short stature, skeletal deformities, blue sclerae, dentinogenesis imperfecta, joint laxity, and deafness later in life (Sillence 1981). This clinical picture varies widely with severity that ranges from perinatal death to asymptomatic individuals. Most cases are due to autosomal dominant inheritance with mutations in COL1A1 and COL1A2 genes encoding the α1 and α2 chains that, when organized into a triplex helix, constitute type I collagen (van Dijk and Sillence 2014). Type I collagen represents the main structural protein of the extracellular matrix of the bone, skin, and tendons, and its biosynthesis is formed by various important steps. COL1A1 and COL1A2 encode the pro-α1 and pro-α2 chains of type I procollagen, respectively. These chains contain at each end N-(amino) and C-(carboxy) terminal propeptides that are essential for the procollagen formation. After translation, pro-α1chains and pro-α2 chains are processed in the rough endoplasmic reticulum starting from the folding process of (pro)collagen into a rigid triple helix in which a posttranslation modification is performed by specific proteins. These proteins are encoded by genes whose mutations have been reported to cause OI. Then, procollagen type I is transported to the Golgi complex and subsequently into the extracellular matrix by exocytosis. The cleavage of the C- and N-propeptides leads to formation of collagen type I. Cross-linking of collagen type I molecules forms fibrils and multiple fibrils form into collagen fibers, important constituents of the bone (van Dijk et al. 2011).

Classification

In 1979 a numerical classification of OI into four types based on clinical and genetic/radiological features has been proposed (Sillence et al. 1979). The type I (autosomal dominant inheritance) is considered as a mild disease with absence of major bone deformity although vertebral fractures can occur causing mild scoliosis. Type II (autosomal recessive inheritance) is a lethal perinatal form in which fractures and severe deformities of the long bone are present early in utero. Usually neonates affected have multiple rib fractures causing restrict lung development and respiratory failure. OI type III (autosomal recessive inheritance) is a progressively deforming disease and represents the most severe form in patients surviving the neonatal period. The clinical picture is characterized by blue sclera, triangular facial shape, very short stature, small chest, and limb and spine deformities secondary to multiple fractures. OI type IV (dominantly inherited) is a moderate to severe form with mild or moderate deformities, variable short stature, and multiple fractures, a phenotype overlapping types I and III (Sillence et al. 1979).

In 2004 this classification has been expanded adding OI types V–VII with an unknown genetic defect and distinct on bone histological feature (Rauch and Glorieux 2004). Thereafter several new mutations in COL1A1 and COL1A2 have been discovered, and new genes have been involved in causing OI so that now it is considered to be genetically heterogeneous with considerable phenotypic variability. In Table 2 there is a reported OI classification listing type, inheritance, phenotype, and genetic defect recently identified (Valadares et al. 2014).

Molecular Genetics

Autosomal dominant form of the disease affects the greatest part of patients with OI which have an identifiable mutation in either the COL1A1 or the COL1A2 genes, whereas about 5–10% have a recessive form (Ben Amor et al. 2013). Two general classes of mutations characterized the majority of patient with autosomal dominant form of OI. The first one is the haploinsufficiency type resulting in a mild type (OI type I) since these mutations lead to a quantitative defect in collagen type I. The second class are usually missense mutations causing structural defects in collagen type I. It has been reported that this mutation "clinically leads to a moderate (OI type IV) or to a severe (OI type III) or even a perinatal lethal-form (OI type II)" (Ben Amor et al. 2013).

Recessive forms of OI result from mutations in genes, recently known, encoding protein involved in the posttranslation modification of type I procollagen. Most recessive cases have null mutations in genes encoding protein acting in hydroxylation of collagen or in those responsible for the correct helical folding (Valadares et al. 2014).

OI type with X-linked inheritance has been recently attributed to mutations in a new gene (van Dijk et al. 2013).

Table 2 Expanded classification of OI. OI classification based on the involved genes with respective inheritance and phenotype. *AD* autosomal dominant, *AR* autosomal recessive (Data are from Valadares et al. (2014), with the permission from the publishers)

Type of OI	Inheritance	Phenotype	Genetic defect
Classical Sillence Types			
I	AD	Mild	*COL1A1*
	X-linked	Mild	*PLS3*
II	AD	Lethal	*COL1A1* or *COL1A2*
III	AD	Progressive deformity	*COL1A1* or *COL1A2*
IV	AD	Moderate	*COL1A1* or *COL1A2*
V	AD	Moderate, hypertrophic callus and ossification of the interosseous membrane	*IFITM5*
VI	AR	Moderate to severe	*SERPINF1*
VII	AR	Severe to lethal	*CRTAP*
VIII	AR	Severe to lethal	*LEPRE1*
IX	AR	Severe to lethal	*PPIB*
X	AR	Severe	*SERPINH1*
XI	AR	Progressive deformity, contractures	*FKBP10*
XII	AR	Moderate	*SP7*
XIII	AR	Severe	*BMP1*
XIV	AR	Variable severity	*TMEM38B*
XV	AR	Variable severity	*WNT1*
	AD	Early-onset osteoporosis	

Pathogenesis

Although many mutations in genes encoding collagen type I are known, the pathogenic mechanism generating bone lesions is still not well understood (van Dijk et al. 2011). It is well known that bone is formed by four active cells (osteoprogenitor cells, osteoblasts, osteocytes, and osteoclasts) and mineralized extracellular matrix (bone matrix). The bone matrix (osteoid) is secreted by osteoblasts and consists of type I collagen and ground substance containing proteoglycans and non-collagenous glycoproteins and resorbed by osteoclasts (Ross et al. 1995).

The active process of resorption and apposition of bone matrix, through the coupled activity of osteoclasts and osteoblasts, the so-called bone remodeling, is essential for calcium homeostasis and integrity of skeleton.

> OI has been defined as a disease of osteoblasts producing an abnormal matrix that does not respond to mechanical loads. A phenomenon of compensation is represented by an increase of osteoblast population and a raising of osteoclast activity leading to high bone turnover rate (Glorieux 2001).

Diagnosis

In most cases of OI patients, the diagnosis is based on clinical and radiological data associated to other investigations as assessment of bone mineral density (BMD) and morphological/structural study of the bone.

Metabolic bone markers relating type I collagen production or degraded products of mature collagen fiber may provide important information on the pathophysiology of OI. Serum determination of markers of bone formation [C-terminal and N-terminal propeptides of procollagen I (PICP, PINP), osteocalcin, and alkaline phosphatase] and bone resorption [C-terminal and N-terminal cross-linked telopeptide of collagen I (NTx, CTx)] may be helpful for decision regarding therapy (Tanaka 2009). Recently it has been reported that serum CKbb could be considered as a marker useful to prevent the risk of overtreatment after long-term nitrogen-containing bisphosphonates (N-BPs), drugs usually used in OI patients (D'Eufemia et al. 2014).

The laboratory confirmation of OI is made by DNA analysis of the genes involved in OI or by decrease or abnormal production of (pro) collagen type I by fibroblast measured on procollagen electrophoresis (van Dijk et al. 2011).

Treatment

When the diagnosis of OI has been considered, the patients should preferentially be evaluated by a multidisciplinary team (Zeitlin et al. 2003). Nowadays the OI therapy consists of nonsurgical management (physical treatment, rehabilitation, bracing, and splinting), surgical management, and medical pharmacological management. The last one includes drugs able to increase the strength of the bone and decrease the number of fractures as bisphosphonate (BPs) or growth hormone, depending on the type of OI (Monti et al. 2010).

Intravenous BPs can be considered the current standard of care for treating moderate to severe forms of OI although several questions remain to be solved (Ben Amor et al. 2013). The classical pharmacological effect of these drugs seems due to their key properties: the affinity for bone mineral and inhibitory effects on osteoclasts. The absorption of BP molecule to bone mineral is responsible for the uptake and retention of these drugs on the skeleton, its diffusion and storage within the bone, and its potential release from the bone (Russell et al. 2008).

Bisphosphonates are synthetic analogs of pyrophosphate that, as reported in Fig. 2, can be divided into two groups: nitrogen-containing and nonnitrogen-containing BPs according to the structure of the side chains, a nitrogen-containing type and a nonnitrogen-containing type. This results in different mechanism of BPs action for antiresorbing activity (Russell et al. 2008).

Nonnitrogen-containing BPs, as etidronate, clodronate, and others, are reported to act through the intracellular accumulation of non-hydrolyzable ATP analogs that exert cytotoxic effects on osteoclasts causing apoptosis.

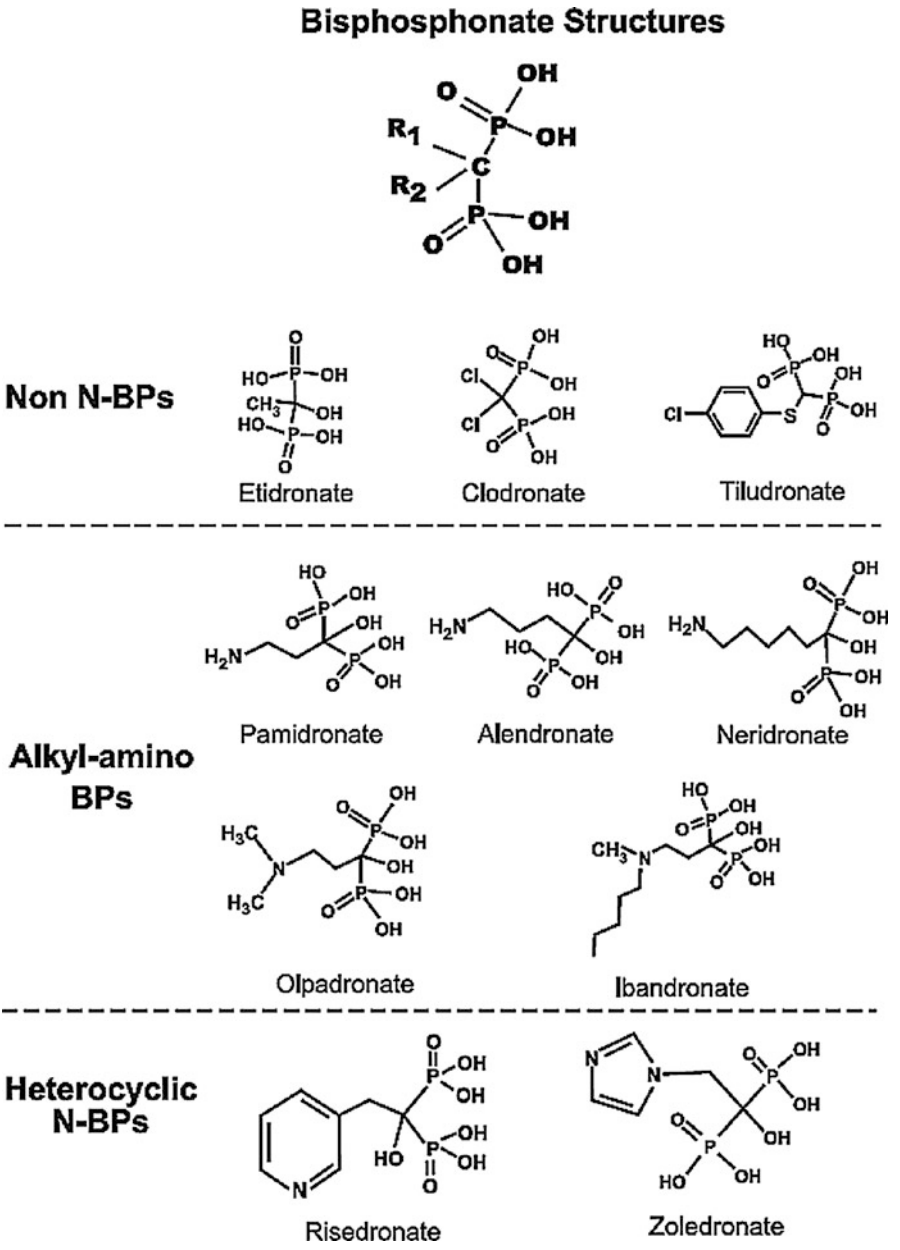

Fig. 2 A new classification of bisphosphonates based on mechanism of action. Nonnitrogen-containing bisphosphonates act by incorporation into ATP, whereas alkyl-amino BPs act by interfering with farnesyl diphosphate synthase (FPPS). The heterocyclic N-BPs also inhibit the FPPS enzyme and in addition stabilize conformational changes that magnify their inhibitory potency (Data are from Russell et al. (2008) with permission from the publisher)

Nitrogen-containing BPs (N-BPs), as risedronate, zoledronate, alendronate, pamidronate, neridronate, and others, are more potent than simple BPs, and they inhibit osteoclasts by different pathways illustrated in Fig. 3 (Russell et al. 2008).

Within osteoclasts, N-BPs inhibit enzymes of the mevalonate enzyme pathway, well known for its role in the biosynthesis of cholesterol and other sterols. After binding to bone mineral, these drugs are internalized into bone-resorbing osteoclasts by endocytosis (Russell et al. 2008). Release of bone-bound N-BPs into osteoclasts is promoted by the activity V-ATPase, localized along the ruffled borders of osteoclasts that pumps protons out into the extracellular resorption area (Takami et al. 2003). When internalized in osteoclasts, N-BPs act as substrate analogs of isoprenoid diphosphates that are crucial substrates of many enzymes of the mevalonate pathway, particularly the farnesyl diphosphate synthase. This action prevents the prenylation of small GTPase forming unprenylated GTPases that accumulate in the cytosol. Small GTPases are a large family of signaling proteins that are fundamentally important for the function and survival of osteoclasts. The accumulation of unprenylated small GTPase seems to impair osteoclast function, reduces osteoclast resorption, and eventually may cause apoptosis (Russell et al. 2008).

Another mechanism by which N-BPs interfere with bone resorption is the increase of osteoprotegerin (OPG) production from osteoblasts. Increase in OPG opposes the binding of RANKL to RANK receptor that are key mediators of osteoclast function and survival. The OPG/RANK/RANKL axis has been considered the hall marker of osteoclastogenesis (Tanaka et al. 2005).

Even if therapeutic effects of N-BPs are based on their inhibitory effects on osteoclasts, new concepts are emerging about a possible role in osteoblastogenesis. Recently it has been reported that N-BPs may stimulate proliferation of osteoblasts and inhibit apoptosis of osteocytes and osteoblasts (Maruotti et al. 2012). This hypothesis, if it will be confirmed by further studies, could help to individuate better the action of the mechanism of N-BPs.

CKbb and OI

In the past, clinical studies reported that serum CKbb value was elevated in patients affected by osteopetrosis (OPT), a genetic bone disease characterized by osteoclastic dysfunction (Gram et al. 1991; Yoneyama et al. 1992; Whyte et al. 1996). In addition to genetic OPT, this biochemical finding has been reported in a child who exhibited acquired OPT due to a long period of high doses of N-BP therapy (Whyte et al. 2003). Evidence of OPT sign was present 18 months after his last dose of N-BPs with persistent high level of serum CKbb. A clinical and biochemical evaluation, performed 6 years later, evidenced a normal serum CKbb showing his osteoclasts resumed functioning (Whyte et al. 2008). Thus the results of all these observations suggested that elevated serum CKbb reflects a fundamental disturbance

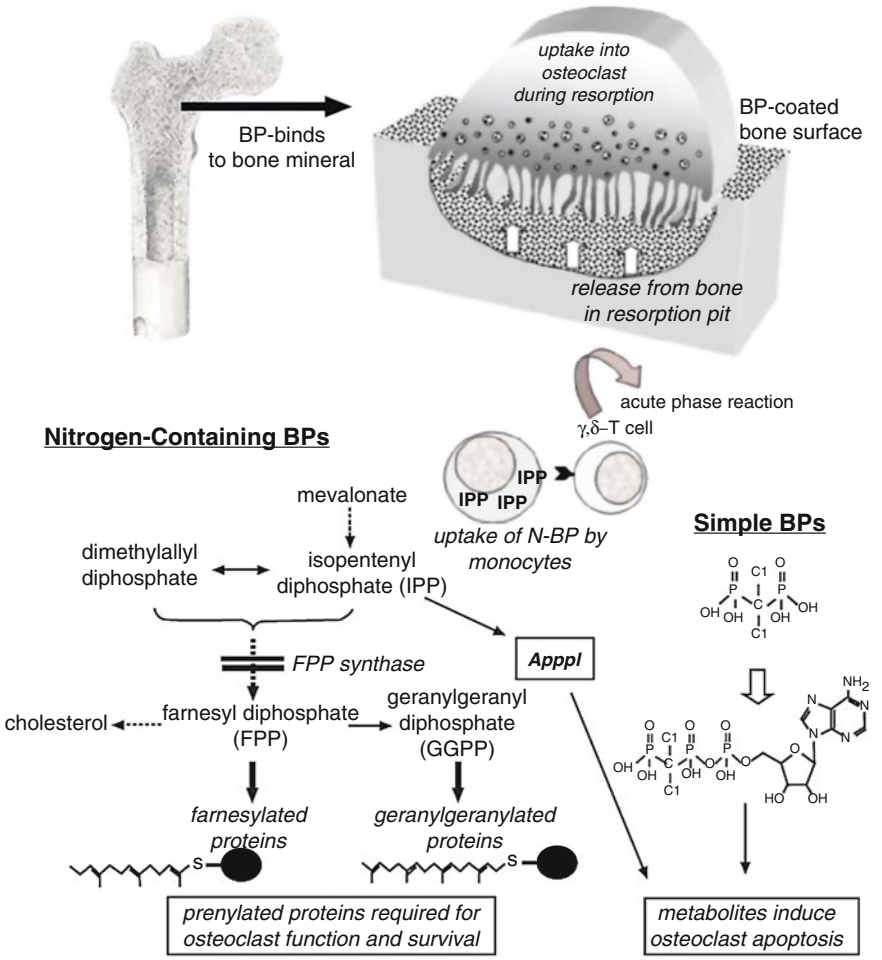

Fig. 3 Mechanism of action of bisphosphonate on osteoclasts. After binding to bone mineral, the drugs are internalized into bone-resorbing osteoclasts by endocytosis. Simple BPs (e.g., clodronate, etidronate) are metabolized in the osteoclast cytosol to ATP analogs that induce osteoclast apoptosis. N-BPs inhibit FPPS (farnesyl diphosphate synthase), thereby preventing the prenylation of small GTPase proteins essential for the function and survival of osteoclasts. Inhibition of FPPS also causes the accumulation of IPP (isopentenyl diphosphate), which is incorporated into Apppl (an analog of ATP capable of inducing osteoclast apoptosis). After IV infusion, transient uptake of N-BPs by monocytes causes accumulation of IPP, which activates gamma/delta T cells and triggers the acute phase reaction (Data are from Russell et al. (2008), with permission from the publisher)

in osteoclast biology, so that the authors proposed it as a potential marker of osteoclast failure.

The hypothesis that osteoclasts could represent an important source of CKbb in the bone has been confirmed in the last two decades. It was shown that CKbb gene is

highly expressed in rabbit osteoclasts (Sakai et al. 1995) and CKbb expression in osteoclast has been shown in bone sections using immunohistochemical analysis (Sistermans et al. 1995). Moreover, using proteomics approaches, it was demonstrated that CKbb was greatly induced in mature osteoclasts representing the predominant isoform and that RANKL upregulates CKbb mRNA expression and protein production during osteoclastogenesis (Chen et al. 2010). The molecular mechanism underlying the regulation of CKbb in osteoclasts involves RANKL-induced cleavage of poly(ADP-ribose) polymerase-1 (PARP-1), a negative regulator of CKbb expression (Chen et al. 2010).

These studies evidenced an important role for CKbb in the bone-resorbing function of osteoclasts and emphasize the importance of temporally and spatially adequate availability of energy in sustaining actin ring and V-ATPase activity in osteoclasts, especially when osteoclast activity is highly required under challenged bone erosive conditions (Chang et al. 2008).

Establishing an important role for CKbb in the bone-resorbing function of osteoclast, these findings underline its potential as a new molecular target for antiresorptive drug development.

A recent report evidenced an increase serum CKbb in OI patients treated with cyclical IV infusion of N-BPs (neridronate) (D'Eufemia et al. 2014). In this study, serum CK isoforms have been determined in 18 prepubertal children affected by OI type I at baseline, during and after 12 months of N-BP therapy. The results, reported in Table 3, demonstrate that baseline serum CKbb value is slightly increased in respect to matched normal controls. This finding could reflect a rise of osteoclast activity in accordance with the increased bone turnover that represents a well-known pathogenic mechanism of OI. During N-BP treatment a statistically significant increase of serum CKbb was observed after the first infusion that triplicates the basal level after 1 year of therapy, probably due to the cumulative effect of N-BPs in suppressing osteoclast function (Fig. 4). As shown in Table 3, CKmm value did not exhibit any significant changes at baseline and during therapy, whereas baseline CKmb level was statistically significantly higher compared with controls, but no changes have been observed during treatment. Interestingly it has been found at basal level an inverse correlation between serum CKbb and CTx, a biochemical marker considered a reliable indicator of bone matrix resorption (Fig. 5). Nevertheless, after the first infusion, no significant correlation was still present between these two biochemical parameters. The lack of this correlation might be explained on the basis of different responses of CKbb and CTx to N-BP therapy. In fact, whereas CKbb showed a progressive increase, CTx rapidly decreased just after 3 months, maintaining successively low levels. This trend is in agreement with the results obtained in recent studies performed on biochemical markers in patients affected by OI and treated with N-BPs (Aström et al. 2010; Li et al. 2011). These results could suggest that the osteoclasts are fully suppressed once the first infusion has been administered. The noteworthy increase of serum CKbb obtained in this study supports previous observations that serum CKbb represents a new molecular marker of osteoclast failure induced either by genetic defects or by N-BP therapy. Moreover a recent experimental researches support the hypothesis that N-BP-induced

Table 3 Serum total CK and isoforms levels in OI type I patients during neridronate treatment (t0-t4). Serum total CK and isoforms levels have been determined in 18 prepubertal children affected by OI type I during 12 months of cyclical IV infusion of N-BPs. The values refer to baseline time before the first infusion (t0) and every 3 months before the second (t1), third (t2), fourth (t3), and fifth (t4) infusion. Note the significant statistically increase of CKbb at the end of therapy in respect to the baseline value. Isoforms CKmm and CKmb did not exhibit any significant changes at baseline and during therapy except for CKmb level that was statistically significant higher compared with controls. Values are given as mean ± SD

	Control Group	Patients					P[a]				
		t0	t1	t2	t3	t4	t0 vs. control group	t1 vs. t0	t2 vs. t1	t3 vs. t2	t4 vs. t3
CK total U/l	89 ± 93	94 ± 23	113 ± 32	116 ± 30	124 ± 41	119 ± 31	0,85	<0.01	0,60	0,30	0,48
CKbb U/l %	2,0 ± 2,2 1,8 ± 3,1	3,0 ± 2,7 3,9 ± 3,3	6,1 ± 5,3 6,5 ± 4,4	7,5 ± 5,9 7,3 ± 6,6	9,2 ± 8,8 7,3 ± 5,8	10,8 ± 8,1 6,1 ± 3,6	0,09	<0,01	<0,01	0,02	<0,01
CKmb U/l %	1,5 ± 1,7 1,7 ± 5,5	4,2 ± 3,1 4,9 ± 2,9	4,8 ± 2,9 4,1 ± 2,3	5,7 ± 3,9 4,9 ± 3,1	4,2 ± 3,4 3,5 ± 3,4	4,7 ± 3,7 4,1 ± 3	<0,01	0,37	0,05	0,73	0,85
CKmm U/l %	86 ± 10 97 ± 5,55	82 ± 33 92 ± 3,8	101 ± 29 89,4 ± 3,2	101 ± 27 87,9 ± 6,1	109 ± 41 89,9 ± 5,3	105 ± 28 89,9 ± 3,4	0,84	0,05	0,97	0,19	0,34

[a]Paired *t*-test (Data are from D'Eufemia et al. (2014) with the permission from the author)

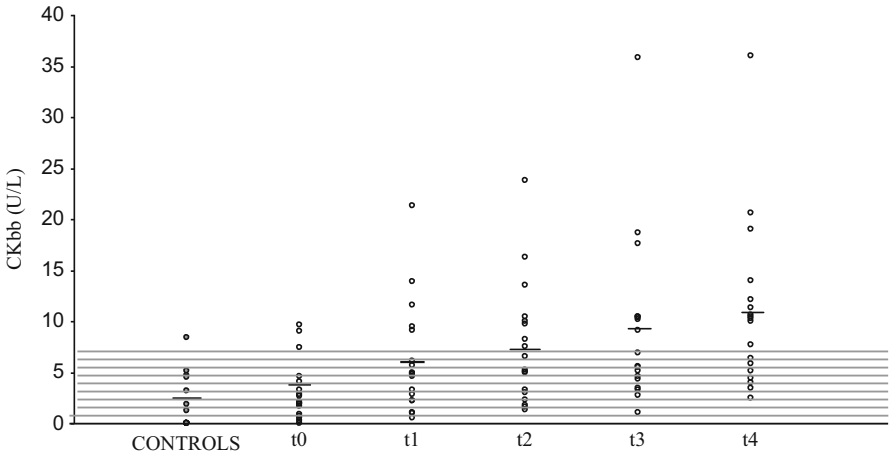

Fig. 4 Serum CKbb values in controls (•) and patients with OI type I (ˆ) at baseline (t0) and during neridronate treatment (t1–t4). Serum CKbb in 18 prepubertal children with OI has been performed at baseline and during 12 months of cyclical IV infusion of N-BPs. Basal serum CKbb (t0) was slightly but not significantly elevated with respect to matched controls, but was statistically significantly elevated before the first (t1), second (t2), third (t3), and fourth infusions (t4). At the end of therapy, serum CKbb reached values that triplicate basal level (Data are from D'Eufemia et al. (2014), with permission from the author)

osteoclast failure is the source of serum CKbb increase described in treated patients (Whyte et al. 2003; D'Eufemia et al. 2014).

In this study, CK release from rabbit osteoclasts induced by N-BPs was examined in an in vitro culture system (Tanaka et al. 2015). The CK activity in culture medium was increased at N-BP concentration which showed antiresorptive activity over 60% inhibition of CTx. The time course CK release in medium culture was compared with histological changes observed in previous studies in osteoclasts, indicating that CK release is an osteoclast apoptotic-related event (Tanaka et al. 2015). Inhibition of V-ATPase abrogated all N-BP actions, including CK release, suggesting that CK is released from osteoclasts via the pharmacological effect of N-BPs. A limitation of this study is that CK isoforms were not determined. However, CKbb is highly expressed in osteoclasts, whereas no CKbb was found in both osteocyte and cartilage (Sistermans et al. 1995). Therefore, it is likely that CKbb isoform could account for the increase of CK activity found in this study.

Potential Application to Prognosis, Other Diseases, or Conditions

All these clinical, biochemical, and experimental studies performed on N-BPs are very remarkable because these drugs are widely used in bone disease and represent the treatment of choice in patients affected by moderate to severe OI forms.

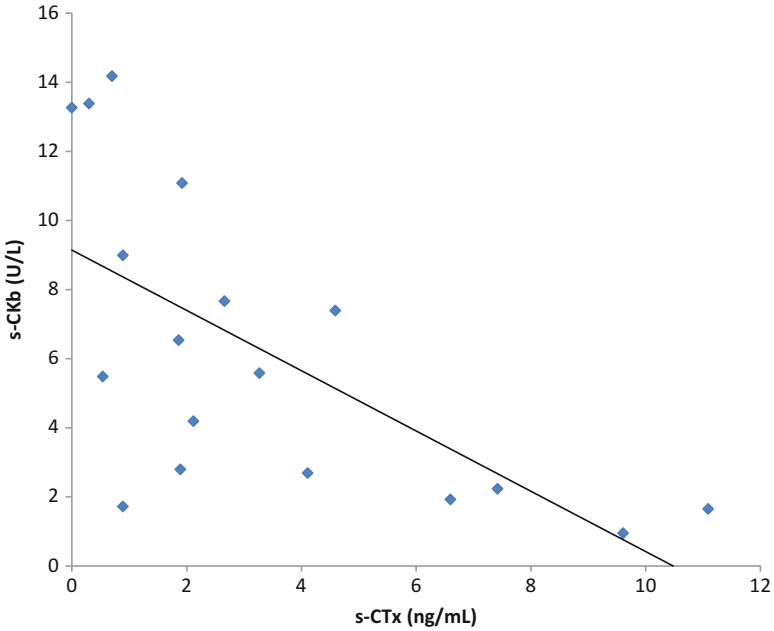

Fig. 5 Correlation between baseline CKbb activity and CTx levels in serum of children with OI type I treated with neridronate. The figure reports pretreatment values showing an inverse correlation (♦) occurred between serum CKbb and CTx, a biochemical marker considered a reliable indicator of bone matrix resorption. * $p < 0.001$ (linear regression analysis); regression line is shown (r = −0,65) (Data are from D'Eufemia et al. (2014), with permission from the author)

In fact it is well known that N-BPs, because of their key properties, potently inhibit skeletal resorption by suppressing the activity of osteoclasts and shortening their life span (Russell et al. 2008).

However the greatest concern about the N-BP use in young patients is long-term suppression of bone turnover. Thus, in spite of therapeutic efficacy reported in OI, many studies suggest that a prolonged administration of commonly used doses of N-BPs could impair bone modeling and remodeling inducing OPT-like changes (Adami et al. 2003; Whyte et al. 2008). Although until now no significant clinical correlation has been found between serum CKbb and parameter of clinical outcome, nevertheless determination of serum CKbb could help to evaluate risk conditions due to oversuppression of osteoclast activity before that clinical evidence of pathological changes of bone density and structure can occur.

Moreover, little is known regarding serum CKbb changes in other OI types as well as in other bone diseases treated with N-BPs like primary and secondary osteoporosis, Paget's disease, and bone malignancies.

Studies are still needed to evaluate possible differences of serum CKbb changes induced by N-BPs in these diseases in respect to OI patients. However, it is reasonable to hypothesize that serum determination of this marker could be useful for monitoring clinical use of N-BPs also in these disorders.

In fact, until now, many questions have to be solved regarding the choice of a particular N-BPs, the route of administration, the lowest effective doses, the appropriate treatment interval, and the overall duration of the treatment in OI as well in other disease of bone remodeling (Ben Amor et al. 2013).

Summary Points

- This chapter focuses on serum creatine kinase (CK), an enzyme involving in cellular metabolism energy, as a biochemical marker in osteogenesis imperfecta (OI).
- The most important feature for the cellular function of CK is the presence of tissue- and cell-specific cytosolic isoforms: sarcomeric muscle-type CK (CKmm), heterodimeric cardiac muscle-type CK (CKmb and CKbb), and ubiquitous brain-type CK (CKbb).
- Recently it has been observed an increase of serum CKbb in children with OI treated with nitrogen-containing bisphosphonates (N-BPs).
- OI is a hereditary connective disorder characterized by an elevated bone turnover with alteration of composition and organization of bone matrix that results in bone fragility.
- N-Bisphosphonates represent the drugs of choice for treatment of moderate to severe types of OI.
- The classical pharmacological effect of N-BPs is due to their affinity for bone mineral and inhibitory action on osteoclasts.
- The CKbb gene is highly expressed in mature osteoclasts during osteoclastogenesis and bone resorption activity.
- Recently it has been postulated that the determination of serum CKbb could be a new biochemical marker useful in monitoring N-BP therapy in pediatric patients affected by OI type I.
- Determination of this marker could evaluate risk condition due to oversuppression of osteoclast activity before the occurring of clinical evidence of bone density and structure changes.
- Future studies regarding serum CKbb determination on other OI types as well as primitive and secondary bone diseases treated with N-BPs could affect the potential role of CKbb in therapeutic decision-making.

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Serum Uric Acid and Biomarkers of Lumbar Spine Bone Mineral Density

9

Yulong Yang and Shinya Ishii

Contents

Abstract

Uric acid is the final product of purine metabolism in humans and higher primates, and its serum level has markedly increased during their evolution. Uric acid is known to be a potent antioxidant, and an increased level of uric acid has been considered advantageous, since it may compensate the inability to synthesize vitamin C, another prominent antioxidative substance, to maintain the total amount of antioxidants in the body. The advantage of a high serum uric acid level through its antioxidant property is observed in its protective effect against various neurological disorders including Alzheimer's disease, Huntington's disease, and stroke. On the other hand, uric acid has been implicated in the

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development of a number of diseases. For example, accumulation of uric acid crystals in joints is the primary pathophysiological cause of gout. In addition, uric acid may contribute to the development of cardiovascular diseases. It has been proposed that uric acid exerts a prooxidant property in certain conditions, and its prooxidant activity is involved in the development of oxidative stress-related diseases. The paradoxical behavior of uric acid is not fully understood, but whether uric acid acts as a prooxidant or an antioxidant may be partially determined by its concentration in the blood and its form.

Recent accumulated studies have provided evidence that oxidative stress is the primary underlying mechanism of age-related bone loss. Oxidative stress enhances osteoclast activity and increases bone resorption. Various antioxidant substances such as vitamin C and carotenoids are favorably associated with bone health. These observations led to the hypothesis that uric acid possesses bone-protective potential, considering its potent antioxidative property. Indeed, recent epidemiological studies suggest that a high uric acid level is associated with higher bone mineral density and decreased risk of bone fractures in various populations, especially in older men and women. Clinical studies of bone turnover markers and experimental studies have lent support to the beneficial effect of uric acid on bone health. These pieces of evidence point toward the clinical utility of serum uric acid as a biomarker of osteoporosis or fracture risk. Further research is needed to prove this contention.

Keywords

Uric acid • Oxidative stress • Antioxidant • Cardiovascular disease • Bone mineral density • Osteoporosis • Bone fracture

List of Abbreviations

BMD	Bone mineral density
CVD	Cardiovascular disease
DEXA	Dual-energy X-ray absorptiometry
eGFR	Estimated glomerular filtration rate
PTH	Parathyroid hormone
QUS	Quantitative ultrasound
ROS	Reactive oxygen species

Key Facts of Uric Acid

- Uric acid is the final product of the purine nucleotide breakdown pathway in humans and higher primates.
- Normal values of serum uric acid will vary from laboratory to laboratory, but in general they are between 3.0 and 7.0 mg/dL.
- The accumulation of uric acid salt in joints causes gout.
- To prevent recurrent gout attacks, treatment with allopurinol is widely employed.

- Allopurinol is a medication that inhibits uric acid production.
- The goal of allopurinol treatment is lowering of uric acid to below 6 mg/dL.

Definitions of Words and Terms

- Bone mineral density: the amount of mineral matter per square centimeter of bone. It is utilized as a clinical indicator to diagnose and evaluate the degree of osteoporosis. It is usually measured by DEXA method.
- DEXA method: the most widely used method to measure bone mineral density. During its measurement, two types of x-ray beams with different energy levels are projected at the bone studied, and bone mineral density is calculated from the absorption of each beam by bone. In the general population, it is recommended to routinely screen for osteoporosis at age 65 in women.
- QUS: a more convenient evaluation tool of bone mineral density than DEXA method. It utilizes sound waves projected through the bone studied to measure the quality and strength of bone.
- Mendelian analysis: uses a genetic instrumental variable, which enables examination of causation in the setting of an association that is potentially complicated by confounding or the possibility of reverse causation (Lawlor et al. 2008).

Introduction

Humans and primates have acquired a markedly increased level of serum uric acid during their evolution. Uric acid is a potent antioxidant, and an increased level of uric acid is presumed to provide an evolutionary advantage because hominoid species lack the ability to synthesize vitamin C, a prominent antioxidant, in the body, and an increased uric acid level may maintain the total amount of antioxidant in their body. While uric acid appears to exert protective effects against various neurological diseases, presumably by acting as an antioxidant, an elevated level of serum uric acid has also been implicated in the pathophysiology of a number of oxidative stress-related diseases in humans. Eminent examples include cardiovascular diseases, for which uric acid is considered a strong risk factor. The mechanism of this apparent contradiction remains to be elucidated, but recent studies suggest that uric acid can be prooxidant in certain situations.

Recent evidence suggests that oxidative stress is a fundamental mechanism of aging-related bone loss. Considering the antioxidant property of uric acid, it is conceivable that uric acid has beneficial effects on bone. Accumulating epidemiological and clinical evidence appears to confirm the beneficial effect of uric acid on bone.

This review discusses the emerging understanding of the antioxidant/prooxidant properties of uric acid, reviews relevant clinical studies in detail, and discusses the clinical application of serum uric acid as a biomarker of decreased bone mineral density or fracture risk.

Uric Acid with Dual Function as Prooxidant or Antioxidant

Uric acid is the final product of the metabolic breakdown of purine nucleotides in humans and higher primates. As described in Fig. 1, purine nucleotides are eventually metabolized to ammonium and carbon dioxide through various intermediate metabolites. The three types of purine, adenosine, inosine and guanosine, are all metabolized to xanthine, followed by oxidization to uric acid. Uric acid is then metabolized to allantoin by urate oxidase, followed by further oxidization to allantoic acid. Allantoic acid is then converted to urea before being finally broken down into ammonium and carbon dioxide. Hominoid species lack urate oxidase, which makes them unable to catalyze uric acid to allantoin, and they produce uric acid as the final product of the

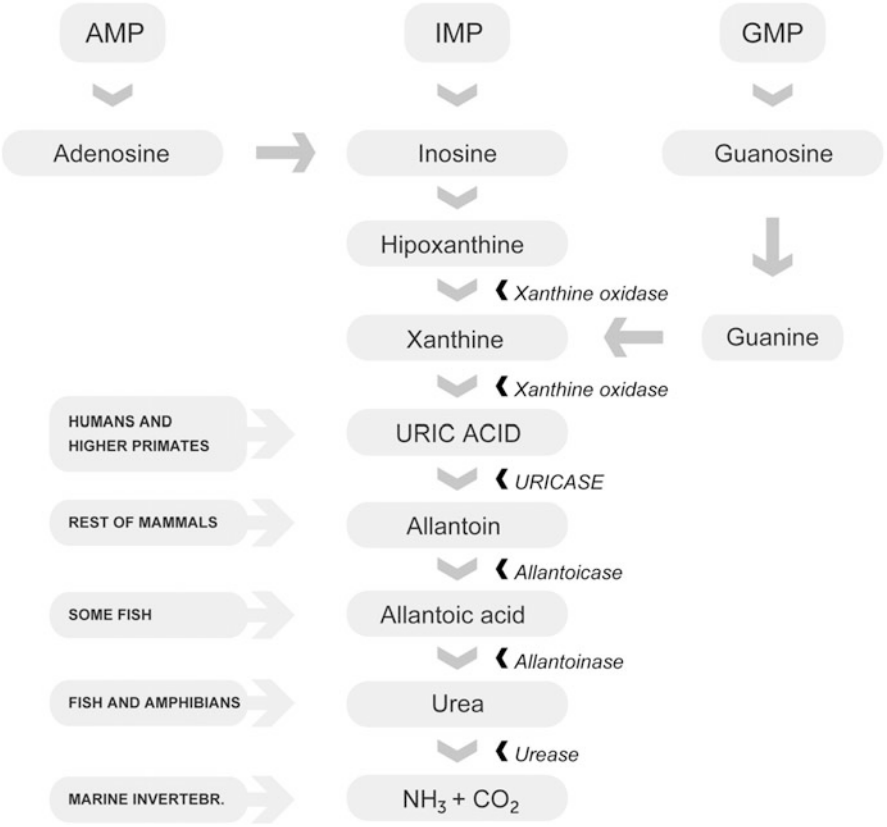

Fig. 1 Schematic representation of purine metabolism. The metabolic pathway of purine breakdown is shown. All three purines are metabolized to xanthine, followed by sequential reaction to produce ammonium and carbon dioxide (Data from Alvarez-Lario and Macarrón-Vicente (2011), with permission from the publishers. *AMP* adenosine monophosphate, *IMP* inosine monophosphate, *GMP* guanosine monophosphate)

purine metabolic pathway. During evolution, these species simultaneously lack the ability to synthesize vitamin C, a prominent antioxidant, in their body. The inability to metabolize uric acid is thus considered beneficial for these species because it increases uric acid, another potent antioxidant, to compensate the inability to synthesize vitamin C and maintain the total amount of antioxidants in the body. In fact, the level of plasma uric acid has markedly increased during primate evolution as a consequence of a series of mutations (Roch-Ramel 1978). In addition, humans have a considerably higher serum concentration of uric acid compared to those of vitamin C and other antioxidant substances (Ames et al. 1981). These two antioxidants, vitamin C and uric acid, have comparable effectiveness as antioxidants (Ames et al. 1981). A recent study showed that uric acid was responsible for neutralizing more than 50% of the free radicals in human blood (Glantzounis et al. 2005).

Several clinical studies suggest a beneficial effect of uric acid, possibly through its antioxidant property. For example, in a large-scale population-based cohort study, Lu et al. reported that the presence of gout was inversely associated with the risk of developing Alzheimer's disease, after adjusting for potential confounding factors such as age, sex, and body mass index (BMI) (Lu et al. 2015). A neuroprotective effect of uric acid was also observed in a study of patients with Huntington's disease, in whom a higher uric acid level was associated with slower disease progression (Auinger et al. 2010). Furthermore, serum uric acid level was lower in patients with Parkinson's disease compared to that in healthy volunteers, and serum uric acid level was correlated with disease severity and duration (Andreadou et al. 2009). Uric acid may also have a protective effect against neurological deficits poststroke (Waring 2002). Such neuroprotective effect has been attributed to the antioxidant property of uric acid (Bowman et al. 2010).

However, recent studies suggest that uric acid may become prooxidant under certain conditions, despite its antioxidant potential. Under what conditions uric acid acts as an antioxidant or prooxidant and how its property shifts between prooxidant and antioxidant is not completely understood, but it appears that the serum level of uric acid and the form of its presence determine its property. Uric acid crystals, which result from a high serum uric acid level and deposit in joints, bones, and soft tissues, are shown to promote an inflammatory response, leading to characteristic acute arthritis or gout. Patterson et al. reported that uric acid can be switched from an antioxidant to a prooxidant by adding a lipid hydroperoxide to native LDL, suggesting that uric acid may confer prooxidant effects even in the physiological range of uric acid concentration, but particularly so when uric acid is supersaturated in blood (Patterson et al. 2003).

Major evidence endorsing the prooxidant property of uric acid comes from studies on its role in the pathophysiology of cardiovascular diseases. During the development of cardiovascular diseases, functional impairment of endothelial activity is the first step in the pathophysiologic changes (Rocha et al. 2010). This condition results from reduced bioavailability of nitric oxide (NO), which is a well-known vasodilator (Rocha et al. 2010). The decrement of bioavailability of NO is mainly caused by increased NO degradation through its reaction with reactive

oxygen species (ROS). Uric acid is thought to facilitate degradation of NO through its prooxidative property inside endothelial cells (Rocha et al. 2010).

Epidemiological studies observed that an increased concentration of serum uric acid was an independent risk factor for cardiovascular diseases in patients with other cardiovascular risk factors, as well as in the general population (Chen et al. 2009). However, there are other studies showing contradictory results. A recent meta-analysis has demonstrated that hyperuricemia increased the risk of coronary events only marginally (Kim et al. 2010). Similarly, in a large-scale clinical trial, the observed association between hyperuricemia and cardiovascular diseases was weak and did not persist when patients with gout were excluded from the analysis (Krishnan et al. 2008), implying that it is not isolated hyperuricemia but the presence of gout that contributes to the increased risk of cardiovascular diseases (Krishnan et al. 2008). The failure to observe an independent association of hyperuricemia with cardiovascular diseases in some studies raises the concern that the observed association between uric acid and cardiovascular diseases is merely a coincidence or a spurious association, rather than a cause-effect relationship (Krishnan and Sokolove 2011). To address the causality issue, Mendelian randomization analysis of two large prospective cohort studies was conducted (Palmer et al. 2013). Mendelian randomization analysis uses a genetic instrumental variable to examine causation of an association (Lawlor et al. 2008). Mendelian randomization analysis did not provide strong evidence for causal associations between uric acid and ischemic heart disease or blood pressure. However, a causal effect was observed between body mass index and uric acid level, indicating that body mass index acts as a confounder in the relationship between uric acid and cardiovascular diseases.

Oxidative Stress as a Mechanism of Age-Related Bone Loss

Human beings typically attain their peak bone mass by the age of 30. Evidence suggests that some bone loss begins thereafter, but bone loss is accelerated around menopause in women (Looker et al. 1998). The precise mechanisms of bone loss are still not known, but various factors including genetics, life style factors (calcium and vitamin D intake, smoking, physical activity), and estrogen deficiency are known to play a role. Among them, estrogen deficiency has been traditionally considered the primary mechanism of bone loss in both men and women (Manolagas 2010).

However, a recent line of evidence gives credence to the notion that oxidative stress is also an important mechanism of age-related bone loss. Oxygen-derived free radicals stimulate the formation of new osteoclasts and enhance bone resorption (Garrett et al. 1990). Oxidative stress decreases osteoblast number and bone formation through antagonizing the Wnt/beta catenin signaling pathway (Manolagas and Almeida 2007). Furthermore, lipoxygenase-mediated lipid oxygenation leads to oxidative stress and affects osteoblast differentiation (Almeida et al. 2009).

Epidemiologic studies have demonstrated that oxidative stress has detrimental effects on bone. In a small cross-sectional study of 101 Swedish men and women aged between 22 and 85, the degree of oxidative stress, which was evaluated by

measuring urinary 8-iso-PGF, was inversely associated with bone mineral density (BMD) at multiple sites (Basu et al. 2001). Another cross-sectional study of 405 older Swedish men replicated the inverse association between urinary 8-iso-PGF and BMD, which was more pronounced in those with a low serum level of vitamin E, another antioxidant (Ostman et al. 2009). In a small case–control study of 94 older Mexicans, oxidative stress, measured with multiple biomarkers including lipoperoxide and superoxide dismutase, was independently associated with osteoporosis (Sanchez-Rodriguez et al. 2007).

Another case–control study of 87 postmenopausal women demonstrated that plasma levels of oxidative stress markers were higher in cases with osteoporosis compared with controls, and oxidative stress was significantly correlated with femoral BMD, emphasizing a role of oxidative stress in postmenopausal bone loss (Sendur et al. 2009).

The proposed link between oxidative stress and bone has been corroborated by studies demonstrating a beneficial effect of antioxidants on bone. In a cross-sectional study of 699 Japanese men and women, the serum concentrations of carotenoids, which are antioxidant micronutrients whose serum levels elevate with increasing intake of fruit and vegetables, were positively associated with radial BMD (Sugiura et al. 2008). However, the association was observed only in postmenopausal women and not in men and premenopausal women, who in general had normal BMD. A case–control study of 150 older Italian women showed that serum levels of exogenous antioxidants (dietary vitamin A, C, and E) and endogenous antioxidants (uric acid, superoxide dismutase, and glutathione peroxidase) were consistently lower in those with osteoporosis compared to those without (Maggio et al. 2003).

The longitudinal association between antioxidants and BMD was examined by Sahni et al. in the Framingham Osteoporosis Study. The Framingham Osteoporosis Study followed 874 older men and women for an average of 4 years and showed that higher total vitamin C intake was associated with higher femoral neck BMD at baseline and that higher dietary vitamin C intake was associated with slower BMD decline over time (Sahni et al. 2008).

The protective effects of antioxidants may extend beyond BMD to fracture risk. Sahni et al. assessed the risk of fracture in the Framingham Osteoporosis Study during a follow-up of more than 15 years and found that subjects in the highest category of total or supplemental vitamin C intake had significantly lower hip and nonvertebral fracture risk compared to subjects in the lower category (Sahni et al. 2009b). A similar protective effect against fractures was also observed for carotenoid intake in the same Framingham Osteoporosis Study (Sahni et al. 2009a).

Studies on Association of Serum Uric Acid with BMD and Fracture Risk

Emerging evidence supports the role of oxidative stress in the pathogenesis of age-related bone loss. Combined with the fact that uric acid has marked antioxidative capacity, it is plausible to assume that uric acid possesses a protective effect on bone.

Tables 1 and 2 show studies that have explored the association of uric acid with bone mineral density and with the risk of fractures, respectively.

As seen in Table 1, a positive association between uric and BMD was consistently observed in various age groups in both men and women with just a few exceptions.

Nabipour et al. for the first time in clinical studies demonstrated the bone-protective effect of uric acid in older men (Nabipour et al. 2011). In this large

Table 1 Studies on association between serum uric acid and bone mineral density. +: positively associated, −: inversely associated, n/a: no association

Author	Year	Design	Patient profile	Result
Nabipour	2011	Cross-sectional	1705 men, aged 70 or older	+
Makovey	2012	Prospective cohort	356 peri- and postmenopausal women	+
Sritara	2012	Cross-sectional	1320 men, 485 women, aged 25–54	+ in men n/a in women
Kuyumuchu	2012	Retrospective	1348 osteoporosis, 643 osteopenia, 199 control	+
Ishii	2013	Cross-sectional	615 women, aged 45–75	+
Ahn	2013	Cross-sectional	7502 healthy postmenopausal women	+
Lane	2014	Prospective cohort	1680 men	+
Dalbeth	2014	Cross-sectional	1265 men, 1236 women	+
Hernandez	2015	Cross-sectional		
Zhang	2015	Cross-sectional	3496 men, 3263 women	n/a

Table 2 Studies on relationship between serum uric acid and bone fractures. +: positively associated, −: inversely associated, n/a: no association

Author	Year	Design	Patient profile	Result
Nabipour	2011	Cross-sectional	1705 men, aged 70 or older	+
Ahn	2013	Cross-sectional	7502 healthy postmenopausal women	+
Kim		Prospective cohort	16,078 Korean men aged 50 or older	
Lane	2014	Prospective cohort	1680 men	+
Mehta		Prospective cohort	1963 men, 2729 women aged 65 or older	− in men n/a in women

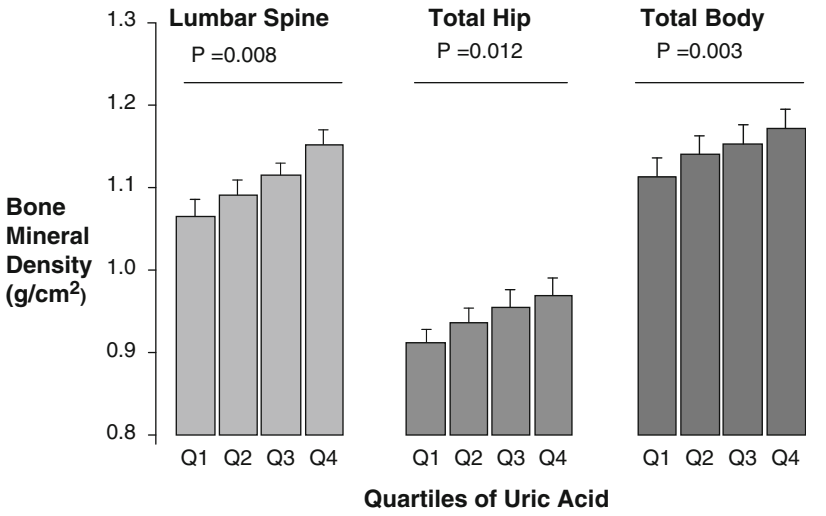

Fig. 2 Association between serum uric acid level and bone mineral density at various sites. Lumbar spine, total-hip, and total-body bone mineral density across quartiles of uric acid are shown ($n = 1387$; multivariate analysis adjusted for age, BMI, and GFR) (Data from Nabipour et al. (2011), with permissions from the publishers)

population-based prospective cohort study consisting of 1705 older men, the authors reported that a higher level of serum uric acid was associated with higher BMD at all skeletal sites including lumbar spine and femoral neck. These associations remained statistically significant after adjusting for possible confounding factors including but not limited to demographic and behavioral factors, comorbid conditions, and relevant biomarkers. The increasingly higher BMD at various sites across quartiles of uric acid is summarized in Fig. 2. Exclusion of subjects with gout or taking allopurinol from the analysis did not alter their conclusions.

Two studies replicated the positive association between uric acid and BMD in older men. In a cross-sectional analysis of 868 men older than 50 years recruited from a larger cohort study, Hernandez et al. demonstrated that men with high serum uric acid above the median had higher lumbar spine, femoral neck, and total hip BMD compared to men with low serum uric acid below the median (Hernandez et al. 2015). The authors did not conduct multivariate analysis for BMD, which raises the possibility that the observed associations might be confounded by covariates such as obesity.

Lane et al. assessed uric acid and BMD in a prospective case-cohort study of older men (Lane et al. 2014); 387 men with incident nonspine fractures and a random sample of 1383 were selected from a larger prospective cohort study, the Osteoporosis Fractures in Men study, and at baseline total hip BMD increased linearly across quartiles of serum uric acid levels after multivariate adjustment.

A beneficial effect of uric acid on BMD was observed in peri- and postmenopausal women as well in several studies.

Makovey et al. demonstrated a bone-protective effect of uric acid in a prospective cohort study of 356 peri- and postmenopausal women with average follow-up duration of 9.7 years (Makovey et al. 2013). Cross-sectional analysis showed that serum uric acid level was positively and independently associated with BMD at all skeletal sites including lumbar spine, hip, forearm, and total body, both at baseline and at the final visit. Longitudinal analysis demonstrated significant associations between serum uric acid level and annual rate of change in BMD at all skeletal sites. The protective effect was most prominent at the lumbar spine, which mostly consists of trabecular bone and is highly vascular and hence may be more susceptible to the effect of uric acid.

Ahn et al. conducted a large cross-sectional study of 7502 Korean healthy postmenopausal women and showed that higher serum uric acid concentration was independently associated with higher BMD at the lumbar spine and all proximal femoral sites (Ahn et al. 2013).

In a cross-sectional study of 615 healthy pre- and postmenopausal Japanese women, Ishii et al. demonstrated that serum uric acid concentration was positively and linearly associated with lumbar spine BMD (Ishii et al. 2014b). Importantly, in this study population, there was a period of rapid age-related changes in uric acid and BMD until the age of 60, and the rate of changes slowed down thereafter. The observed trajectory of uric acid and BMD against age is shown in Fig. 3. The observed age-related change in uric acid is consistent with menopause-related changes, rather than changes secondary to chronological aging, which is congruent with previous studies showing that uric acid level was higher in postmenopausal women compared with pre- or perimenopausal women (Hak and Choi 2008). This suggests the importance of taking menopause into account when examining the association between uric acid and BMD in women. In this study, the presence of menopause and/or years after menopause did not modify the association between uric acid and BMD.

The association between uric acid and BMD was also observed in older subjects. Kuyumcu et al. reported in a case control study of 2190 older men and women that patients with osteoporosis had a lower concentration of serum uric acid compared to subjects without osteoporosis, and that uric acid was independently and inversely associated with the prevalence of osteoporosis in multivariate analysis (Kuyumcu et al. 2012). Patients with osteoporosis had lower levels of endogenous antioxidants and higher levels of homocystein and gamma glutamyltransferase, both of which are involved in oxidative stress mechanisms, highlighting the role of oxidative stress in the development of osteoporosis.

The beneficial role of uric acid in bone health was also observed in a younger population. In a cross-sectional study of 1320 men and 485 women, young to middle aged, Sritara demonstrated that uric acid level was positively and independently associated with BMD at the lumbar spine in men only (Sritara et al. 2013). Interestingly, a significant association was observed in women, but in the opposite direction at the femoral neck. This was contradictory to the previous studies conducted by Makovey et al. (2013), Ahn et al. (2013) and Ishii et al. (2014b). This may have been due to the difference in age of the study subjects. The mean age of women included

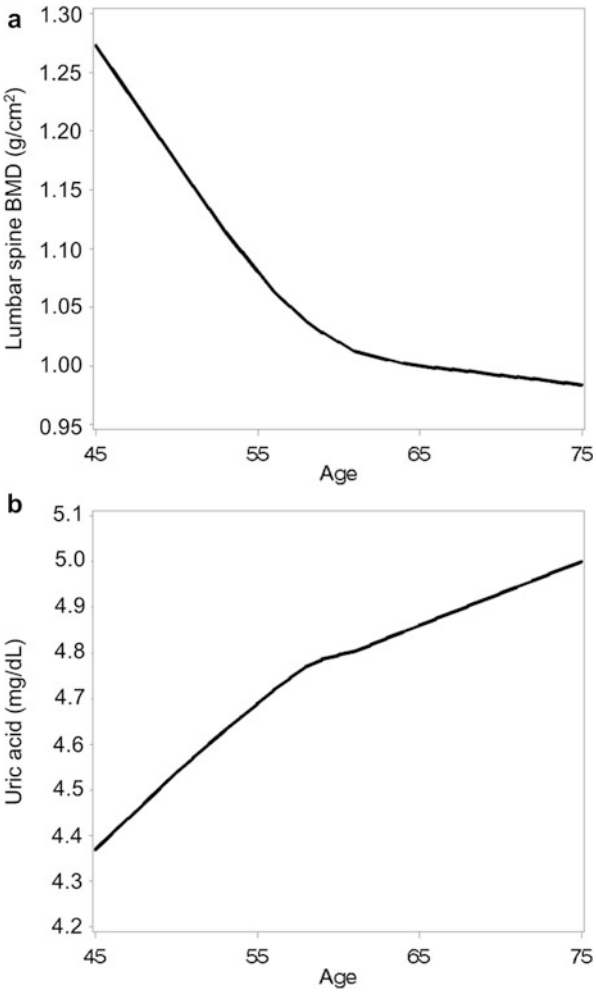

Fig. 3 Chronological changes in bone mineral density and uric acid with age. Local polynomial regression fitting plots of (**a**) lumbar spine bone mineral density against age and (**b**) serum uric acid against age are shown (Data from Ishii (2013), with permission from the publishers)

in Sritara's study was 40.2 years, who were mostly premenopausal. Another possible explanation is confounding by estrogen. Estrogen may be a potential confounder, since uric acid level is lower in the premenopausal period, as shown by Ishii's study (Ishii et al. 2014b), and it is well established that estrogen improves BMD. However, neither estrogen level nor menopausal status was properly accounted for in the analysis.

Dalbeth et al. conducted Mendelian randomization analysis to explore the causal relationship between serum uric acid and BMD (Dalbeth et al. 2015). The data were taken from the Framingham Heart Study, and the analysis included 1265 men and 1236 women in their forties. Although the cross-sectional analysis showed a strong positive association between serum uric acid level and BMD at the total femur and lumbar spine after adjusting for confounders including menopause status, Mendelian

randomization analysis did not provide evidence for causality between uric acid and BMD. Mendelian randomization analysis relies on a number of assumptions, and this result needs to be confirmed in other cohorts. However, this finding suggests that the association between uric acid and BMD observed in multiple previous studies is just a spurious association due to mere confounding, despite the fact that the majority of previous studies controlled for an extensive list of confounders.

In sharp contrast to the previous studies demonstrating a positive association between uric acid and BMD, there is a recent cross-sectional study that failed to show a positive association. The data come from the nationally representative National Health and Nutrition Examination Survey (NHANES) study 2005–2010, and the analytic sample included 6759 men and women aged 30 years or older (Zhang et al. 2015). BMD at the total hip, femoral neck, and lumbar spine increased significantly across quartiles of serum uric acid in men and women, regardless of their menopausal status. However, after adjustment for potential confounders, the association was not statistically significant. The authors corroborated this finding by conducting an animal study, which showed that induction of chronic mild hyperuricemia in a rodent model did not result in significant differences in BMD and bone biochemical properties. The reasons for the discrepancy between this finding and previous study results may include the difference in age of study subjects, gender and ethnic/racial composition, and covariates included in the analysis.

Several studies have assessed the effect of uric acid on bone metabolism by measuring bone turnover markers. In healthy postmenopausal women, serum uric acid level was inversely correlated with both serum C-terminal telopeptide of type 1 collagen (CTX) and osteocalcin levels (Ahn et al. 2013). Another study replicated the inverse relationship between serum CTX and serum uric acid levels in young men (Sritara et al. 2013). In older men, serum uric acid level was negatively correlated with urinary amino-terminal cross-linked telopeptide of collagen type 1 (NTX-1) (Nabipour et al. 2011). Another study of older men showed that men with higher serum uric acid levels had lower bone turnover makers, serum C-terminal telopeptide of type 1 collagen and aminoterminal propeptide of type 1 collage (P1NP), than men with lower uric acid levels (Hernandez et al. 2015). All of these studies suggest that a higher serum uric acid level is associated with lowering of both bone resorption (as seen by lower NTX-1 and CTX-1 levels) and bone formation (as seen by lower osteocalcin and P1NP levels). Taking these findings together, uric acid may suppress bone turnover, which is closely related to the coupling processes of osteoclast-mediated bone resorption and osteoblast-mediated bone formation. This is consistent with an experimental study using rodents (Ahn et al. 2013), which demonstrated that uric acid itself suppresses osteoclast generation as well as decreases the production of intracellular reactive oxygen species. These results suggest that the bone-protective effect of uric acid may be due to suppression of osteoclast activity, which is partially explained by the antioxidative property of uric acid.

Two studies have utilized qualitative bone ultrasonography (QUS) measurements at the calcaneus to assess the effect of uric acid on bone quality. One study was a population-based study of older men and showed that serum uric acid level was

significantly associated with all QUS parameters after adjusting for confounders in older men (Hernandez et al. 2015). The other study was a cross-sectional study conducted by Sritara et al. of young to middle-aged men and women (Sritara et al. 2013). In this study, a positive association between serum uric acid level and BMD was observed only in men, as mentioned above, and the results were similar for QUS. Uric acid level was related to bone quality assessed by QUS in men only.

Bone strength is determined by bone mass, geometry, and quality. There has been no study describing the effect of uric acid on bone geometry to our knowledge. Obese individuals have more robust bone geometry (Beck et al. 2009), and, on the other hand, hyperuricemia is more prevalent in obese individuals (Lee et al. 2011; Loenen et al. 1990; Palmer et al. 2013). Therefore, the link between bone strength and uric acid may be partially via the effect of obesity on bone strength. Future research is needed to examine the effect of uric acid on bone geometry.

There are a limited number of studies exploring the potential benefit of uric acid for reducing the risk of bone fractures. Among them, two studies, which were cross-sectional in design, assessed the association of prevalent fractures with uric acid.

Nabipour et al., in the cross-sectional study mentioned above, demonstrated that a higher serum uric acid level was associated with lower prevalence of osteoporosis and vertebral and nonvertebral fractures in older men (Nabipour et al. 2011). Vertebral fractures were ascertained using a DEXA scanner, and history of nonvertebral fracture was obtained at interview. The association between serum uric acid and prevalence of osteoporosis and bone fractures at various sites is summarized in Table 3.

In the cross-sectional study on healthy postmenopausal women, also mentioned above, Ahn et al. demonstrated that subjects with prevalent vertebral fracture, assessed by obtaining radiographs of the thoracolumbar spine, had lower serum uric acid levels than those without, after adjustment for potential confounders (Ahn et al. 2013). It should be noted that the average age of subjects in this study was 57.4 years, and consequently the prevalence of vertebral fracture was quite low, 3.4%. This finding needs to be replicated in older women with a higher prevalence of vertebral fracture.

Three studies assessed the longitudinal association between uric acid and incident fractures.

In the prospective case-cohort study mentioned above, Lane et al. reported an 18% decreased risk (hazard) of nonvertebral fracture per 1 standard deviation increment in baseline serum uric acid in the analytic sample of 1680 older men after adjustment (387 cases with incident nonvertebral fractures over 5.3 years of follow-up and a random sample of 1383) (Lane et al. 2014). Hip fractures were not significantly associated with uric acid, but this may have been due to insufficient power, because only 73 hip fractures occurred in cases during the follow-up.

Kim et al. conducted a longitudinal study of 16,078 Korean men aged 50 years or older (Kim et al. 2014). During the follow-up of 3 years, 158 (1.0%) incident fragility fractures, including both vertebral and nonvertebral fractures, were identified using the health insurance database. In this study, the risk (hazard) of fragility fractures decreased by approximately 17% per 1 standard deviation increment in baseline serum uric acid level after adjusting for confounders.

Table 3 Adjusted odds ratios (*OR*) and 95% confidence intervals (*CI*) for high serum uric acid (above median) and osteoporosis at femoral neck, total hip, and lumbar spine and prevalence of vertebral fractures ($n = 1387$, men with gout, excluding allopurinol users). Model 1 included serum uric acid, age, GFR, and body mass index (*BMI*). Model 2 included serum uric acid, age, GFR, BMI, drinking, smoking, and physical activity score. Model 3 included serum uric acid, age, GFR, BMI, drinking, smoking, physical activity score, comorbid conditions, and medication use. Model 4 included serum uric acid, age, GFR, BMI, drinking, smoking, physical activity score, comorbid conditions, medication use, PTH, calcium, and 25(*OH*) vitamin D levels

	Femoral neck osteoporosis			Total hip osteoporosis			Lumbar spine osteoporosis			Vertebral fracture		
	OR	CI	p	OR	CI	p	OR	CI	p	OR	CI	p
Model1	0.54	0.32–0.91	0.22	0.34	0.11–0.99	0.50	0.44	0.25–0.78	0.05	0.65	0.47–0.91	0.012
Model2	0.62	0.36–1.06	0.84	0.27	0.88–0.91	0.35	0.44	0.24–0.80	0.08	0.67	0.48–0.94	0.021
Model3	0.47	0.25–0.86	0.16	0.29	0.08–1.03	0.56	0.43	0.23–0.82	0.11	0.66	0.46–0.96	0.029
Model4	0.42	0.22–0.81	0.10	0.30	0.08–1.12	0.75	0.44	0.23–0.86	0.16	0.62	0.43–0.91	0.015

Data from Nabipour et al. (2011), with permission from the publishers

Mehta et al. reported results conflicting with previous study findings, in the Cardiovascular Health Study (Mehta et al. 2015). The Cardiovascular Health Study is a prospective cohort study of men and women aged 65 or older; 4692 were included in the analysis and 430 (22%) had hyperuricemia (serum uric acid level higher than 7 mg/dL). During the average follow-up of 9.6 years, 156 hip fractures (7.9%) occurred in men. Interestingly, in an unadjusted model, a U-shaped relationship was observed between serum uric acid level and risk of hip fracture. However, in the multivariate model, the highest serum uric acid quartile was associated with an increased risk of hip fracture, but such an association was not observed for the lowest serum uric acid quartile. In women, 363 (13.3%) hip fractures occurred during the average follow-up of 10.9 years, and the association of uric acid with hip fracture was not significant. The reason for the increased fracture risk associated with higher uric acid in men was not clear. If uric acid compromises bone health, the risk of fractures in the spine would have been much more greatly increased in the highest serum uric acid group since the spine is highly vascular and considered more susceptible to the effect of uric acid. Unfortunately, vertebral fractures were not assessed in this study. Considering the U-shaped relationship between uric acid and hip fracture risk, it is possible that there are multiple underlying mechanisms by which uric acid affects bone health.

There are several proposed mechanisms for the relationship between bone and uric acid other than the antioxidant capacity of uric acid. First, uric acid clearance is affected by osteoporosis-induced metabolic changes. Renal clearance of uric acid appears to be partly dependent on parathyroid hormone (Laspa et al. 2004), which is affected by osteoporosis treatment such as calcium supplementation. Serum uric acid is also weakly associated with dietary calcium intake (Dalbeth et al. 2009). Second, serum vitamin D level may play a role. A cross-sectional study demonstrated a significant correlation between serum 25(OH)D and serum uric acid levels (Nabipour et al. 2011). The underlying mechanism of this relation may be due to age-related impairment of hepatic function. Xanthine oxidase, an enzyme in uric acid production, is found in hepatocytes. Therefore, age-related impairment of hepatic function can result in both lower uric acid production and impaired 25-hydroxylation of vitamin D in the liver. Third, body weight might confound the association of uric acid with BMD and fracture risk. Body weight is associated with both BMD and uric acid (Lee et al. 2011; Loenen et al. 1990). The association of body weight with fracture risk involves multiple mechanisms and may not be linear (Ishii et al. 2014a). Therefore, residual confounding is a concern particularly when the association between fracture and uric acid is examined.

In summary, accumulating evidence supports the beneficial effect of uric acid on bone, especially in older men and women. There may be multiple underlying mechanisms for the association between uric acid and bone. The primary mechanism is considered to be the antioxidant property of uric acid, but future research is warranted to understand the complex relationship between uric acid and bone.

Potential Applications to Prognosis, Other Diseases or Conditions

This review highlights the complex relationship between serum uric acid and bone health. While a few recent studies reported conflicting findings, most studies to date demonstrated a protective effect of uric acid on bone. The underlying mechanism is believed to involve the antioxidant property of uric acid, but needs further elucidation. At this point, there is insufficient evidence to determine whether the observed association between uric acid and bone is causal, and it will most likely take a long time to resolve this issue. However, the fundamental question we should ask is whether we can apply the findings on uric acid from previous studies to actual patients in order to improve the clinical care for osteoporosis, and the answer seems to be yes. Uric acid measurement may provide information on the balance between oxidative stress and antioxidant levels, which would be otherwise unavailable in daily clinical practice. If the bone-protective effect of uric acid is real and indeed due to the antioxidant property of uric acid, measurement of serum uric acid level would provide additional prognostic insight for osteoporosis or bone fractures. For example, it might be worthwhile to incorporate the serum level of uric acid into the FRAX score, which evaluates the 10-year probability of developing bone fractures, to improve the accuracy of its prediction. Serum uric acid concentration might also be used to select high-risk subjects to be screened for osteoporosis. However, the clinical utility of uric acid as a marker of osteoporosis/bone fracture needs to be proven in clinical studies.

Another implication of the beneficial effect of uric acid is that hyperuricemia might be preferable as long as it remains asymptomatic. The optimal goal of uric acid-lowering therapy might need to be reconsidered, taking into account the potential benefit of a high uric acid level. Nonetheless, future research is warranted to *first* confirm and establish the association between the serum level of uric acid and bone health.

Uric Acid Measurement

For serum uric acid measurement, in research settings, a blood sample is usually drawn after an overnight fast to minimize the effects of diet and diurnal variation. In healthy people with a normal diet, serum uric acid levels are higher at dawn and lower in the evening, with a diurnal variation of approximately 0.5 mg/dL (Kanabrocki et al. 2000). In addition, serum uric acid levels increase after ingesting purines, soy beans, or alcohol and decrease after ingesting animal-derived proteins (Garrel et al. 1991). In clinical practice, such meticulous care is usually not warranted and a blood sample is collected at any time of the day. However, if hyperuricemia is observed, repeat uric acid determination is recommended after an interval of at least a week to confirm the presence of hyperuricemia.

After the blood sample is collected, serum is separated by a standard centrifugation procedure. Serum samples can be stored at room temperature for several days without influencing uric acid levels (Henriksen et al. 2004).

Serum uric acid levels are usually measured using an enzymatic method. Uric acid is oxidized in the presence of the enzyme uricase to allatoin, carbon dioxide, and hydrogen peroxide. Several assays, including a colorimetric assay (Fossati et al. 1980) and a fluorometric assay, are employed to quantify the amount of hydrogen peroxide formed in this reaction. The colorimetric assay is utilized widely in autoanalyzers. High performance liquid chromatography may also be used to determine serum uric acid levels with high accuracy and precision but is not widely employed (Cooper et al. 2006).

Uric acid measurement is accurate and reliable, with a measurement error of approximately 2% (Fuentes-Arderiu 2001).

Summary Points

- This chapter focuses on the bone-protective effect of uric acid.
- Uric acid is a potent antioxidant in humans.
- Under certain situations, it acts as a prooxidant substance.
- Uric acid is associated with increased risk of cardiovascular diseases, but the causality remains controversial.
- Most of the recent studies showed that a higher level of uric acid was associated with higher bone mineral density and lower incidence of bone fractures.
- These associations may be attributable to the antioxidant property of uric acid.
- Further study is needed to fully understand the underlying mechanism of the bone-protective effect of uric acid.

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Circulating Sclerostin in Bone Sclerosing Disorders

10

Antoon H. van Lierop and Socrates E. Papapoulos

Contents

Abstract

Sclerostin is a negative regulator of bone formation, which is produced by osteocytes and acts on osteoblasts where it binds the LRP5/6 co-receptors and antagonizes canonical Wnt signaling in these cells. The availability of commercial assays to measure sclerostin in blood initiated numerous clinical studies to

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elucidate the role of this protein in physiological and disordered bone metabolism. In interpreting results of such studies it is important to consider that neither the active form of sclerostin nor its metabolism are currently known and values obtained with available assays are moderately correlated. However, measurement of circulating sclerostin can assist the differential diagnosis of patients with rare bone sclerosing disorders and high bone mass such as sclerosteosis, van Buchem disease, and the high bone mass phenotype.

Keywords

DKK1 • High bone mass phenotype • Bone turnover • LRP4 • Sclerosteosis • Sclerostin • van Buchem disease • Wnt signaling

List of Abbreviations

CTX	Carboxyterminal collagen crosslinks
DAN	Differential screening-selected gene abberative in neuroblastoma
DKK1	Dickkopf-related protein 1
HBM	High bone mass
LRP	Low-density lipoprotein receptor-related protein
P1NP	Procollagen type 1 N-terminal propeptide
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor kappa-B ligand
SCL	Sclerostin
VBD	Van Buchem disease
Wnt	Wingless-related integration site

Key Facts of Osteocytes

- They constitute more than 95% of bone cells in the adult skeleton.
- They are one of the longest living cells in the body (half-life 25 years).
- They reside in lacunae within the mineralized bone matrix and communicate with neighboring osteocytes and cells at the bone surface and vasculature through cytoplasmic processes (dendrites) that run through tiny channels (canaliculi).
- They sense mechanical signals (mechanosensors), and they control the adaptive responses of the skeleton to mechanical loading.
- They orchestrate bone remodeling by regulating the function of osteoclasts and osteoblasts and, hence, bone resorption and bone formation.
- They integrate hormonal and mechanical signals in the regulation of bone mass.
- They are the target cells for PTH action on bone, and they mediate the anabolic actions of the canonical Wnt signaling pathway.
- They have an endocrine function and produce FGF23, a factor, which acts in the kidney and controls phosphate homeostasis.
- Osteocyte-produced molecules are targets of therapeutics for bone disorders (e.g., RANKL, sclerostin, FGF23).

Definition of Words and Terms

DKK1	An inhibitor of the canonical Wnt signaling pathway that binds to LRP5/6, co-receptors preventing the activation of Wnt signaling
High bone mass phenotype	A bone sclerosing disorder due to mutations of the LRP5 gene, rendering the LRP5 receptor resistant to the antagonist actions of sclerostin and DKK1
LRP5 receptor	A transmembrane receptor that forms a complex with the Frizzled receptor to transduce signals by Wnt proteins through the canonical Wnt pathway
Sclerosteosis	A bone sclerosing disorder due to loss-of-function mutations of the SOST gene that encodes sclerostin
Sclerostin	A 22.5-KDa secreted glycoprotein that is synthesized in bone by osteocytes and functions as a negative regulator of bone formation by inhibiting the canonical, β catenin-dependent, Wnt signaling pathway
Van Buchem disease	A bone sclerosing disorder due to a large genomic deletion downstream of the SOST gene resulting in impaired synthesis of sclerostin

Introduction

During the past two decades, studies of rare bone disorders have greatly enhanced our understanding of the local regulation of bone metabolism. The finding of gain-of-function mutations in the low density lipoprotein receptor-related protein 5 (LRP5) gene in patients with the high bone mass (HBM) phenotype (Boyden et al. 2002; Little et al. 2002) and loss-of-function mutations of LRP5 in patients with the osteoporosis-pseudoglioma syndrome (Gong et al. 2001) revealed the crucial role of canonical Wnt signaling pathway in osteoblast development and function. Subsequently, a number of regulators of the Wnt signaling pathway in bone was identified of which the most extensively studied is sclerostin. Commercial assays for the measurement of circulating sclerostin were developed and are used in studies of patients with metabolic bone disorders. We review here the value of circulating sclerostin as biochemical marker of bone metabolism in patients with rare skeletal disorders associated with high bone mass.

Sclerostin Molecule and Actions

Sclerostin is a secreted glycoprotein with sequence similarity to the DAN (differential screening-selected gene aberrative in neuroblastoma) family of proteins. It has 190 amino acid residues, which form a three loop-like structure with a cystine knot at

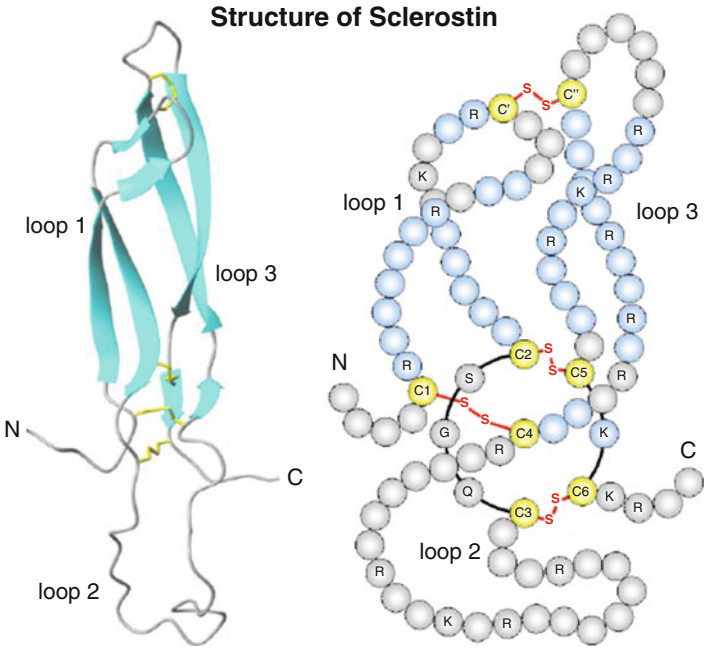

Fig. 1 *Left panel* shows a ribbon representation of the backbone topology of the structured core of the protein (residues 52 to 147) in the same orientation. *Right panel* shows a schematic representation of the protein, highlighting the positions of disulphide bonds, the resulting 3 loop structure, and residues involved in regular sheets (Reproduced with permission from Veverka et al. 2009)

its central core (Fig. 1). While the first and third loops are well structured, the second loop appears disordered on nuclear molecular resonance imaging. The long N- and C-termini are also flexible but appear to have a functional role (Weidauer et al. 2009; Veverka et al. 2009). The third loop of the sclerostin molecule is the presumed binding site to its target receptor LRP5/6 (Veverka et al. 2009), although parts of the cystine knot might also be involved (Boschert et al. 2013). A neutralizing monoclonal antibody against sclerostin binds the second loop, suggesting that this loop is also important for sclerostin activity.

Sclerostin is produced in the skeleton exclusively by osteocytes and is transported by the osteocyte canaliculi to act on osteoblasts at the bone surface but also on neighboring osteocytes (van Bezooijen et al. 2004; Poole et al. 2005). At the bone surface, sclerostin inhibits bone formation by osteoblasts while it increases bone resorption by stimulating the production of RANKL by osteocytes. Sclerostin antagonizes the canonical Wnt signaling pathway in osteoblasts through binding to the first propeller of LRP5/6 (Li et al. 2005; Bourhis et al. 2011), thereby preventing the binding of Wnt ligands to the LRP5/6 and frizzled co-receptor complex and blocking Wnt signaling. The action of sclerostin on Wnt signaling pathway is facilitated by LRP4, a negative regulator of LRP5/6 signaling which acts as co-receptor for sclerostin (Choi et al. 2009; Leupin et al. 2011; Chang et al. 2014; Fig. 2).

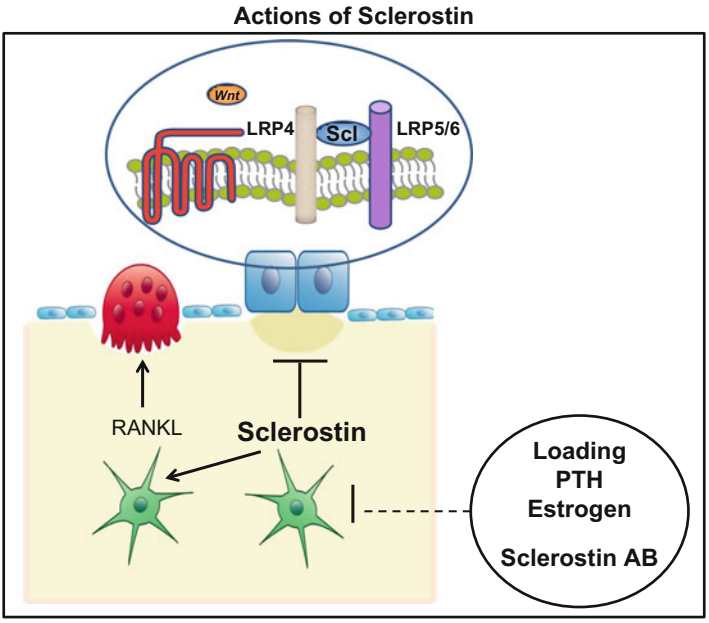

Fig. 2 Osteocyte-produced sclerostin inhibits the proliferation, differentiation, and survival of osteoblasts and reduces bone formation; it also stimulates the production of RANKL by neighboring osteocytes and bone resorption. In osteoblasts, sclerostin binds to LRP5/6 and inhibits the Wnt signaling pathway, an action facilitated by LRP4. Production of sclerostin is decreased by mechanical loading, PTH, estrogens, and antisclerostin antibodies. *LRP* low-density lipoprotein receptor-related protein, *PTH* parathyroid hormone, *RANKL* receptor activator of nuclear factor kappa-B ligand (Reproduced from Appelman-Dijkstra and Papapoulos 2015)

Sclerostin Assays

During the past few years, there has been mounting interest in the role of sclerostin in bone remodeling, and the availability of assays to measure this protein in blood facilitated clinical studies to understand its role in disorders of bone and mineral metabolism (reviewed by Clarke and Drake 2013). These studies have mainly been conducted with three commercially available sclerostin assays: the enzyme-linked immunosorbent sclerostin assays of Biomedica Medizinprodukt (Wien, Austria), TECO medical (Sissach, Switzerland), and the electro-chemiluminescence sclerostin assay of Meso-Scale diagnostics (Rockville, USA). In interpreting results obtained with these assays it should be noted that the active form of sclerostin is not known and there is no information about its metabolism and, hence, of circulating fragments that may be immunoreactive.

The Biomedica assay uses a polyclonal goat antihuman sclerostin capture antibody and a murine monoclonal antisclerostin detection antibody. It has a reported detection range between 3.2 pmol/ml (72 pg/ml) and 240 pmol/ml (5400 pg/ml) and

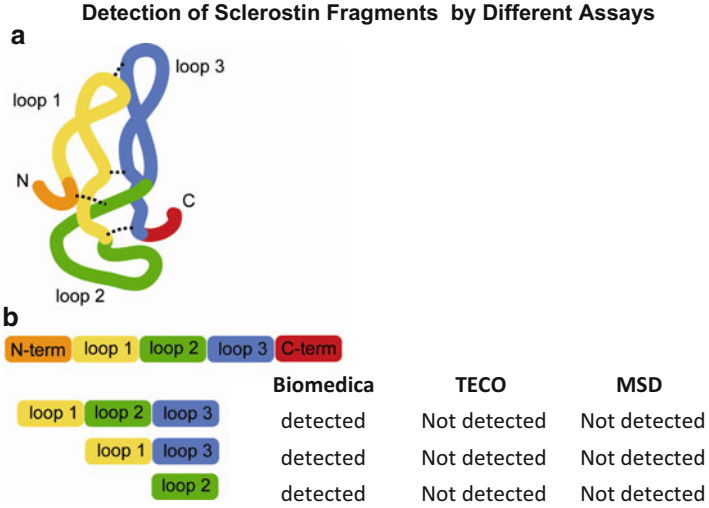

Fig. 3 Detection of fragments of the sclerostin molecule using different sclerostin assays. (**a**) Simplified representation of the sclerostin molecule. (**b**) Detection of sclerostin fragments of different sizes with the Biomedica, TECO, and the MSD sclerostin assays. All fragments were undectable in the TECO and MSD assay, but were detected by the Biomedica assay (Modified from van Lierop et al. 2012a)

a lower limit of quantification of 7.5 pmol/l (170 pg/ml). Validation of the assay showed intra- and interassay coefficients of variation of 5% and 6%, respectively, and linearity of values of serially diluted samples. Recovery of sclerostin in serum spiked with the protein was 92–108%. Serum and EDTA plasma samples showed stable results when kept at room temperature for up to 24 h and after four freeze/thaw cycles (Biomedica n.d.). In earlier developed assays, sclerostin levels were higher in EDTA and heparin plasma compared with serum samples (McNulty et al. 2011), but later assays showed no difference between sample matrixes (Biomedica n.d.). The Biomedica assay detects recombinant sclerostin fragments of different length (the three loop core without the N- and C-termini, the 1st and 3rd loop without the N- and C-termini, and the 2nd loop alone; Fig. 3) (van Lierop et al. 2012a). This suggests that the assay does not recognize the entire sclerostin molecule. Although the assay did not detect structurally related proteins such as noggin and wise (Biomedica n.d.), sclerostin was detectable in serum of 3 of 7 patients with sclerosteosis, suggesting possible cross-reactivity with other proteins (van Lierop et al. 2012a).

The TECO sclerostin assay uses a polyclonal goat antihuman sclerostin capture antibody and a monoclonal antihuman detection antibody. Antibodies recognize epitopes that reside at the amino terminus and the midregion of the protein (Durosier et al. 2013). The detection range is between 50 pg/ml and 3000 pg/ml, with a lower limit of quantification of 130 pg/ml (Garnero et al. 2013). The intra- and interassay coefficients of variation range between 3.8 and 8.0% and 3.3–9.0%, respectively (Durosier et al. 2013). Serial dilutions of samples showed good linearity, and

recovery in serum spiked with sclerostin was 96–102% (Garnero et al. 2013). Sclerostin levels are stable at room temperature for more than 24 h and after three freeze/thaw cycles (TECOmedical n.d.). The sclerostin fragments described in the previous paragraph were not detected by the TECO assay. Sclerostin was measurable, however, in serum samples of all six patients with sclerosteosis tested but values were close to the limit of detection (own unpublished observations).

The electro-chemiluminescence sclerostin assay of MSD uses a polyclonal goat capture antibody against murine sclerostin and a polyclonal goat antihuman sclerostin detection antibody. Epitope mapping of the capture and detection antibodies revealed binding sites for the capture antibody at the N-terminus, and one at the distal end of the C-terminus of human sclerostin, while for the detection antibody three epitopes were identified, at the N-terminus, at the third loop, and at the C-terminus, respectively (van Lierop et al. 2011; Fig. 4). The MSD sclerostin assay has a very broad detection range from 1 pg/ml to 10.000 pg/ml and a very low limit of quantification (5 pg/ml). The intra- and interassay coefficients of variation are 4 and 7%, respectively. The recovery of added sclerostin in serum

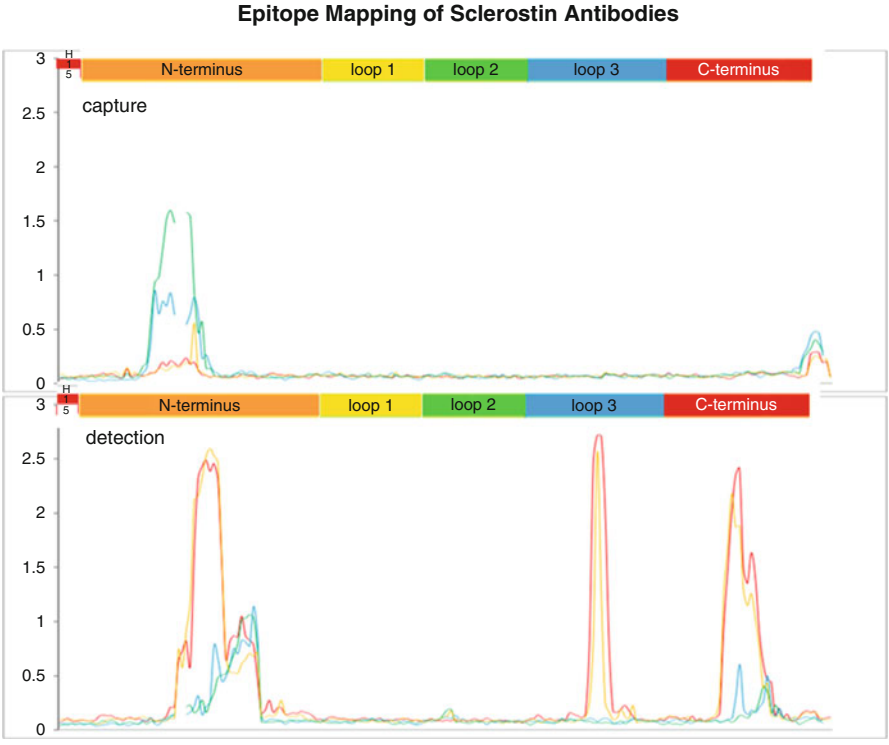

Fig. 4 Epitope mapping of the MSD capture antibody (*upper panel*) and detection antibody (*lower panel*). Spikes in the baseline represent increased binding affinity of the antibodies for the sclerostin molecule. Above each panel is a schematic linear representation of the sclerostin molecule (Unpublished)

samples was 82–93%. The measured concentrations of serial dilutions of recombinant sclerostin were highly concordant with that of the sclerostin standard provided in the assay (ratio 1.01, range 0.8–1.3) (van Lierop et al. 2011). When samples of the sclerostin standard provided with the Biomedica assay were measured by the MSD assay, sclerostin values were on average six-fold lower than expected (own unpublished observations). Sclerostin levels remained stable after multiple freeze/thawing cycles, but levels declined by about 50% when samples were kept at room temperature for more than 24 h (Durosier et al. 2013). The MSD sclerostin assay did not detect any of the above described recombinant sclerostin fragments and did not detect any immunoreactive sclerostin in the serum of 19 patients with sclerosteosis (van Lierop et al. 2011). These findings suggest that this assay is specific for the entire sclerostin molecule and does not appear to cross react with other circulating proteins.

Differences Between Sclerostin Assays

Comparison of measured sclerostin levels in the same samples with the three assays showed considerable differences (McNulty et al. 2011; Durosier et al. 2013). The Biomedica assay detected about two-fold and 30-fold higher levels than the TECO and the MSD assays, respectively, and serum sclerostin levels were moderately correlated (Spearman's correlation coefficient between 0.53 and 0.71) (Durosier et al. 2013). Observed differences between measured values by the three assays are presumably the result of different epitope recognition. The lower stability of the N- and C-terminus compared to the central core of the protein, and their easy cleavage, may explain the diminished stability of measured sclerostin with the MSD assay in samples kept at room temperature for more than 24 h. Furthermore, this may also explain the much lower levels of circulating sclerostin measured by the MSD assay compared with the Biomedica and TECO assays. In addition, binding of circulating sclerostin to proteins, that may obscure binding sites crucial for antibody detection, may also contribute to differences in measured values. Sclerostin was found to have a binding site for heparin (Veverka et al. 2009), and the addition of heparin to sclerostin-producing osteoblastic cell cultures increased the amount of sclerostin in the supernatant, without affecting its production. In line with these observations, measurement of sclerostin with the Biomedica and TECO assays revealed higher levels in heparin samples than serum samples (McNulty et al. 2011). Since the presence of heparin in the samples matrix results in higher sclerostin concentrations, it has been suggested that the heparin displaces sclerostin from other proteins, which obscured the binding sites to the detection or capture antibodies. Differences in reported sclerostin values with the three assays may, therefore, also result from differences in the sensitivity of specific epitopes of antibodies to protein-bound and free-circulating sclerostin. Notably, Biomedica in their last generation of the sclerostin ELISA has resolved the differences in measured sclerostin levels between sample matrixes.

Circulating Sclerostin in Sclerosing Bone Disorders

Sclerosteosis

Sclerosteosis is an autosomal recessive bone sclerosing disorder caused by loss-of-function mutations in SOST, the gene encoding sclerostin (Balemans et al. 2001; Brunkow et al. 2001). So far, eight different mutations have been identified resulting either in impaired synthesis of sclerostin or in the synthesis of a nonfunctioning protein (Balemans et al. 2001, 2005; Brunkow et al. 2001; Kim et al. 2008; Piters et al. 2010; Bhadada et al. 2013; Belkhribchia et al. 2014; He et al. 2016). The majority of patients are members of the Afrikaner population in South Africa, but isolated cases have been reported in other countries around the world. In the absence of sclerostin, bone formation is increased in these patients, while bone resorption is mostly unaffected or decreased (van Lierop et al. 2011). As a result, patients develop generalized osteosclerosis, with bone mineral density Z-scores sometimes exceeding +10 (Gardner et al. 2005). The excess bone formed in patients with sclerosteosis is of excellent quality (Hassler et al. 2014) evidenced also by the lack of any reports of fractures in these patients (Hamersma et al. 2003; van Lierop et al. 2011). Serious complications of sclerosteosis are due to overgrowth of the skull bones and include facial distortion with elongation of the forehead and mandibular overgrowth and nerve entrapment syndromes, such as facial palsy due to narrowing of cranial foramina. Another frequently encountered complication is hearing loss, resulting from narrowing of the external auditory canal, fixation of ossicles, or impingement of the acoustic nerves. Moreover, the thickening of calvaria and skull base can lead to increased intracranial pressure, prompting the need for craniotomy in a substantial number of patients, being also a cause of sudden death (Hamersma et al. 2003; van Lierop et al. 2011). Other characteristic findings of sclerosteosis include tall stature and syndactyly, which can be used to clinically differentiate these patients from those with van Buchem disease and HBM (Beighton et al. 1988). Although carriers of sclerosteosis mutations have increased bone formation and a higher bone mineral density compared with healthy controls, they do not develop any of the complications of sclerosteosis (Gardner et al. 2005; van Lierop et al. 2011).

In line with the underlying genetic defect, no sclerostin could be detected in serum of 19 South African patients with sclerosteosis with the MSD sclerostin assay (van Lierop et al. 2011). In contrast, sclerostin was detected in serum of all 26 measured disease carriers, but mean levels were about 50% lower than those of healthy controls (16 pg/ml vs. 40 pg/ml), indicating a gene-dose effect. There was, however, considerable overlap between values of carriers and healthy controls (Fig. 5). As already discussed, sclerostin was also measured in a few patients with sclerosteosis with the Biomedica and TECO assays.

As expected by the inhibitory action of sclerostin on bone formation, patients with sclerosteosis and sclerostin deficiency have elevated levels of the bone formation marker procollagen type 1 N-terminal propeptide (P1NP), while P1NP levels in carriers are within the reference range but higher than those of healthy controls

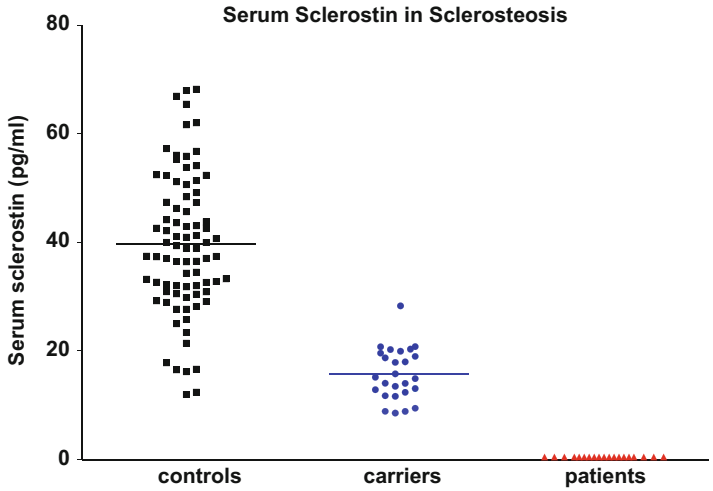

Fig. 5 Serum levels of sclerostin in patients and carriers with sclerosteosis and healthy controls. Sclerostin levels were measured with the MSD sclerostin assay. No sclerostin was detected in serum samples of 19 patients with sclerosteosis (Reproduced from van Lierop et al. 2011)

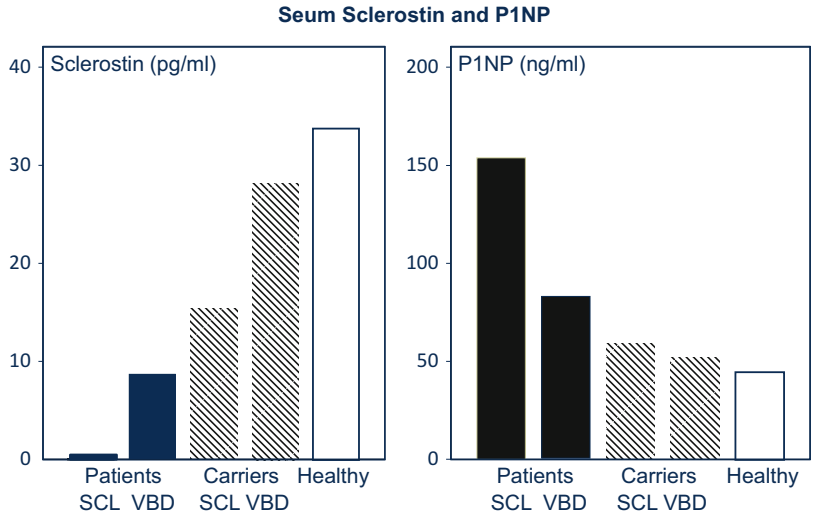

Fig. 6 Serum sclerostin and P1NP levels in patients and heterozygous carriers of sclerosteosis (*SCL*), van Buchem disease (*VBD*), and healthy subjects (Reproduced from Appelman-Dijkstra and Papapoulos 2014)

(Fig. 6). There were no differences in levels of the bone resorption marker carboxy-terminal collagen crosslinks (CTX) between patients, carriers, and controls (van Lierop et al. 2011). Interestingly, serum levels of Dickkopf 1 (DKK1), another Wnt

signaling inhibitor which also acts on the LRP5 receptor, is increased in patients with sclerosteosis (van Lierop et al. 2014), possibly as a compensatory response to sclerostin deficiency.

Van Buchem Disease

Van Buchem disease (VBD), or "hyperostosis corticalis generalisata familiaris," is an autosomal recessive disorder due to a 52 kb deletion downstream of the SOST gene, which contains an essential regulatory element for postnatal SOST transcription (Balemans et al. 2002; Staehling-Hampton et al. 2002). About 30 cases of VBD have been described, the majority inhabitants of a small village in the Netherlands. Clinical manifestations of patients with VBD are very similar to those of patients with sclerosteosis including complications due to bone overgrowth of the skull, such as facial distortion, facial nerve palsy, and hearing loss. However, increased intracranial pressure is a rare finding (van Lierop et al. 2010), and patients have normal stature and no digit abnormities (van Lierop et al. 2013). Bone mass is greatly increased to values comparable to those of patients with sclerosteosis, with bone mineral density Z-scores sometimes exceeding +10 (van Lierop et al. 2013).

In patients with VBD, circulating sclerostin levels measured with the MSD assay were low, but detectable in nearly all patients (range <5 to 17 pg/ml). This is in agreement with the slightly milder phenotype of these patients compared with that of patients with sclerosteosis. Carriers of VBD had levels lower than controls (29 vs. 40 pg/ml) with considerable overlap of values (Fig. 6; van Lierop et al. 2013). There are no reported values of circulating sclerostin in carriers or patients with VBD with either the Biomedica or the TECO assays.

Patients with VBD had increased levels of P1NP in serum, while in carriers these were slightly higher than in healthy controls; in contrast, serum CTX levels were similar in patients and carriers (van Lierop et al. 2013). As in sclerosteosis, patients with VBD had increased levels of DKK1 in serum (van Lierop et al. 2014).

High Bone Mass Phenotype

In contrast to sclerosteosis and VBD, the HBM phenotype is inherited as an autosomal dominant trait (Boyden et al. 2002). Twelve different mutations have been identified, all clustered within the region encoding for the first propeller domain of LRP5 (Balemans et al. 2007; Boyden et al. 2002; Gregson et al. 2015; Kwee et al. 2005; Little et al. 2002; Rickels et al. 2005; van WesenBeeck et al. 2003; Whyte et al. 2004). These mutations render the LRP5 receptor resistant to the inhibitory action of sclerostin and DKK1 (Ai et al. 2005; Semenov et al. 2005; Balemans et al. 2007; Niziolek et al. 2015). About one hundred cases have been identified in different parts of the world.

HBM is characterized by generalized endosteal hyperostosis, although to a lesser extent than sclerosteosis or VBD, with bone mineral density Z- scores

ranging between +5 and +10 (Boyden et al. 2002; Little et al. 2002; Rickels et al. 2005; Balemans et al. 2007). Enlargement of the jaw is a frequent finding but complications resulting from cranial nerve entrapment are rare (Kwee et al. 2005). Furthermore, patients have normal stature and no digit abnormalities but almost all have torus pallatinus, a bone protrusion of the palate, which has also been described in some patients with sclerosteosis but not in patients with VBD (Beighton et al. 1988).

Three studies reported sclerostin levels measured with the Biomedica assay in patients with HBM. In 19 patients with HBM due to a T253I mutation, Frost et al. found higher sclerostin levels than in 19 control subjects (22 vs. 13 pmol/l or 500 vs. 296 pg/ml) (Frost et al. 2011). Similarly, Gregson et al. reported higher serum sclerostin levels in six HBM patients compared with 196 controls (129 vs. 66 pmol/l; 2900 vs. 1500 pg/ml) (Gregson et al. 2014). In contrast, Simpson and colleagues found comparable serum sclerostin levels in 16 HBM patients with G171V or N198S LRP5 mutations and 24 controls (53 vs. 55 pmol; 1204 vs. 1250 pg/ml) (Simpson et al. 2014).

Boyden et al. reported increased serum osteocalcin levels in HBM patients (Boyden et al. 2002) while others found normal levels of bone formation markers (bone specific alkaline phosphatase, osteocalcin, P1NP) in the serum of such patients; no differences in serum levels of bone resorption markers between patients and healthy controls have been observed (Frost et al. 2011; Boyden et al. 2002; Gregson et al. 2014; Simpson et al. 2014). In addition, and in contrast to sclerosteosis and VBD, patients with HBM have normal levels of DKK1 (Simpson et al. 2014). In population studies, serum sclerostin levels are positively correlated with bone mass (Durosier et al. 2013), reflecting probably the total number of functioning osteocytes. Increased circulating sclerostin levels in HBM might, therefore, result either from increased synthesis as a compensatory response to sclerostin resistance and/or to increased osteocyte numbers due to the high bone mass.

Sclerosing Bone Dysplasia due to Mutations of LRP4

In two cases with generalized osteosclerosis, syndactyly, facial palsy, and hearing loss, no mutations in either SOST or LRP5 could be identified. Instead, in these patients homozygous and heterozygous mutations of LRP4 were found. These mutations diminish the inhibitory function of sclerostin on LRP5/6, underscoring the important role of LRP4 for sclerostin action (Leupin et al. 2011). In one patient, serum sclerostin levels were increased (Fijalkowski et al. 2016). In addition, in mice harboring LRP4 mutation, sclerostin levels were highly increased due to increased expression and decreased binding of sclerostin (Chang et al. 2014). Because of these findings and the mechanism of action of LRP4, it is expected that patients with loss-of-function mutations of LRP4 will have increased circulating sclerostin levels.

Table 1 Serum biomarkers in bone sclerosing disorders

Disorder	Genetic defect	Sclerostin	P1NP	DKK1
Sclerosteosis	Loss-of-function mutations SOST	Undetectable	Increased	Increased
VBD	52 kb deletion downstream SOST	Very low	Increased	Increased
HBM	Gain-of-function mutations LRP5	Normal or increased	Normal	Normal
Sclerosteosis phenotype	Loss-of-function mutations LRP4	Increased	Normal	?

VBD Van Buchem disease, **HBM** high bone mass phenotype

Practical Guidance

Circulating sclerostin can be measured in the initial assessment of patients with similar bone phenotypes associated with high bone mass to distinguish patients with defective sclerostin production from those with resistance to sclerostin action (Table 1). Undetectable sclerostin levels in serum are pathognomic for sclerosteosis, while very low levels are suggestive of VBD. Because the MSD sclerostin assay has a very low limit of detection with appropriate accuracy, even at the lower end of the reference range, it is presently the preferred assay to distinguish between absent or low sclerostin levels in serum. This initial screening can direct genetic analyses to specific genes avoiding examination of a panel of candidate genes, a cost-effective approach. In addition, because disease carriers of sclerosteosis have lower sclerostin levels than controls, serum sclerostin measurements can also be used to detect carriers in family members of patients. On the other hand, high serum levels of sclerostin exclude the diagnoses of sclerosteosis and VBD and point to different genetic defects involving the Wnt signaling pathway. Standardization of the assays and appropriate determination of reference range for sclerostin levels in the normal population are essential for proper interpretation of measured values.

Potential Application to Prognosis, Other Diseases or Conditions

There is no recognized value of serum sclerostin measurements to the prognosis of bone sclerosing dysplasias. Serum sclerostin has been measured in various disorders affecting the skeleton but consistent changes were not always detected precluding its use as biomarker in routine clinical practice (reviewed by Clarke and Drake 2013). Examples include osteoporosis (Ardawi et al. 2012b; Lapauw et al. 2013, Garnero 2014; Guañabens et al. 2014), hyper- and hypoparathyroidism (van Lierop et al. 2010; Costa et al. 2011; Ardawi et al. 2012a), hypercortisolism (van Lierop et al. 2012b; Belaya et al. 2013), Paget's disease of bone (Yavropoulou et al. 2012), osteogenesis imperfecta (Kocijan et al. 2016), diabetes mellitus type 2 (van Lierop et al. 2012c; Garcia-Martin et al. 2012), chronic renal disease (Moysés and Schiavi

2015), multiple myeloma (Terpos et al. 2012), and prostate cancer (Yavropoulou et al. 2012; García-Fontana et al. 2014).

Summary Points

- Sclerostin, a protein produced in bone by osteocytes, decreases bone formation by inhibiting the Wnt signaling pathway in osteoblasts.
- Deficient production or impaired action of sclerostin are associated with distinct clinical phenotypes associated with high bone mass.
- Sclerostin measurements in blood are not standardized and available assays provide different results.
- Sclerostin is undetectable in serum of patients with sclerosteosis while patients with van Buchem disease have very low levels.
- Phenotypically healthy carriers of these diseases have serum sclerostin levels lower than healthy controls.
- Patients with the high bone mass phenotype reported serum sclerostin levels are either normal or increased.
- Measurement of circulating sclerostin can be used in the differential diagnosis of bone sclerosing disorders.

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Utilization and Reference Values of Bone Turnover Markers: Osteocalcin and Procollagen Type 1 N-Propeptide

11

Milan Bayer and Vladimir Palicka

Contents

Abstract

All body bones undergo continual remodeling. This process consists of bone resorption and bone formation which are closely coupled actions. Changes in bone tissue are accompanied by changes of biochemical markers. Plasma (and/or urine) concentration of these markers depends on bone resorption/formation activity, i.e., bone turnover rate. Osteocalcin is important noncollagenous protein

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in bone matrix, synthetized by osteoblasts and osteocytes. It is a good marker of bone turnover. Its undercarboxylated form has a role in regulation of energy metabolism. Osteocalcin is also involved in biosynthesis of testosterone or neurotransmitter production. Procollagen type 1 N-propeptide is a aminoterminal part of procollagen precursor cleaved by proteases during bone formation process. Its serum concentration is significantly related to newly formed collagen. Procollagen type 1 N-propeptide is supposed to be the most reliable biochemical marker of bone formation at present time. Reference values of both laboratory markers for central European population of children and adolescents are presented, and some data about reference intervals in adults from different ethnic groups are mentioned.

Keywords

Bone markers • Osteocalcin • Procollagen type 1 N-propeptide • Reference values • Children

List of Abbreviations

AMP	5′-adenosinmonophosphate
Atf4	Activating transcription factor 4
BMI	Body mass index
EDTA	Ethylenediaminetetraacetic acid
FoxO1	Forkhead box protein O1
mRNA	Messenger ribonucleic acid
OC	Osteocalcin
P1NP	Procollagen type 1 N-propeptide

Key Facts of Bone Turnover Markers

- For the prevention of osteoporosis in the future, it may be important to achieve a high amount and quality of bone tissue during childhood and adolescence.
- Bone turnover rate depends on activity of bone resorption and bone formation which are lifelong continual processes running in the whole skeleton.
- Bone turnover is minimally affected by some factors – for example, seasonality, daily meals, premenopausal menstrual cycles – while other individual lifestyle factors including lack of physical activity, smoking, and excessive alcohol intake could significantly damage bone health.
- Biochemical bone turnover markers are substances, measurable in blood and/or urine, whose concentrations are dynamically changed with bone turnover rate.
- Interpretation of results requires reliable age- and gender-specific reference databases. Detailed evaluation should be performed with regard to individual pubertal stage.

- Biochemical bone turnover markers could help to monitor changes of bone turnover very early, before significant changes of bone mineral density are measurable.
- They are very useful, but there is impossible to make a diagnosis of osteoporosis only according their values.

Definition of Words and Terms

Bone remodeling	Microscopic parts of bone tissue undergo bone resorption and followed bone formation. Thereby, the old or damaged bone tissue is replaced. This process is lifelong, genetically determined but influenceable by a lot of intrinsic and extrinsic factors.
Bone turnover markers	Bone-derived compounds, measurable in blood and/or urine, reflecting bone remodeling activity.
Bone turnover	In healthy skeleton, bone resorption and bone formation activities are closely coupled. Bone turnover rate depends on actual growth velocity, nutritional status, hormonal balance, and physical loading. Bone turnover is physiologically high during childhood with ascendant bone formation. Increased bone turnover with enhanced bone resorption is typical for postmenopausal bone loss.
Osteoblasts	Bone cells of mesenchymal origin. They are producing substances of bone matrix. They are responsible for bone formation. Osteoblasts embedded into bone tissue are called osteocytes.
Osteoclasts	Bone cells originated from monocyte-macrophage lineage. They are multinucleated and their main role is bone tissue degradation, that is, bone resorption.
Pubertal growth spurt	Significant increase of growth in height and weight during puberty. Pubertal growth spurt is managed by sex steroids and growth hormone and lasts for 2–3 years. There is very important role of estrogens which promote growth but also have maturational effect on long bones leading to epiphyseal fusion and final growth cessation in both genders.
Tanner stages	The most manifested somatic pubertal changes are growth in stature and development of sexual characteristics (it means development of external genitalia in boys, breast development in girls, and pubic hair development in boys and girls). They appear in typical sequences. It allows determine the staging system. Marshall and Tanner published the most frequently utilized system, commonly called "Tanner stages."

Introduction

The skeleton provides protection for organs, represents a storage system for minerals, and creates points of attachment for skeletal muscles. It is the largest organ in organism. Findings from recent years support theory that skeleton is a true endocrine organ (Oldknow et al. 2015). The whole skeleton undergoes permanent remodeling process. Bone resorption is coupled to bone formation. Bone-derived compounds reflecting bone remodeling activity represent two main categories – bone formation and bone resorption markers. In adults, approximately 20% of bone tissue is replaced annually varying by site and type (Carey et al. 2006). The remodeling of cortical bone is slower than trabecular one. To monitor these dynamic changes, laboratory bone turnover markers are measured in blood and urine. In postmenopausal women, they have shown association with bone loss and fracture risk (Garnero 2000). The bone turnover rate in healthy premenopausal women was considered to be ideal metabolic situation of skeletal tissue. Significant changes of biochemical markers could be found within several months, whereas changes in bone mineral density could be recorded after about 2 years at least. Therefore, biochemical markers are useful tool for treatment monitoring. But there is no possibility to make a diagnosis of osteoporosis only according their values. In children, rapid bone turnover and high growth of bone mass during childhood result in significantly elevated bone marker levels. Bone markers in children reach the circulation during bone growth, modeling, and remodeling. They are changed in time according to growth velocity. Measurement in urine brings about some problems in childhood: the child should be able to comply with the instructions for obtaining a second void fasting urine (Mora et al. 1998); there are significant circadian and intraindividual variation in urinary bone markers (Schönau and Rauch 1997); their concentration should be expressed in relation to creatinine which is subject to change with muscle mass accrual (Szulc et al. 2000). Thus, the measurement of bone markers in blood seems to be more convenient in children and adolescents and become the preferred. It is necessary to take in account that markers of bone turnover have different degree of intraindividual variation. Their level could be influenced by a lot of factors: age, gender, ethnicity, fasting, physical activity, calcium supplementation, and others. Long-term corticosteroid therapy results in suppression of bone formation (van Staa et al. 2002). Its role could play menstrual cycle, with high osteoblastic activity during the luteal phase (Nielsen et al. 1990) and higher bone resorption within the follicular period (Chiu et al. 1999). There are different opinions of seasonal variation from insignificant changes (Blumsohn et al. 2003) to significant increase during the winter (Woitge et al. 1998). Regarding interindividual variation, parameters of bone metabolism are highest in infants up to 3 years of age. Then they are stable (but higher than in adults) until pubertal growth spurt, in which they are influenced by pubertal stage rather than age (Mora et al. 1999). Somatic growth in childhood and adolescence comprises bone modeling, bone remodeling, epiphyseal bone growth, and soft tissue accrual. There is no marker in children specific for these processes. Thus, results should be corrected to growth velocity and pubertal development,

and large population of healthy children is needed to obtain sex- and age-specific pediatric reference intervals for bone markers.

Osteocalcin

Osteocalcin – Characteristics

OC was isolated from bone tissue 40 years ago (Hauschka et al. 1975). It is a small protein (49 amino acids in human, 46–50 amino acid residues varying from different species) synthetized by osteoblasts and osteocytes. OC gene activity is regulated by 1-alpha, 25-hydroxyvitamin D3. Translation results in prepro-OC containing 98 amino acid residues. Following proteolysis will form the mature OC (Lee et al. 2000). Three vitamin K-dependent γ-carboxyglutamic acid residues are added. They are important for OC activity. OC is one of most important noncollagenous proteins in bone matrix. Carboxylated OC is able, in the presence of calcium, to bind to hydroxyapatite and regulate bone mineralization. Osteoblasts can secret OC to stimulate osteoblastic differentiation and osteocytic maturation (Shao et al. 2015). Small amount of OC circulates in blood in carboxylated or undercarboxylated form (Ferron et al. 2010). OC carboxylation status has an important role in energy metabolism management. Undercarboxylated OC is acting as a hormone with only little affinity to bone. Its effect is realized through several activating transcription factors, for example, FoxO1 and activating Atf4. They are involved in more processes including regulation of insulin sensitivity and glucose tolerance (Kode et al. 2012). According to recent research, undercarboxylated OC is able to stimulate beta cell proliferation (Klein 2014). OC is stored in bone tissue and during bone resorption released to circulation. Local pH is decreased during osteoclastic activity and allows OC decarboxylation (Shao et al. 2015). Thus, osteoclasts are able to regulate glucose metabolism indirectly by osteocalcin decarboxylation (Kanazawa 2015). Glucocorticoids suppress osteoblast activity and OC production (Ferron and Lacombe 2014); thyroid hormone stimulate OC synthesis in osteoblasts under regulation of AMP-activated protein kinase. Thus, energy metabolism has a direct link to bone tissue (Kondo et al. 2013). The evidence of expression of OC mRNA in adipose tissue (it means ability to product OC) contributes to the complex picture (Foresta et al. 2010). OC participates also in management of testosterone biosynthesis in males (Oury et al. 2011). It is insulin signaling in osteoblast which has positive influence to OC stimulated testosterone synthesis illustrating the existence of pancreas-bone-testis axis (Oury et al. 2013). Finally, osteocalcin is involved in regulation of neurotransmitter production and could play a role in brain function (Zoch et al. 2016). OC is stable as EDTA sample for up to 8 h at room temperature (Stokes et al. 2011), but has a short half-life (Blumsohn et al. 1995) with large interlab variation (Vasikaran et al. 2011b). If there is no possibility of immediate analysis, samples should be stored at −20 °C or lower. OC has a circadian variability with maximal levels in early morning and nadir between 11:00 and 15:00 h. Difference is about 20% (Wheater et al. 2013). The ratio of undercarboxylated and

carboxylated OC is not changed, but the total amount of undercarboxylated OC in circulation had the similar changes as the circadian rhythm of OC (Lee et al. 2000; Nishimura et al. 2007). It could be also influenced by vitamin K status and renal functions (Brown et al. 2009). On the other hand, OC shows negligible dietary influences – its analytical coefficient of variation is below 5% in both the fed and fasting states (Clowes et al. 2002).

Osteocalcin – Utilization

Serum concentration of OC has relation to osteoblast number and bone formation, and it was repeatedly documented to use it as laboratory marker of bone formation (Gundberg et al. 2012), but it is possible to say that OC reflects entire skeletal metabolic activity (Wheater et al. 2013). At present time, OC is supposed to be very good marker of bone turnover rather than bone formation. In healthy adults, OC levels correlate negatively with sclerostin (Amrein et al. 2012). Some authors studied the possibility to use OC plasma concentrations as a predictor of cardiovascular disease risk in seniors. In population older than 75 years, higher plasma OC concentration was related to higher risk of cardiovascular disease in women, while significant inverse relation was found in men (Holvik et al. 2014). In male adolescents, OC has inverse relation to leptin values and body adiposity (Jürimäe et al. 2015).

Osteocalcin – Reference Values

Serum concentration of OC is highest in the early morning and lowest in the afternoon. Rauchenzauner et al. gathered morning blood samples from 572 healthy children and adolescents to form reference curves for bone markers including serum osteocalcin. Values correlated to age and pubertal stage. Taller and heavier individuals for age had greater bone marker concentrations, which may be due to greater growth velocity (Rauchenzauner et al. 2007). In a group of Brazilian male adolescents, OC levels were related to bone age with maximal values between 13 and 15 years (da Silva et al. 2012). Reference values of OC in relation to age, sex, and pubertal stage were recently established in a group of 439 healthy children and adolescents in central Europe. OC missed postnatal peak, but its levels were higher than the adult reference interval throughout childhood (Bayer 2014). See Tables 1 and 2. OC peaked with the pubertal growth spurt at 2nd–3rd Tanner stage (Marshall and Tanner 1969; 1970) of breast development in girls (Fig. 1) and at 2nd–3rd Tanner stage of genital development in boys (Fig. 2). In group of 638 healthy premenopausal women, serum OC decreased with advancing age and independently and negatively correlated with BMI ($P<0.001$). The use of contraceptive pills in healthy premenopausal women was associated with 14–26% decrease of OC in comparison with non users ($P<0.005$) (Adami et al. 2008). Nabipour et al. found in group of 785 healthy adult Iranian individuals consecutive decrease in serum osteocalcin in women from second to third decade of life with following increase in women older than fifty. In men, the highest

Table 1 Osteocalcin (µg/l) and P1NP (µg/l) serum concentration in healthy children 0–9 years (Bayer 2014) (With permission of Springer Science + Business Media)

Age	n	Osteocalcin		P1NP	
		Lower	Upper	Lower	Upper
0–1	18	20,8	144,3	227,2	4762,8
1.1–2	16	28,3	126,1	346,6	1088,0
2.1–3	16	30,7	85,4	178,3	645,7
3.1–4	15	23,9	98,4	135,2	746,1
4.1–5	17	22,8	129,3	85,7	901,7
5.1–6	18	42,1	128,2	111,5	768,4
6.1–7	17	30,9	122,2	187,6	887,3
7.1–8	15	12,5	232,5	49,9	1200,0
8.1–9	19	25,7	151,1	120,4	1021,0

Lower and upper limits correspond to 2.5 and 97.5 percentile

Table 2 Serum concentration of osteocalcin in healthy children 9.1–18 years (Bayer 2014) (With permission of Springer Science + Business Media)

Age	Osteocalcin µg/l						P	Test
	Boys			Girls				
	n	Lower	Upper	n	Lower	Upper		
9.1–10	16	12,2	110,6	15	18,4	251,7	0,847	KS
10.1–11	17	12,6	145,7	15	18,5	154,2	0,334	t
11.1–12	16	31,9	200,9	15	11,9	140,4	0,554	t
12.1–13	14	19,8	164,9	15	13,1	186,7	0,749	t
13.1–14	17	58,7	236,2	15	16,8	238,9	0,122	MW
14.1–15	16	25,7	241,0	16	15,4	88,8	0,000068	t
15.1–16	17	30,1	186,9	16	16,9	96,3	0,0169	KS
16.1–17	17	32,0	124,2	16	5,7	66,7	0,00004	t
17.1–18	19	13,5	160,1	16	20,7	45,6	0,003	KS

Lower and upper limits correspond to 2.5 and 97.5 percentile Statistical evaluation: *KS* .... Kolmogorov–Smirnov test, *t* . . ..two-sample *t*-test, *MW* . . .Mann–Whitney test, *P* . . .significance level (comparing boys and girls)

serum osteocalcin concentration was revealed in second decade, showing important differences according gender (Nabipour et al. 2008). Not only very young age is in relation to higher levels of OC but also low BMI could be another contributing factor in healthy adult women (Glover et al. 2008).

Procollagen Type 1 N-Propeptide

P1NP – Characteristics

Procollagen precursor is secreted from proliferating osteoblast to extracellular space. Then, the amino- and carboxy-terminals are split off by proteases and are released into the blood. Trimeric structure of P1NP is very quickly broken down by thermal

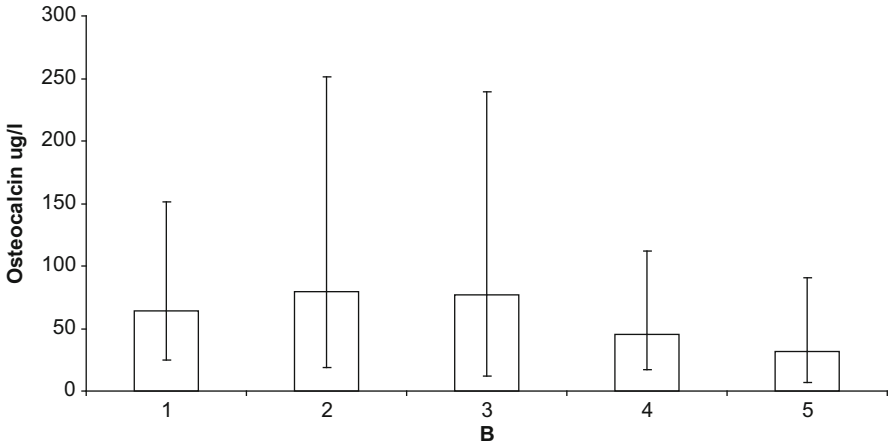

Fig. 1 Serum concentration of osteocalcin – relation to pubertal stage in girls (Bayer 2014) B. grade of breast development in girls (Tanner B) Lower and upper deviations correspond to 2.5 and 97.5 percentile (With permission of Springer Science + Business Media)

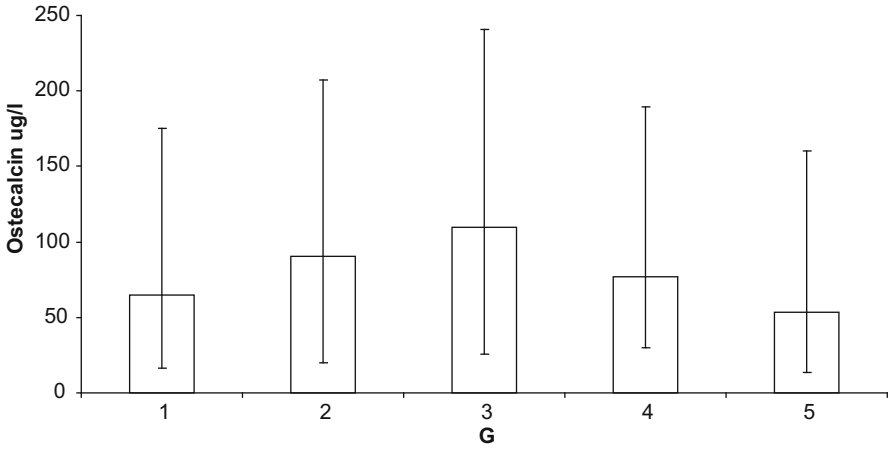

Fig. 2 Serum concentration of osteocalcin – relation to pubertal stage in boys (Bayer 2014) G grade of genital status in boys (Tanner G) Lower and upper deviations correspond to 2.5 and 97.5 percentile (With permission of Springer Science + Business Media)

degradation resulting in monomeric structure (Brandt et al. 1999). Trimeric form or both structure, so-called total P1NP, could be measured by current immunoassays (Wheater et al. 2013). The serum concentration of P1NP is directly related to the amount of newly formed collagen laid down in the bone – it is a very good bone formation marker. P1NP was confirmed as a more sensitive marker of type I collagen synthesis than P1CP (Crofton et al. 2004). P1NP has advantageous laboratory properties – it is stable in serum at room temperature (Stokes et al. 2011) – has

low interindividual variability and good assay precision (Vasikaran et al. 2011b). P1NP did not vary with season (Munday et al. 2006) and only small circadian rhythm was reported (Brown et al. 2009). But it is necessary to bear in mind that total P1NP concentration could be affected by delayed clearance of monomeric fraction, for example, in renal failure or metastatic bone disease (Marin et al. 2011). P1NP also shows imponderable dietary influences with coefficient of variation similar to OC (Clowes et al. 2002).

P1NP – Utilization

According Bone Marker Standards Working Group, P1NP represents a reliable laboratory marker of bone formation (Vasikaran et al. 2011a) due to its characteristics and good assay precision. During pubertal spurt, bone modeling increases with high P1NP levels corresponding to peak of growth hormone secretion but without significant association to systemic IGF-1 (Russell et al. 2011). Serum P1NP levels significantly correlated with total body bone mineral density changes in children after successful living-related liver transplantation and could be used as predictor of bone status in these patients (Kryskiewicz et al. 2012).

P1NP – Reference Values

Reference values of P1NP in relation to age, sex, and pubertal stage were established in a group of 439 healthy children and adolescents in central Europe. The highest levels of P1NP were observed during the first year of life. It slows down until 3 years of age and is then relatively stable up to the pubertal growth spurt (Bayer 2014). See Tables 1 and 3. P1NP peaks during 2nd–3rd Tanner stage of breast development in girls (Fig. 3) and during 2nd–4th Tanner stage of genital development in boys (Fig. 4). High bone turnover rate, reflecting bone growth, is decreased in young adult age with the end of puberty and seems to be stable until hormonal changes in advanced age. In adult Spanish men aged 65 ± 9 years, 95% P1NP ranges were 15–78 ug/l (Olmos et al. 2010). Similar age-related reference P1NP values were recently obtained in Australian population. In men aged 25–70 years, the interval was 15–80 ug/l. Values increased in older men, possibly due to changes in bone turnover (Jenkins et al. 2013). In the female Spain population aged 63 ± 9 years, 95% P1NP reference interval reached 19–100 ug/l (Martínez et al. 2009), and in Australian women with the corresponding age it was 15–75 ug/l. P1NP values in younger women were 25–90 ug/l (age less than 30 years), 15–80 ug/l (30–39 years), and 15-60ug/l (40–49 years) (Jenkins et al. 2013). Analogous results were found in a group of healthy Thai women – 95% confident interval was 40.79–48.35 ug/l in women with mean age 38.5 years (Bunyaratavej and Kittimanon 2005) as well as in premenopausal healthy Japanese women aged 30–44 years. Their P1NP plasma levels were 39.4 ± 15.4 ug/l (Nomura et al. 2013). Values generally increased in older women with probable high bone turnover.

Table 3 Serum concentration of P1NP in children 9.1–18 years (Bayer 2014) (With permission of Springer Science + Business Media)

	P1NP µg/l							
	Boys			Girls				
Age	n	Lower	Upper	n	Lower	Upper	P	Test
9.1–10	16	45,2	552,5	15	42,7	952,1	0,962	t
10.1–11	17	48,3	769,6	15	62,5	914,7	0,457	t
11.1–12	16	142,5	2501,7	15	65,3	855,8	0,938	KS
12.1–13	14	67,2	854,8	15	47,0	984,9	0,623	t
13.1–14	17	267,9	1514,6	15	37,1	1195,6	0,047	t
14.1–15	16	148,1	1200,0	16	58,5	451,4	0,0018	KS
15.1–16	17	81,8	961,4	16	45,6	600,2	0	KS
16.1–17	17	77,7	430,3	16	14,6	238,3	0,00021	t
17.1–18	19	38,7	494,5	16	36,3	143,9	0,0036	KS

Lower and upper limits correspond to 2.5 and 97.5 percentile Statistical evaluation: *KS* .... Kolmogorov–Smirnov test, *t* . . ..two-sample *t*-test, *MW* . . .Mann–Whitney test, *P* . . .significance level (comparing boys and girls)

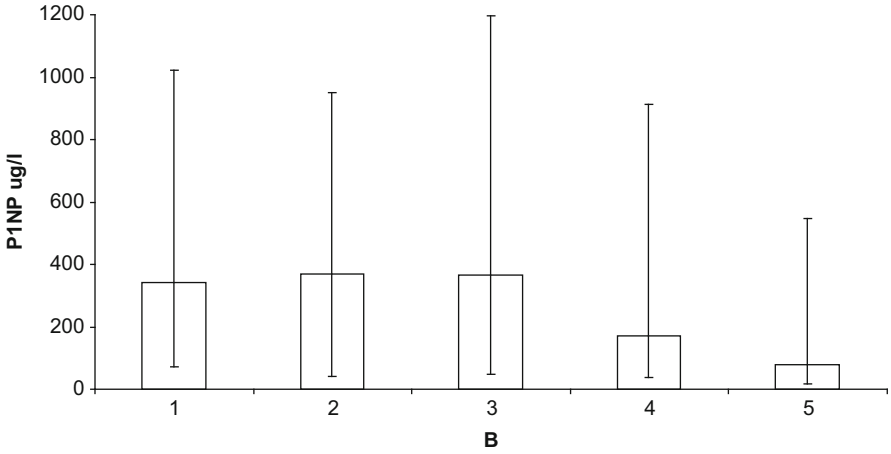

Fig. 3 Serum concentration of P1NP – relation to pubertal stage in girls (Bayer 2014) B grade of breast development in girls (Tanner B) Lower and upper deviations correspond to 2.5 and 97.5 percentile (With permission of Springer Science + Business Media)

Potential Applications to Prognosis, Other Diseases, or Conditions

Referred laboratory markers (as well as other bone turnover markers) could provide good information about bone metabolism rate and on the efficacy of treatment in osteoporotic patients. However, their large biological variation limits their predictive value in individual patient. They would not be suitable to estimate bone loss as

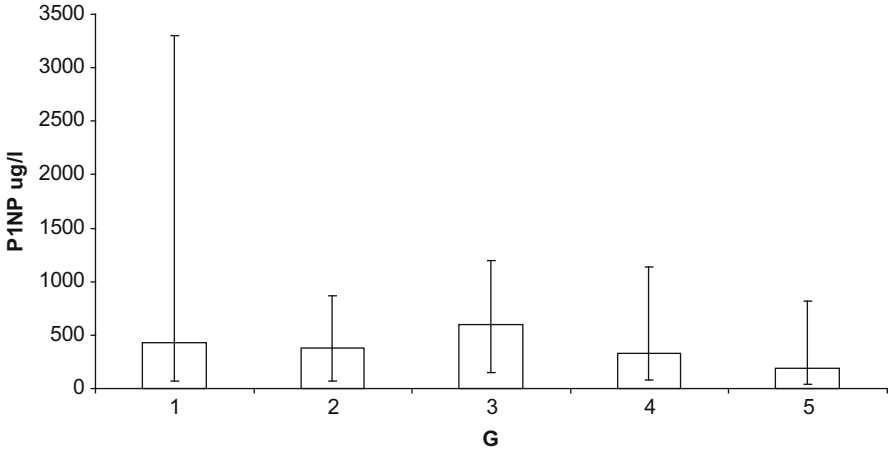

Fig. 4 Serum concentration of P1NP – relation to pubertal stage in boys (Bayer 2014) G grade of genital status in boys (Tanner G) Lower and upper deviations correspond to 2.5 and 97.5 percentile (With permission of Springer Science + Business Media)

alone, but they are very useful supplement to bone mineral density measurement. Laboratory markers can reflect treatment efficacy before bone mineral density changes reach significancy. Their early changes can be used to measure the clinical efficacy of an antiresorptive treatment and to reinforce patient compliance (Bergmann et al. 2009) and are broadly used as a surrogate for bone mineral density changes. During the treatment with anabolic agent such as parathormone, markers of bone formation increase very early after the initiation of therapy. Due to the coupling these changes are followed by increase in resorption markers (Finkelstein et al. 2010). Bone turnover markers can also monitor patients during treatment holidays (Wheater et al. 2013). According to recent research, OC plasma concentrations could serve as predictor of cardiovascular disease risk in women older than 75 years (Holvik et al. 2014).

Summary Points

- This chapter focuses on osteocalcin and procollagen type 1 N-propeptide, which are biochemical markers of bone metabolism.
- Osteocalcin is most important noncollagenous protein in bone matrix, synthetized by osteoblasts and osteocytes.
- Serum concentration of osteocalcin reflects activity of bone turnover.
- Undercarboxylated form of osteocalcin participates in regulation of energy metabolism.
- Osteocalcin is also involved in management of testosterone secretion and neurotransmitter production.

- Procollagen type 1 N-propeptide is a peptide split off in the process of collagen formation.
- Serum concentration of procollagen type 1 N-propeptide is relevant to new collagen amount.
- Procollagen type 1 N-propeptide is a reliable marker of bone formation.
- Reference values osteocalcin and procollagen type 1 N-propeptide for children, adolescents, and some data about adult population are presented.

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Pentraxin 3 (PTX3) as a Biomarker of Bone Disease

12

Bongkun Choi and Eun-Ju Chang

Contents

Abstract

Pentraxin 3 (PTX3) is the prototype protein of the long pentraxin group and a critical component of innate immunity. PTX3 is produced by various types of cells in response to proinflammatory signals and Toll-like receptor engagement. Albeit the protective functions of PTX3 in several infection, the persistent elevation in PTX3 levels is associated with disease severity and increased morbidity in diverse clinical pathological conditions such as psoriasis, unstable angina pectoris, atherosclerosis, acute myocardial infarction, and ischemic heart

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disorders. However, the presence of PTX3 in bone-related cells and the role of PTX3 in bone-associated diseases have not been well elucidated. Osteoblasts derived from bone marrow stromal cells highly express PTX3. PTX3 induces production of receptor activator of NFkB ligand (RANKL) from osteoblasts, thereby contributing to the osteolysis as an inflammatory mediator in the bone environment. Moreover, PTX3 expression is elevated in the distant bone metastases of breast cancer, and PTX3 plays a key role in the inflammation-associated osteolytic complications of breast cancer. Here, we review the key properties of PTX3 as a mediator of bone pathogenesis, with an emphasis on PTX3 as a prototypic member of the long pentraxin family and recent data suggesting that persistently elevated PTX3 may represent a new and useful biomarker for clinical outcomes in bone pathologic condition.

Keywords

PTX3 • Bone • Osteoblasts • Osteoclasts • RANKL • Biomarker • Inflammation • Bone metastasis

List of Abbreviations

AgP	Aggressive periodontitis
CKD	Chronic kidney disease
CMV	Cytomegalovirus
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
KO	Knockout
M-CSF	Macrophage-colony-stimulating factor
OB	Osteoblast
OC	Osteoclast
PTX3	Pentraxin 3
RANKL	Receptor activator of NFkB ligand
Runx2	Runt-related transcription factor 2
SAP	Serum amyloid P component
siRNA	Small interfering RNA
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Key Facts

- Pentraxin 3 (PTX3) is the prototype protein of the long pentraxin group and an essential component of the innate immunity.
- PTX3 is produced by various types of cells in response to proinflammatory signals, Toll-like receptor (TLR) engagement, microbial moieties, and intact microorganisms.

- PTX3 is involved in complement activation, opsonization, inflammation regulation, extracellular matrix construction, and female fertility.
- An elevated circulating PTX3 concentration is associated with various clinical conditions, including liver disease, cardiovascular diseases, kidney disease, infectious disease, autoimmune diseases, and various malignant tumors.
- Likewise, elevated PTX3 concentrations are also related to bone-associated pathological conditions.
- Thus, the combination of PTX3 and classic biomarkers may provide additional diagnostic and prognostic value to several clinical conditions.

Definitions of Words and Terms

Biomarker	A biomarker is a biological molecule found in the body fluids that is used as a sign of normal or abnormal condition or disease. Biomarkers play an important role in measuring and indicating biological and pathological conditions.
Bone metastasis	Bone metastasis is a class of cancer metastases that results from primary tumor invasion to bone.
Osteoblast	An osteoblast is mononuclear cell that produces bone matrix proteins and responsible for bone mineralization. Osteoblasts share a common progenitor – the undifferentiated mesenchymal cell –with chondrocytes, myocytes, and adipocytes.
Osteoclast	An osteoclast is multinucleated cell that specializes in bone-degrading processes. Osteoclasts secrete acid and hydrolytic enzymes, which dissolve the mineralized and organic components of the bone matrix. Osteoclasts are closely associated with the macrophage lineage, which is located in bone marrow and formed by fusion of mononuclear precursors.
Osteoclastogenesis	Osteoclastogenesis refers to the process of osteoclast development, which includes initiation, proliferation, differentiation, and maturation.
Pentraxins	Pentraxins are a family characterized by the presence of pattern-recognizing proteins in the carboxy-terminal region, which contains a homologous pentraxin domain. Based on the primary structure of the composing promoters, pentraxins are divided into two groups: short and long pentraxins. The C-reactive protein (CRP) and serum amyloid P component (SAP) are typical short pentraxins. PTX3 is a typical long pentraxin.

RANKL Receptor activator of NFkB ligand (RANKL) (also called ODF, OPGL, or TRANCE) is a tumor necrosis factor (TNF)-like family cytokine and activates NFkB through the kinases JNK and c-Jun. RANKL induces the differentiation of bone marrow hematopoietic precursor cells into bone-resorbing osteoclasts.

Introduction

Pentraxin 3 (PTX3) is the prototype protein of the long pentraxin group and an essential component of the innate immunity. PTX3 is produced by various tissues and cells in response to proinflammatory signals and Toll-like receptor (TLR) engagement (Alles et al. 1994). Moreover, PTX3 is involved in regulating inflammation, extracellular matrix construction, and female fertility (Garlanda et al. 2005; Jeannin et al. 2005; Bottazzi et al. 2006). However, persistently elevated PTX3 levels in patients with bone metastatic breast cancer are associated with disease severity and increased morbidity (Choi et al. 2014). A growing body of evidence suggests that PTX3 may be a useful serological marker of diverse clinical conditions (Latini et al. 2004; Bevelacqua et al. 2006; Inoue et al. 2007; Suzuki et al. 2008), but a limited number of studies have been conducted to define the presence of PTX3 in bone-related cells and the role of PTX3 in bone-associated disease (Table 1).

Table 1 Elevated PTX3 expression in various clinical diseases or conditions

Disease category	Disease	Ref
Liver disease	Acute liver disease	(Craig et al. 2013)
	Nonalcoholic steatohepatitis	(Yoneda et al. 2007)
	Infection associated with liver transplant	(Razonable 2008)
Cardiovascular disease	Unstable angina pectoris	(Inoue et al. 2007)
	Acute myocardial infarction	(Latini et al. 2004)
	Ischemic heart disorders	(Suzuki et al. 2008)
	Atherosclerosis	(Zanetti et al. 2009)
Kidney disease	Chronic kidney disease	(Tong et al. 2007)
Infectious disease	Sepsis	(Ketter et al. 2014)
	Cytomegalovirus infection	(Rollag et al. 2012)
Autoimmune disease	Rheumatoid arthritis	(Luchetti et al. 2000)
	Systemic sclerosis	(Shirai et al. 2015)
	Small vessel vasculitis	(Fazzini et al. 2001)
Malignant cancer	Breast cancer bone metastasis	(Choi et al. 2014)
	Gliomas	(Locatelli et al. 2013)
	Liposarcoma	(Germano et al. 2010)
	Lung cancer	(Diamandis et al. 2011)
	Prostate carcinoma	(Stallone et al. 2014)
	Pancreatic carcinoma	(Kondo et al. 2013)

Here, we review the key properties of PTX3 related to bone pathology and discuss recent data suggesting that PTX3 demonstrates bone pathogenic effects. Persistently elevated PTX3 may represent a novel and promising biomarker of bone disease, which correlates with the risk of developing bone injury and pathologic events, thereby providing useful prognostic information for clinical outcomes in patients with this pathological condition.

Cellular Sources of PTX3

The short pentraxins including C-reactive protein (CRP) and serum amyloid P component (SAP) are primarily produced by hepatocytes in response to inflammatory signals (Jaillon et al. 2007; Mantovani et al. 2008). However, PTX3 is locally expressed at the site of inflammation from various types of cells, including neutrophils, dendritic cells, macrophages, fibroblasts, vascular endothelial cells, smooth muscle cells, adipocytes, chondrocytes, and epithelial cells (Breviario et al. 1992; Alles et al. 1994; Vouret-Craviari et al. 1997; Abderrahim-Ferkoune et al. 2003; Doni et al. 2003; Klouche et al. 2004; Han et al. 2005; He et al. 2007) in response to stimulation by proinflammatory signals or TLR engagement (Garlanda et al. 2005). PTX3 plays a critical role in defense mechanisms as well as physiological and pathological function in various organ systems (Doni et al. 2003). However, the presence of PTX3 in bone-related cells and the role of PTX3 in bone-associated diseases have not been well elucidated.

Bone Cells

Using monodimensional electrophoresis and tandem mass spectrometry, Chiellini et al. identified several proteins that are released from human mesenchymal stem cells during the commitment to osteoblasts and found that PTX3 expression is upregulated during osteoblast differentiation (Chiellini et al. 2008). Similarly, Lee et al. reported that human bone marrow-derived stromal cells highly express PTX3 during osteoblast differentiation and proinflammatory signals further enhance PTX3 expression (Lee et al. 2014). Lee et al. also observed PTX3 protein expression in the murine bone environment, as demonstrated on immunohistochemistry. Likewise, murine osteoblasts express PTX3 in bone-related cells (Lee et al. 2014), confirming the presence of PTX3 in osteoblasts.

In addition, bone metastatic breast cancer cells express higher levels of PTX3 in comparison with non-bone metastatic breast cancer cells, and TNF-α and IL-1β significantly enhance PTX3 expression (Choi et al. 2014). Similarly, bone metastatic gastric cancer cells also produce more abundant amounts of PTX3, thereby indicating that PTX3 may play a specific role in bone metastasis (Choi et al. 2015). In contrast, non-bone metastatic prostate cancer cells express PTX3 in the absence of proinflammatory stimuli (Choi et al. 2014).

PTX3 as Biomarkers of Bone Pathology

PTX3 in Physiological Bone Formation

Kelava et al. reported that PTX3-knockout (KO) mice have a lower bone mass (bone volume/total volume = 2.72 ± 1.23 for females vs. 5.39 ± 1.73 for males) than their wild-type (WT) littermates (bone volume/total volume = 5.03 ± 0.87 for females vs. 7.04 ± 0.87 for males; $p < 0.05$) (Kelava et al. 2014). In addition, PTX3-KO mice demonstrate lower trabecular thickness and greater trabecular separation. Although the number of osteoblast and osteoclast progenitor cells in the bone marrow compartments does not differ between WT and PTX3-KO mice, a lower bone formation rate was observed in PTX3-KO mice. PTX3-KO mice formed significantly fewer mineralized calluses following bone fracture than WT mice (bone volume/total volume = 15.33 ± 2.32 for PTX3-KO vs. 19.66 ± 4.32 for WT mice; $p < 0.05$), and nonhematopoietic periosteal cells highly upregulate PTX3 expression during the initial phase of fracture healing, thereby suggesting that PTX3 has a positive impact on bone mass.

PTX3 in Aggressive Periodontitis

Periodontitis is an inflammatory disease initiated by gram-negative, anaerobic, and microaerophilic bacteria and characterized by gingival inflammation, alveolar bone resorption, and attachment loss (Smalley 1994). Significantly elevated salivary concentrations of PTX3 were detected in the aggressive periodontitis (AgP) group in comparison with the periodontally healthy group ($p < 0.05$). PTX3 levels in sera and saliva were correlated with the IL-1β level in the AgP group ($p < 0.05$), suggesting that PTX3 is related to periodontal tissue inflammation that results in alveolar bone destruction (Gumus et al. 2014). Consistent with these results, the PTX3 concentrations in gingival crevicular fluid and plasma were elevated in the periodontal disease group in comparison with the healthy control group, thereby suggesting that PTX3 could be considered a marker of inflammatory periodontitis disease (Pradeep et al. 2011). Moreover, a rat model of ligature-induced periodontitis developed significant alveolar bone resorption and periodontal inflammation and concomitantly exhibited a higher level of PTX3 in gingival tissue (Keles et al. 2012).

Elevated PTX3 Expression in Inflammatory Condition of Bone Resorption

Bone homeostasis is maintained by the well-coordinated activities of bone-forming osteoblasts (OBs) and bone-resorbing osteoclasts (OCs) (Theoleyre et al. 2004). During differentiation, OBs produce macrophage colony-stimulating factor (M-CSF), RANK ligand (RANKL), and osteoclastogenic factors to differentiate

and activate OC, thereby resulting in increased bone resorption (Theoleyre et al. 2004). Inflammation causes imbalances in bone homeostasis, thereby leading to excessive bone resorption and inflammatory bone erosive diseases such as rheumatoid arthritis, spondyloarthritis, psoriatic arthritis, and osteoporosis. It is also known that proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 directly stimulate osteoclastogenesis, and OBs also overproduce RANKL.

In an animal study, Lee et al. found that injecting mice with lipopolysaccharide in order to induce an inflammatory osteolytic condition leads to trabecular bone destruction and the simultaneous elevation in PTX3 expression within the bone tissue. PTX3 significantly increased Runt-related transcription factor 2 (Runx2), a key transcription factor that activates RANKL expression (Lee et al. 2014). PTX3 in turn enhances RANKL production and secretion from OBs, and subsequently RANKL binds to RANK during precursor osteoclast-stimulating osteoclastogenesis and thereby leads to increased bone resorption (Lee et al. 2014; Fig. 1). PTX3 silencing with PTX3-specific small interfering RNA (siRNA) suppresses RANKL production and reduces osteoclastogenesis, suggesting that PTX3 contributes to osteolytic conditions as an inflammatory mediator in the bone environment (Lee et al. 2014).

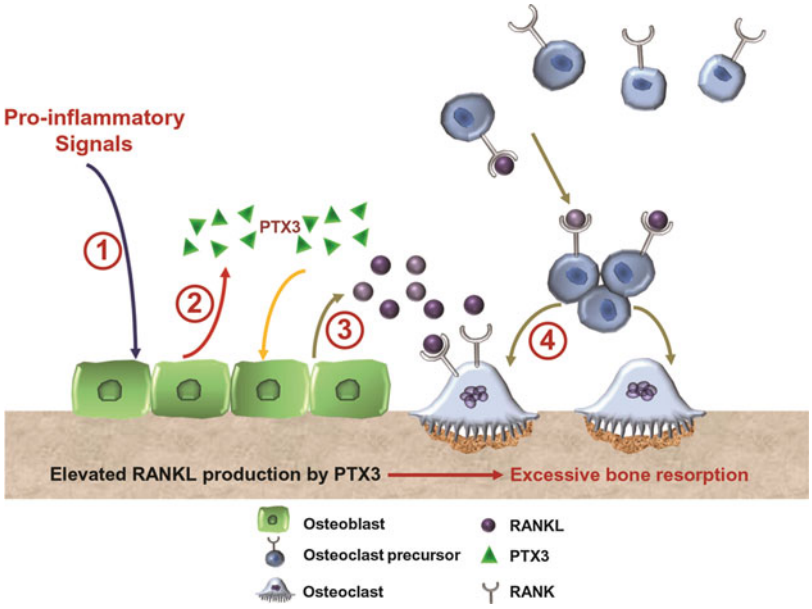

Fig. 1 Model of PTX3-mediated osteolysis. (*1*) Proinflammatory signals stimulate osteoblasts to overproduce PTX3. (*2*) The PTX3 protein is secreted from osteoblasts. (*3*) PTX3 in turn enhances RANKL production and secretion from osteoblasts. (*4*) Finally, RANKL binds to RANK in precursor osteoclasts and stimulates osteoclast differentiation and activity, leading to excess bone destruction.

Elevated PTX3 Expression in Bone Metastatic Cancer with Osteolytic Function

Bone has a unique environment that contains a variety of growth factors and is one of the common target sites for the distant metastases of breast and lung cancer (Mundy 2002). Bone metastases from breast and lung cancer are typically osteolytic and thus represent bone resorption (Mundy 2002). Bone metastatic cancer cells secrete OC-activating cytokines, which elevate the expression of RANKL from OBs in the bone microenvironment (Yoneda et al. 2013). As a result, OCs degrade bone and release various bone-storing growth factors from bone. These growth factors in turn promote tumor growth, thus establishing a "vicious cycle" (Mundy 2002).

Elevated PTX3 expression was observed in the distant bone metastases of breast cancer in comparison with the lung, liver, and brain metastases, as well as primary tumors in human breast cancer patients, and correlated with poor survival in human breast cancer patients (Choi et al. 2014). PTX3 expression is also upregulated in a bone metastatic gastric cell line (article in preparation) as well as a bone metastatic breast cancer cell line (Choi et al. 2014). PTX3 enhances the mobilization of bone metastatic breast cancer cells and the migratory potential of macrophages (a precursor of OCs) toward breast cancer cells. Importantly, elevated PTX3 expression led to enhanced OC formation, suggesting the distinct role of PTX3 in the osteolytic bone metastasis of breast cancer cells (Fig. 2) and implying that PTX3 may at least in part participate in the vicious cycle of osteolysis. Moreover, PTX3 silencing using PTX3 siRNA decreased the migratory potential of bone metastatic breast cancer cells and subsequently inhibited OC formation, suggesting that PTX3 plays a key role in the inflammation-associated osteolytic complications of breast cancer and could be a biomarker of inflammation-mediated bone pathology (Choi et al. 2014).

Potential Applications of Predicting Prognosis and Other Diseases or Conditions

PTX3 expression is elevated in liver-associated pathological conditions. Analgesic drug-induced acute liver injury leads to elevation of PTX3 level (Craig et al. 2013; Yaman et al. 2013). PTX3 expression is also profoundly elevated in nonalcoholic steatohepatitis patients (Yoneda et al. 2007) and infection associated with liver pathology and transplant (Razonable 2008; Ketter et al. 2014), suggesting that PTX3 could be a novel biomarker of liver pathology (Choi et al. in press).

In addition, highly elevated level of PTX3 was demonstrated in patients with atherosclerosis (Alberti et al. 2009), and the macrophages that infiltrate atherosclerotic plaques show elevated expression of PTX3 (Savchenko et al. 2008). Besides atherosclerosis, a growing body of evidence suggests that measurement of PTX3 level in the blood can be applied as a diagnostic or prognostic marker of heart disorders and vascular pathologies such as unstable angina pectoris, acute

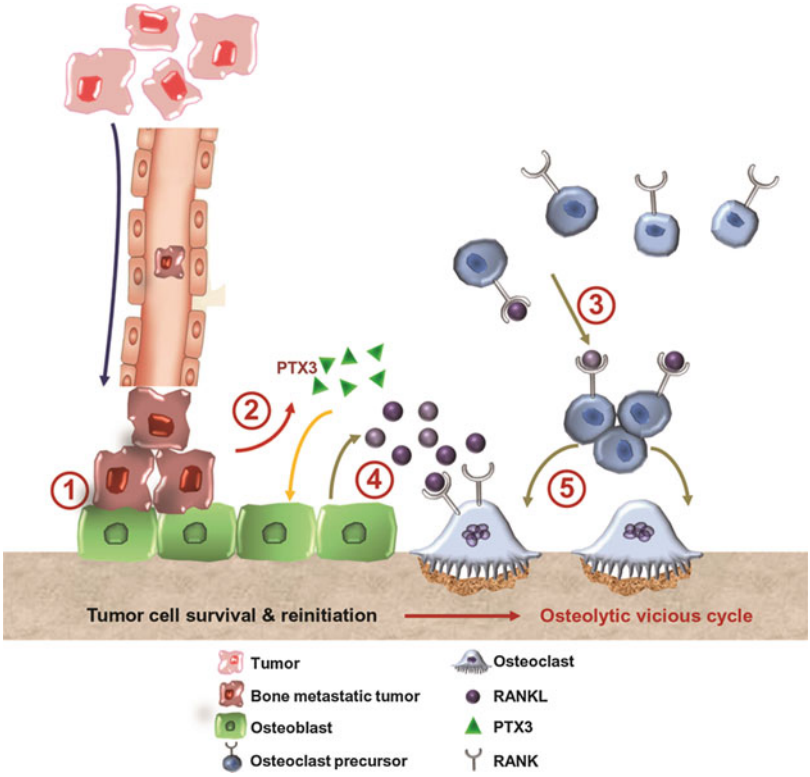

Fig. 2 Model of PTX3-mediated osteolysis of bone metastatic cancer. (*1*) PTX3 promotes the migratory capacity of bone metastatic cancer cells toward osteoblasts and enhances interactions between these cells. (*2*) Bone metastatic cancer cells produce and secrete PTX3. (*3*) PTX3 also enhances the migration of osteoclast precursor. (*4*) PTX3 enhances RANKL production and secretion from osteoblasts. (*5*) Finally, RANKL stimulates osteoclastogenesis, leading to the vicious cycle of osteolysis.

myocardial infarction, atherosclerosis, and ischemic heart disorders (Peri et al. 2000; Latini et al. 2004; Inoue et al. 2007; Suzuki et al. 2008; Zanetti et al. 2009).

Human breast cancers expressing elevated PTX3 demonstrate higher rates of bone metastasis (Choi et al. 2014). Several lines of evidence suggested elevated PTX3 expression in malignant cancer from multiple tissues such as gliomas, liposarcoma, lung cancer, prostate carcinoma, and pancreatic carcinoma, supporting a potential role of PTX3 as a biomarker of cancer (Germano et al. 2010; Diamandis et al. 2011; Kondo et al. 2013; Locatelli et al. 2013; Stallone et al. 2014).

Moreover, circulating PTX3 concentration is significantly correlated with disease severity in patients with chronic kidney disease (CKD) (Tong et al. 2007). Several reports show that PTX3 levels are increased in autoimmune disorders such as small vessel vasculitis, systemic sclerosis, and rheumatoid arthritis (Luchetti et al. 2000; Fazzini et al. 2001; Shirai et al. 2015) which correlate with the clinical activity. The

rapid and dramatic elevation in PTX3 expression was observed in sepsis, endotoxic shock, and other infectious disorders (Nauta et al. 2003; Razonable 2008; Kang et al. 2010; Table 1). Thus, PTX3 in association with other classic biomarkers may provide diagnostic and prognostic value toward determining the clinical outcomes in different pathologic conditions.

Conclusion

We have here focused on the role of PTX3 as a mediator and marker of bone pathology. In the bone environment, osteoblasts derived from bone marrow stromal cells highly express PTX3 upon proinflammatory signals. PTX3 secreted from osteoblasts enhances RANKL production and, in turn, stimulates osteoclastogenesis, thereby contributing to osteolytic conditions as an inflammatory mediator (Fig. 1). PTX3 expression is elevated in the distant bone metastases of breast cancer, suggesting that PTX3 may play a key role in the inflammation-associated osteolytic complications of breast cancer (Fig. 2). PTX3 could be a novel biomarker in combination with other biomarkers for the diagnosis of bone disease. A growing body of evidence suggests that PTX3 may be a promising biomarker that provides useful prognostic information regarding the clinical outcomes of different pathologic conditions. Future studies will be necessary to verify the value of PTX3 as a biomarker of clinical bone pathologic events.

Summary Points

- This chapter focuses on the key properties of pentraxin 3 (PTX3) in relation to bone pathology and its potential role as a biomarker of this disease.
- A number of studies have suggested that PTX3 may function as a marker for disease activity and a diagnostic biomarker for the pathogenesis of diverse human pathologies.
- However, a limited number of studies have been conducted to define the presence of PTX3 in bone-related cells and the role of PTX3 in bone-associated disease.
- Osteoblasts derived from bone marrow stromal cells highly express PTX3, and proinflammatory signals further enhance PTX3 expression.
- Receptor activator of NFkB ligand (RANKL) induced by PTX3 stimulates osteoclastogenesis, thereby leading to increased bone resorption in the bone environment.
- PTX3 expression is elevated in the distant bone metastases of breast cancer, and PTX3 plays a key role in the inflammation-associated osteolytic complications of breast cancer.
- PTX3 expression is significantly elevated in aggressive periodontitis.
- Therefore, PTX3 may be a novel, promising biomarker that provides useful prognostic information on the clinical outcomes in patients with bone pathological conditions.

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Ameloblastin as Biomarker of Bone

13

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Abstract

Regional bone diversity has major public health implications. This is exemplified by the tissue incompatibility problems associated with bone ectopic autografts or the puzzling jaw osteonecrosis induced by antiresorptive agents that are otherwise effective in treating long-bone osteoporosis or metastatic resorptive lesions. Identifying bone site-specific biomarkers is therefore essential, firstly to determine why or how bone cell phenotypes vary depending on the anatomical site and secondly to implement new bone site-specific therapeutics. The present chapter summarizes findings on site-specific bone cell profiles and highlights ameloblastin (AMBN) as an exemplary peptide for jaw bone site-specificity. AMBN was originally discovered in tooth enamel matrix, extracts of which have been successfully applied clinically for regeneration of mineralized tissue. AMBN has also been detected outside the enamel in both mineralized and nonmineralized tissues. In bone, functional studies have demonstrated crucial functions of AMBN in the control of bone balance, notably processes associated with a high bone remodeling rate. In contrast to appendicular and axial bones, jaw bones are highly affected by AMBN. For example, AMBN participates in the physiological control of alveolar bone integrity in response to tooth-associated biomechanical stimulation. Based on these observations, AMBN-based

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treatments have promising clinical potential for craniofacial tissue repair and more specifically for alveolar bone regeneration.

Keywords

Ameloblastin • Enamel • Osteoblast • Bone remodeling • Alveolar bone • Site-specificity • Bone regeneration

List of Abbreviations

ADAS	Adipose-derived adult stem cell
ALP	Alkaline phosphatase
AMBN	Ameloblastin
AMEL	Amelogenin
BMMC	Bone marrow-derived monocyte/macrophage cell
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cell
BP	Bisphosphonate
BRONJ	BP-related osteonecrosis of the jaw
DF	Dental follicle
DFDBA	Demineralized freeze dried bone allograft
ECM	Extracellular matrix
EMD	Enamel matrix derivative
EP	Enamel protein
ERM	Epithelial rest of Malassez
ES	Embryonic stem
FDBA	Freeze dried bone allograft
HERS	Hertwig's epithelial root sheath
hOMSC	Human oral mucosa stem cell
HOX	Homeobox
IHC	Immunohistochemistry
ISH	In situ hybridization
IUP	Intrinsically unstructured protein
KLK4	Kallikrein 4
LCM	Laser capture microdissection
LNA	Lock nucleic acid
LRAP	Leucine rich amelogenin peptide
MMP	Metalloprotease
MSC	Mesenchymal stem cell
MSX	Muscle segment homeobox
NB	Northern blot
NBM	Natural bovine mineral
NHO	Normal human osteoblast
Ocn	Osteocalcin
Opg	Osteoprotegerin
Opn	Osteopontin
PBMC	Peripheral blood mononuclear cell

PDL	Periodontal ligament
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa b
RANKL	Receptor activator of nuclear factor kappa b ligand
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SCC	Squamous cell carcinoma
SCPP	Secretory calcium-binding phosphoprotein
Seq	Sequencing
SIBLING	Small integrin binding ligand N-linked glycoprotein
SPARC	Secreted protein, acidic, cysteine-rich
STAT	Signal transducer and activator of transcription
WB	Western blot

Key Facts of Ameloblastin

The following are the key facts of ameloblastin (AMBN) and provides a brief description of this protein, its expression and functions in enamel and dental tissues, its role in bone cells, its differential expression in bone compartments, its expression and functions in craniofacial bones, and its putative clinical application to alveolar bone regeneration.

- Ameloblastin (AMBN) is the second most abundant protein in the enamel matrix after amelogenin (AMEL) (~5–10% and 90% of organic matrix proteins for AMBN and AMEL, respectively).
- The gene encoding AMBN as well as many other genes involved in vertebrate skeletal mineralization form the secretory calcium-binding phosphoprotein (SCPP) gene cluster located on human chromosome 4q21 and murine chromosome 5q (for rat and mouse).
- In enamel, AMBN promotes the growth of a crystalline layer and regulates the matrix binding, proliferation, and differentiation of ameloblasts.
- Besides its expression in enamel, AMBN is expressed during early stages of embryogenesis and has been shown to affect a wide variety of cells in mineralized and soft tissues. This suggests that AMBN may act as a signaling molecule in various bodily processes, including epithelial–mesenchymal interactions.
- In bone, AMBN stimulates both osteogenesis and osteoclastogenesis.
- AMBN is considered as a biomarker of the craniofacial bones due to its relatively higher expression in jaw and skull bones compared to long bones.
- AMBN is almost exclusively expressed in bone compartments with neural crest-derived osteoblasts. This suggests that AMBN could be a specific marker of the embryonic origin of bone organs (neuroectoderm versus mesoderm-derived bones).

- AMBN is strongly expressed in jaw bone processes associated with high remodeling rates, such as bone formation, repair, and regeneration.
- AMBN is involved in biomechanical responsiveness of the alveolar bone, and AMBN and Msx2 interact in this process.
- The application of AMBN to mandibular bone defects has been shown to stimulate bone repair in vivo; AMBN-based treatments might therefore have promising clinical potentials for alveolar bone regeneration.

Definition of Words and Terms

Adenomatoid odontogenic tumor	Tumor originating from the enamel organ or dental lamina.
Ameloblastoma	Odontogenic tumor of the buccal cavity, derived from ameloblasts that have lost their ability to build enamel.
Amelogenesis	The process of enamel formation.
Blastema	A group/mass of cells able to grow and differentiate into organs or body parts. Blastemas are composed of undifferentiated pluripotent cells.
Calvaria	The part of the skull consisting of the frontal bone, occipital bone, temporal bone, parietal bones, and sphenoid.
Chondrogenesis	The process of cartilage formation.
LNA	Abbreviation for lock nucleic acid. LNA is a modified nucleic acid with a methyl bridge between 2'-O and 4'-C.
Odontogenic tumor	Refers to a neoplasm and tumor-like malformation originating from cells of the odontogenic apparatus.
Perichondrium	Layer of fibrous connective tissue that surrounds the developing bone cartilage.
Squamous odontogenic tumor	Any group of rare odontogenic tumors originating in alveolar bone.

Introduction

During the processes of bone growth, homeostasis, and healing, terminally differentiated mesenchymal stem cells known as osteoblasts regulate bone apposition and participate in bone remodeling by controlling osteoclast differentiation and activity. Driven by the master genes Runx2 and Osterix (Sp7), osteoblasts synthesize a panel of extracellular matrix (ECM) proteins controlling biomineralization as well as

various other proteins governing cell fate (e.g., WNTs/BMPs for osteoblast differentiation or RANK/RANKL for osteoclastogenesis). Although osteoblasts are traditionally considered to be a unique, differentiated mesenchymal entity, irrespective of their location in the skeleton, several studies have highlighted that major differences in bone physiopathology – specifically between long and craniofacial bones – are associated with bone site-specific osteoblast phenotypes.

For example, disparate hormonal sensitivities have been demonstrated in jaw versus long bones. Using transgenic mouse models, Liu et al. (2009) showed that the mandible is less sensitive to the anabolic action of increased endogenous parathyroid hormone (PTH) than are long bones, and that this differential sensitivity is associated with differential expression of PTH receptor and subsequent regulation of insulin growth factor-1 (IGF1) by PTH in osteoblasts. In addition, estrogen deficiency (modeled using ovariectomy) induced an extensive reduction in bone mineral density and deterioration of the trabecular structure in appendicular and axial bones while jaw and cranial bones showed no or only minor changes (Rawlinson et al. 2009a; Liu et al. 2015). Furthermore, when compared with the tibia, alveolar bone developed ovariectomy-induced bone loss at a later stage (Du et al. 2015).

The site-specificity of bone physiopathology is also exemplified by the localized side effects of certain drugs used to treat bone-destructive disorders, such as the deleterious effect of bisphosphonates (BPs) on jaw bones. Indeed, BPs have been shown to induce BP-related osteonecrosis of the jaw (BRONJ), while no adverse effects in long bones have been observed (Marx 2003). Interestingly, a recent study highlighted that the jaw-specific impairment of bone remodeling by BPs is associated with differential expression of markers of osteoblast activity, including Msx1, Dlx5, Bmp2, Rankl, and Osteopontin (Wehrhan et al. 2015).

Finally, this bone site-specificity is exemplified in the limitations of regional autograft procedures: grafts obtained from craniofacial donor sites (mandible, maxilla, calvaria) show superior therapeutic efficacy in bone regeneration, independently of the bone acceptor-site, compared to grafts obtained from iliac crest, femur, or rib donor sites (Zins and Whitaker 1983; Casey et al. 1995; Leucht et al. 2008). Furthermore, even when successfully incorporated within the jaw, the noncraniofacial grafts are resorbed with time, probably due to inappropriate communication between donor-site osteoblast/matrix and acceptor-site bone cells such as osteoclasts or endothelial cells. In line with these observations, both osteoblasts and bone marrow stromal cells isolated from alveolar bone (mandible or maxilla) and cranial bones show increased osteogenic capacities in vitro when compared to osteoblastic cells isolated from axial or appendicular bone (Kasperk et al. 1995; Leucht et al. 2008; Reichert et al. 2013).

Taken together, these observations demonstrate that osteoblast responsiveness under physiopathological circumstances is bone site-dependent and that osteoblast behavior should no longer be considered independently of the bone site physiologic environment. The mechanisms underlying the site-specific responsiveness of osteoblasts remain poorly understood. However, recent studies have identified factors that are involved in osteoblast site-specificity and highlight ameloblastin (AMBN) as a putative molecular marker of the jaw bones.

This chapter aims to provide an interdisciplinary state-of-the-art review on enamel peptides, with a focus on AMBN and its expression and functions in bone tissue. Because for years amelogenins and AMBN were considered to be exclusive to the tooth compartment, section "Part I" gives an overview of these proteins and their structural and regulatory roles in dental tissues. In the last decade, the detection of the enamel proteins has been expanded to other mineralized and nonmineralized tissues. In this context, section "Part II" provides a detailed description of AMBN expression, functions, and pathways beyond enamel. Section "Part III" focuses on AMBN expression in various bone compartments and bone cells. This part provides supportive data that AMBN is a bone site-specific marker, strongly involved in cranial and jaw bone formation and healing, and their biomechanical responsiveness. Finally, based on the data covered in this chapter, section "Part IV" introduces perspectives on the potential of AMBN-based therapies for orofacial bone regeneration.

Part I: Enamel Matrix and Enamel Proteins

Enamel Matrix, Amelogenesis, and Enamel Proteins

Dental enamel acts as a solid barrier that protects the inner layers of the tooth from masticatory impacts, bacterial invasion, and acid exposure. Enamel is the most mineralized tissue in the human body. Mature enamel is mostly composed of bioapatite crystals (97%) [$Ca_5(PO_4)_3(OH)$, with a high number of ion substitutions (e.g., Ca^{2+} by Sr^{2+} and PO_4^{3-} by CO_3^{2-} or F^-)]. The remainder consists of organic material. Because of its unique composition (hypermineralized, acellular, avascular, no innervation, and very low content of protein), and also its mechanical properties and ectodermal embryonic origin, enamel differs from other mineralized tissues like dentin, cementum, or bone. Finally, enamel is a distinctive tissue because its formation begins with a labile protein scaffold devoid of collagen, which is gradually replaced by highly organized crystalline architecture.

Amelogenesis

Enamel is one of the rare mineralized tissues in the human body that is deposited by epithelial cells. Amelogenesis – the process of enamel formation – is orchestrated by the ameloblasts, which are derived from the basal layer of the oral ectoderm.

Enamel formation per se consists of two steps: (1) secretion of a partially mineralized organic matrix by ameloblasts, and (2) degradation of this matrix temporally linked to termination of the mineralization process [reviewed in Nanci (2013), Lignon et al. (2015)]. Briefly, enamel formation begins at the bell stage of odontogenesis and involves the differentiation of cells of the inner enamel epithelium into polarized ameloblasts. The deposition of enamel matrix requires the functional differentiation of the ameloblasts into effector/secreting cells.

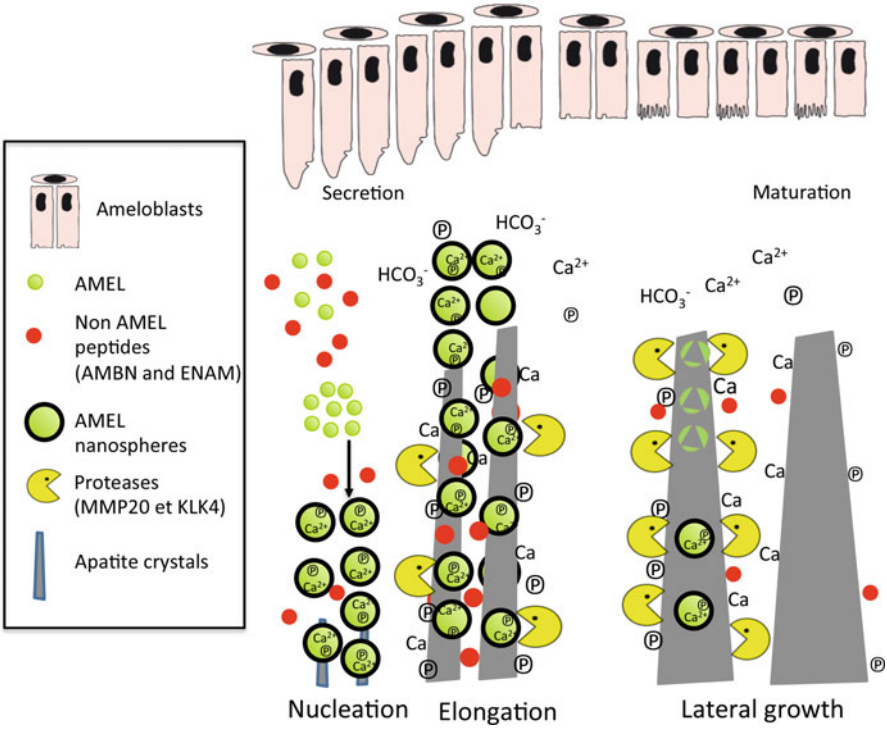

Fig. 1 Schematic representation of crystal growth during amelogenesis. Crystalline nucleation begins with the secretion of enamel peptides – mainly AMEL, AMBN, and ENAM – by ameloblasts at the secretion stage. AMEL self-assembles into nanospheres while the other enamel peptides remain as monomers. The AMEL nanosphere supramolecular structure promotes calcium phosphate formation by concentrating the charge at the nanosphere surface and acting as a nucleation template. At the crystal level, the nucleation step is followed by an elongation step. During this step, crystal elongation and inhibition of its lateral growth are controlled by the adsorption of enamel proteins onto the growing crystal. From the secretion stage to the end of the maturation stage, ameloblasts secrete proteases such as MMPs and KLK4 to degrade the enamel peptides; this results in crystal lateral growth. To counteract acidification of the medium secondary to the crystal growth during amelogenesis, bicarbonate ions are secreted to maintain pH

Amelogenesis is divided into three main sequential cell stages: presecretion/secretion, transition, and maturation (Fig. 1). During the secretion stage, the production and secretion of enamel proteins by the ameloblasts is maximal, while their protease production is limited. The resulting secreted enamel is composed of an organic matrix that interacts with calcium and phosphate to initiate bioapatite crystal formation. The longitudinal growth of crystals is controlled by a transient protein scaffold (Fig. 1). Differential orientation of these elongated crystals generates distinct areas: (1) the innermost and outermost layers called the “aprismatic” enamel and (2) the “prismatic” area of the bulk of enamel composed of intra-prismatic and inter-prismatic zones. Once the final thickness of the enamel is established, ameloblasts

undergo a morphological transition; they become shorter and reduce the volume of their organelles for preparation of the next stage, enamel maturation. This stage is characterized by decreased matrix protein secretion and increased protease synthesis and activity. These proteases [essentially kallikrein 4 (KLK4) and metalloprotease 20 (MMP)] are necessary to clear proteins from the enamel matrix. The matrix proteins are digested and removed, thereby allowing growth and densification of the bioapatite crystals (Hu et al. 2005). The mineral quantity increases from 35% in the secretion stage to 97% in the final mineralized matrix of mature enamel.

Of the matrix peptides (including matrix proteins and their enzymatic degradation products) produced by the ameloblasts during amelogenesis, this chapter focuses on the two main species, amelogenins and AMBN. The synchronized secretion of these enamel proteins is crucial for the formation of organized enamel crystals and the regulation of their growth.

Enamel Proteins and Their Structural Roles in Mineralizing Enamel

Amelogenins in enamel are secreted by ameloblasts and constitute ~90% of the organic matrix during enamel secretion [reviewed in Nanci (2013)]. They are members of a highly conserved protein family (produced by translation of at least 16 mRNAs). Two human amelogenin genes have been mapped to Xp22.1-p22.3 and Yp11.2 (AMELX and AMELY), with ~90% of the transcripts being expressed from the X chromosome. In experimental animal species, such as mouse and rat, only the X-chromosomal amelogenin gene is found, which will be referred to as "AMEL" in this chapter. In addition to the number of isoforms produced by alternative splicing of RNA, AMEL proteins are cleaved into different peptides that differentially regulate crystal growth. Amelogenins are the key proteins of enamel mineralization control: they aggregate into nanospheres that are reservoir of Ca^{2+} and PO_4^{3-} ions and participate in the regulation of crystal development. These nanospheres inhibit lateral crystal growth, guiding their tri-dimensional structure (Fig. 1). The quality of enamel (thickness, molecular organization) is conditioned by the amount and quality of amelogenins (Gibson et al. 2001; Molla et al. 2010).

AMBN is the second most abundant protein in enamel and represents ~5–10% of the organic matrix during enamel secretion. In the enamel matrix, AMBN is located at the boundary between inter- and intra-prismatic enamel crystals, mostly in the newly externally formed enamel. This protein initiates and regulates enamel mineralization and maintains the supracrystalline structure of enamel by inhibiting lateral crystal growth (Fig. 1). Using transgenic mouse models, Lu et al. showed that AMBN promotes the growth of a crystalline enamel layer with short and randomly oriented crystals but lacks the ability to facilitate the formation of mature oriented apatite crystals. This suggests that AMBN is not involved in enamel apatite crystal elongation (Lu et al. 2011). At the cellular level, AMBN was shown to regulate the matrix binding, proliferation, and differentiation of ameloblasts (Fukumoto

et al. 2004; Sonoda et al. 2009). The molecular structure of AMBN and its functions will be detailed in section "Part II."

The major roles of these two proteins in enamel structure are supported by clinical enamel phenotypes. Mutations in the human AMEL and AMBN genes lead to various forms of amelogenesis imperfecta (Lagerstrom et al. 1991; Poulter et al. 2014).

A First Overview of Enamel Protein Expression Outside the Enamel Tissue

For years, enamel proteins were considered to be exclusively produced by the ameloblasts and to specifically control enamel formation and biomineralization. However, more recently, their expression and functions once thought to be restricted to the ectodermal tooth compartment have now been extended to a second odontogenic compartment, the mesenchyme. This localization was definitively established for AMEL when two specific cDNAs comprising AMEL gene exons were cloned and sequenced from a rat tooth odontoblast pulp cDNA library (Veis et al. 2000). Enamel protein expression outside the enamel tissue is now widely documented in the literature. However, detection and quantification of enamel proteins in mineralized tissues is difficult with conventional techniques, and some authors still consider enamel proteins to be exclusive to enamel, with no extra-enamel distribution.

Many studies have demonstrated the presence of AMEL in dentin and dental ligament. For example, using immunohistochemistry (IHC), AMEL peptides were detected in the outermost layer of the dentin during the enamel presecretion stage (Inai et al. 1991). RNA studies have further demonstrated that AMEL is expressed by odontoblasts (Oida et al. 2002). In 2006, Ye et al. suggested that this latter expression is transitory during the first steps of dentinogenesis, and they showed that recombinant AMEL protein enhances pulp cell proliferation (Ye et al. 2006). Other studies have reported sequential AMEL expression during tooth root formation (Diekwisch 2001), both in cementoblasts (Nunez et al. 2010) and in Hertwig's epithelial root sheath (HERS) cells in response to inflammation (Fong and Hammarstrom 2000). In addition to these dental tissues, AMEL has been detected in many nondental tissues, including bone and soft tissues such as the eye, tongue, testis, heart, colon, ovary, and kidney (Deutsch et al. 2006; Li et al. 2006; Haze et al. 2007; Gruenbaum-Cohen et al. 2009; Jacques et al. 2014b) (Table 1).

Shortly after its discovery in secretory ameloblasts (Cerny et al. 1996; Krebsbach et al. 1996), AMBN was detected in dentin matrix, preodontoblasts, polarized odontoblasts, and pulp cells (Begue-Kirn et al. 1998; Fong et al. 1998). AMBN was also shown to be transitorily expressed during mineralization of the dentin outermost layer (Fong et al. 1998) and in trauma-induced reparative dentin (Spahr et al. 2002). AMBN was also detected in HERS cells (Fong and Hammarstrom 2000; Lu et al. 2013). Similar to AMEL, AMBN was identified in epithelial cell rests of

Table 1 The expression of ameloblastin and amelogenin in extra-dental tissues. This table reviews the literature reporting on the expression of ameloblastin and amelogenin in extra-dental tissues. *AMBN* ameloblastin, *AMEL* amelogenin, *ENAM* enamelin, *LRAP* leucine rich amelogenin peptide, *D* day, *E* Theiler sage, *M* month, *RT-qPCR* reverse transcriptase quantitative polymerase chain reaction, *IHC* immunohistochemistry, *ISH* in situ hybridization, *WB* Western blot, *NB* Northern blot, *Seq* sequencing, *MSC* mesenchymal stem cell, *BMSC* bone marrow stromal cell, *ADAS* adipose derived adult stem cell, *PBMC* peripheral blood mononuclear cell, *NHO* normal human osteoblasts, *Saos-2* sarcoma osteogenic cells, *ES* embryonic stem, *hOMSC* human oral mucosa stem cell, *LCM* laser capture microdissection

Reference	Model	Species/cells	Stage	Tissue	Evaluation method	Main conclusion
Deutsch D., 2006	In vitro	RAW 264.7 cells (mouse macrophages)			RT-PCR/seq	Identification of AMEL M180 transcript
	In vivo	Rat (Sabra) and dog	W5-6	Brain and long bones	RT-PCR/seq/ IHC	Identification and localization of AMEL transcripts (M180 and M194) in brain (glial cells) and in bone marrow cells
Spahr A., 2006	In vivo	Rat (Sprague Dawley)	E18, D2-28	Skull, vertebrae, mandible	RT-PCR/HIS/ IHC	Higher expression of AMBN in newly formed bone matrix during endochondral and intramembranous ossification. Decreased expression with aging
Li Y., 2006	In vivo	Mouse (C57Bl/6/ 129)	D2 and D4	Variety of hard and soft tissues	RT-PCR/seq	Identification of AMEL isoforms (LRAP and E4) and detection of AMEL in bones (cavarial and long bones) and in soft tissues (e.g., skin, eye, brain)
Haze A., 2007	In vitro	Human bone cells and bone marrow stromal cells			RT-PCR/seq	Identification of AMEL M180 transcript
	In vivo	Rat (Sabra), Dog	W5-10 (rat), M2 (dog)	Long bones	RT-PCR/seq/ WB/ISH/IHC	Identification of AMEL isoforms (LRAP) and detection in bone cells (osteoblasts, chondrocytes, osteoclasts) and bone marrow (mesenchymal stem cells)
				Bone marrow		Identification of AMEL isoform (10–15 kDa)
Müller W., 2007	In vitro	Saos-2 cells (human osteosarcoma cells)			NB	Detection of ARN coding for AMEL, AMBN, and ENAM

(*continued*)

Table 1 (continued)

Reference	Model	Species/cells	Stage	Tissue	Evaluation method	Main conclusion
Gruenbaum-Cohen Y., 2009	In vivo	Mouse	E10.5-17.5	Whole craniofacial complex	RT-PCR/seq/ IHC	Identification of two AMEL isoforms and detection in all the analyzed tissues. Expression of AMEL in brain and eye before tooth formation
Haze A., 2009	In vivo	Human	Fetus	Mandible	IHC	Higher expression of AMEL in preameloblasts compared to alveolar bone cells
		Dog (Beagle)	M13-16		IHC	High expression of AMEL in newly formed bone with high bone turnover and activity
		Rat (Sabra)	W5-10		ISH	High expression of LRAP in alveolar bone at the boundary of periodontal ligament (PDL)
Rawlinson SC., 2009	In vivo	Rat (CD) (100 g)		Parietal skull bone and ulnar limb	microarray	Higher mRNA levels of AMEL and AMBN in skull bone when compared to limb bone
Iizuka S., 2011	In vitro	Calvaria bone cells (Wistar rat fetuses)			RT-PCR	Higher levels of AMBN during proliferation stages compared to differentiation and maturation stages
		NOS-1, Saos-2, MG63, HOS cells			RT-PCR/IHC	Detection of AMBN in NOS-1 cells
	In vivo	Human		Samples of osteosarcoma	IHC	Expression of AMBN in osteosarcoma tissues
Tamburstuen M., 2011	In vitro	Human and mouse cells (MSC, BMSC, ADAS, PBMC, NHO, pulp and PDL cells, LS8, MC3T3-E1)			RT-PCR/seq	AMBN expression in human hematopoietic and mesenchymal cells, from calcified and noncalcified tissues
					Dot blot/WB	Expression in cell lysate and secretion in cell culture medium of AMBN protein in the tested cells
	In vivo	Rat (Sprague Dawley)	Adult	Ramus	IHC	AMBN expression in newly formed bone after mandibular ramus defect

Landin M., 2012	In vivo	Mouse (CD1)	E12.5-P2	Whole craniofacial complex	ISH	AMBN and ENAM mRNA expression in forming facial bone structures, adjacent to molars
Alikhani M., 2012	In vivo	Rat (Sprague Dawley)	D120	Maxillar bone	RT-qPCR/ Biomechanical assay	Increased mRNA expression of AMBN and ENAM in alveolar bone after high-frequency acceleration
Treves-Manusevitz S., 2013	In vivo	Implantation of human cells from oral gingiva and mucosa (hOMSC) in SCID mice			IHC	Detection of AMEL and AMBN proteins in hOMSC 8 weeks after implantation in mice and in the newly acellular mineralized matrix formed by hOMSC
		Sections obtained from human periodontal tissues			IHC	Detection of AMEL and AMBN proteins in human cementum and PDL tissues
Atsawasuwan P., 2013	In vivo	Mouse (CD1)	D1-20	Calvarial vault and sutures	RT-qPCR/IHC/ WB	AMBN mRNA detection in sutures. Detection of AMBN protein in bone extracts and localization in calvarial bone matrix, dura mater, and adjacent mesenchyme
	In vitro	Skull suture mesenchymal cells (mouse)			RT-qPCR/WB	AMBN expression in suture mesenchymal cells in vitro
Atsawasuwan P., 2013	In vivo	Mouse (CD1)	D3-35	Calvarial vault	RT-qPCR/IHC/ WB	Detection of AMEL, AMBN, and ENAM in calvarial tissues at lower levels compared to teeth. Variation of expression in calvaria with developmental stages
	In vitro	MC3T3-E1 cells/primary calvarial osteoblasts (mouse)			RT-qPCR	Variation of expression of AMEL, AMBN, and ENAM in cultured bone cells during osteoblastic differentiation and matrix mineralization stages

(*continued*)

Table 1 (continued)

Reference	Model	Species/cells	Stage	Tissue	Evaluation method	Main conclusion
Jacques J., 2014	In vivo	Mouse (CD1)	E10.5	Whole body and head	RT-qPCR	Detection of AMBN mRNA before tooth formation in embryos, with higher expression in body compared to head
			D3 and D105	Mandible, tibia, occipital, and frontal bone	RT-qPCR/seq/ LCM/IHC	AMBN expression in both craniofacial and long bone. Variation of expression with anatomical site and ontogenic stage
Jacques J., 2014	In vivo	Mouse (CD1)	W1, W8, and W15	Dental, mandibular, and a variety of soft tissues	RT-qPCR/seq/ ISH	Detection of AMEL and AMBN in both epithelia and mesenchymal-derived tissues. Variation of expression with tissue type, anatomical region, and age
					IHC/WB	Expression of AMEL and AMBN proteins in mineralized matrix and in solubilized form in mandible. Variation of expression with anatomical region

Malassez (ERM), and this expression was increased in response to physiopathological events such as pulp inflammation (Hasegawa et al. 2003) or developmental disorders (Molla et al. 2010). AMBN expression was also detected in cementoblasts at the level of the root (Nunez et al. 2010). Together, these observations suggest that AMBN may have a signaling function in epithelial–mesenchymal interactions occurring during the formation of the tooth/parodontium complex and under physiopathological circumstances.

As seen in this first part, the history of these two enamel proteins is quite different. In contrast to AMEL, as soon as AMBN was discovered, it was detected outside the enamel, notably in mesenchymal cells. In addition, again in contrast to AMEL, AMBN is not involved in the elongation of the apatite crystals of enamel; rather, it acts mostly during the initial steps of crystal growth and regulates cell adhesion and differentiation. These observations therefore suggest that AMBN may act as both a cell adhesion molecule and a signaling factor and be involved in various bodily processes, including epithelial–mesenchymal interactions.

Part II: Ameloblastin

Ameloblastin Gene and Proteins

AMBN, the second most abundant protein in the enamel matrix, was discovered in 1996 (Cerny et al. 1996; Krebsbach et al. 1996). This occurred long after an AMEL cDNA had been cloned (Snead et al. 1983), consistent with a low amount of AMBN in enamel matrix (comprising ~5–10% of the organic matrix proteins, versus 90% for AMEL). AMBN, AMEL, and many genes involved in vertebrate skeletal mineralization (as the noncollagenous SIBLINGs family members [DSPP, DMP1, IBSP, MEPE, SPP1, etc.]) form the secretory calcium-binding phosphoprotein (SCPP) gene cluster. The SCPP genes are evolutionarily related as they all originate from duplications of a common ancestral SPARC gene. With the single exception of the genes encoding the amelogenins, which are located on the X and Y chromosomes (see section "Part I"), AMBN and the other SCPP genes form a cluster located on human chromosome 4q21 and murine chromosome 5q (for rat and mouse).

The AMBN gene contains 11 exons in rat, mouse, and pig, whereas the human AMBN gene consists of 13 exons. Two isoforms have been described for AMBN protein (Krebsbach et al. 1996). AMBN protein is first synthesized as a polypeptide of 44–55 kDa, and after glycosylation it has a molecular weight of 65 kDa; this form is considered as the native protein. In enamel, AMBN is first secreted by ameloblasts, then cleaved by enamel proteases (e.g., MMP20 and KLK4), resulting in two types of peptides: (1) basic peptides with an N-terminus of 13–17 kDa and (2) acidic peptides with a C-terminus of 27–29 kDa. The AMBN protein sequence features binding sites for calcium, heparin, CD63, fibronectin, and integrins (Beyeler et al. 2010; Zhang et al. 2011a). Based on bioinformatics analysis, AMBN is an

intrinsically unstructured protein (IUP); it lacks a fixed 3D structure and contains long disordered regions (Vymetal et al. 2008).

AMBN Expression

Besides its expression in dental tissues (detailed in section "Part I"), some studies have demonstrated that AMBN is also expressed in various body compartments (mineralized and nonmineralized) during embryonic development – AMBN is detected as early as E10.5 in both mouse body and head segments (Jacques et al. 2014a) – and after birth (Table 1).

In bone tissues, different patterns of AMBN expression were found in the processes of intramembranous and endochondral ossification during craniofacial bone formation at embryonic and early postnatal stages (Spahr et al. 2006; Landin et al. 2012). At E18, in intramembranous ossification (e.g., in alveolar bone of developing mandible, frontal, and parietal bones), AMBN RNA and protein were detected in the superficial layer of the condensed vascularized primitive connective tissue and in the cellular layer covering the surface of the newly formed woven bone (Spahr et al. 2006). A similar expression pattern was reported in calvaria by Atsawasuwan et al. (2013a). In endochondral ossification (e.g., in ethmoid, parts of the sphenoid, vertebra), AMBN was expressed within the ECM of the cartilage templates and in the perichondrium. Between postnatal days 2 and 28 the expression decreased markedly, concordant with the maturation of the bone, and was not detected after completion of bone remodeling (Spahr et al. 2006). AMBN RNA expression was also detected in postnatal cranial sutures (Atsawasuwan et al. 2013b). In line with Jacques et al. (2014a), unambiguous AMBN RNA expression was shown in alveolar bone using laser capture microdissection of mandible from 3-day-old mice (Fig. 2). At day 3, AMBN protein was detected in bone-lining cells and recently embedded osteoblasts/osteocytes of the mandibular alveolar bone (Fig. 3). AMBN RNA in adult mice showed a similar localization pattern (Fig. 4). In recent studies, AMBN RNA expression has been measured and compared in different bone compartments, including in craniofacial and long bones isolated from young and old mice (Jacques et al. 2014a, b). In line with studies on AMEL expression in mandible (Haze et al. 2009) and on AMBN expression in appendicular, axial, and cranial bones (Spahr et al. 2006; Atsawasuwan et al. 2013a), Jacques et al. showed that AMBN RNA quantity in jaw bones decreased with age, suggesting that AMBN expression tends to phase out during the differentiation of bone cells, a process which is reduced after completion of bone remodeling (Jacques et al. 2014b). In addition, using PCR analysis, the same group demonstrated that AMBN expression is not restricted to mineralized tissues; it is also expressed in several soft tissues, at a high level in dental follicle of the mouse incisor and at low levels in eye, tongue, and testis (Jacques et al. 2014b). Interestingly, using Western blot on proteins extracted by nondissociative means, Jacques et al. showed that AMBN in mandibular bone is present both in an aggregate form incorporated into

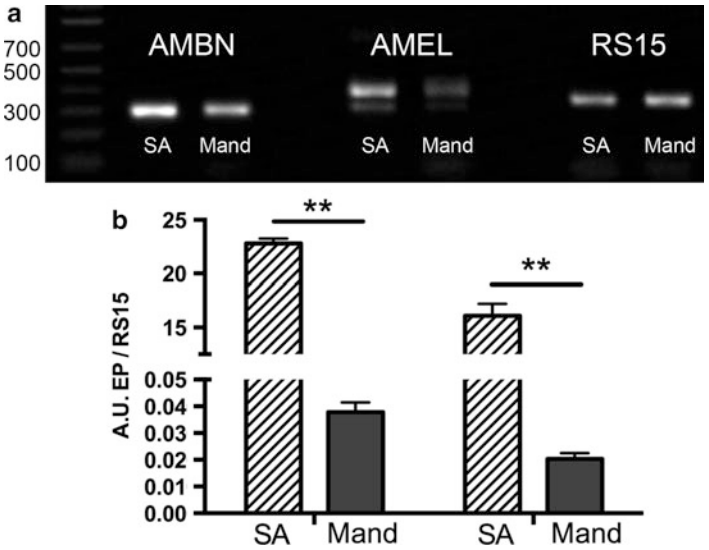

Fig. 2 AMBN and AMEL RNA expression in mandibular bone in 3-day-old mice. Mandibular bone (*Mand*) was isolated from 3-day-old mice by using laser capture microdissection and subjected to RT-qPCR to quantify AMBN and AMEL. Secreting ameloblasts (*SA*) were used as positive control. The expression levels of AMBN and AMEL were significantly higher in SA compared to mandible. The migration of the amplicon products (2% agarose gel) and their sequencing confirmed AMBN and AMEL RNA expression in SA and mandibular bone. RNA levels in these tissues were compared using RT-qPCR, which showed that expression of both AMBN and AMEL was significantly higher in SA compared to mandibular bone (≈600:1 and 700:1, respectively). The DNA molecular size marker is indicated on the side of the gels. RNA levels were normalized to the expression of the housekeeping gene RS15. Significance was evaluated using the Mann–Whitney test (*$p < 0.05$, **$p < 0.01$). *EP* enamel protein, *AU* arbitrary units

matrix and also in a solubilized state (Jacques et al. 2014b). These data support the notion that AMBN has some capacity to diffuse and may function as a growth factor-like molecule solubilized in the aqueous bone microenvironment.

In vitro, Iizuka et al. investigated the expression of AMBN during osteogenesis by RT-PCR using rat embryonic calvaria cells (Iizuka et al. 2011). AMBN RNA was detected during cell growth, and its expression was downregulated when the cells reached confluence. By screening various cell culture models, Tamburstuen et al. showed that AMBN was expressed in many primary cells and in cell lines that had originated from mesenchymal tissues such as bone and adipose tissues (e.g., MSC, BMSC, ADAS, PBMC, and MC3T3) (Tamburstuen et al. 2010, 2011). Of note, AMBN gene expression was also detected in human osteosarcoma tissue (Iizuka et al. 2011) and tumor cell lines such as Saos-2 (Muller et al. 2007) and NOS-1 (Iizuka et al. 2011). Osteoclasts were also shown to contain AMBN protein (Tamburstuen et al. 2010).

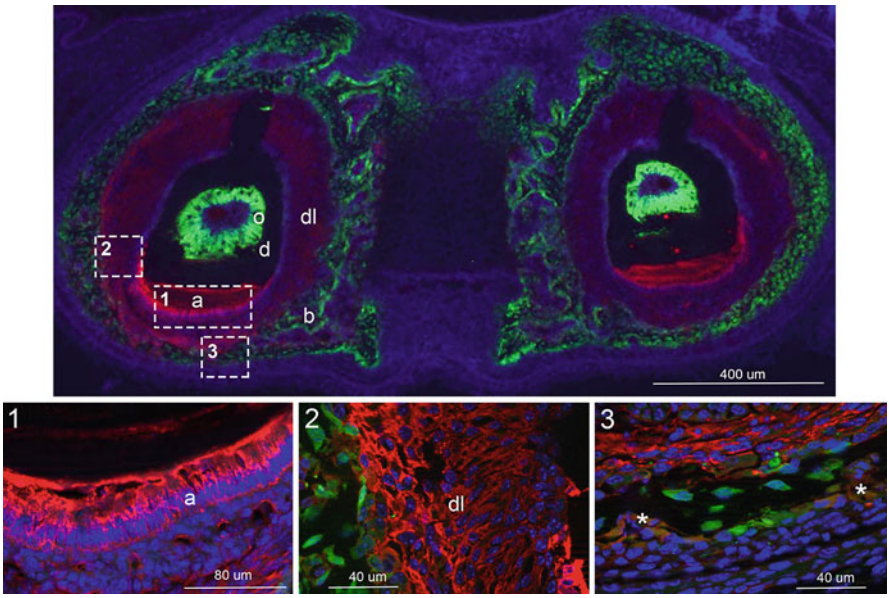

Fig. 3 AMBN protein expression in incisor/parodontium complex in 3-day-old mice. Frontal sections of 3-day-old mouse head were labeled with an antibody to AMBN (*red signal*). Collagen I-expressing cells (e.g., odontoblasts and osteoblasts) appear *green* and nuclei appear *blue*. AMBN protein is strongly expressed in ameloblasts (*a*) and in dental ligament (*dl*). No protein expression was detected in odontoblasts (*o*) or in dentin (*d*). Higher magnification shows strong AMBN signal in ameloblasts (*1*) and in cells of the dental ligament (*2*). Higher magnification of AMBN protein expression in bone (*3*) shows AMBN-positive osteoblastic cells lining bone trabeculae (*white asterisk*) (*3*). *a* ameloblast, *b* bone, *o* odontoblast, *dl* dental ligament, *d* dentin

AMBN Functions and Pathways

The signaling function of enamel peptides was demonstrated for the first time in 1999 when Nebgen et al. showed that implantation into muscle of a peptide fraction derived from bovine dentin matrix induced ectopic chondrogenesis and osteogenesis (Nebgen et al. 1999). These experiments aimed to duplicate in teeth the success story of the bone morphogenetic proteins (BMPs), which are osteoinductive growth factors isolated from decalcified bone matrix. In vitro, a peptide fraction distinct from the BMP family was shown to induce the synthesis of type II collagen and proteoglycans and to stimulate the expression of Sox9 and Runx2 in embryonic muscle-derived fibroblasts (Nebgen et al. 1999). Since then, many studies have reported that recombinant or purified enamel proteins and peptides affect a wide variety of cell types, suggesting direct roles for these molecules in both epithelial and mesenchymal tissues [reviewed in Grandin et al. (2012)]. For some authors, the "unstructured" characteristics of AMEL and AMBN proteins and their cleavage products may explain their signaling potential and their ability to promote cell interactions (Gibson 2008; Vymetal et al. 2008). However, although the biological

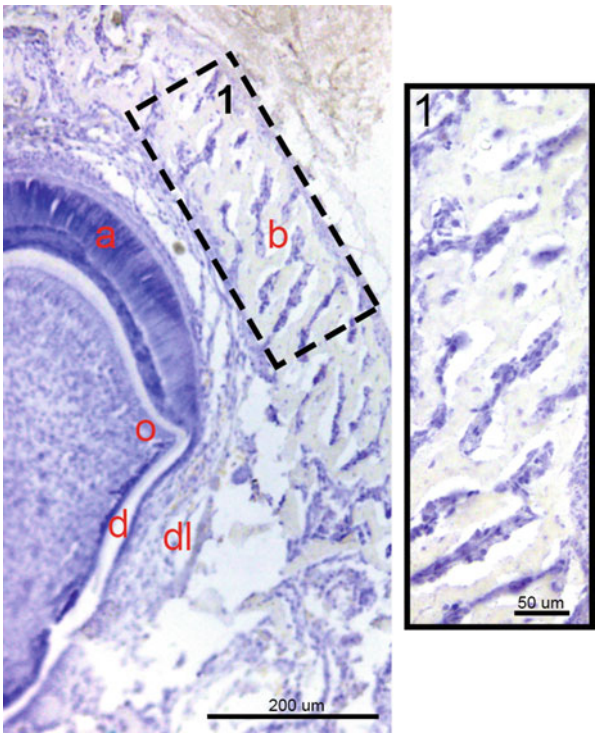

Fig. 4 AMBN RNA expression in incisor/parodontium complex in 3-day-old mice. In situ hybridization was performed using AMBN LNA probes. A strong AMBN RNA signal is observed in ameloblasts (*a*) and odontoblasts (*o*) and in cells from the dental ligament (*dl*). Higher magnification of the bone tissue shows AMBN RNA in osteoblasts and in some recently embedded osteocytes. *a* ameloblast, *b* bone, *o* odontoblast, *dl* dental ligament, *d* dentin

functions of enamel proteins have been widely studied, their mechanisms of action in a physiological context are still under investigation.

The use of transgenic mouse models has provided new insight into the in vivo functions and mechanisms of AMBN.

As mentioned above, results showing AMBN expression inside and outside the enamel have suggested that this protein acts as a signaling molecule in epithelial–mesenchymal interactions and may play a role during the cellular processes of commitment and differentiation, including early tooth development (Begue-Kirn et al. 1998; Fong et al. 1998). In that context, AMBN expression was found to be associated with epithelial odontogenic tumors. In 2004, Fukumoto et al. developed a transgenic mouse model expressing a truncated form of AMBN lacking the portion encoded by exons 5 and 6 (AMBNΔ5–6 mouse) (Fukumoto et al. 2004). Using this mouse model, Fukumoto et al. showed that about 20% of the mutant mice developed an odontogenic tumor of dental epithelium originating in the buccal vestibule of the maxilla (Fukumoto et al. 2004). AMBN mutations were also reported in ameloblastomas, adenomatoid odontogenic tumors, and squamous odontogenic tumors (Perdigao et al. 2004), suggesting that AMBN regulates odontogenic tumor formation. This assertion was confirmed by Sonoda et al., who showed that overexpression of recombinant AMBN protein (rAMBN) inhibited proliferation of human ameloblastoma cells (Sonoda et al. 2009). This inhibition required the AMBN

heparin-binding sites, indicating that the presence of heparin sulfate on the cell surface is important for AMBN to adhere to and act on target cells. Moreover, the AMBN-induced inhibition of proliferation was associated with dysregulation of Msx2, p21, and p27. These data suggest that AMBN acts as an inhibitor of ameloblastoma cell proliferation by regulating cellular signaling through its heparin-binding domains and Msx2/p21/p27 pathways. Recently, the role of AMBN as an inhibitor of proliferation in epithelial carcinoma cells was confirmed by Saito et al., who showed that rAMBN treatment suppressed the proliferation of squamous cell carcinoma of tongue cells (SCC-25) via cell-cycle arrest in G1 phase (Saito et al. 2014).

In 2011, Lu et al. developed a transgenic mouse line (AMBN-K14-Tg) in which AMBN overexpression was under the control of the human keratin 14 promoter (Lu et al. 2011). In 2013, the same group showed that AMBN-K14-Tg mice displayed fivefold elevated AMBN levels in mandibles and suffered from root cementum resorption, delamination, and reduced alveolar bone thickness (Lu et al. 2013). AMBN gain of function resulted in a significant reduction in trabecular bone volume and bone mass in the postnatal mouse jaw. In addition, AMBN-overexpressing mice displayed increased thickness of the periodontal ligament (PDL) as well as significant bone loss at the alveolar bone crest. To investigate the specific role of AMBN in osteoclastogenesis, the same authors used an in vitro model of osteoclastogenesis in which bone marrow derived-monocyte/macrophage cells (BMMCs) were cultured in the presence of rAMBN. AMBN dramatically increased the osteoclast number and modulated osteoclast differentiation, as shown by the upregulation of the osteoclastogenic genes NFATc1 and cFos. AMBN also increased adhesion and accelerated cell spreading of BMMCs; this effect was associated with enhanced RhoA protein expression and elevated ERK1/2 and AKT phosphorylation. Furthermore, blocking the integrin α2β1 and ERK1/2 pathways inhibited the effects of AMBN on osteoclast adhesion and differentiation. The authors therefore concluded that AMBN acts as a signaling molecule that enhances osteoclastogenesis and resorption of mineralized tissue by regulating adhesion of osteoclastic cells via integrin-dependent ECM signaling cascades involving integrins, ERK 1/2, RhoA, MAPK, and phosphorylation of AKT (Lu et al. 2013).

Using the same AMBN-K14-Tg mouse model, Atsawasuwan et al. showed that overexpression of AMBN also resulted in delayed posterior frontal suture fusion and incomplete suture closure. In addition, mutant mice displayed lighter skulls, thinner interfrontal bones, and morphological alterations of the craniofacial bones and their sutures (Atsawasuwan et al. 2013b). These mice also featured reduced cell proliferation in suture blastemas and in mesenchymal cells from frontal sutures. In addition, calvaria and suture mesenchymal cells isolated from these mice displayed significantly reduced expression of Msx2 and its downstream target genes, including the osteogenic transcription factors Runx2 and Sp7, the bone matrix proteins Ibsp, Col1, Ocn, and Opn, and the cell-cycle-related gene CcnD1. Furthermore, using the AMBNΔ5–6 mouse model developed by Fukumoto et al. (2004), Atsawasuwan et al. demonstrated that AMBN expression varied with osteoblast differentiation and affected bone development and mineralization (Atsawasuwan et al. 2013a). Indeed, skulls from AMBNΔ5–6 mice were approximately 15% shorter. In addition,

analysis of calvaria from AMBNΔ5–6 mice and calvaria osteoblast cultures revealed a dramatic reduction in mineralized nodules associated with reduced expression of Runx2, Sp7, Ibsp, and Msx2 and a delay in suture closing. Taken together, these studies suggest that AMBN plays a crucial role in the regulation of cranial bone growth and suture closure and affects osteoblast differentiation and mineralization. AMBN acts on craniofacial bone and suture formation and homeostasis by regulating Msx2 and controlling the proliferation of progenitor cells (Atsawasuwan et al. 2013a, b). Involvement of AMBN in cell proliferation associated with Msx2 alteration, which was identified in an Msx2-knockout transgenic mouse model (Aioub et al. 2007), is supported by the studies on odontogenic tumor mentioned above (Fukumoto et al. 2004; Perdigao et al. 2004; Sonoda et al. 2009).

To further determine the mechanisms involved in AMBN regulation of mesenchymal cells, several studies using rAMBN have been performed in vitro.

In 2010, Tamburstuen et al. demonstrated that rAMBN enhanced the proliferation and migration of osteoprogenitors and osteoblasts (Tamburstuen et al. 2010). Using gene-screening techniques, they showed that rAMBN treatment was associated with increased expression of markers of bone cell differentiation (e.g., Ocn, CD44) as well as immune responses (e.g., IL6, IL8, IL1). Importantly, the genes encoding the transcription factors STAT1 and STAT2 were among the stimulated genes involved in signaling pathways. The authors therefore suggested that AMBN would have the potential to induce osteoprogenitor cell recruitment and growth via the interferon pathway and its downstream factors STAT1, STAT2, and CD44. Using Western and dot blot, the same group demonstrated that mesenchymal stem cells and primary human osteoblasts not only expressed AMBN RNA but also secreted the protein into the cell culture medium (Tamburstuen et al. 2011). Moreover, human rAMBN was shown to stimulate high levels of AMBN RNA expression in primary human mesenchymal stem cells, suggesting there is positive feedback regulation of AMBN expression in these cells. Feedback regulation is a well-recognized mechanism for controlling cell signaling and cell homing; these observations therefore suggested a role for AMBN protein, or its derivatives, in signaling pathways. Interestingly, and in line with an in vivo study described earlier (Lu et al. 2013), Tamburstuen et al. also showed that rAMBN treatment was associated with increased differentiation of osteoclast precursors cells in vitro (Tamburstuen et al. 2010).

To further investigate the role of AMBN in osteoblastic differentiation and mineralization, human oral primary squamous cell osteocarcinoma (NOS-1) cells were treated with AMBN siRNA (Iizuka et al. 2011). Downregulation of Alp, Col1, and Ibsp was observed, suggesting that AMBN is involved in the mineralization process. Furthermore, ectopic overexpression of AMBN in Saos-2 cells resulted in enhanced matrix mineralization and increased Runx2 and Ibsp gene expression. In addition, AMBN was shown to bind to CD63 and promote CD63 binding to integrin β1 in osteosarcoma cells. The interaction between CD63 and integrin β1 led to inactivation of Src kinase. These results suggested that AMBN acts as a promoting factor for osteogenic differentiation of osteosarcoma cells via a pathway involving CD63, integrin β1, and Src.

Furthermore, AMBN was shown to affect cell attachment and mineralization of mouse PDL cells (Zeichner-David et al. 2006). In vitro, rAMBN treatment resulted

in an increased level of cell attachment of PDL cells, modulation of BMP expression, downregulation of Col1 expression, and induction of de novo Ocn expression. These data demonstrated that AMBN has growth factor activity during periodontium development and regeneration. More recently, Zhang et al. provided new insight into the growth factor activity of AMBN in mouse PDL cells and dental follicle (DF) cells (Zhang et al. 2011b). Using rAMBN and AMBN siRNA treatments, the authors showed that AMBN reduced both PDL and DF cell proliferation. This was associated with upregulation of the cell-cycle inhibitor p27. Furthermore, adhesion and spreading of both cell types were significantly enhanced in AMBN-coated dishes, and AMBN treatment resulted in increased expression of RhoA, one of the classic intracellular mediators of ECM-induced cell adhesion. Finally, blocking of CD63, integrin β1 and ERK pathways reduced the effects of AMBN on RhoA expression, cell adhesion, and proliferation. This study indicates that AMBN affects cell-cycle progression through p27 modulation and stimulates cell adhesion by increasing RhoA activity via the already mentioned cross-talk between CD63, integrin β1, and ERK pathways (Iizuka et al. 2011; Lu et al. 2013).

To summarize, the results detailed in section "Part II" demonstrate that AMBN has roles beyond the control of enamel mineralization. Most of the newer articles dedicated to the study of AMBN indicate its presence in bone. During bone growth, AMBN is highly expressed at the boundaries of the bone matrix, including of DF, PDL, and endosteal and periosteal surfaces. Subsequently, AMBN expression fades in mature bone, except for bone with a high remodeling rate, such as the alveolar bone, where AMBN presence is maintained at all stages. Interestingly, even if some of AMBN protein appears to be incorporated into bone matrix, the protein is also detected in a solubilized state in the microenvironment of AMBN-producing superficial mesenchymal dental and bone cells. The role of AMBN in bone formation and remodeling has been demonstrated using various in vivo and in vitro models. AMBN stimulates the recruitment, proliferation, osteoblastic differentiation, and mineralization of many mesenchymal cells, including osteoprogenitors, osteoblasts, and also PDL and DF cells. Besides its osteogenic role, AMBN has been shown to directly stimulate osteoclastogenesis and to be a key factor in the regulation of cell adhesion to the ECM. Taken together, these data demonstrate the crucial function of AMBN in the control of bone balance. However, although AMBN activities have been shown to involve many molecular mechanisms (e.g., interferon pathway, cross-talk between CD63 and integrins, Msx2/p21/p27 pathway, etc.), full knockout and bone-specific conditional transgenic mouse models are now required to address the controversy over AMBN function in bone tissues (Kuroda et al. 2011) and to fully elucidate its mechanisms of action.

Part III: AMBN as a Bone Site-Specific Marker

Enamel is distinct from bone and dentin in its tissue origin, mineralization matrix composition, and mineralization process. As shown in section "Part I," enamel forms by bioapatite crystallization on a noncollagenous protein matrix secreted from

ameloblasts of epithelial origin, whereas bone and dentin form on a collagenous matrix deposited by cells of mesenchymal origin. The enamel matrix mineralizes immediately after secretion, whereas bone and dentin both mineralize on preformed unmineralized collagenous matrix called osteoid and predentin, respectively. Afterwards, the enamel matrix, mainly composed of specialized proteins such as amelogenins and AMBN, matures into a hypermineralized inorganic tissue (~97% inorganic content). However, this traditional distinction between enamel (noncollagenous protein matrix) versus bone and dentin (collagenous protein matrix) as well as the enamel-specificity of AMEL and AMBN has recently been challenged. Indeed, recent studies (detailed in section "Part II") have shown that expression of AMEL and AMBN is not simply restricted to enamel and ameloblasts but also occurs in many nonenamel tissues and cells, notably bone and osteoblasts. Interestingly, recent quantitative studies focusing on bone tissue have confirmed the expression of AMBN in various bone compartments and further demonstrated that AMBN shows significantly higher expression in jaws and skull bones compared to long bones (Rawlinson et al. 2009b; Jacques et al. 2014a, b).

As emphasized in the introduction to this chapter, the osteodiversity evident between different anatomical regions has major public health implications. This is exemplified by the tissue incompatibility problems associated with bone ectopic autografts or the puzzling jaw osteonecrosis induced by antiresorptive agents that are otherwise effective in treating long-bone osteoporosis or metastatic resorptive lesions. Identifying bone site-specific biomarkers is therefore essential to determine why bone cell behaviors vary depending on their anatomical site and to propose new bone site-specific therapeutics.

Bone Site-Specific Molecular Fingerprint and AMBN

In vivo and ex vivo transcriptomic and proteomic studies have shown molecular fingerprint variations between bone sites. These include differential gene and protein expression profiles of bone morphogens (e.g., BMPs, TGFs, IGFs, FGFs), ECM proteins (e.g., collagens, enamel matrix proteins), and remodeling factors (i.e., OPG, RANKL, CSF1) (Kasperk et al. 1995; van den Bos et al. 2008; Rawlinson et al. 2009b; Kingsmill et al. 2013; Reichert et al. 2013; Isaac et al. 2014).

Transcription factors encoded by homeogenes such as Hox genes are also differentially expressed among bone sites (Leucht et al. 2008; Rawlinson et al. 2009b). Differentiation of the vertebral and appendicular segments during patterning is driven by a Hox code that is maintained in adult bones, whereas the craniofacial skeleton is free of Hox-gene expression and mainly depends on divergent homeoproteins (e.g., MSXs and DLXs families). Among these divergent homeoproteins, Msx2, a major actor of osteoblast differentiation, is one of the key markers of the bone site-specific molecular fingerprint. Msx2 is more highly expressed in jaws when compared to long bones (Kingsmill et al. 2013), and human Msx2 mutations specifically impact craniofacial bone morphogenesis (craniosynostosis OMIM #604757, parietal foramina OMIM #168500). In addition,

Msx2-knockout transgenic mice show alterations exclusively in jaw bone remodeling (Aioub et al. 2007). The role of divergent homeoproteins in bone site-specificity was also recently illustrated with the Dlx3 gene. Indeed, in vivo and in vitro models of Dlx3 deletion revealed that Dlx3 has differential bone site-specific gene targets (Isaac et al. 2014).

Based on these observations, the spatio-temporal expression of AMBN was further explored and compared at the RNA and protein levels in various bone compartments of wild-type and knockout mice. Among the investigated enamel proteins, AMBN was identified as a bone site-specific marker, with both AMBN RNA and protein showing higher expression in jaws and skull bones when compared to long bones (Jacques et al. 2014a, b).

AMBN and Embryonic Origin

The regionalized bone physiopathology and associated osteoblast site-specific molecular fingerprint may result from the dual embryonic origin of the different bone compartments. Developmentally, osteoblasts derive from two distinct tissues. The osteoblasts of most of the craniofacial bones, including the mandible, maxillar, and interparietal bones, as well as the dental mesenchyme are derived from neural crest (Chai et al. 2000). In contrast, the osteoblasts of the parietal and occipital bones and of the axial and appendicular skeleton are mesoderm derived. Of note, several studies in both mouse and chicken suggest that frontal bone could have a mixed embryonic origin, with osteoblasts from both neural crest and paraxial mesoderm contributing to this formation (Deckelbaum et al. 2012).

By exploring enamel proteins in bones, Jacques et al. recently demonstrated that AMBN RNA levels in adult mice were graded, from mandibular (1:1), to frontal (≈1:10), occipital (≈1:100), and tibial (≈1:2,000) bones (Fig. 5). Bones with neural crest-derived cell contributions (e.g., mandibular and frontal bones) therefore displayed higher AMBN RNA expression levels compared to mesoderm-derived bones (occipital and tibial bones). These observations, in line with those of Rawlinson et al. (2009b), suggest that AMBN expression in osteoblasts varies depending on their embryonic origin. AMBN may therefore be a useful marker of neural crest-derived bone compartments.

AMBN and Craniofacial Bone Healing

In craniofacial bones, AMBN is involved in processes associated with high remodeling rates, such as bone formation or healing. In calvaria, AMBN expression varied with age: it is increased during bone formation and reduced in mature bone (Atsawasuwan et al. 2013a). In line with these observations, Jacques et al. showed that AMBN RNA expression was 50-fold higher in alveolar bone in young mice (1 week) compared to adults (15 weeks). Notably, no significant variation with age was detected in basal bone, a bone with a lower remodeling rate (Jacques

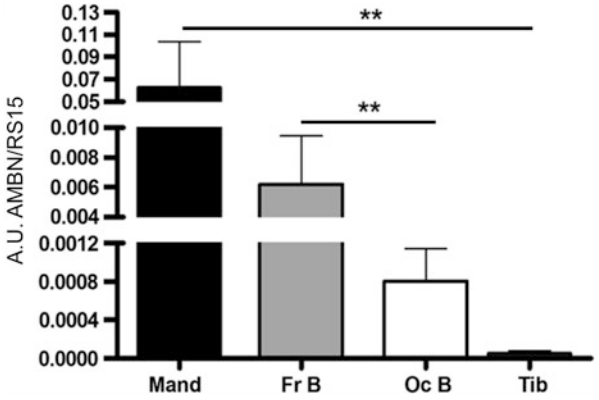

Fig. 5 AMBN expression in different bone compartments in 15-week-old mice. Whole mandible (*Mand*), tibia (*Ti*), frontal bone (*Fr B*), and occipital bone (*Oc B*) from 15-week-old mice were dissected with the aid of a stereomicroscope and transcript expression was analyzed using RT-qPCR. RT-qPCR analyses show that AMBN expression is significantly higher in mandibular bone compared to the other bone compartments: frontal bone (≈10:1), occipital bone (≈100:1), and tibia (≈2,000:1). Moreover, frontal bone shows significantly higher expression when compared to occipital bone (≈10:1) and tibia (≈200:1). Finally, occipital bone displays an increased RNA expression level when compared to tibia (≈20:1). AMBN mRNA levels were normalized to the expression of the housekeeping gene RS15. Significance was evaluated using the Mann–Whitney test (*$p < 0.05$, **$p < 0.01$). *AU* arbitrary unit

et al. 2014b). Using experimentally induced bilateral defects in the mandibular ramus of adult rats as a model, Tamburstuen et al. reported AMBN protein expression in newly formed bone (Tamburstuen et al. 2010). During the bone healing process, AMBN was located in the immature bone ECM adjacent to lining cells, osteoblasts, and perivascular cells. Interestingly, osteocytes were AMBN negative as was the matrix located in the more mature parts of the formed bone and the surrounding original bone. In 2013, Trevez-Manusevitz et al. showed de novo expression of AMBN protein in bone cells, and newly mineralized matrix formed 8 weeks after the implantation of human oral mucosa and gingiva stem cells (hOMSC) in mouse calvaria (Treves-Manusevitz et al. 2013). The role of AMBN in craniofacial bone healing was further consolidated by Tamburstuen et al., who showed that rAMBN treatment of rat mandibular bone defects enhanced bone regeneration (Tamburstuen et al. 2010). Together these findings demonstrate a crucial role for AMBN in early jaw bone formation, repair, and regeneration.

AMBN and Jaw Bone Biomechanical Responsiveness and Plasticity

Bone tissue formation and homeostasis are conditioned by site-specific biomechanical loading to which they are constantly subjected. Weight-bearing bones (e.g., appendicular and axial bones) and non-weight-bearing bones (e.g., jaws and cranial bones) have different ossification processes. Weight-bearing bones form from

endochondral ossification, which enables bone growth to occur under heavy mechanical loads, whereas most of the craniofacial bones, which are not exposed to heavy loads, form directly from mesenchymal cell condensation (intramembranous ossification). During growth and homeostasis, while the weight-bearing bones are exposed to direct loading, the alveolar bone is exposed to indirect loading via the teeth.

Alveolar bone morphology (shape and volume) is conditioned by the existence of the tooth, which is considered to be the organizing "signaling center" of the alveolar bone anlage during jaw development (Jernvall and Thesleff 2000). Thus, tooth extraction results in alveolar bone loss (height and thickness) (Atwood and Coy 1971). In addition, the morphology and properties of the adjacent alveolar bone, and also of the antagonist jaw, are affected by the variations in occlusal stress levels (e.g., mastication, chewing, orthodontic treatments, etc.). The rapid remodeling of the alveolar bone and its exceptional lability may result, at least in part, from the intrinsic specificity of the alveolar bone cells compared to the cranial, axial, and appendicular bone cells. Particularly, the role of the osteoblasts in this site-specific mechanical sensitivity has been confirmed in vitro by Rawlinson et al., who showed that the molecular responses of osteoblasts to mechanical stress vary according to bone localization (Rawlinson et al. 1995).

Interestingly, Alikhani et al. demonstrated that local application of high-frequency acceleration on the occlusal surface of the rat maxillary first molar jointly increased alveolar bone formation and AMBN RNA expression levels (Alikhani et al. 2012). Using a biomechanical assay in which the crowns of the right maxillary molars of mice were reduced occlusally by grinding to simulate the unopposed teeth, Jacques et al. showed a threefold increase in AMBN RNA expression 72 h after altering the tooth occlusion (Jacques et al. 2014b). Based on these observations, the authors hypothesized that the AMBN expression gradient observed in bone compartments (mandible > frontal bone > occipital bone > tibia) (Fig. 5) may result from mechanical stress diffusion in the craniofacial apparatus during mastication and swallowing. This hypothesis is in line with the low AMBN RNA level in tibia and the loss of AMBN protein in edentulous animals such as avian species (Meredith et al. 2014).

The high level of AMBN, described as an osteogenic factor (see section "Part II"), during biomechanical stimulations may contribute to the increase in alveolar bone mineralization consequent to unilateral mandibular hypofunction (removal of maxillary molars) (Rawlinson et al. 2009a). The AMBN increase observed in the mandibular alveolar bone after occlusal alteration (Jacques et al. 2014b) would therefore trigger new alveolar bone formation, allowing mandibular molar "overeruption" in response to the loss of contact with the opposite, grinded molar.

Interestingly, Jacques et al. demonstrated that the increase in AMBN level in response to alteration of tooth occlusion was associated with a threefold decrease in Msx2, one of the candidates for the molecular signature of craniofacial osteoblasts (Jacques et al. 2014b). This observation is in line with those of previous studies showing that Msx2 was affected when mechanical loading was applied in vitro on craniofacial osteoblastic cells (Gonzalez et al. 2008; Fushiki et al. 2015). As detailed

in section "Part II," previous studies have reported that AMBN and Msx2 reciprocally control their expression and are involved in common signaling pathways in the regulation of both mineralized tissues and tumors (Aioub et al. 2007; Sonoda et al. 2009; Molla et al. 2010; Atsawasuwan et al. 2013a, b; Jacques et al. 2014b). Taken together, these results demonstrate that AMBN is involved in the biomechanical responsiveness of the craniofacial osteoblasts and that there could be interplay between AMBN and Msx2 in this process.

Part IV: Potential Applications of AMBN in Jaw Bone Reconstruction

Many studies have demonstrated the high potential of enamel peptides for periodontal tissue healing, including the regeneration of alveolar bone [reviewed in Grandin et al. (2012)]. Considered as "bioactive," the application of these peptides during periodontal healing would recapitulate the epithelial–mesenchymal signaling interactions that orchestrate the normal development of the dento-alveolar complex. This growth factor activity has motivated the development of protein products derived from enamel matrix (Enamel Matrix Derivatives = EMD) in an attempt to regenerate functional periodontal tissues.

The first clinical EMD-based treatment, a commercial formulation of purified extract of porcine fetal tooth enamel matrix called Emdogain® (Straumann, Basel, Switzerland), stimulated the regeneration of tooth-supportive tissues, including alveolar bone, ligament, and cementum, when applied to periodontal defects (Heijl 1997). Since that time, EMD has been shown to stimulate in vitro the proliferation and differentiation of osteoprogenitor cells [reviewed in Grandin et al. (2012)] and to promote the clinical regeneration of alveolar bone and periodontal tissues in patients with intrabony defects (Dori et al. 2013). In order to improve the physicochemical properties for the adsorption of EMD, new formulations of EMD such as Osteogain® are under development (Zhang et al. 2015). Of note, since 2006, Xelma® (Mölnlycke, Göteborg, Sweden), another commercial EMD, has been used to promote fibroblast and keratinocyte cell adhesion in the treatment of soft-tissue wounds, including venous leg and foot ulcers (Vowden et al. 2006).

Today, although EMD is widely used in clinics, knowledge of its exact composition and its cellular and molecular mechanisms of action is far from complete. Because AMEL is the major component (>95%) of EMD, most studies of EMD have focused on AMEL [reviewed in Lyngstadaas et al. (2009)]. However, EMD is a complex mix of proteins, including growth factors and proteases that could also contribute to the clinical bioactivity (Maycock et al. 2002). This composition could explain the contrasting effects of EMD extracts and AMEL observed on osteoclast activity. Indeed, whereas AMEL negatively regulated osteoclastogenesis in vivo (Yagi et al. 2009) and in vitro (Hatakeyama et al. 2006; Nishiguchi et al. 2007), EMD has been shown to promote osteoclastogenesis [reviewed in Grandin et al. (2012)]. Both osteoblastogenesis and osteoclastogenesis are essential to bone regeneration because these two differentiation processes jointly interact in the remodeling of

newly formed bone to functional mature bone. Thus, the beneficial effects of EMD-based treatment on alveolar bone regeneration are likely a consequence of its dual actions; EMD not only enhances bone formation via stimulation of osteoblastic bone-forming activity but it also stimulates osteoclastic bone-resorptive activity.

Similar to EMD, AMBN has been shown to stimulate both osteogenesis and osteoclastogenesis (see section "Part II"). And, as described in section "Part III," craniofacial bone regeneration activity of AMBN (initially hypothesized to be due to enhanced AMBN expression during bone formation, remodeling, and repair) was demonstrated by Tamburstuen et al., who showed that application of rAMBN to rat mandibular bone defects stimulated bone regeneration (Tamburstuen et al. 2010). Together, these data strongly suggest that (1) the clinical effect of EMD in alveolar bone reconstruction might also be associated with its AMBN content and (2) the use of "pure" AMBN protein could optimize the beneficial activity of EMD on bone regeneration. AMBN-based treatment might therefore enhance alveolar bone reconstruction (healing time, bone volume, mineral density) and provide the new bone with high adaptability properties.

The clinical potential of AMBN for alveolar bone regeneration is further supported by in vivo studies showing that AMBN-based treatments stimulated the regeneration of two other mineralized tissues: dentin and cementum. Thus, local application of rat rAMBN (native protein) in a pig pulpotomy model enhanced pulpal wound healing and reparative dentin formation compared with calcium hydroxide treatment (Nakamura et al. 2006). Interestingly, Fukae et al. demonstrated that the 17-kDa N-terminal cleavage product of AMBN (either isolated from developing porcine incisors or commercially synthesized based on human and porcine peptide sequences) showed ALP-inducing activity in vitro on human PDL cells (Fukae et al. 2006). Using a dog mandible bone-defect model, the same group further showed that this peptide also exhibits cementum regeneration activity in vivo (Kanazashi et al. 2006).

In addition to its high potential for alveolar bone reconstruction, the use of human rAMBN (native form or cleavage peptides) could circumvent clinical problems potentially associated with treatment based on animal products (e.g., immune response, disease transmission, ethical issues). From a clinical perspective and similar to EMD (Dori et al. 2013), the bone-regeneration activity of rAMBN-based treatments might later be optimized by combining rAMBN with osteoconductive/osteoinductive scaffolds such as synthetic bone substitutes (e.g., calcium phosphates, bioactive glasses), allografts [e.g., freeze dried bone allograft (FDBA) or demineralized freeze dried bone allograft (DFDBA)], or xenografts [e.g., natural bovine bone mineral (NBM)].

Beyond its stimulatory activity in osteogenesis and osteoclastogenesis, AMBN is also a biomarker of the craniofacial bones and specifically of alveolar bone (see sections "Part II" and "Part III"). Thus, it can be speculated that AMBN-based treatment may be used to direct/redirect the molecular and phenotypic profile of osteoblastic progenitor cells into osteoblasts with either a "craniofacial" or an "alveolar" site-specific fingerprint. Modifying the osteoblast profile would have

many clinical applications. In bone grafting, for example, AMBN treatment might induce the differentiation of progenitor cells isolated from the appendicular and axial skeleton into craniofacial osteoblasts. Theoretically, AMBN-based treatment could thereby improve the clinical efficacy of heterotopic autograft performed in maxillo-facial reconstructive surgeries (see section "Part III") by increasing the adaptability/plasticity of the extra-craniofacial donor site. As previously mentioned in section "Part III," alveolar bone has been shown to be less sensitive to hormonal alterations (such as PTH increase and estrogen deficiency) compared to the appendicular and axial skeleton. Preventive AMBN-based treatment might therefore be used to limit the bone loss associated with postmenopausal osteoporosis.

However, as detailed in section "Part II," mouse AMBN is not only expressed in mineralized tissues it is also detected during early stages of embryogenesis and in many soft tissues in the adult, including reproductive organs such as the testis (Jacques et al. 2014a, b). This expression pattern suggests that AMBN may play additional roles in the organism which are still unknown. These observations, in line with the association between AMBN alterations and ameloblastoma or odontogenic tumor (see section "Part II"), highlight that the delivery of rAMBN (and EMD) to damaged tissues has to be perfectly controlled in terms of localization and quantity. Gene therapy might be used to help circumvent rAMBN delivery problems. Indeed, AMBN DNA could be locally delivered to the damaged bone tissues to transduce the resident bone cells and increase (or induce) regional secretion of AMBN protein over extended periods. Based on tissue engineering and hybrid biomaterials strategies, AMBN DNA could also be used to transduce in vitro autologous stem cells isolated from the patient in order to later graft AMBN-overexpressing osteoprogenitors into the damaged bone site. The feasibility of AMBN gene therapy is supported by Wazen et al., who showed that bone cells could be efficiently transduced by lentiviral vectors encoding AMBN both in vitro and in vivo (Wazen et al. 2006).

As illustrated by the alveolar bone loss subsequent to tooth extraction, mechanical stimulations are essential to maintain a functional alveolar bone (see section "Part III"). Interestingly, recent studies detailed above have shown that local variations of tooth occlusal forces induce increased AMBN RNA expression in the adjacent alveolar bone (Alikhani et al. 2012; Jacques et al. 2014b). These recent results suggest that mechanical therapy using the osteogenic effect of these stimuli could stimulate alveolar bone formation/regeneration in a physiopathological context. Thus, local application of biomechanical stimulation could be a promising alternative method to induce regionally controlled cell secretion of osteogenic factors such as AMBN without using biologically active factors or DNA transduction. Together, these data encourage the use of mechanical therapies for alveolar bone regeneration such as low intensity pulsed ultrasound stimulation (LIPUS) and orthodontic extrusion.

In conclusion, AMBN expression in bone tissues depends on the anatomical site and the ontogenic stage. AMBN is almost exclusively expressed in bone compartments with a neural crest-derived osteoblast contribution, suggesting that this protein could serve as a specific marker of the embryonic origin of bone organs (neuroectoderm- versus mesorderm-derived bones). In addition, in vitro and

in vivo studies support that AMBN (1) is strongly involved in bone processes associated with high remodeling rates, such as bone formation, repair, and regeneration and (2) controls the physiological response of peri-dento-alveolar bone to tooth-associated biomechanical stimulation, thereby maintaining alveolar bone integrity. Owing to its physiological properties, AMBN-based treatments may have promising clinical potential for craniofacial tissue repair and most specifically for alveolar bone regeneration.

Summary Points

- This chapter focuses on ameloblastin (AMBN), which is an extracellular matrix protein initially discovered in enamel matrix and ameloblasts.
- In enamel, AMBN promotes the growth of a crystalline enamel layer and regulates the matrix binding, proliferation, and differentiation of ameloblasts.
- Mutation of human AMBN leads to various forms of amelogenesis imperfecta.
- AMBN is also detected outside the enamel in both mineralized and nonmineralized tissues, including bone.
- In bone, AMBN stimulates osteoblastogenesis and osteoclastogenesis and participates in the regulation of cell adhesion to the extracellular matrix.
- Although many molecular mechanisms have been shown to be involved in AMBN function (e.g., interferon pathway, cross-talk between CD63 and integrins, Msx2/p21/p27 pathway, etc.), full knockout and bone-specific conditional transgenic mouse models are now required to fully elucidate its mechanisms of action.
- AMBN expression in bone tissues depends on the anatomical site and the ontogenic stage; it shows higher expression in jaw and skull bones compared to long bones, and it is highly expressed during processes associated with high levels of bone remodeling, such as bone formation, repair, and regeneration.
- AMBN is a bone site-specific marker that participates in the physiological control of alveolar bone integrity in response to tooth-associated biomechanical stimulation.
- Owing to its physiological properties, AMBN-based treatments may have promising clinical potential for craniofacial tissue repair, and most specifically for alveolar bone regeneration.

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Chitinases as Biomarkers in Bone Studies 14

Michelino Di Rosa and Lucia Malaguarnera

Contents

Abstract

Despite the hardness and strength characteristic, the bone is not a static tissue, but is constantly changing and constantly repairing. This process is called *bone remodeling*. In this cyclical process, the oldest bone tissue is removed and replaced with another new tissue. There are two types of cells responsible for

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the bone turnover: osteoclasts and osteoblasts. The different phases of bone remodeling are controlled by numerous local and systemic factors. The alteration of balance between these factors can lead to different types of bone disorders. In the recent years, the bone turnover markers underwent extensive development. Pathological bone resorption is a cause of significant morbidity in diseases affecting the skeleton, such as rheumatoid arthritis, osteoporosis, periodontitis, and cancer metastasis. Biochemical monitoring of bone metabolism depends upon the measurement of enzymes and proteins released during bone formation and of degradation products formed during bone resorption. The mammalian chitinases can be considered new bone resorption markers. These molecules belong to the families 18 glycosyl hydrolase (GH) superfamily. Although, these enzymes have been widely implicated in a variety of diseases involving immune dysfunction, their biologic role in bone resorption is poorly understood.

Herein we will focus on what is known in the role chitinase family in bone disease development, as well as the potential of some of the engaged molecules as prognostic or diagnostic markers and their perspective in developing new therapeutic strategies against bone disease.

Keywords

Bone disease • CHIT1 • CHI3L1 • Osteoclasts • Osteoblasts • Macrophages • Monocytes

List of Abbreviations

BSP	Bone sialoprotein
Ca	Calcium
CBM18	Carbohydrate-binding module family 18
CCL2	Chemokine (C-C motif) ligand 2
CHI3L1	Chitinase 3 like 1
CHI3L2	Chitinase 3 like 2
CHIA	Chitinase, acidic
CHID1	Chitinase domain containing 1
CHIT1	Chitotriosidase
CLPs	Chitinase-like-proteins
CPhoDs	Calcium phosphate thin film disks
CTX and NTX	C-telopeptide of type 1 collagen and cross-linked N-terminal telopeptide of type 1 collagen
CXCL2	Chemokine (C-X-C motif) ligand 2
DDs	Dentin disks
GH	Glycosyl hydrolase
GlcNAc	β-1,4-linked *N*-acetyl-D-glucosamine
GMCSF	Granulocyte-macrophage colony-stimulating factor
IL-6	Interleukin 6
MMP9	Matrix metallopeptidase 9
MØs	Macrophages
OA	Osteoarthritis

OC	Osteoclast
OVGP1	Oviductin
P	Phosphate
P1CP and P1NP	C- and N-terminal pro-peptides of procollagen type 1
PDB	Protein data bank
PTH	*Parathyroid hormone*
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
siRNA	Small interfering RNA
Th1	Lymphocyte T helper 1
TNFα	Tumor necrosis factor alpha
TRAP	Tartrate-resistant acid phosphatase

Key Facts

1. The chitinases (18 glycosyl hydrolase (GH18) superfamily) are able to hydrolyze the *glycosidic bond*, a covalent bond that connect a sugar (carbohydrate) molecule to another sugar or molecule (chitin, chitosan, and peptidoglycan).
2. The chitin is a polymer of *N*-acetylglucosamine, a sugar derivate (glucose), present in the exoskeleton of arthropods, in fungi and cell walls.
3. The GH18 family includes both catalytically active and nonhydrolytic proteins that function as carbohydrate binding modules (CBM).
4. The chitinases are pleiotropic molecules distributed in the human body. The main chitinases is chitotriosidase (CHIT1).
5. The innate immunity cells (monocyte, macrophages, dendritic cells, microglia) defend the host from infection by other organisms.
6. The monocyte derived cells produce different chitinases (CHIT1, CHI3L1, CHI3L2, CHIA, and CHID1).
7. The chitinases are related to various disease
8. The CHIT1activity is a prognostic marker for *Gaucher disease, sarcoidosis, and acute stroke.*
9. The chitinases are polymorphic proteins. The CHIT1 polymorphism (24-bp duplication in exon 10) inhibits its hydrolase activity.
10. The heterozygosis for a 24-bp duplication in the CHIT1 gene has a protective effect in human longevity.

Definition of Words and Terms

Bone tissue — is the main representative connective tissue of the body. It is by a remarkable hardness and resistance and composed of cells dispersed in an abundant extracellular matrix, formed of

	fibers and amorphous substance of glycoproteins.
Calcium	is an alkaline earth metal tender. In the human body is present about one kilogram of calcium, of which 99% is fixed in the bones and the rest circulating free in the blood. Vitamin D is needed to absorb calcium from food.
Calcium phosphate thin film disks (CPhoDs)	consists of submicron synthetic calcium phosphate thin films coated onto various culture vessels. This system has been used as an alternative method for compound screening for direct assessment of osteoclast and osteoblast activity in vitro.
Carbohydrate-binding domain	is a common region between the chitinase with chitinolytic activity and chitinase-like proteins (CLPs) and is involved in the recognition or binding of chitin subunits.
Chitinases	The chitinase are glycolytic enzymes that destroy chitin. When pathogens colonize or attack various organisms (such as plants), they defend themselves by secreting enzymes capable of destroying chitin. In the human, the chitinases play a role in the immunity.
Chitosan	is a linear polysaccharide composed by D-glucosamine and *N*-acetyl-D-glucosamine, linked via β (1–4) bonds. It is obtained by treating chitin, generally obtained from the exoskeleton of crustaceans (crabs, shrimp, etc.) with a basic aqueous solution.
Collagen	is the main protein of connective tissue in animals. It is the most abundant protein in mammals (about 25% of the total protein mass), in human represents approximately 6% of body weight.
Dentin disk	is slide of animal tusk (ivory) the most frequently used in vitro model for the osteoclast resorption assay.
Hydroxyapatite crystals	is produced and absorbed by body tissues. It is one of the main components of the bone lying in the form of calcium salts: CaCO3 (calcium carbonate), Ca3 (PO4) 2 (calcium phosphate) and CaF2 (calcium fluoride).
Osteoblasts	cells that process the bone extracellular matrix. Their function is to produce the organic matrix

	of bone tissue, consisting of fibers of type I collagen, proteoglycans and glycoproteins.
Osteoclasts	are very large cells, multinucleated (syncytia) and lysosomes rich. The osteoclasts are places to the bone matrix and have the function to reabsorb the bone by exocytosis enzymes and alkaline pH.
Vitamin D	a group of fat-soluble prohormones consisting of 5 different vitamins: vitamin D1, D2, D3, D4, and D5. The two most important forms of vitamin D are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). The cholecalciferol (D3) is synthesized in animals, while the ergocalciferol (D2) from vegetable origin.

Introduction

In the last few years, the bone degeneration has become an increasingly treated topic by the scientific community. To live better is important to know the function of the human body and the problems behind it. The most frequent disorders and diseases, in particular in older people, are related to the bones and joints. They play a key role in the body supporting and the movements allowing. In order to understand the mechanisms that regulate the bone degeneration processes and the main molecules involved, it is necessary firstly understand the bone tissue structure.

The skeleton that supports the human body consists of 206 bones that are held together by 68 joints. That apparatus has three main functions: supporting the body, protecting internal organs and soft tissues of the body, and allowing the muscle movement. The bones are composed of two portions: organic and inorganic. When the skeleton is affected by varying severity diseases, motor skills and balance, it may be compromised and can also occur difficulty in grasping objects. Bone is a metabolically active tissue, in which the processes of formation and resorption are continued throughout life (le Noble and le Noble 2014; Martin 2014).

The bone tissue, the most predominant connective tissue in the human body, is composed of specialized cells and an extracellular matrix comprising an *organic* component (35%, with the 90% of type I collagen) and an *inorganic* component (65%, composed of Calcium (Ca) and phosphate (P) in the form of hydroxyapatite crystals) (Buckwalter et al. 1996). There are three principal types of cells involved in bone tissue homeostasis:

– ***Osteoblasts***. These cells origin from mesenchymal stem cells and synthesize the cross-linked collagen and several specialized proteins in much smaller quantities (*osteocalcin* and *osteopontin*), which comprise the organic matrix of bone.

Moreover, these cells produce Ca and P minerals that are deposited into the organic matrix forming a very strong and dense mineralized tissue. In addition, they are equipped with receptors for parathyroid hormone (Arnett 2003).

- ***Osteoclasts***. These cells belong to the monocytes/macrophages family. They are derived from bone marrow. The mononuclear precursors self-fusion originates giant multinucleate cells having several nuclei. They are rich in mitochondria and lysosomes (just to prove their catabolic activity) and have a large number of receptors for the calcitonin. Designate to active bone resorption, the osteoclast forms a "ruffled border" that opposes the surface of the bone tissue. Among their lysosomal enzymes, it is important to include their acid phosphatase. This permits osteoclasts characterization by their staining for high expression of tartrate-resistant acid phosphatase (TRAP) and cathepsin K (Holtrop and King 1977).
- ***Osteocyte:*** the most abundant cell in the bone tissue. They are osteoblasts that, after processing the bone substance, remain imprisoned in the calcified matrix of gaps inside bone cavities shaped lenticular dug in the slats. Osteocytes remain connected between them through extensions that extend to the haversian canal that allows the nourishment. Former osteoblast in case of trauma or bone fracture can resume its synthetic activity (because freed) transforming then in osteoblast.

Regarding the ***organic component***, the type I collagen represents about 90% of the organic matrix of bone. It is a triple helix structure made from three chains, rich in proline and hydroxyproline. The collagen is synthesized as a precursor to having a long series of carboxy and amminoterminal AA. These extensions are then cleaved during the secretion and formation process (Viguet-Carrin et al. 2006).

As regards the ***inorganic component***, the mineral phase of calcium and phosphorus ions are under the control of three different hormones (Raisz 1988):

Parathyroid hormone (PTH) regulates the level of calcium ion in extracellular fluids in three ways: (*) increases bone resorption, (**) increases the renal tubules reabsorption, (***) increases the intestinal reabsorption and the vitamin D transformation in its active form, the ***calcitriol*** (1,25 $(OH)_2D_3$).

Calcitriol is a powerful stimulator of calcium and phosphorus absorption in the intestine. In normal conditions, the calcitriol promotes bone growth and bone formation by providing adequate mineral levels. However, if the dietary intake is insufficient, calcitriol acts directly on the skeleton by promoting the Ca and P reabsorption.

Calcitonin inhibits the Ca reabsorption from the bones, thereby reducing the serum Ca concentration; it decreases Ca fecal excretion and exercises, similar to PTH, a phosphaturia effect.

The different phases of bone remodeling are controlled by numerous local and systemic factors. The systemic factors include the calcium metabolism (parathyroid hormone, calcitonin, vitamin D), sex hormones, and glucocorticoids (Jilka et al. 1998). These possess a modulating activity on the growth and differentiation of osteoblasts and osteoclasts. Local factors include the growth factors, cytokines, prostaglandins, and leukotrienes (de Vernejoul 1996). The role of these factors is very complex because none of these acts independently, but several of them works together.

The alteration of this balance between growth factors, hormones, cytokines, minerals, and specialized cells can lead to different types of bone disorders.

Bone Disease

Bone diseases are disorders and conditions that cause abnormal development and/or impairment in normal bone development. This can result in weakened bones, inflamed joints, and pain. In these conditions, bones naturally lose density after the age of 20 due to the aging process; however, some the bone diseases result in excessive loss of bone strength and density. Nutrient deficiencies, including lack vitamin D or C deficiency, hormonal imbalances, and cell abnormalities can also cause bone disorders in both children and adults. There are many kinds of bone problems:

- Low bone density and osteoporosis
- Osteogenesis imperfecta
- Paget's disease
- Bone disease can make bones easy to break
- Bones can also develop cancer and infections
- Other bone diseases are caused by poor nutrition, genetic factors or problems with the rate of bone growth or rebuilding

Bone remodeling is exposed to environmental, mechanical injury (Chen et al. 2010). Alterations at different phases of this process result in bone disease onset (Kular et al. 2012). Older people are predisposed to bone tissue atrophy as a result of decreased levels of physical exercise and stationary lifestyle (Kaneko et al. 2014). Bone and cartilage cells are susceptible to mechanical signals and respond through mechano-transduction pathways modifying bone remodeling in proportion to the nature of the mechanical signals received. Different circumstances affecting bone and cartilage biology deregulate bone homeostasis and function. Thus, a constant solicitation of mechanical signals that origin high tension or irregular cargo distribution results in increased bone synthesis (Robling et al. 2002). In contrast, static load reduces bone synthesis and increases bone resorption (Ehrlich and Lanyon 2002). The most frequent disorder where decreased bone mass is a result of hormonal status or disuse is osteoporosis. Other common conditions where bone architecture is subverted are Paget's disease, osteogenesis imperfecta, osteopetrosis, and osteosarcoma. Bone health can also be affected by changes in cartilage biology. Osteoarthritis (OA) is a disease involving both cartilage and bone tissue. Other conditions affecting the cartilage, but could be possibly the results of altered bone remodeling are achondroplasia, costochondritis, spinal disk herniation, relapsing polychondritis, chondromas, and chondrosarcomas. Additionally, altered remodeling process can be the result of pathological factors like distorted mechano-responsiveness of osteocyte, osteoblast, osteoclast or chondrocyte cells, deregulated mechano-transduction pathways, or altered bone matrix mechanical properties. One or a combination of these factors could induce pathological bone remodeling and consequent

disease progression (Cox et al. 2011). Osteolysis is a hallmark of various and etiologically different diseases of bone and joint, which is mediated by osteoclasts and osteoblasts (Sims and Gooi 2008). It is known that osteoclast plays a role in pathological bone resorption. The osteoclasts' activity is controlled by local factors produced in the bone microenvironment. In addition, osteoclasts are autocrine/paracrine, intracrine regulatory cells able to produce factors such as IL-6, annexin II, TGF-beta, and OIP-1/hSca, which influence their own formation and activity (Yavropoulou and Yovos 2008). A critical initiating event in osteolysis is the activation of pro-inflammatory cytokine signaling within periprosthetic macrophages, which, in turn, leads to an imbalance in the levels of the key osteoclastogenesis regulators RANKL and OPG (Zaidi 2007). Recent investigation indicates that protein groups called chitinases are involved in different processes such as tissue remodeling and injury and osteoclastogenesis (Lee et al. 2011).

Bone Turnovers Markers

In the past decade, the field of bone turnover markers achieved considerable advancement. Biochemical monitoring of bone metabolism depends upon the measurement of enzymes and proteins released during bone formation and of degradation products produced during bone resorption. Various biochemical markers are now available that allow a specific and sensitive assessment of the rate of bone formation and bone resorption (Pagani et al. 2005). Although these markers are not yet recommended for use in diagnosis of osteoporosis, they appear to be useful for the individual monitoring of osteoporotic patients treated with antiresorptive agents. The bone markers constitute a new and potentially important clinical tool for the diagnosis and monitoring of bone metabolism, being not invasive, not expensive, and giving answers much faster than the classic diagnostic tool. The bone mineral density employs 2 or 3 years to show significant changes, for example, in response to an antiresorptive therapy. In contrast, the bone markers indicate the variations of bone metabolism in minor time: i.e., from 1 to 3 months for markers of resorption, and 6–9 months for formation markers. The bone markers reflect the pathophysiological mechanisms of bone disease. They generally increase in patients with diseases in which bone turnover increases as Paget's disease, hyperparathyroidism, and hypothyroidism and conversely decrease in patients with slower turnover, such as hypothyroidism or hypopituitarism.

It is possible to distinguish two types of bone remodeling markers (Fig. 1).

The Bone Formation Markers

The principal bone formation markers are:

- ***Alkaline phosphatase***: has been clinically available for several years as a marker for bone metabolism. Various tissues, such as liver, bone, intestine, spleen,

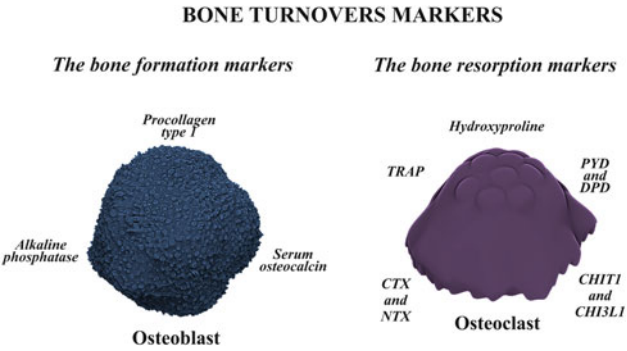


Fig. 1 Bone turnovers markers. In the plasma or urine are detectable different molecules, which allow determining the correct balance between bone formation and resorption. These markers can be the product of the principal cells of bone remodeling, osteoclast and osteoblast

kidney, and placenta, secrete serum alkaline phosphatase. In adults with normal liver function, approximately 50% of the total alkaline phosphatase activity arises from the liver and 50% from the bone. The development of monoclonal antibodies directed to the bone-specific isoform of alkaline phosphatase has improved specificity and sensitivity.

- ***Serum osteocalcin***: Osteocalcin is synthesized by mature osteoblasts, odontoblasts, and hypertrophic chondrocytes. Serum osteocalcin is considered a specific marker of osteoblast function. Its levels correlate with the bone formation rate. Nevertheless, the molecule is rapidly degraded in the serum, and intact structure and fragmented segments coexist in the serum. Notwithstanding the use of these markers, the resulting heterogeneity of the osteocalcin fragments in the serum clues some limitations. In general, serum levels are elevated in patients with diseases characterized by a high bone turnover rate, and the serum levels reflect the expected changes in bone formation following surgical and therapeutic intervention. Serum osteocalcin levels fluctuate significantly during the menstrual cycle, with the highest levels observed during the luteal phase (Gundberg et al. 1985).
- ***Procollagen type 1***: Procollagen type 1 contains N- and C-terminal extensions, which are removed by specific proteases during conversion of procollagen to collagen. The extensions are the C- and N-terminal pro-peptides of procollagen type 1 (P1CP and P1NP). Measurement of P1NP appears to be a more sensitive marker of bone formation rate in osteoporosis. These assays are being developed for clinical use.

The Bone Resorption Markers

The principal bone resorption markers are:

- ***The acid phosphatase 5, tartrate resistant (TRAP)***: Under normal condition, TRAP is highly expressed by osteoclasts and in activated macrophages. In

osteoclasts, TRAP is localized in the ruffled border area, in the lysosomes, and in the Golgi vesicles (Ljusberg et al. 2005). Plasma levels are altered in various bone diseases. It is easily degradable in the frozen samples. This condition has limited their use in clinical analytical.

- ***Collagen-derived assays***: Hydroxyproline is a component of the bone collagen. During degradation of bone, it is released into the serum and is detectable in the urine in free and bound forms. Nowadays, serum hydroxyproline is considered a nonspecific marker of bone turnover. It originates from the degradation of newly synthesized collagens, from collagens of tissues other than bone, and from the diet. From a practical standpoint, a major drawback of urinary hydroxyproline is the necessity for dietary restrictions on gelatine intake before applying the test. Therefore, urinary hydroxyproline analysis has been replaced by assays that are more specific.
- ***Cross-link assays***: The pyridinium compounds, PYD and DPD, are formed during the extracellular maturation of fibrillar collagens and are released upon the degradation of mature collagens. The PYD and DPD production are significantly elevated in diseases with an increased bone turnover. Paget's disease, the bone disease secondary to carcinoma, primary hyperparathyroidism, renal osteodystrophy, osteomalacia, hyperthyroidism, hypercalcemia, and osteoporosis by immobilization are related to the PYD and DPD levels. The PYD-to-DPD ratio in urine is similar to the ratio of these two cross-links in bone, which suggests that both of the cross-links are derived predominantly from bone. PYD and DPD are present in urine as free moieties (40%) or peptide bound (60%). Free forms can be detected by direct immunoassays (free DPD, Pyrilinks-D).
- ***Peptide assays***: Several groups have developed assays based on specific antibodies raised against isolated collagen peptides containing cross-links. These fragments detected by radioimmunoassay technique are available for C-telopeptide of type 1 collagen (CTX, CrossLaps) and cross-linked N-terminal telopeptide of type 1 collagen by ELISA technique (NTX, Osteomark). The monoclonal antibody used for NTX assay is directed against the urinary pool of collagen cross-links derived from a patient with Paget disease. Only β-isomer of CTX is measured in the serum CrossLaps assay, while α- and β-isomers of CTX are measured in the urine CrossLaps assay. These assays have shown detectable reaction with urine from healthy individuals, as well as large increases associated with elevated turnover. It has been shown that the NTX constitutes an effective and sensitive marker in early changes in the bone resorption that occur in physiological conditions, such as the onset of menopause. In osteoporotic patients in late menopause, it was frequently observed an increase level of 171% compared to the average premenopausal.
- ***Noncollagenous markers***: Few noncollagenous proteins or glycoproteins have sufficient specificity for bone to be considered potential markers. Bone sialoprotein (BSP) is thought to be involved in the mineralization of newly deposited bone matrix and/or the calcification of extra skeletal tissues. BSP is a

highly acidic protein with strong affinity for hydroxylapatite crystals. BSP may be a sensitive marker of bone turnover, and clinical data suggest that its serum levels predominantly reflect processes related to bone resorption. The discovery that the type 5b Isotype is specific for bone osteoclasts has facilitated an antibody capture activity assay for tartrate-resistant acid phosphatase 5b as a bone resorption marker but is still under development. Another protein group that can be considered markers of resorption bone is the ***Chitinases (CHIT1 and CHI3L1)***.

Chitinase a New Potential Noncollagenous Markers of Bone Disease

Characteristics of Human Chitinase Family

Chitinases are classified into families 18 and 19 of glycosyl hydrolase (GH) superfamily based on their amino acid sequence similarities (Henrissat and Bairoch 1993). The glycoside hydrolase 18 is widely expressed in different organisms such as archaea, prokaryotes, and eukaryotes. Some of these enzymes are capable of hydrolyzing the chitin, a β-1, 4-linked *N*-acetyl-D-glucosamine (GlcNAc) oligosaccharide. Their structure has revealed a common ancestor gene from which they are subsequently divided (Robertus et al. 1998). Chitin is the common element among all the GH18. Chitin is a major structural component of the fungal cell walls, insect exoskeletons, and shellfish and in parasitic nematodes, but is not present in vertebrates. Belonging to the human chitinases family are eight members. Seven of the eight human GH18 family members are located on chromosome 1, including chitotriosidase (CHIT1, 1q32.1) (Renkema et al. 1995; Boot et al. 1995), acidic mammalian chitinase (AMCase, CHIA, 1p13.2) (Boot et al. 2001), chitinase 3-like protein 1 (CHI3L1, YKL-40, GP-39, 1q32.1) (Johansen et al. 1992; Rehli et al. 1997), chitinase 3-like protein 2 (CHI3L2, YKL-39, 1q13.3) (Hu et al. 1996), oviductin (OVGP1, 1p13.2) (Arias et al. 1994), di-N acetylchitobiase (CTBS, 1p22) (Fisher and Aronson 1992), and chitinase acidic pseudogene 2 (CHIAP2, 1p13.2) (Bussink et al. 2007). Located on chromosome 11 in position p15.5, there is the chitinase domain containing 1, also know stabilin-1 (CHID1, SI-CLP) (Kzhyshkowska et al. 2006) (Fig. 2). The human major histocompatibility complex paralogon is located on chromosome 1, close the human GH18 gene family. This position related to the modulation of chitinase-like chitotriosidase, CHI3L1 and CHIA in the immune responses (Welch et al. 2002) confer GH18 gene family a role in innate and adaptive immunity (Funkhouser and Aronson 2007). There are only three chitinases with the ability to hydrolyze the chitin: CHIA, CHIT1, and CTBS. The CHIA functions mainly as an exo-chitinase, while CHIT1 as an endo-chitinase. As regards CTBS, it is a lysosomal enzyme with lower sequence homology to CHIA and CHIT1. It hydrolyses the reducing end of GlcNAc from the chitooligosaccharides following the ordered degradation of

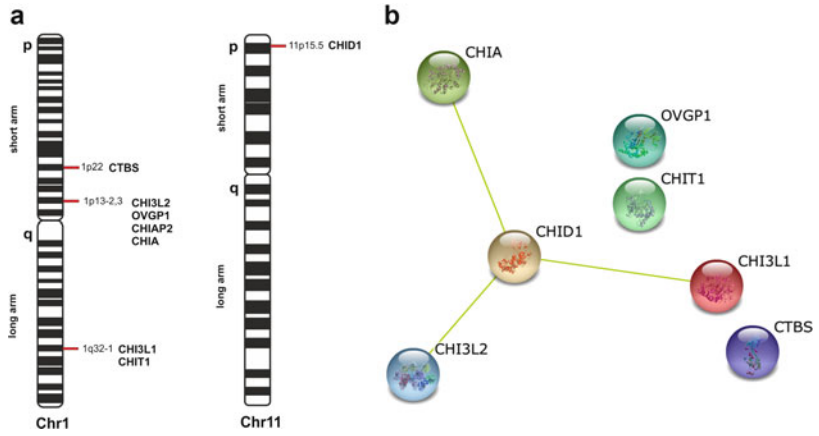


Fig. 2 Chromosome and network chitinases. (**a**) Schematic representation of Chr1 and Chr11. Seven of the eight human GH18 family members are located on chromosome 1(CTBS, CHI3L2, OVGP1, CHIAP2, CHIA, CHI3L1, and CHIT1) instead CHID1 is located in chromosome 11. (**b**) Differentially expressed genes are depicted: links have been predicted using STRING (http://string.embl.de/). Predicted interactions are depicted according to the type of available evidence. The interactions (see *color* labels) include direct (physical) and indirect (functional) associations; they are derived from four sources: genomic context, high-throughput experiments, conserved co-expression, and previous knowledge from literature (doi: 10.1371/journal.ppat.1000781.g002)

asparagine-linked glycoproteins by lysosomal glycosyl-asparaginase. The rest of chitinases lack the catalytic glutamic acid residue and are collectively termed chitinase-like proteins or chitinase-like lectins (chitolectins) as they retain lectin-binding capabilities. The main representatives among chitolectins are YKL-40 and YKL-39. They bind strongly to chitin and chitooligosaccharides due to the presence of a hydrophobic substrate binding cleft (Fusetti et al. 2003). Most likely YKL-40 and YKL-39 may play a role in tissue remodeling and in cancer development. As regards human OVGP1, it is secreted by oviductal epithelium and is a much larger protein (~120 kDa) than other chitinases (Rapisarda et al. 1993). OVGP1 may play a role during the early embryonic development (Buhi 2002). CHID1 is a highly conserved GH18 family member of unknown function. It is known to interact with the protein STAB1(stabilin 1), a type 1 transmembrane endocytic receptor involved in angiogenesis, lymphocyte homing, cell adhesion, trafficking between early/sorting endosomes and the trans-Golgi network in human sinusoidal endothelial cells, and macrophages (Kzhyshkowska et al. 2004). Finally, CHIAP2 is a chitinase-like pseudo gene that does not produce a functional protein. It is a novel chitolectin member and does not align well with other human members of the GH18 family. It is located in the proximity of CHIA and OVGP1 genes, and the role in health and in disease is still unknown (Table 1).

Herein we will focus on what is known in the role chitinase family in bone disease development, as well as the potential of some of the engaged molecules as

Table 1 Human glycosyl hydrolase 18 family. The table shows the main human chitinase with their symbol official, chromosome locus, the presence of activity, and the molecular weight

Chitinase	Symbol	Locus	Activity	M.W.
Chitotriosidase	CHIT1	1q32.1	Yes	50–39 kDa
Acid mammalian chitinases	CHIA	1q13.2	Yes	50 kDa
Chitinase-3-like 1 protein	CHI3L1	1q32.1	No	40 kDa
Chitinase-3-like 2 protein	CHI3L2	1q13.3	No	39 kDa
Oviductin	OVGP1	1q13.2	No	120 kDa
Di-*N*-Acetyl-Chitobiase	CTBS	1p22	Yes	40 kDa
Chitinase acidic pseudogene 2	CHIAP2	1q13.2	No	No protein codin
Stabilin-1-interacting chitinase-like protein	CHID1	11p15.5	No	44.9 kDa

prognostic or diagnostic markers and their perspective in developing new therapeutic strategies against bone disease.

Carbohydrate-Binding Domain

Carbohydrate-binding module family 18 (CBM18) (or chitin binding 1 or chitin recognition protein), which binds N-acetylglucosamine, is a common region between the chitinase with chitinolytic activity and chitinase-like-proteins (CLPs) (Henrissat and Davies 1997). This domain may be present in one or more copies and is involved in the recognition or binding of chitin subunits. The CBM of both CHIT1 and CHI3L1 could be a critical region for the interaction with bone target molecules (Fig. 3). The finding that CHIT1 and CHI3L1 are crucial in the osteoclastogenesis and in the osteolysis suggests that their CBM could be also involved in the tumor metastasis of osteolytic lesions. This observation is consistent with the evidence showing that CHI3L1 inhibition restrains tumor growth and metastasis by its own CBM (Chen et al. 2011). Increased concentrations of CHI3L1 have been detected also in serum of patients with rheumatoid arthritis (RA). It has been suggested that neutrophil-released CHI3L1 acts as an autoantigen in RA. Local release of CHI3L1 in the arthritic joint is followed by a secondary increase of CHI3L1 concentration in serum. In contrast to healthy individuals, who show strong bias in the regulatory response to CHI3L1, 50% of patients with RA exhibit polarization towards Th1 phenotype (van Bilsen et al. 2004). At the same time, CHI3L1 is able to suppress the TNFα and IL-1-induced secretion of matrix metalloproteases and IL-8 in both human skin fibroblasts and articular chondrocytes. In contrast, in RA the serum levels of CHI3L1 positively correlated with serum levels of IL-6. Increased levels of CHI3L1 in serum reflect the degree of the synovial inflammation, and joint destruction in patients with RA and CHI3L1 promotes proliferation of human synovial cells (Johansen 2006).

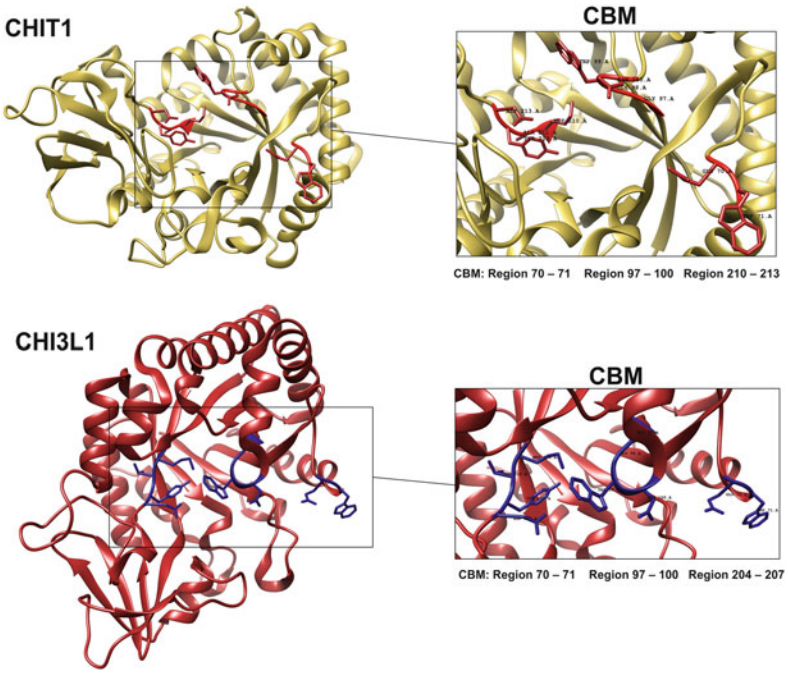


Fig. 3 CBM in CHIT1 and CHI3L1. Schematic representation of CHIT1 (1GUV) and CHI3L1 (1HJV) by CHIMERA software. It is possible to distinguish (in the box) the AA of CBM

Chitinases and Osteolysis

As above mentioned, the chitinases are a very heterogeneous protein group. Most of the human chitinases have been associated with a wide variety of diseases, and they can be potentially used as diagnostic and prognostic markers for those diseases, but their role remains obscure. In the last decade, it has been focusing attention to the physiological and pathological role of any of the human GH18 chitinases in disease conditions. It has been shown that CHIT1 and CHI3L1 were modulated during the osteoclasts differentiation derived from human monocyte/macrophages and play a role in the bone resorption (Di Rosa et al. 2014b) (Fig. 4).

This discovery indicates that these molecules could play a crucial role in the bone degeneration processes.

The Role of CHIT1 in Osteolytic Process

In the 1995, Boot and colleagues discovered and characterized the most representative of GH18 chitinases, named chitotriosidase. This enzyme has been widely implicated in a variety of diseases involving immune dysfunction (Nair

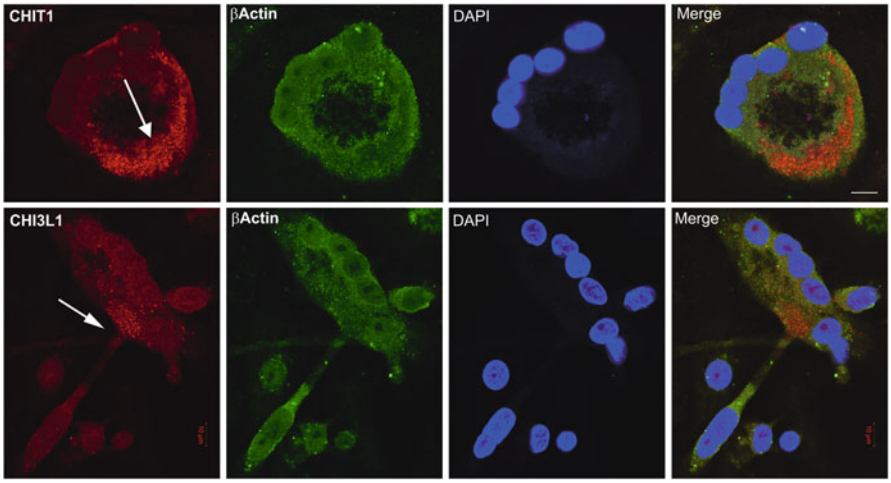


Fig. 4 Intracellular distribution of CHIT1 and CHI3L1in mature osteoclasts. In order to obtain mature osteoclasts, the monocyte was treated with conditioned medium (RANKL and GMCSF) for 21 days and used for immunofluorescent microscopy examination. Most of the cells in immunostaining for CHIT1 and CHI3L1 show a characteristic distribution pattern, the signal being localized in the cytoplasm. *Blue* represents DAPI nuclear counterstain. Merge of *green* and *red* is shown in *blue* and *pink*; merge of *blue* and *yellow* is shown in *red* and *blue*, and *green*. It is important to note the number of nuclei present per cell. The *white arrows* indicate the greater levels of concentration in the cells contact proximity. Scale bars equal 10 μm

et al. 2005). Recently, CHIT1 has come under increasing scrutiny due to their excess secretion into the serum or overexpressed in tissues, which are chronically inflamed (Lee et al. 2011). The CHIT1 gene is localized on chromosome 1q31-q32 and consists of 12 exons and spans approximately 20 kb of genomic DNA (Boot et al. 1998) and is expressed in prevalence in mature macrophages, but a recent publication showed the presence in osteoclast (Fig. 5). Interestingly, the 71-bp exon 11 can be alternatively spliced. This exon is usually skipped in the splicing process, generating the predominant mRNA species encoding the 50-kDa protein stored in the granules of neutrophilic granulocyte progenitors. Exon 11 introduces a premature stop codon; the alternatively spliced mRNA encodes a 40-kDa CHIT1 that is almost identical to the 39-kDa isoform generated by proteolytic processing of the 50-kDa CHIT1 (Renkema et al. 1997). The N terminal of both isoforms is identical as disclosed by the cloned CHIT1 cDNA. CHIT1 is an enzymatically active chitinase that shows transglycosylation activity toward chitin (Aguilera et al. 2003) and is the major chitinase measured in disease states (Malaguarnera 2006). CHIT1 has been included as one of the secreted biomarkers for Gaucher's disease (Hollak et al. 1994). The elevation of CHIT1 in these patients may reflect a particular state of activation of macrophages (Boven et al. 2004). In a healthy population, CHIT1 activity is very low and originates in the circulating polymorphonuclear cell. Conversely, during the development of acute/chronic inflammatory

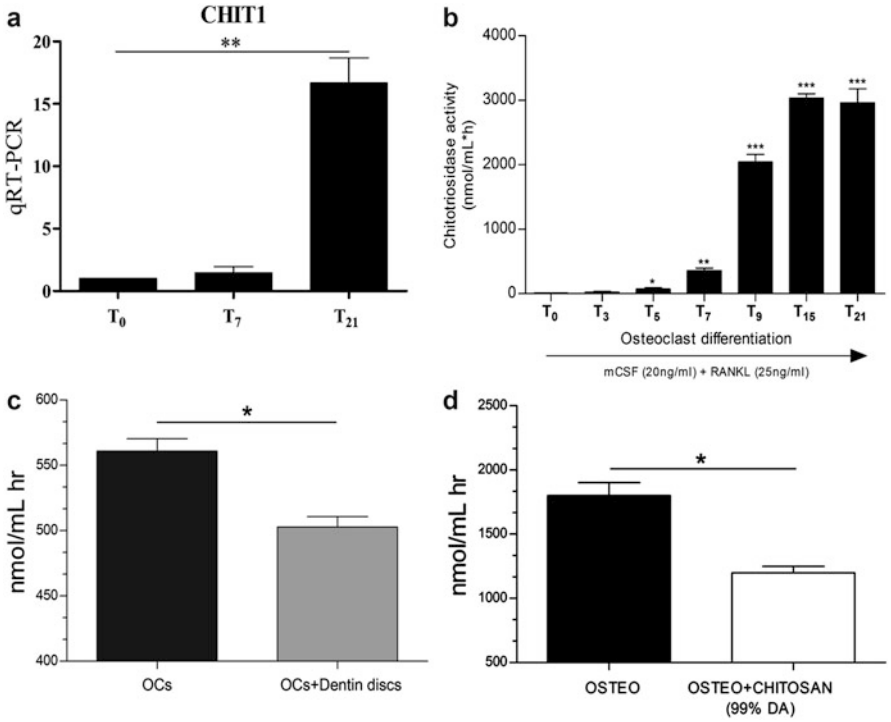


Fig. 5 CHIT1 related to osteoclast activity. (**a**, **b**) During the osteoclast differentiation, the CHIT1 expression and activity are increased. (**c**) During the osteoclast dentin disk digestion, the activity of CHIT1 is reduced also under the treatment with chitosan. Data are expressed as mean ± SD of at least three independent experiments. $*P < 0.01$,$**P < 0.001$, $***P < 0.0001$ compared to monocytes untreated (**a** and **b**) and OCs (osteoclasts) untreated (**c** and **d**)

disorders, the enzymatic activity of CHIT1 increases significantly (Malaguarnera 2006). A conspicuous number of evidence indicates that CHIT1 possesses an active role in disease states where inflammatory responses overcome (Di Rosa et al. 2005). It has been reported that CHIT1 is strongly correlated with Gaucher's disease symptoms and is used to monitor the efficacy of therapy (Pacheco and Uribe 2013). Cellular alteration in Gaucher's disease produced a pro-inflammatory milieu leading to bone destruction through enhancement of monocyte differentiation to osteoclasts and the improvement of osteoclasts resorption activity (Mucci et al. 2012). Therefore, it was hypothesized that CHIT1 play a crucial role in the disruption of bone homeostatic balance in Gaucher's disease, implying dysfunction of osteoclasts, osteoblasts, and mesenchymal cells (Campeau et al. 2009). A study showed that in periprosthetic soft tissue from patients with osteolysis the expression of alternative macrophage activation markers (CHIT1, CCL18) was increased in comparison to osteoarthritis controls (Koulouvaris et al. 2008), indicating a correlation between CHIT1 and osteolytic lesions (Koulouvaris et al. 2008). Nowadays, only little evidence shows that CHIT1 may play a role in the osteolytic process. As

previously mentioned, there are two enzymatically active isoforms of CHIT1, respectively of 39 kDa and 50 kDa (Kawada et al. 2007). The C-terminal domain of 50-kDa CHIT1 mediates a strong binding of this enzyme to chitin, enabling it not only to cleave chitotriose but also hydrolyzes colloidal chitin to yield chitobiose, a feature that is not shown by the 39-kDa isoform. This isoform, formed during the posttranslational process, is expressed and stored in intracellular lysosomes, since lack of the domino C-terminal chitin binding represents the truncated part of the whole protein (Renkema et al. 1995), whereas the 50 kDa moiety is the predominantly secreted isoform. The recent investigation, determining which of these subunits were responsible for the bone resorption, was observed that the 50 kDa subunit seems to be the ruling isoform competent to digest the bone matrix by osteoclast. The involvement of the CHIT1 in the osteolytic process was confirmed by the reduced level of CHIT1 activity in osteoclasts placed on dentine disk and after the treatment with carboxymethyl chitosan molecules (Fig. 5).

In addition, the silencing of CHIT1 provided a strong validation that CHIT1 activity is essential in the osteolytic process (Fig. 6). This recent evidence strongly supported an evolving concept regarding the roles of CHIT1 in osteolysis (Norberg et al. 2010). The high expression of CHIT1 observed in osteoclasts could have a detrimental role in the osteolytic processes occurring in the conditions where bone

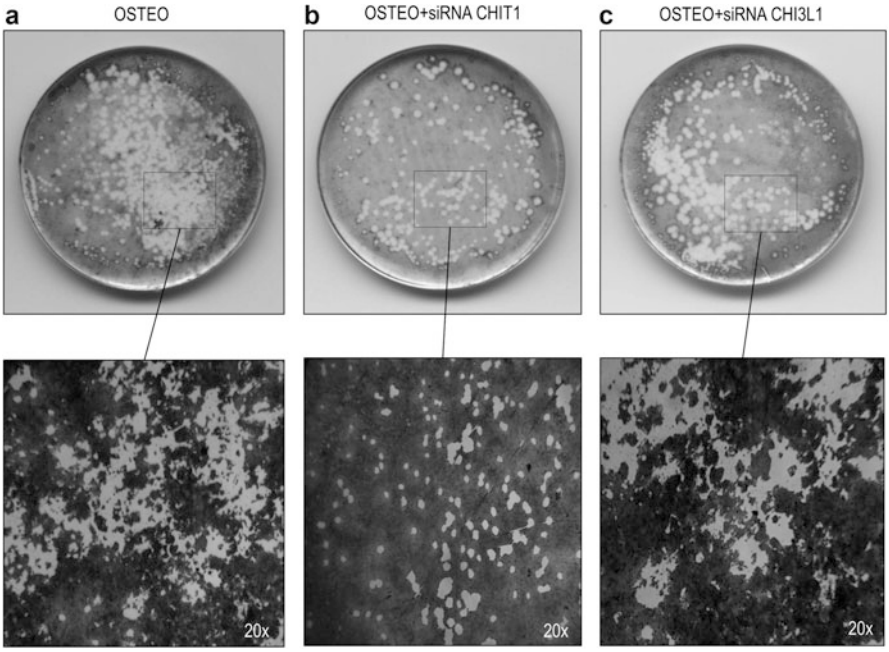


Fig. 6 Osteoclast digestion reduction by CHIT1 and CHI3L1 silencing. Resorption pits that appeared clear on the disk surface demonstrate the ability of osteoclasts (21 days of culture) to digest the bone matrix; (**b**) the treatment with the CHIT1 siRNA shows a reduction in the digestion of CPhoDs; (**c**) the treatment with the CHI3L1 siRNA shows a reduction in the digestion

architecture is subverted. Additional studies demonstrated that CHIT1 is expressed in an osteoarthritic rat cartilage model. These findings suggested that patients with elevated serum levels of CHIT1 may have a more increased osteolytic activity and a faster progression of the disease (Di Rosa et al. 2014a). Indeed, silencing CHIT1 with siRNA resulted in a significant decrease in bone resorption activity and transfection with CHIT1 or siRNA and cotransfection with both decreased the levels of the pro-differentiative marker MMP9 (Di Rosa et al. 2014b) (Fig. 6).

Therefore, patients with elevated serum levels of CHIT1 may have a more increased osteolytic activity and a faster progression of degenerative skeletal diseases.

Chitinase-3-Like-1 as an Inflammation Marker in Osteolysis Process

CHI3L1 protein also called YLK-40, based on its three N-terminal amino acids: tyrosine (Y), lysine (K), and leucine (L), is a 40 kDa mammalian glycoprotein which is a heparin, chitin, and collagen binding member of the mammalian chitinase-like proteins by CBM (Fig. 3). Unlike CHIA and CHIT1, CHI3L1 binds chitin polymers but lacks the active site residues necessary for cleavage. CHI3L1 has been the best investigated human chitinase-like protein regarding its biological activity and association with various disorders. Biological activities of CHI3L1 embrace regulation of cell proliferation, adhesion, migration, and activation. CHI3L1 is produced by a variety of cells, including neutrophils, monocytes/macrophages, and osteoclasts (Fig. 4) (Johansen 2006). It has been shown that CHI3L1 silencing by siRNA in mature osteoclast reduces the ability of these cells to digest the matrix (dentin disk or calcium phosphate thin film disks) (Norberg et al. 2010) (Fig. 6). CHI3L1 stimulates production of inflammatory mediators (e.g., CCL2, CXCL2, MMP-9) and has been proposed as a pro-inflammatory biomarker. Induction of CHI3L1 has been reported in patients suffering from a surprisingly vast array of diseases, including a number of autoimmune disorders. In addition, elevated plasma levels of CHI3L1 have been found in rheumatoid arthritis and inflammatory-related illnesses in humans (Vaananen et al. 2014). In 2007, Pozzuoli and colleague showed that CHI3L1 was released by intervertebral disk culture. In this condition, CHI3L1 may contribute to the pathophysiology of discal degeneration and inflammation as confirmed by its relationships with COX-2 and NO in disk tissue culture (Pozzuoli et al. 2007). The expression of CHI3L1 has been reported to be significantly associated with migration of human macrophages (Kawada et al. 2012). Immune cells, including tissue macrophages (MØs) that are activated locally, have been considered as major CHI3L1 producers. CHI3L1 is involved in the modulation of the extracellular matrix affecting cell adhesion and migration during the tissue remodeling processes that take place in fibrogenesis (Johansen et al. 2006). In addition, CHI3L1 promotes the proliferation and antagonizes catabolic or degradative processes during the inflammatory response of connective tissues (Ling and Recklies 2004). The ability of CHI3L1 to regulate cell proliferation, adhesion, migration, and activation, as well as to regulate extracellular matrix assembly, correlates well with elevated level of

CHI3L1 in the sites of chronic inflammation and active connective tissue turnover. CHI3L1 had been linked to tissue remodeling (Mucci et al. 2012), joint injury (Di Rosa et al. 2014a), and significantly elevated levels of CHI3L1 protein have been detected in serum and synovial fluid from OA patients (Recklies et al. 2002). It has been reported that miR-24 participates in osteogenic differentiation by targeting and regulating Tcf-1 expression in osteoblastic cells (Zhao et al. 2015). In the light of these results, Tao JIN and colleagues showed that miR-24 suppresses the expression of CHI3L1 in osteomyelitis caused by *Staphylococcus aureus* (Jin et al. 2015). A number of evidence showing that CHI3L1 stimulates proliferation of connective tissue cells, modulates expression levels of chemokines and metalloproteases in inflammatory fibroblasts, and enhances chemotaxis of endothelial cells (Recklies et al. 2005) strongly indicate that CHI3L1 plays crucial role in stromal cells not only in inflammatory conditions. Additionally, in vitro studies demonstrated that CHI3L1 is secreted by osteosarcoma (Johansen et al. 1993). The findings show a correlation between CHI3L1 expression and the development of primary and metastatic tumors that further support the idea that CHI3L1 plays a role in the development and progression of a variety of malignancies.

Chitinase-Like Protein and Bone Disease

To date, there is no evidence that the remaining chitinase is related to bone degeneration. It is also true that CHI3L2, CHID1, and CHIA are closely related to inflammatory processes that may be related to bone degeneration.

Chitinase Versus Chitosan for Bone Tissue Engineering

In recent years, many achievements have been obtained in organ transplantation, surgical reconstruction and the use of artificial protheses to treat the loss or failure of an organ or bone tissue. Chitosan is a new promising natural substance used in biomaterials research that can be applied in tissue engineering. This polymer can be easily combined with other biomaterials in order to obtain a greater tolerance in transplantation. It is obtained in a rapid and economic method from chitin, which forms a major component of crustacean exoskeleton. Thanks to its high tolerance, to an intrinsic antibacterial nature, biocompatibility, biodegradability, and the ability to be folded into various geometric forms such as porous structures, it is an excellent candidate for tissue engineering applications. Chito-oligomers are derivatives from chitosan and chitin. The application of chito-oligomers is highly used to antitumor activity and inhibition of angiogenesis (Xiong et al. 2009). This evidence may be related to the role played by the chitosan to chitinases. It seems reasonable to assume that the chitinases bind the chitosan and these molecules reduce the ability to improve the inflammation, which is the leading cause of the osteolytic process. It has been shown that osteoclasts treated with carboxymethyl chitosan during the dentin disk and CPhoD digestion reduce substrate digestion and CHIT1 activity

(Fig. 5 C/D) (Norberg et al. 2010). This result suggests that the action undertaken by CHIT1 in the digestion process of the CPhoDs or dentin disks can be mediated by the binding between its own CBM and dentin disk or CPhoD substratum. Most likely, a similar effect could be also ascribed to CHI3L1. In 2009, Nam KS and colleagues showed that treatment of human breast cancer cells with increasing concentrations of chitosan oligosaccharides led to a concentration-dependent decrease in cell migration and reduced the amounts of secreted MMP-9 (Nam and Shon 2009). The antimetastatic property of chitosan oligosaccharides mediated by CHI3L1 binding was further confirmed by experimental animal data that reveal a decreased tumor dissemination in response to administration of chitosan nanoparticles, which may capitalize on the conserved binding to CHI3L1 (Hamilton et al. 2015). In the light of this, the use of Chitosan scaffold as materials for artificial bone and bone regeneration in tissue engineering could be a solution for reducing the degenerative processes mediated by chitinase.

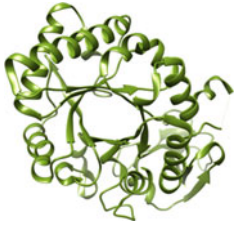
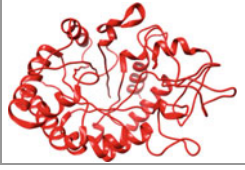
Potential Application to Prognosis, Other Disease, or Conditions

Most of the human GH18 chitinases, except for those identified recently, have been linked to a variety of diseases, and they can potentially be used as diagnostic and prognostic markers for various diseases (Table 2). Nevertheless, causal relationships between any of these chitinases and corresponding diseases have not been established. Numerous studies have confirmed the correlation between CHIT1, CHI3L1, and osteolytic disease. The presence of polymorphism in CHIT1 and CHI3L1 makes it even more interesting from a clinical point of view. It has been that heterozygous for a 24-bp duplication in the CHIT1 gene could have a protective effect in human longevity (Malaguarnera et al. 2010). Furthermore, the CHI3L1 polymorphism is associated with asthma and with sarcoidosis (Kruit et al. 2007). Studies are still needed to validate the efficacy of evaluating CHIT1 and CHI3L1 as an early diagnostic test for osteolytic disease and its prognostic potential in the identification of bone degeneration.

Summary Points

1. This chapter focuses on role of chitinases in bone disease.
2. The bone tissue is characterized by a process called the *bone remodeling* (bone resorption and bone formation).
3. The two main cells involved in these process are the *osteoblast*s (bone formation) and *osteoclasts* (bone resorption).
4. Bone disorders show an underlying balance alteration in bone remodeling factors.
5. There are many diseases related to bone resorption as rheumatoid arthritis, osteoporosis, periodontitis, and cancer metastasis. All of these diseases are related to the human chitinases expression.
6. The mammalian chitinases can be considered new bone resorption markers.

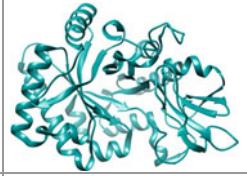
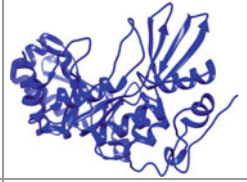
Table 2 Chitinases and corresponding diseases. The table shows all human chitinase with their official symbol, biological activity, related disease, PDB code, and 3D images by CHIMERA software

Symbol	PDB code 3D Structure Name	Biological activity	Character disease	Disease
A: Chitinase with activity				
CHIT1		Carbohydrate binding Chitin binding Chitinase activity	Inherited lysosomal storage disorders	*Niemann-Pick Gaucher*
			Sphingolipidosis	b-*Glucocerebrosidase deficiency*
			Atherosclerosis	*Atherothrombotic stroke Ischemic heart disease*
			Hematological disorders involving activated macrophages	*Malaria (Plasmodium falciparum)* b-*thalassemia major*
			Neuro-degenerative disorders	*Alzheimer's disease Ischemic cerebrovascular dementia Amyotrophic lateral sclerosis (ALS)*
			Inflammation	*Nonalcoholic steatohepatitis Sarcoidosis*
CHIA (AMCase)		Carbohydrate binding Chitin binding Chitinase activity Food processor Parasites defense	Th2-mediated Inflammation	*Asthma Rhinosinusitis with nasal polyps Allergic conjunctivitis Eosinophilia Basophilia*

(*continued*)

Table 2 (continued)

Symbol	PDB code 3D Structure Name	Biological activity	Character disease	Disease
CTBS	*chitobiase, di-N-acetyl*	Chitin binding Chitinase activity Chitin catabolic process Oligosaccharide catabolic process	Involved in degradation of asparagine-linked oligosaccharides on glycoproteins	*Spermatogenesis dysfunction ?*
B: Chitinase-like protein				
			Cancer	*Adenocarcinoma Small cell lung carcinoma Glioblastoma Myxoid chondrosarcoma Papillary thyroid carcinoma Melanoma*
CHI3L1		Carbohydrate binding Chitin binding Not Chitinase activity Food processor Parasites defense Proliferation Adhesion Migration	Joint diseases	*Rheumatoid arthritis Osteoarthritis Joint diseases Ankylosing spondylitis*
			Chronic inflammation	*Giant cell arteritis Inflammatory bowel disease Sarcoidosis Systemic sclerosis*
			Infection	*Human endotoxemia Streptococcus pneumoniae pneumonia S. pneumoniae bacteraemia Purulent meningitis and encephalitis*
			Liver fibrosis	*Alcoholic liver disease Chronic hepatitis C virus infection*

CHI3L2		Carbohydrate binding Chitin binding No chitinase activity	Joint diseases	*Rheumatoid arthritis Joint diseases Osteoarthritis*
			HIV	*Induction by HIV TAT*
CHID1		Oligosaccharide binding Chitin binding Chitinase activity Protein binding	Inflammation	*Activated Sinusoidal endothelial cells Sarcoidosis*
OVGP1	*oviductal glycoprotein 1,*	Chitin binding Chitin catabolic process Female pregnancy Reproduction Single fertilization	hormone dysfunction	*Endometrial hyperplasia and endometrial cancer*

7. Chitosan is a natural molecule able to reduce chitinases' activity and function.
8. Chitinases regulation may be a new strategy for bone degenerative processes.

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Hormone Relaxin as Biomarker for Bone Health and Disease

15

Yukiho Kobayashi, Carolina Duarte, and Keiji Moriyama

Contents

Abstract

Bone homeostasis is maintained by fine-tuning of the dynamic balance between bone resorption via osteoclasts and bone formation via osteoblasts. Bone metabolism-related biomarkers such as a soluble factor or type I collagen

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metabolism product specifically secreted by osteoblasts or osteoclasts are useful for evaluating the change in bone metabolism in a noninvasive manner in real time. Monitoring of bone metabolism-related biomarkers that are excreted in the urine or secreted into the bloodstream is quite useful for the diagnosis of various kinds of skeletal metabolism abnormalities. For example, an elevated level of a bone metabolism marker is a risk factor of bone fracture independent of bone density, as well as for bone density loss in the future. Relaxin (RLN) is a pleiotropic hormone of the insulin-like peptide hormone family, which is mainly secreted into the bloodstream from the ovary, uterus, and placenta during pregnancy. Therefore, RLN helps labor to progress by softening and widening the pubic symphysis and cervix, owing to its ability of remodeling the extracellular matrix by degrading collagen. The physiological roles of RLNs and relaxin family peptides through their receptors, relaxin family peptide receptors (RXFPs), in the reproductive system have been extensively studied. However, recent studies have shown that RLNs/RXFPs also play a key role in the cardiovascular system, renal function, organ protection, metabolism, cancer metastasis, and the central nervous system. The effectiveness of RLN for the treatment of acute heart failure is now assessed under phase III clinical trials. In addition to these broad physiological activities, its role in bone metabolism was also recently highlighted because of its ability to induce osteoclastogenesis, activate osteoclast function, and enhance osteoblast differentiation in vitro. In addition, the majority of men with *RXFP2* mutations presented with symptoms of osteoporosis, and *Rxfp2*-deficient mice showed a lower bone mass and reduced osteoclast surface compared to their wild-type littermates. This chapter provides an overview of the biological functions of RLN and its receptors (RXFPs), with particular focus on bone metabolism. In addition, the utility and possibility of RLNs/RXFPs as biomarkers for bone health and disease are discussed.

Keywords

Bone • Relaxin • Relaxin family peptide receptor • Osteoblast • Osteoclast • Bone remodeling • Collagen

List of Abbreviations

ALP	Alkaline phosphatase
BAP	Bone-specific alkaline phosphatase
BCE	Bone collagen equivalents
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
BSP	Bone sialoprotein
BTM	Bone turnover marker
cAMP	Cyclic adenosine monophosphate
c-FMS	Colony-stimulating factor 1 receptor
CLIA	Chemiluminescence immunoassay
COL1α1	Collagen type I alpha 1
CREA	Urinary creatinine
CTX	Carboxy-terminal cross-linking telopeptide of type 1 collagen

DPD	Deoxypyridinoline
DXA	Dual-energy X-ray absorptiometry
ECLIA	Electrochemiluminescence immunoassay
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
ICTP	Carboxy-terminal cross-linking telopeptide of type 1 collagen generated by MMPs
IGF-1	Insulin-like growth factor-1
INSL	Insulin-like peptide
M-CSF	Macrophage colony-stimulating factor
MMPs	Matrix metalloproteinase
mRNA	Messenger RNA
NFATc1	Nuclear factor of activated T-cells cytoplasmic 1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
N-MID	Amino-terminal mid-fragment
NO	Nitric oxide
NTX	Amino-terminal cross-linking telopeptide of type 1 collagen
OC	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
PBMC	Peripheral blood monocyte cells
PDL	Periodontal ligament
PICP	Carboxy-terminal propeptide of type 1 collagen
PINP	Amino-terminal propeptide of type 1 collagen
PTH	Parathyroid hormone
PYD	Pyridinoline
RANK	Receptor activator of NF-kB
RANKL	Receptor activator of NF-kappaB ligand
RIA	Radioimmunoassay
RLN (Rln)	Relaxin
RUNX2	Runt-related transcription factor 2
RXFP (Rxfp)	Relaxin family peptide receptor
sRANKL	Soluble RANKL
TGF-β	Transforming growth factor-β
TRAP	Tartrate-resistant acid phosphatase
TRAP5b	Tartrate-resistant acid phosphatase type 5b

Key Facts of Relaxin

- It is reported that relaxin is associated with increased knee joint laxity in pregnant women toward the end of the pregnancy period (Blecher and Richmond 1998).
- Phase III clinical trials have shown that recombinant human relaxin-2 (serelaxin) can be safely administered and has a promising pharmacological effect on patients with acute heart failure (Ponikowski et al. 2014; Teerlink et al. 2013).

- *Rxfp1* and *Rxfp2* mRNAs were expressed in developing mouse embryonic facial tissues, including Meckel's cartilage, the tongue, and tooth primordia (see Fig. 6) (Duarte et al. 2014a).
- RNL did not have any significant effects on human orthodontic tooth movement, and there was short-term relapse presumably because of systemic administration of this hormone, resulting in low concentrations in the local periodontal tissues. On the other hand, RLN reportedly prevents relapse after orthodontic tooth movement in rats (Hirate et al. 2012).
- RLN3 and RXFP3 control metabolism in humans via regulation of appetite, food intake, and body weight. A previous study revealed higher RLN3 levels in the serum of female patients with metabolic syndrome compared to those in the controls; thus, this hormone might be a candidate biomarker of metabolic disorder syndrome (Ghattas et al. 2013).

Definition of Words and Terms

Bone modeling	Bone modeling is the process of shaping bones during the developing stage and growing period. It changes the size and morphology of bones. In many instances, bone resorption and bone formation can take place independently.
Bone remodeling	Bone remodeling is the substitutive system of bone matrix, which involves removal of old bone matrix via osteoclast and new bone formation via osteoblasts. The bone remodeling process can be classified into five stages: (1) activation, (2) resorption, (3) reversal, (4) formation, and (5) termination. Osteoclasts migrate, become activated, and resorb on the surface of the bone that is covered with the bone-lining osteoblasts. Then, osteoblast precursor cells migrate to the resorption site, differentiate, and perform active bone matrix synthesis. The borderlines between old bone and new bone are called cement lines.
Osteoblast	Osteoblasts are derived from mesenchymal stem cells as a result of the action of multiple cytokines including BMPs, TGF-β, PHT, FGFs, IGFs, Wnts, and hedgehogs. Osteoblasts express specific markers depending on the cell differentiation stage. Runx 2 is a transcription factor, which is indispensable for osteoblast differentiation. Osteoblasts play an important role in extracellular matrix deposition, matrix mineralization, and osteoclast differentiation.
Osteoclast	Osteoclasts, a member of the monocyte/macrophage family, are the only cells that control mineralized bone resorption.

	The differentiation and the functions are strictly controlled by M-CSF, OPG, and RANK/RANKL signaling. Disorder in osteoclast function results in bone metabolic disorders, such as osteopetrosis, Paget's disease of bone, and osteoporosis.
Relaxin	Relaxin (RLN) is an insulin-like hormone that was first described as a factor that facilitates parturition by softening and lengthening the pubic symphysis and softening the cervix. This hormone is released in the bloodstream from the corpus luteum of the ovary, breast, placenta, and decidua in pregnant/nonpregnant females and from prostate in males. RLN is known as a mediator of the hemodynamic changes during pregnancy. Recombinant human RLN2 is considered to have a promising pharmacological therapeutic effect on patients with acute heart failure.

Introduction

Bone is a supporting tissue and an organ of locomotion particular to vertebrates and also plays a role as a storage organ for several bioactive substances required for the living body, including differentiation or growth factors (e.g., TGF-β or IGF-1) (Tang et al. 2009; Xian et al. 2012) inside the bone matrix as well as minerals such as calcium or phosphorus. In addition, immune system cells and hematopoietic cells that control the biological defense mechanism are contained in the bone medullary cavity. Bone homeostasis is maintained by fine-tuning of the dynamic balance between bone resorption via osteoclasts and bone formation via osteoblasts. With regard to mammalian bone, bone formation is maintained by "modeling" and "remodeling" phenomena (Parfitt 1984). Bone modeling mainly occurs during periods of growth and is a mechanism of bone formation at the new site. On the other hand, bone remodeling is a reconstructive phenomenon involving the cylindrically structured basic multicellular unit (BMU) (Frost and Straatsma 1964; Matsuo and Irie 2008) called an "osteon" in the cortical bones or a "packet" in the cancellous bones. The strength and flexibility of the bone are maintained by bone remodeling; however, disruption of the balance of remodeling results in bone mass abnormality and the consequent development of various bone-related diseases. Bone remodeling is initiated by activation of the osteoclasts (Hattner et al. 1965; Martin and Rodan 2001; Parfitt 1984; Takahashi et al. 1964) and followed by the phases of resorption, reversal, and bone formation, which is characterized by collagenous matrix production and mineralization via osteoblasts (Raggatt and Partridge 2010), and then the termination phase. A series of processes from the activation to termination stage are repeated within approximately 3 months (Rosen et al. 2009). The

soluble molecules that are specifically secreted from osteoblasts or osteoclasts and the metabolic products of type I collagen are generally used as biomarkers of bone metabolism in clinical examinations (Table 1). Bone remodeling biomarkers in the blood or urine reflect the momentary sum of systemic bone resorption and formation progressing at the BMU. There are currently two major known groups of biochemical bone turnover markers, namely, bone formation markers and bone resorption markers. Bone formation is assessed with bone-specific alkaline phosphatase (BAP) (Ahmed and Gibbons 2015) and osteocalcin (OC), which are secreted by the osteoblasts at the early or late stage of differentiation, respectively. In addition, N- and C-terminal type I propeptides (PINP and PICP) (Pagani et al. 2005) reflect the activity of osteoblasts at the early differentiation stage. On the other hand, bone resorption is assessed by N- and C-terminal cross-linking telopeptides of type I collagen (NTX-I and CTX-I) (Pagani et al. 2005) generated by cathepsin K and C-terminal cross-linking telopeptides of type I collagen generated by metalloproteinases (MMPs; CTX-MMP or ICTP), which is a bone metastatic marker (Pagani et al. 2005). Moreover, the concentrations of deoxypyridinoline (DPD) in the urine and isoform 5b of tartrate-resistant acid phosphatase (TRAP5b), specifically secreted from osteoclasts into the bloodstream, are the bone resorption markers associated with increased fracture risk (Ivaska et al. 2010). The bone turnover markers (BTMs) provide a useful assessment of a clinical condition caused by abnormal bone metabolism such as osteoporosis and bone metastases. In addition, BTMs are a helpful index for the prediction of fragility fractures, response evaluations, and establishing an appropriate dose of anti-osteoporotic treatment (e.g., determining the metabolic effects and anti-fracture efficacy), which could improve compliance to treatment for osteoporosis.

Relaxin (RLN) is an insulin-like peptide hormone that is well known to facilitate parturition by inducing the softening and lengthening of the pubic symphysis and softening of the cervix (Hisaw 1926; Lu et al. 2005). RLN belongs to the relaxin family of peptides; among members of this family, RLN, insulin-like peptide 3 (INSL3), RLN3, and INSL5 interact with relaxin family peptide receptors (RXFPs) 1–4, respectively (Bathgate et al. 2005, 2006, 2013). Humans and apes possess the *RLN1–RLN3* and *RLN3–RLN6* genes, which encode H1–H3 relaxins and INSL3–INSL6 proteins, respectively (Bathgate et al. 2005). On the other hand, mice possess only *Rln2*, *Rln3*, *Insl3*, *Ins5*, and *Ins6* (Bathgate et al. 2005). Human RLN1 and RLN2, as well as RLN2 in other mammals, are commonly referred to as "relaxin" (Bathgate et al. 2005). RLN exerts a variety of effects in different types of cells. RLN blood levels are highest in the first trimester of pregnancy for the initiation of cardiovascular changes, although RLN is also produced in both males and females to exert broad physiological roles in paracrine- or autocrine-regulated mechanisms (Bathgate et al. 2006, 2013; Fig. 1). In particular, RLN inhibits fibroblast proliferation and differentiation (Samuel et al. 2004) but increases the production of matrix MMP1, MMP2, MMP9, and MMP13 (Ahmad et al. 2012;

Table 1 Biomarkers of bone turnover and reference intervals of each assay (Data from Jung and Lein (2014) with permission from the publisher)

Bone formation marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
BAP	Serum plasma	Access ostase/ CLIA, automated	Beckman Coulter, Brea, CA, USA	Women, premenopausal, 3.2–18.8 μg/L Women, postmenopausal, 5.3–22.7 μg/L Men, 5.0–22.8 μg/L
		IDS-iSYS Ostase BAP/CLIA, automated Ostase BAP EIA/ELISA	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 6.0–22.7 μg/L Women, postmenopausal, 8.1–31.6 μg/L Men (>45 years), 7.5–26.4 μg/L as access ostase assay
		MicroVue BAP/ELISA	Quidel Corp., San Diego, CA, USA	Women, premenopausal, 11.6–29.6 U/L Women, postmenopausal, 14.2–42.7 U/L Men, 15.0–41.3 U/L
OC	Serum plasma	N-MID osteocalcin/ ECLIA, automated	Roche Diagnostics, Mannheim, Germany	Women, premenopausal, 11–43 μg/L Women, postmenopausal, 15–46 μg/L Men (>50 years),14–46 μg/L
		N-Mid Osteocalcin/ ELISAIDS-iSYSN-MID Osteocalcin/ CLIA, automated	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 12.8–55.0 μg/L Women, postmenopausal, 8.4–33.9 μg/L Men, 9.6–40.8 μg/L
		MicroVue Osteocalcin/ELISA	Quidel Corp., San Diego, CA, USA	Adults (>25 years), 3.7–10 μg/L
		Undercarboxylated Osteocalcin EIA Kit/ELISA	TaKaRa Bio Inc., Shiga, Japan	ca. 20% of total OC in serum; no further data
PICP	Serum plasma	MicroVue CICP/ ELISA	Quidel Corp., San Diego, CA, USA	Women, postmenopausal, 69–147 μg/L Men (>25 years), 76–163 μg/L
		Procollagen Type I C-Peptide (PIP)/ ELISA	TaKaRa Bio Inc., Shiga, Japan	Adults, 161–757 μg/L

(*continued*)

Table 1 (continued)

Bone formation marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
PINP	Serum plasma	UniQ PINP/RIA	Orion Diagnostica Oy, Espoo, Finland	Women, 19–83 μg/L Men, 22–87 μg/L
		IDS-iSYS Intact PINP/CLIA, automated	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 19.3–76.3 μg/L Women, postmenopausal, 18.2–102.3 μg/L Men (>45 years), 19.1–77.0 μg/L
		Total P1NP/ECLIA, automated	Roche Diagnostics, Mannheim, Germany	Women, premenopausal, 13.8–60.9 μg/L Men, 13.9–85.5 μg/L
Bone resorption and osteoclastogenesis marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
DPD	Urine	MicroVue DPD/ELISA	Quidel Corp., San Diego, CA, USA	Women (25–44 years), 3.0–7.4 nmol/mmol CREA Men (25–55 years), 2.3–5.4 nmol/mmol CREA
Total DPD	Serum urine	MicroVue Total DPD/ELISA	Quidel Corp., San Diego, CA, USA	Women, (25–44 years), 2.18–4.68 nmol/L Men (25–55 years), 1.95–4.54 nmol/L 19–325 nmol/L
	Serum	MicroVue Serum PYD (only free)/ ELISA	Quidel Corp., San Diego, CA, USA	Adults, 1.09–2.79 nmol/L
	Urine	MicroVue PYD (free PYD + DPD)/ ELISA	Quidel Corp., San Diego, CA, USA	Women, premenopausal, 16.0–37.0 nmol/mmol CREA Men, 12.8–25.6 nmol/ mmol CREA
CTX	Serum plasma	ß-CrossLaps/ ECLIA, automated	Roche Diagnostics, Mannheim, Germany	Women, premenopausal, 31–568 ng/L Women, postmenopausal, 113–999 ng/L Men (30–50 years), 22–578 ng/L (>50–70 years), until to 692 ng/L (>70 years), until to 855 ng/L
		Serum CrossLaps/ ELISA	IBL, Toronto, Canada	Women, premenopausal, 112–738 ng/L Women, postmenopausal,

(*continued*)

Table 1 (continued)

Bone formation marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
				142–1351 ng/L Men (30–50 years), 115–748 ng/L
		Serum CrossLaps (CTX-I)/ELISA	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 0.39–3.20 nmol/L Women, postmenopausal, 0.36–5.55 nmol/L
		IDS-iSYS CTX-I (CrossLaps)/ ECLIA, automated		Women, premenopausal, 50–670 ng/L Women, postmenopausal, 90–1050 ng/L Men (>45 years), 90–730 ng/L
	Urine	CrossLaps Urine/ ELISA	IBL, Toronto, Canada	Women, premenopausal, 67–544 μg/mmol CREA
		Urine BETA CrossLaps (CTX-I)/ ELISA	IDS Inc., Scottsdale, AZ, USA	Women, postmenopausal, 121–874 μg/mmol CREA Men (31–80 years), 54–559 μg/mmol CREA
		Alpha CrossLaps EIA/ELISA	IDS Inc., Scottsdale, AZ, USA	Women, premenopausal, 0.10–0.99 μg/mmol CREA Women, postmenopausal, 0.17–2.26 μg/mmol CREA Men, 0.13–1.13 μg/mmol CREA
NTX	Serum plasma	Osteomark NTx Serum/ELISA	Alere Inc., Waltham, MA, USA	Women, premenopausal, 7.7–19.3 nmol BCE/L Men (31–80 years), 8.1–24.8 nmol BCE/L
	Urine	Osteomark NTx Urine/ELISA	Alere Inc., Waltham, MA, USA	Women, premenopausal, 14–74 nmol BCE/mmol CREA Men (31–87 years), 13–78 nmol BCE/mmol CREA
ICTP	Serum plasma	UniQ ICTP/ELISA and RIA	Orion Diagnostica Oy, Espoo, Finland	Women, 1.6–4.2 μg/L Men, 1.5–4.3 μg/L
TRAP5b	Serum plasma	MicroVue TRAP5B EIA/ELISA	Quidel Corp., San Diego, CA, USA	Women, premenopausal, 0.25–5.64 U/L Women, premenopausal, until to 9.12 U/L Men, 1.26–6.74 U/L

(*continued*)

Table 1 (continued)

Bone formation marker				
Analyte	Sample	Assay/principle	Producer	Reference intervals
BSP	Serum plasma	Bone Sialoprotein (BSP)/ELISA	Immundiagnostik AG, Bensheim, Germany	Without data
OPN	EDTA-plasma	Quantikine ELISA Human Osteopontin	R&D Systems, Minneapolis, MN, USA	Adults, 46–144 μg/L
OPG	Serum plasma	Osteoprotegerin/ ELISA	Immundiagnostik AG, Bensheim, Germany	Adults, until 3.60 pmol/L
RANKL	Serum plasma	Total sRANKL/ ELISA	Immundiagnostik AG, Bensheim, Germany	Women, premenopausal, until 3.29 pmol/L Men until 1.66 pmol/L

Abbreviations: *BAP* bone-specific alkaline phosphatase, *BCE* bone collagen equivalents, *BSP* bone sialoprotein, *CLIA* chemiluminescence immunoassay, *CREA* urinary creatinine, *CTX* carboxy-terminal cross-linking telopeptide of type 1 collagen, *DPD* deoxypyridinoline, *ECLIA* electrochemiluminescence immunoassay, *ICTP* carboxy-terminal cross-linking telopeptide of type 1 collagen, generated by MMPs, *MMPs* matrix metalloproteinases, *NTX* amino-terminal cross-linking telopeptide of type 1 collagen, *OC* osteocalcin, *OPG* osteoprotegerin, *OPN* osteopontin, *PICP* carboxy-terminal propeptide of type 1 collagen, *PINP* amino-terminal propeptide of type 1 collagen, *PYD* pyridinoline, *RANKL* receptor activator of NF-kappaB ligand, *RIA* radioimmunoassay, *TRAP5b* tartrate-resistant acid phosphatase type 5b

Chow et al. 2012), resulting in a strong antifibrotic effect on various kinds of organs. RLN also influences bone metabolism by inducing osteoclastogenesis and the activation of mature osteoclasts expressing RXFP1 through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), receptor activator of NF-kB (RANK), tartrate-resistant acid phosphatase (TRAP), and cathepsins (Ferlin et al. 2010; Hombach-Klonisch et al. 2006). The physiological roles of RXFP, RLN, and INSL3 in bone metabolism have been elucidated in recent studies. Ferlin et al. reported that 64% of young men with *RXFP2* mutations had significantly reduced bone mass density without any apparent cause of osteoporosis and no aberration of testosterone levels and gonadal function (Ferlin et al. 2008). Interestingly, *Rxfp2*-deficient mice also presented a decreased bone mass, mineralizing surface, bone formation, and osteoclast surface in comparison with their wild-type littermates (Ferlin et al. 2008). The authors concluded that INSL3/RXFP2 signaling is essential for bone metabolism. In another study, Duarte et al. reported the expression of *Rxfp1* and *Rxfp2* during mouse craniofacial skeletal development and tooth development (Fig. 6) (Duarte et al. 2014a) and found that RLN enhanced osteoblastic differentiation and caused abnormal mineralization and extracellular matrix metabolism in vitro through *Rxfp2*, which was predominant over *Rxfp1* in MC3T3-E1 mouse calvarial osteoblasts (Duarte et al. 2014b). In addition, Moon et al. showed that in vivo administration of Rln enhanced bone morphogenetic protein (BMP)-2-induced bone formation and synergistically enhanced the BMP-2-induced osteoblast differentiation of mouse bone marrow stem cells and mouse embryonic C3H/10 T1/2

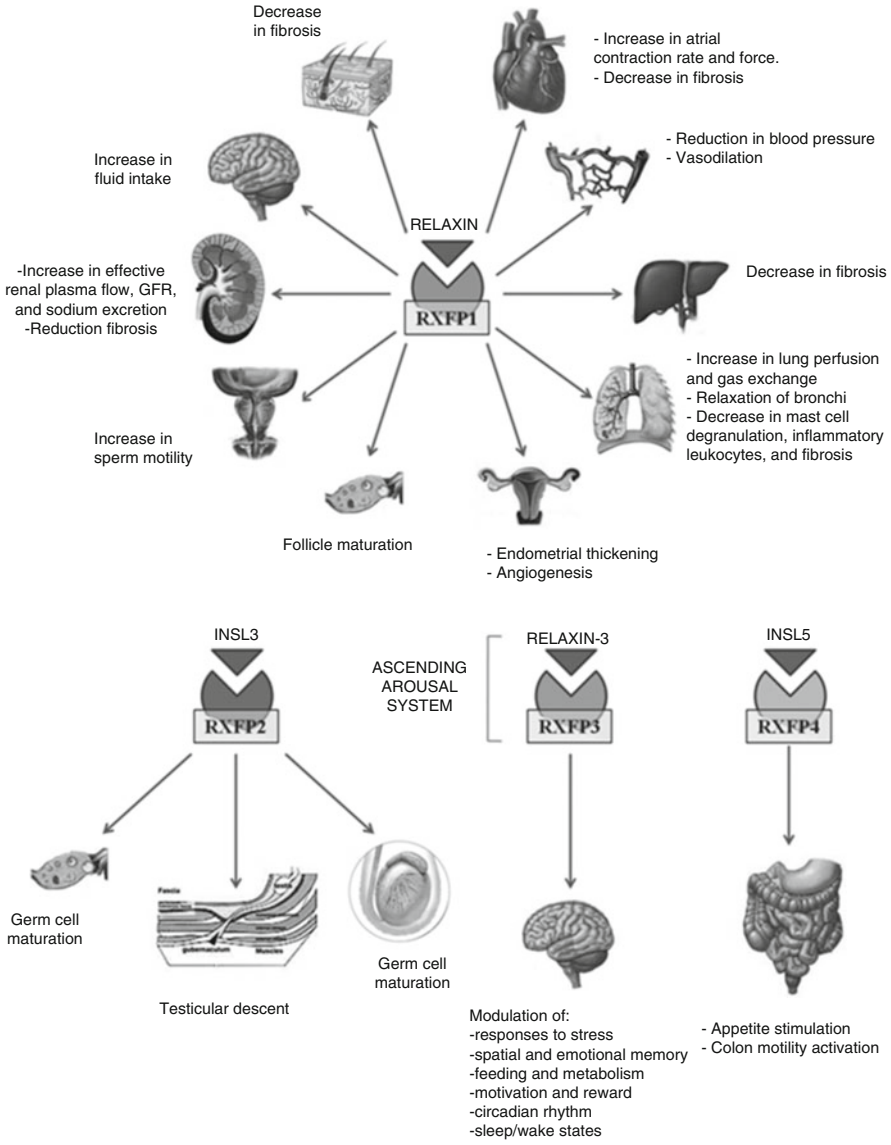

Fig. 1 Pattern diagram of physiological role of RLN on multiple organs Data from Cernaro et al. (Cernaro et al. 2014), with permission from the publisher.

fibroblasts through *Rxfp1* by augmenting and sustaining Smad and p38 phosphorylation, which upregulated runt-related transcription factor 2 (Runx2) expression and activity (Moon et al. 2014). These results strongly suggest that RLNs might be useful for therapeutic applications in bone metabolic disorders and that RLNs and RXFPs

might be candidate biomarkers to reflect the condition of bone health and disease status.

Overview of Bone Turnover Biomarkers in the Serum/Plasma and Urine

Bone turnover biomarkers are generally classified into bone formation and bone resorption markers (Delmas et al. 2000). Analysis of the dynamics of bone turnover markers in the serum and/or urine is extremely useful as a diagnostic tool and for evaluation of the prognosis and treatment of patients with skeletal disorders. Table 1 shows the common bone markers that are tested in clinical settings (Jung and Lein 2014). The following sections provide a brief description of each marker listed in Table 1.

Biomarkers for Evaluating Bone Formation

An increased concentration of bone-specific alkaline phosphatase (BAP) in the serum primarily reflects accelerated osteoblast activity or secondarily indicates elevated bone resorption. There are two effective methods for measuring either the protein mass or enzyme activity of BAP (Gomez et al. 1995). Osteocalcin (OC) is secreted by mature osteoblasts in a vitamin K- and D3-dependent manner and is a calcium-binding protein, accounting for 25% of the non-collagenous proteins in the skeletal tissue. Proteolytic cleavage between amino acids 43 and 44 produces an intact molecule of 49 amino acids in the bloodstream (Garnero et al. 1994), and the mostly stable N-terminal N-MID fragment from amino acids 1–43 can be detected with an N-MID osteocalcin assay (see Table 1). Bone has a complex structure consisting of minerals based on hydroxyapatite and organic constituents based on type I collagen. Type I collagen is a triple helical-structured molecule containing two identical α1 (I) and α2 (I) chains. Type I procollagen is separated by proteases on the N- and C-terminal ends, accordingly referred to as the C-terminal or N-terminal propeptides of type I procollagen (PICP and PINP, respectively), which reflect the activity of osteoblasts at the early stage of differentiation.

Biomarkers for Evaluating Bone Resorption

The helix shape of bone collagen is stabilized by cross-links of lysine or hydroxylysine residues called pyridinoline (PYD) and deoxypyridinoline (DPD), which are released during bone resorption (Delmas et al. 2000). DPD is almost solely found in the bone and is thus a specific indicator of bone resorption. Methods have

been developed for the determination of free as well as total cross-links in the urine and serum. Type I collagen releases carboxy- as well as amino-terminally cross-linked telopeptides (CTX and NTX, respectively) into the bloodstream during bone resorption (Herrmann and Seibel 2008). CTX and NTX can be measured in both the serum and urine. Carboxy-terminal cross-linking telopeptide of type I collagen (ICTP) is a specific telopeptide that is separated from collagen by metalloproteinases. ICTP is a trivalent cross-linked telopeptide of 8.5 kDa, with two phenylalanine-rich domains between the two α1 collagen chains.

Osteoclasts specifically contain tartrate-resistant acid phosphatase type 5b (TRAP5b) as a bone-specific isoenzyme and release it into the bloodstream during bone resorption. The TRAP5b concentration in the serum reflects the number and activity of osteoclasts involved in the resorption process (Chao et al. 2010). TRAP5b has been specifically proposed for the diagnosis and monitoring of the treatment of metastatic bone disease, including breast, prostate, lung, and multiple myeloma (Chao et al. 2010). Bone sialoprotein (BSP) and osteopontin (OPN) are components of the non-collagenous bone matrix and are both expressed in not only osteoclasts and osteoclast-like cells but also in osteoblasts. BSP and OPN have been shown to be essential factors in the bone metastasis of osteotropic cancers from the breast, prostate, and lung (Kruger et al. 2014).

Although various types of cells express receptor activator of nuclear factor kappa B ligand (RANKL), chondrocytes, osteoblasts, and osteocytes are the main RANKL-expressing cells in the bone microenvironment under normal physiological conditions (Nakashima et al. 2012). The expression of RANKL is induced by bone resorption factors such as activated vitamin D_3, PTH, and inflammatory cytokines (Nakashima et al. 2012). RANK, the receptor of RANKL, is expressed on osteoclast precursor cells, and RANK/RANKL signaling activates the transcription factor nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) and controls osteoclastogenesis (Nakashima et al. 2012). Osteoprotegerin (OPG) is a soluble decoy receptor for RANK/RANKL signaling by binding to RANKL (Nakashima et al. 2012), indicating that RANK/RANKL signaling and its control mechanism mediated by OPG play indispensable roles in the bone resorption level by osteoclasts. The free RANKL and OPG-soluble RANKL (sRANKL) complex can be detected in the blood, and the so-called total sRANKL level can be differentially measured as an effective bone resorption biomarker.

Overview of Relaxin Family Peptides and Receptors

Relaxin is well known for its physiological role in the growth and differentiation of the reproductive tract during gestation (Bathgate et al. 2005, 2006, 2013). Relaxin plays roles in reproduction, the cardiovascular system, organ protection, metabolism, cancer metastasis, bone metabolism (including osteoclastogenesis and osteoblast differentiation), and as a neuropeptide in the brain (Fig. 1). Relaxin is a peptide

hormone that belongs to the relaxin-like hormone family, which is part of the insulin/insulin-like growth factor/relaxin-like hormone superfamily (Lu et al. 2005). The relaxin-like hormone family comprises relaxins 1, 2, and 3 (RLN1–RLN3) and insulin-like factors 3, 4, 5, and 6 (INSL3–INSL6). A group of G-protein-coupled receptors known as relaxin family peptide receptors 1–4 (RXFP1–RXFP4) shows tissue- and species-specific affinities to relaxin-like hormones. Relaxin, insulin-like peptide 3 (INSL3), RLN3, and INSL5 interact with RXFP1–RXFP4, respectively (Bathgate et al. 2005, 2006, 2013). Humans and apes possess the *RLN1–RLN3* and *INSL3–INSL6* genes, which encode H1–H3 relaxins and INSL3–INSL6 proteins, respectively (Bathgate et al. 2005). On the other hand, mice possess only *Rln2*, *Rln3*, *Insl3*, *Insl5*, and *Insl6* (Bathgate et al. 2005). Human RLN1 and RLN2, as well as RLN2 in other mammals, are commonly referred to as "relaxin" (Bathgate et al. 2005). RXFPs are G-protein-coupled receptors with seven transmembrane domains anchored to the cell membrane. RXFP1 and RXFP2 have large extracellular domains with characteristic leucine-rich repeats (LRRs) that allow them to bind to RLN1 and RLN2. Accordingly, RXFP1 and RXFP2 were previously referred to as LRR-containing G-protein-coupled receptors 7 and 8 (LGR7 and LGR8), respectively. Because RXFP3 and RXFP4 do not possess a large extracellular domain, they cannot bind to relaxin but they can bind to RLN3 and INSL5. Relaxin inhibits TGF-β-mediated collagen synthesis and increases the expression of MMPs in lung and kidney fibroblasts (Unemori et al. 1996). It also inhibits TGF-β-mediated cardiac fibroblast proliferation and differentiation, as well as collagen synthesis by increasing the expression of MMPs (Mookerjee et al. 2005). RLN3 is most concentrated in the brain, which suggests that it may have neurological effects. RLN3 is involved in stress responses and in the regulation of food intake. INSL4 is most abundant in the maternal decidua and can inhibit fetal growth by increasing cell apoptosis and reducing cell viability. INSL5 is abundant in the colon and is a possible marker of colorectal and neuroendocrine tumors (Bathgate et al. 2006). The activation of RXFP1 increases the accumulation of cyclic adenosine monophosphate (cAMP) and the rapid phosphorylation of mitogen-activated protein kinases 1 and 2 (ERK1/ERK2), resulting in activation of the nitric oxide (NO) signaling pathway. In the connective tissue, the activation of NO inactivates pSMAD2 and transforming growth factor-beta (TGF-β) to produce matrix metalloproteinase (MMP) 1, 2, 9, and 13, resulting in the degradation of collagen in the extracellular matrix. In osteoblast progenitor cells, RXFP2/INSL3 signaling induces ALP activity, extracellular matrix mineralization, and the activation of mitogen-activated kinase (MEK) and ERK1/ERK2. This results in increased production of type I collagen, osteonectin, OPN, TGF-β, macrophage colony-stimulating factor (M-CSF), and the PTH receptor, leading to osteoblast differentiation and osteoclastogenesis (Ferlin et al. 2011). In contrast to RXFP1 and RXFP2, RXFP3 and RXFP4 inhibit the activation of cAMP. RXFP3 activation induces the phosphorylation of MEK and ERK1/ERK2 and the activation of nuclear factor kappa B, subunit 1. RXFP4 increases intracellular calcium ion concentrations, although the mechanism of this effect remains unclear.

The Roles of Relaxin in Bone Development, Metabolism, and Disease

The Effects of Relaxin and Its Receptor on Craniofacial Skeletal Development and Osteoblast Activity

The craniofacial skeletal tissues are of mesenchymal origin, derived from the cranial neural crest and/or mesoderm. The boundary between these two types of mesenchyme is reported to lie between the parietal and frontal bones in the cranial vault and between the basisphenoid and basioccipital bones in the cranial base (Chai and Maxson 2006; Morriss-Kay 2001). The mechanism of skeletal development is divided into two main categories: intramembranous ossification and endochondral ossification. Calvarial bones, with the exceptions of a part of the great wing of the sphenoid bone, and facial bones, with the exception of the inferior nasal concha, ethmoid bone, and hyoid bone, are developed by the intramembranous ossification, which is characterized by direct ossification by the osteoblasts contained in mesenchymal cellular aggregates. The membranous bones are connected to each other by dense fibrous connections called sutures. Despite the wide-ranging effects of RLNs and RXFPs, their specific expression pattern and their effects on craniofacial skeletal development have scarcely been examined. A large number of signaling molecules have been described as modulators of craniofacial skeletal morphogenesis. Bone morphogenetic protein (BMP) 2 and 4 induce intramembranous ossification and guide the morphological changes of the craniofacial bones (Farhadieh et al. 2004). Runx2 is a signaling molecule exclusive to osteoblasts (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997) that is found in the cells of the osteoblastic lineage in embryonic mesenchymal condensations (Ducy et al. 1997) and mediates the commitment of mesenchymal cells into osteoblasts. The embryonic formation of craniofacial bones, especially the calvarial vault and mandibular bones, depends on the specific temporospatial expression of Runx2 (Maeno et al. 2011). After an osteoblast progenitor is committed to differentiate into an osteoblast, its maturation is characterized by BAP and collagen type 1 α1 (COL1α1) secretion into the extracellular matrix. BAP, a bone formation biomarker, is expressed in the osteoid and mineralized bone matrix of craniofacial bones and is induced by BMPs (Kim et al. 2004). COL1 is the main component of the bone matrix where minerals are deposited. The expression of *Col1α1*, the gene encoding Col1, is induced by both BMPs and Runx2 (Ortuno et al. 2013). OPN is expressed in well-differentiated osteoblasts and in more mature osteogenic tissues (Strauss et al. 1990). Along with OPN, mature osteoblasts express OCN, another bone formation marker, and bone sialoprotein (BSP). OCN is the protein responsible for the binding of calcium ions to the osteoid, and OPN induces the formation of mineral crystals. Fine-tuning of the tempo-spatial expression and function of OCN and OPN is indispensable for the proper development and growth of the craniofacial bones. Relaxin synergistically enhances BMP-2-induced osteoblast differentiation and bone formation through its receptor

Rxfp1 by augmenting and sustaining Smad and p38 phosphorylation, which in turn enhances Runx2 expression and activity (Moon et al. 2014). In addition, it has been shown that *Rxfp1* and *Rxfp2* mRNAs are expressed in the developing calvarial frontal bones (Duarte et al. 2014b; Fig. 2) and facial bones (Duarte et al. 2014b; Fig. 3). Relaxin was shown to enhance osteoblastic differentiation, mediated by enhanced *Runx2* expression and upregulation of BAP activity, which was

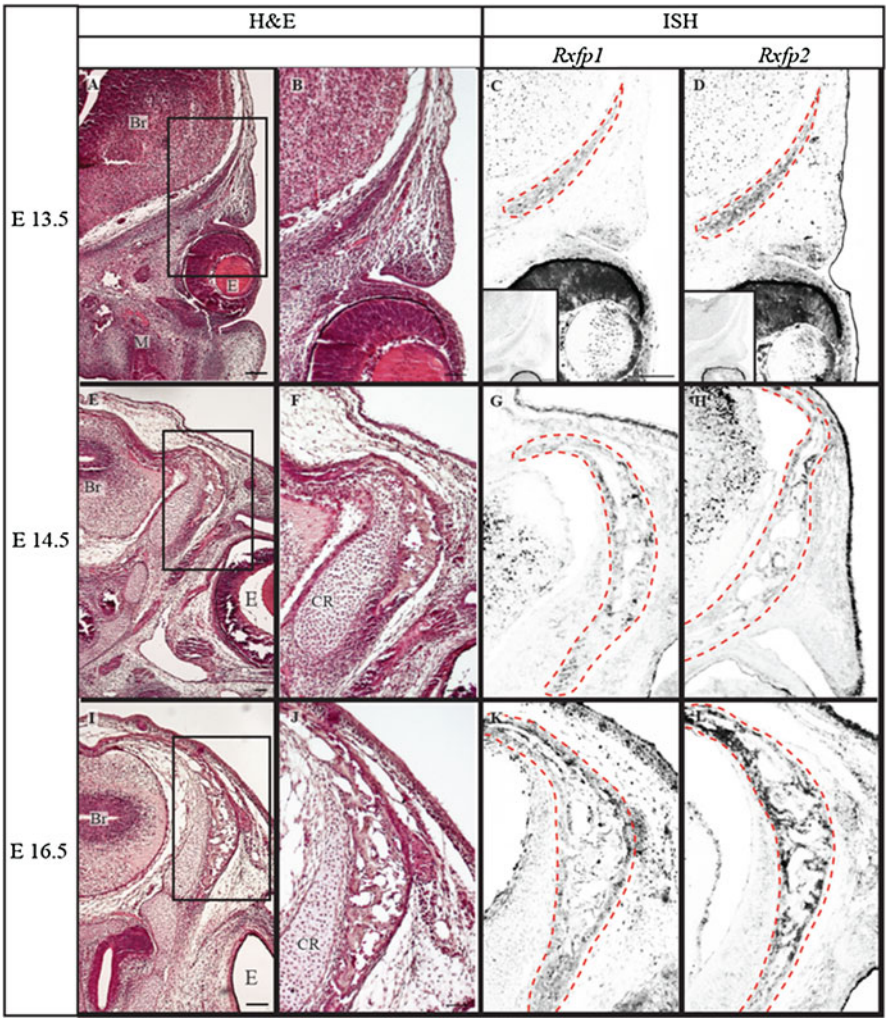

Fig. 2 Expression of Relaxin/insulin-like family peptide receptors (Rxfp) 1 and 2 in developing mouse calvarial frontal bones. Both Rxfp1 and 2 mRNA in the osteoblasts of the developing murine calvarial frontal bone from E13.5 to E16.5 by in situ hybridization (C, D, G, H, K, and L). Br, brain; CR, cartilaginous precursor of the cranial base; E, eye; and M, molar. Data from Duarte et al (Duarte et al. 2014a) with permission from the publisher.

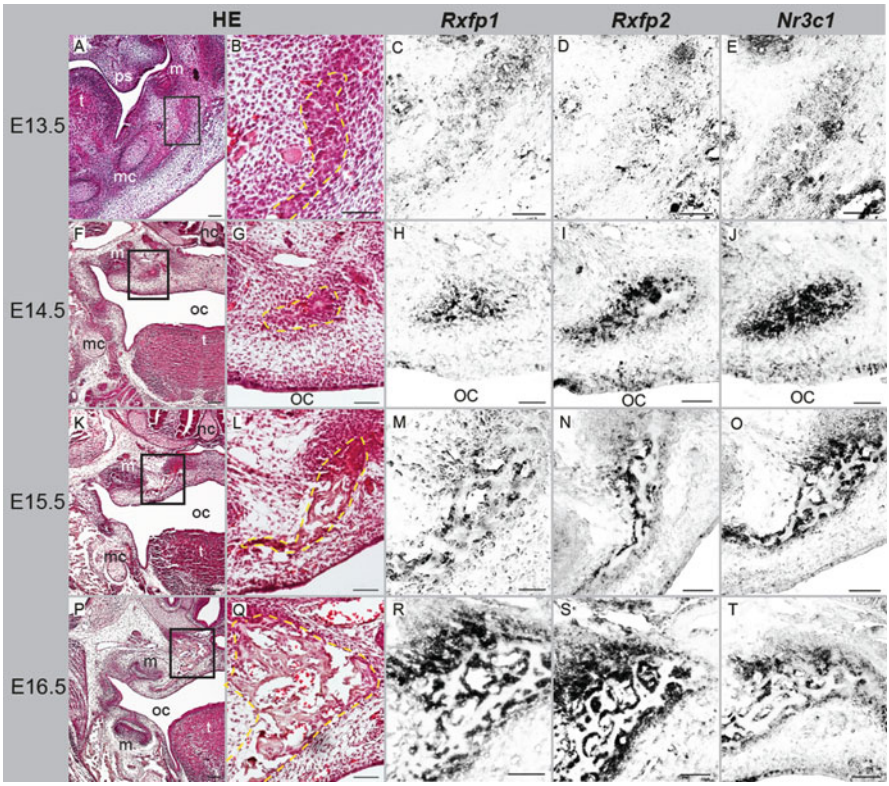

Fig. 3 Expression patterns of Rxfp1 and Rxfp2 during the development of the mandibular bone (E13.5) and maxillary bone (E14.5–E18.5) determined by in situ hybridization. Expression pattern of Rxfp1, Rxfp2 was observed in the mesenchymal condensations of the mandibular bone at E13.5 (C, D), and in the ossifying maxilla from E14.5 to E16.5 (H, I, M, N, R, and S). m, molar; mc, Meckel's cartilage; nc, nasal cartilage; oc, oral cavity; ps, palatal shelf; t, tongue. Data from Duarte et al (Duarte et al. 2014a) with permission from the publisher.

accompanied by ERK1/ERK2 phosphorylation through *Rxfp2* expression (predominant over *Rxfp1*) in MC3T3-E1 mouse calvarial osteoblasts, and thereby enhanced in vitro mineralization (Duarte et al. 2014b; Figs. 4 and 5). Moreover, relaxin has an effect on osteoblast mineralization, which is presumably derived from its capacity to increase the activities of MMP2 and MMP13 in osteoblasts (Duarte et al. 2014b; Fig. 5). These findings suggest a novel role for relaxin in craniofacial skeletal development and metabolism through Rxfp.

The Effects of Relaxin on Osteoclast Activation

The mature osteoclast, which arises from hematopoietic precursors, exhibits multinucleated giant cells and plays an indispensable role in bone resorption in the

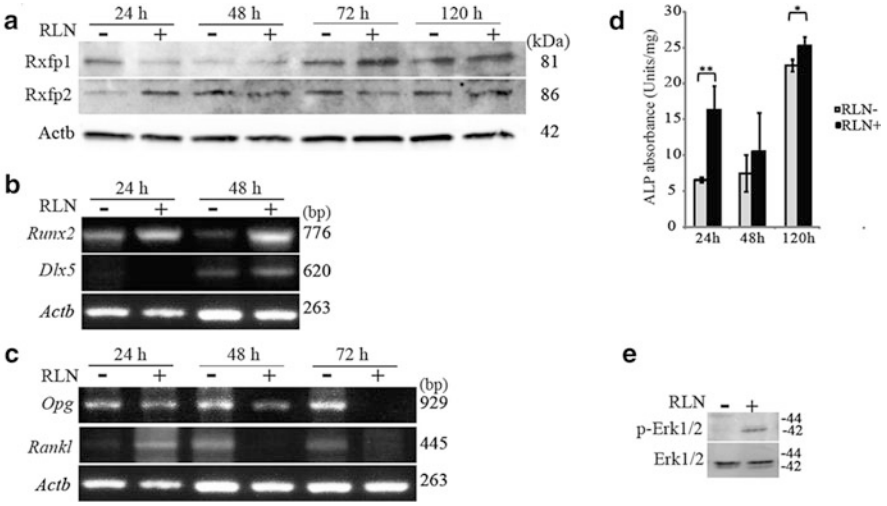

Fig. 4 RLN enhanced alkaline phosphatase (ALP) activity and Erk1/2 phosphorylation of mouse calvarial osteoblast cell line MC3T3-E1 cells. (A) RLN (20 ng/mL) enhanced Rxfp2 expression in MC3T3-E1 mouse calvarial osteoblasts, but inhibited expression of Rxfp1 after 24 h determined by Western blot analysis. (B and C) RLN enhanced expression of Runx2 after 48 h and Bmp2 after 72 and 120 h in MC3T3-E1 cells cultured in differentiation medium. On the other hand, RLN inhibited Opg and Rankl expression after 48 and 120 h culture period. (D) ALP activity and (E) ERK 1/2 phosphorylation were significantly increased by RLN (20 ng/mL) after 24 h culture in differentiation medium. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Data from Duarte et al (Duarte et al. 2014b) with permission from the publisher.

skeletal tissues. The differentiation and function of osteoclasts are strictly controlled by osteoblastic lineage cells, including osteoblasts, osteocytes, and stromal marrow cells. These cells express important cytokines such as M-CSF and RANKL. On the other hand, osteoclast progenitor cells express the transmembrane receptor tyrosine kinase c-FMS, which binds to M-CSF and RANK. The majority of the calcium-regulating hormones and cytokines that promote bone resorption induce the expression of RANKL in osteoblastic lineage cells. Osteoclasts secrete the soluble receptor OPG, which inhibits the RANK-RANKL interaction as a decoy receptor for RANKL. RANKL and M-CSF activate various intercellular signaling molecules to ultimately promote the expression of NFATc1, a master transcriptional factor for inducing osteoclast differentiation. Interestingly, human peripheral blood monocyte cells (PBMCs), the precursors of osteoclasts, were found to express *RXFP1* mRNA and respond to RLN2 by increasing the levels of tumor necrosis factor-α and interleukin-1β secretion (Kristiansson et al. 2005). Furthermore, primary cultured mature human osteoclasts derived from human PBMCs were shown to express *RXFP1* mRNA (Facciolli et al. 2009). Relaxin regulates the recruitment of leukocytes to the sites of inflammation and plays a role in the substrate adhesion and migration of mononuclear leukocytes (Figueiredo et al. 2009). RLN also influences bone metabolism by inducing osteoclastogenesis and the activation of mature

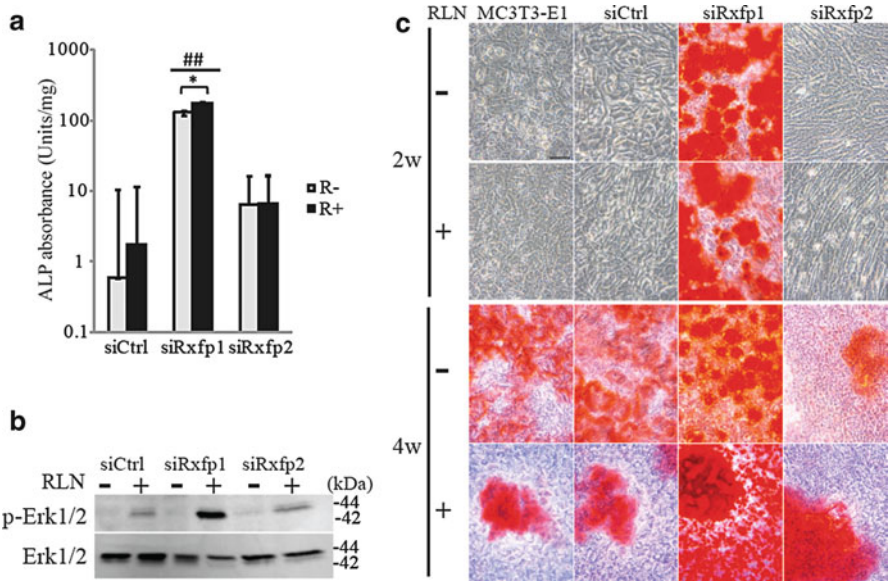

Fig. 5 RLN/Rxfp2 signaling enhanced ALP activity through Erk1/2 phosphorylation and enhanced mineralization of MC3T3-E1 cells. (A) ALP activity in siRxfp1 and siRxfp1/2, which means partially down regulation of Rxfp1/2 in cells, was significantly higher than in control and siRxfp2. RLN significantly increased ALP activity in siRxfp1 cells after 24 h culture. (B) Erk1/2 phosphorylation was observed in control, siRxfp1, and siRxfp2 samples after 24 h RLN (20 ng/mL) treatment; however, it was markedly higher in Rxfp1 cells. Phosphorylation of ERK1/2 in siRxfp1/2 cells was observed independent of RLN administration. (C) Significantly enhanced matrix mineralization was observed in siRxfp1 and siRxfp1/2 cells after 2 weeks culture with or without RLN administration. Data from Duarte et al (Duarte et al. 2014b) with permission from the publisher.

osteoclasts expressing RXFP1 through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), RANK, TRAP, and cathepsins (Ferlin et al. 2010; Hombach-Klonisch et al. 2006). Administration of RLN combined with estrogen increased OPG and decreased the RANKL/OPG protein ratio more than administration of estrogen alone in an adjuvant-induced arthritis rat model of rheumatoid arthritis (Ho et al. 2011). These observations indicate a new pharmacological role of relaxin in controlling bone resorption.

The relationship between INSL3/RXFP2 signaling and osteoporosis has been a topic of research interest. A comprehensive study involving clinical, biochemical, and hormonal analyses, including bone densitometry analysis through DXA, showed that 64% of young men with an *RXFP2* gene mutation resulting in a T222P amino acid substitution presented significantly reduced bone mineral density; their testosterone levels and gonadal function were normal, and no other apparent cause of osteoporosis was evident (Ferlin et al. 2008). Human and mouse osteoblasts express *RXFP2/Rxfp2*, and administration of INSL3 to these osteoblasts results in cAMP production to affect cell proliferation in a dose- and time-dependent manner. In support of the human phenotype, bone histomorphometric and computed tomography analyses of

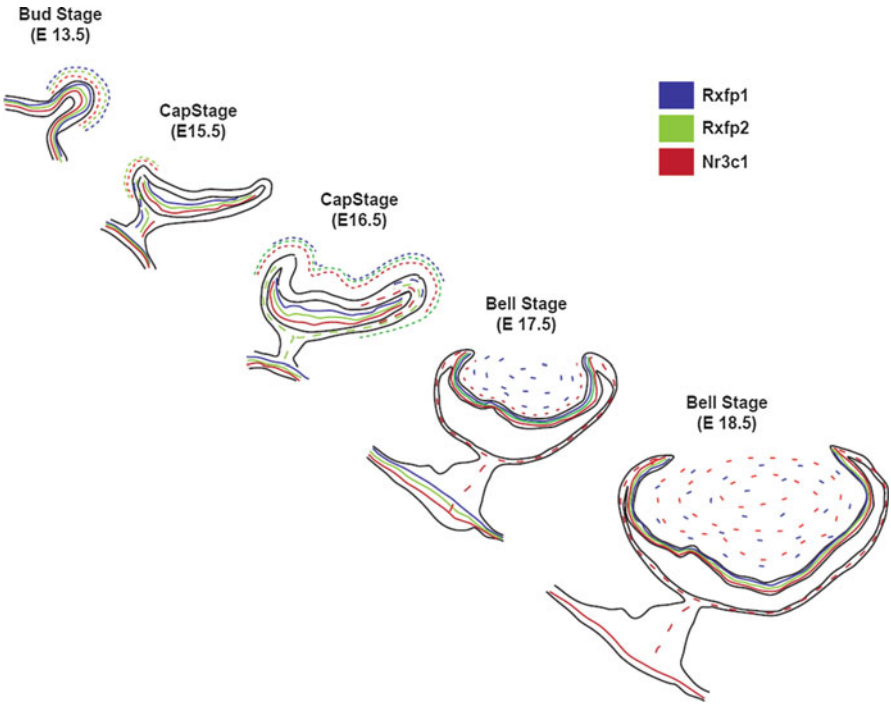

Fig. 6 Diagram of the mRNA expression patterns of relaxin family peptide receptors 1 and 2 (Rxfp1 and Rxfp2) and the glucocorticoid receptor (Nr3c1) during upper molar development. Data from Duarte et al (Duarte et al. 2014a) with permission from the publisher.

Rxfp2-deficient mice revealed a decreased bone mass, mineralizing surface, bone formation, and osteoclast surface compared with their wild-type littermates. These results suggest a significant role of INSL3/RXFP2 signaling in bone metabolism and link *RXFP2* gene mutations with human osteoporosis (Ferlin et al. 2008).

Given the physiological function of relaxin family peptides and RXFP2 on bone development, remodeling, metastasis, and metabolic disorders, these molecules are promising candidates as biomarkers to accurately reflect bone health and multiple bone-related disorders.

Potential Applications of Relaxin to the Prognosis of Other Diseases or Conditions

Relaxin and Orthodontic Treatment

A necessary and adequate force is required for orthodontic tooth movement, which is accompanied by remodeling of the soft tissue, including the periodontal ligament (PDL) and gingival tissue, and the alveolar bone supporting the teeth. Many

molecules, including local and systemic factors, have been suggested to be involved in the remodeling process of the alveolar bone and PDL during and after tooth movement, which influence on the rate of tooth movement, stability, and relapse after the treatment. The orthodontic compressive force induces alveolar bone resorption by osteoclasts, which is accompanied by the reorganization of periodontal soft tissues. Simultaneously, bone formation from active osteoblasts occurs at the alveolar bone surface of the tension side. Given the broad physiological activity of RLN, several experiments have been performed to test its clinical feasibility in orthodontic treatment (Hirate et al. 2012; Mcgorray et al. 2012; Yang et al. 2011). There are conflicting views regarding the action of relaxin on periodontal tissues. Relaxin was shown to reduce the level of PDL organization and mechanical strength, and to increase tooth mobility at early time points, but did not accelerate orthodontic tooth movement in rats (Madan et al. 2007). Relaxin receptors are localized in PDL fibroblasts (Stewart et al. 2005) and were found to decrease the expression of the collagen type I gene and increase the expression of MMP1 in stretched human PDL cells (Takano et al. 2009). The orthodontic force enhanced the expression of *Rln1* mRNA and the synthesis of Rln1 protein in the granulosa cells of the rat ovary (Yang et al. 2011). Accordingly, it is tempting to speculate that the RLN1 expressed in the ovary during orthodontic tooth movement might affect osteoclastogenesis on the pressure side and collagen turnover on the tension side. This speculation can be supported by other reports showing that relaxin may accelerate orthodontic tooth movement (Liu et al. 2005). The velocity of orthodontic tooth movement has been shown to be influenced by the hormones released during pregnancy (Hellsing and Hammarstrom 1991), and the levels of relaxin mRNA are altered in the rat ovary during pregnancy (Crish et al. 1986). However, it is not yet certain whether the increased expression of *Rln1* may directly affect tooth movement. Relaxin has been reported to be present in the cranial suture and PDL (Nicozisis et al. 2000). It has also been suggested that the effect of relaxin on PDL remodeling might reduce the rate of relapse after orthodontic treatment (Masella and Meister 2006). However, a randomized clinical trial was performed on humans given weekly injections of 50 μg of RLN or a placebo control for 8 weeks. Tooth movement was measured weekly by impressions. There was no significant difference between the RLN and placebo control groups regarding the acceleration and relapse rates, presumably quite low dose of RLN at the periodontal area (Mcgorray et al. 2012). The concentration of RLN in the bloodstream might reflect the efficacy of tooth movement, the adequacy of the orthodontic force, or the likelihood of relapse after orthodontic treatment. Nevertheless, the precise mechanism and effects of relaxin and RXFPs on orthodontic tooth movement or relapse should be elucidated with detailed investigations.

Summary Points

- Bone homeostasis is maintained by fine-tuning of the dynamic balance of "remodeling," which refers to the removal of old bone and subsequent formation of new bone by osteoclasts and osteoblasts, respectively, at the basic multicellular unit.

- Currently, the biomarkers used to assess bone metabolism are categorized into two groups: bone resorption markers and bone formation markers. The soluble molecules that are specifically secreted from osteoblasts or osteoclasts and the metabolic products of type 1 collagen are generally used as biomarkers of bone metabolism.
- RLN is an insulin-like peptide hormone that facilitates parturition by inducing the softening and lengthening of the pubic symphysis and softening of the cervix.
- RLN inhibits fibroblast proliferation and differentiation, but increases the production of matrix MMPs, resulting in a strong antifibrotic effect.
- RLN also influences bone metabolism by inducing osteoclastogenesis and activates mature osteoclasts through RXFP1.
- Significant bone loss, which can lead to osteoporosis, was observed in a large number of men with mutated *RXFP2* and in *Rxfp2*-deficient mice.
- In vivo administration of Rln enhanced BMP-2-induced bone formation through Rxfp1 by augmenting and sustaining Smad and p38 phosphorylation induced by BMP-2. This activated intracellular signaling upregulated Runx expression and activity, which enhanced osteoblast differentiation.
- These results suggest that RLNs can be useful for therapeutic applications in bone metabolic disorders and that RLN and RXFPs might be candidate biomarkers that reflect bone health and disease status.

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Pentosidine as a Biomarker for Poor Bone Quality and Elevated Fracture Risk

16

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Abstract

The purpose of this chapter is to explore the concept of pentosidine, an advanced glycation end product, as a biomarker of bone quality and bone fragility. Bone quality is a term used to describe the many factors that contribute to fracture risk that are not necessarily captured during clinical fracture assessment. Pentosidine formation in the body requires glycation and oxidation of proteins. In bone, the protein that is most affected is type 1 collagen. Pentosidine forms divalent cross-links between adjacent type 1 collagen molecules. Pentosidine content can be assessed in systemic fluids (urine and serum) and in bone tissue using various methods, including high-performance liquid chromatography (HPLC) and more recently, mass spectrometry (MS). When assessed in urine and serum, most studies report an association between pentosidine and prevalent or incident fracture. The risk of incident fracture appears to be 3–42% higher for elevated levels of pentosidine in serum or urine. However, these results may be confounded by overall bone turnover rate, as gold standard bone turnover markers, procollagen type 1 N propeptide of type 1 collagen (P1NP), and C-terminal cross-linking telopeptide of type 1 collagen (CTX) are not accounted for in regression models. There are other inconsistencies in the results related to disease status (i.e., type 2 diabetes diagnosis), ethnicity (i.e., Japanese vs. Caucasian), and sex. When assessed in bone samples, pentosidine accumulation in cortical bone is negatively related to measures of bone toughness (such as ductility, work-to-fracture), but inconsistent results have been reported in trabecular bone. Also, correlation is not causation and in vitro models do not completely account for all changes occurring in the bone material with aging and disease. Pentosidine content in bone tends to increase in an age-dependent manner, and different diseases can accelerate the accumulation of pentosidine. Studies in animal models of type 2 diabetes, type 1 diabetes, low and high turnover chronic kidney disease, and postmenopausal osteoporosis have shown elevated levels of bone pentosidine and altered amounts of enzymatic cross-links (lysyl oxidase [LOX]-dependent cross-links). As these diseases are also associated with higher fracture risk, the hypothesis is that pentosidine contributes to fracture risk. However, whether pentosidine plays a causal role in degrading bone mechanical properties and increasing fracture risk remains to be elucidated. The role of pentosidine as a biomarker for bone quality and fracture risk requires more research in the areas of determining whether systemic pentosidine is better at predicting fractures and is superior to other *gold standard* bone turnover markers (s-CTX, s-P1NP), determining whether the link between bone-specific pentosidine and bone mechanical properties is consistent in trabecular and cortical bone, determining relationships between bone-specific and systemic pentosidine in diseases with high and low bone turnover, and accounting for differences in resorption rate, ethnicity, and sex. In addition, standard quality assurance measures must be addressed (i.e., specimen stability, variability) for pentosidine to be a biomarker for bone quality and fracture risk.

Keywords

Pentosidine • Bone quality • Fracture • Fracture risk • Osteoporosis • Biomarker • Bone turnover markers • Advanced glycation end product • Nonenzymatic cross-link

List of Abbreviations

AGE	Advanced glycation end product
BAP	Serum bone-specific alkaline phosphatase
BMD	Bone mineral density
CTX	C-terminal cross-linking telopeptide of type 1 collagen
deH-DHLNL	Dehydro-dihydroxylysinonorleucine
deH-HLNL	Dehydro-hydroxylysinonorleucine
DPD	Deoxypyridinoline
DPL	Deoxypyrrololine
DXA	Dual x-ray absorptiometry
ELISA	Enzyme-linked immunosorbent assay
HPLC	High-performance liquid chromatography
HR	Hazard ratio
LOX	Lysyl oxidase
OR	Odds ratio
P1NP	Procollagen type 1 N propeptide of type 1 collagen
PYD	Pyridinoline
PYL	Pyrrololine
RR	Relative risk
u-NTX	Urinary N-terminal telopeptide of type 1 collagen

Key Facts About Biomarkers, Bone Quality, and Fracture Risk

1. A biomarker must be easily accessible and sampled from peripheral fluids or tissue, easily measured using affordable and good quality assays, and levels must be associated with pathology.
2. Fractures due to osteoporosis and other bone-affecting diseases are associated with a significant burden to the healthcare system, are debilitating, and increase the risk of death.
3. Fracture risk depends on various factors, including clinical risk factors, and the structural and material composition of bone.
4. Enzymatic cross-linking of type 1 collagen in bone contributes to overall bone tissue strength and toughness and is necessary for good bone quality.
5. Nonenzymatic cross-linking of type 1 collagen is caused by glycation of protein and/or damage associated with oxidative stress.
6. Current bone biomarkers used to predict fracture risk are actually bone turnover markers rather than biomarkers for bone quality.

Key Facts about Pentosidine

1. Pentosidine is an advanced glycation end product also known as a nonenzymatic cross-link that forms between type 1 collagen molecules in bone (and many other tissues).
2. One way that pentosidine is formed in the body is through the Maillard reaction.
3. Pentosidine accumulates in bone and other tissues and therefore when the tissue is turned over, pentosidine can be measured in systemic circulation (urine and serum).
4. Systemic pentosidine is positively related to other bone turnover markers, such as serum procollagen type 1 N propeptide of type 1 collagen (s-P1NP) and serum C-terminal cross-linking telopeptide of type 1 collagen (s-CTX).
5. Most studies report a positive relationship between systemic pentosidine and incident fracture; however, they do not account for bone turnover.
6. Other factors such disease status, ethnicity, and sex also influence the relationship between systemic pentosidine and fracture.
7. When measured in bone tissue (in situ), the accumulation of pentosidine in bone is negatively correlated with bone quality, strength, and toughness.
8. To improve systemic pentosidine measured in urine or serum for use as a biomarker for bone quality and fracture prediction, future research should investigate normalizing its concentration to the concentration of an established bone resorption biomarker such as CTX.

Definition of Words and Terms

Advanced glycation end product	Spontaneously forming reaction products initiated by glycation by glucose and metabolic products.
Biomarker	A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (NIH 2001).
Bone mineral density (BMD)	A clinical measure of bone content/mass/volume detected using the attenuation of x-rays caused by the mineral phase of bone and measured using a dual-energy x-ray attenuation (DEXA) instrument.
Bone quality	An umbrella term for characteristics of bone at the tissue level that influence the bone's resistance to failure or fracture and properties that influence the unpredicted portion of fracture risk in assessment tools relying on BMD.

Bone turnover marker	Biomarkers detected in the serum and urine as a result of bone formation or resorption processes.
Enzymatic cross-link	A protein cross-link formed due to catalysis by an enzyme. In bone collagen, the enzyme is lysyl oxidase (LOX).
Fracture risk	Statistical risk of fracturing a bone.
Immature enzymatic cross-link	Early-stage divalent cross-links catalyzed by LOX
Mature enzymatic cross-link	Later-stage trivalent cross-links resulting from the reaction of immature LOX cross-links with an additional lysine or hydroxylysine.
Nonenzymatic cross-link	A covalent cross-link formed between or within proteins by spontaneous organic chemistry, usually associated with advanced glycation end products.
Osteoporosis	A disease characterized by low bone mineral density (BMD) and bone micro-architectural deterioration resulting in an increased risk of fracture.
Pentosidine	A divalent, naturally fluorescent advanced glycation end product that cross-links between a lysine and an arginine, found in collagen.

Introduction

This chapter explores the concept of pentosidine as a biomarker of poor bone quality and fracture risk. Given the growing global burden of diseases that are associated with fractures, such as osteoporosis and type 2 diabetes, there is a need to improve the detection of poor bone quality and improve fracture risk prediction in order to prevent future fractures. The National Institutes of Health (NIH) Biomarkers Definitions Working Group defines a biomarker (biological marker) as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (NIH 2001). According to the Working Group, a good biomarker has three attributes: (1) it must be present in easily accessible and sampled peripheral body tissue and/or fluid; (2) it must be easy to detect or quantify in affordable and robust assays; and (3) its appearance must be associated as specifically as possible with quantifiable damage of a particular tissue (NIH 2001).

Pentosidine is an advanced glycation end product that can accumulate in bone and is released into systemic fluids (urine and serum) when bone is turned over. Borrowing from a commentary about pentosidine as a biomarker for osteoarthritis (Felson and Lohmander 2009), we can similarly state that pentosidine is a biomarker

for poor bone quality and elevated fracture risk, as the accumulation of pentosidine in bone is correlated with a loss of bone strength and toughness, and systemic levels of pentosidine are related to elevated fracture risk.

Bone-Affecting Diseases, Fracture Risk, and Bone Quality

Motivation

The costs associated with musculoskeletal diseases in the United States are approximately $874 billion USD annually, as over 50% of older adults are affected by musculoskeletal diseases causing injury (Yelin and Watkins-Castillo 2015). Osteoporosis is a chronic musculoskeletal disease that is common among older adults and is characterized by low bone mineral density (BMD) and microarchitecture deterioration leading to increased fracture risk (Tenenhouse et al. 2000). In men and women, fractures due to osteoporosis include nontraumatic fractures of the hip, vertebrae, wrist, proximal humerus, rib, pelvis, clavicle, scapula, and sternum (Kanis et al. 2001). The most serious health and economic consequences are associated with hip and vertebral fractures, as the cost per care of hip and vertebral fracture is highest compared to other fracture types (Tarride et al. 2012; Kaffashian et al. 2011) and hip and vertebral fractures decrease quality of life and increase the risk of death (Ioannidis et al. 2009).

Clinical Assessment of Fracture Risk

Given the economic and health consequences associated with fractures, understanding a patient's fracture risk is an essential step to preventing fractures through the use of pharmacotherapy and other interventions. The most commonly used and internationally recognized tool is the FRAX^{TM} 10-year fracture risk prediction algorithm (Kanis et al. 2011). This tool takes into consideration the presence of various clinical risk factors (i.e., age, previous fracture, use of glucocorticoid medication, etc.) to provide a 10-year probability of hip and major osteoporotic fracture. One of the commonly used indicators of skeletal health is bone mineral density (BMD), which can be factored into the FRAX algorithm. BMD is most often assessed using dual x-ray absorptiometry (DXA), which measures the attenuation of x-rays at the hip and lumbar spine (L1–L4), providing an indirect measure of amount of bone mineral content in a projected area (g/cm^2) (Pacifici et al. 1988). BMD plays an important role in overall fracture risk prediction, as with each standard deviation (SD) decrease in femoral neck BMD, the risk of hip fracture increases by 2–3 times in men and women (risk ratio [RR] 2.94 [95% confidence interval (CI) 2.02–4.27] in men and RR 2.88 [95% CI 2.31–3.59] in women) (Johnell et al. 2005). However, BMD does not entirely explain variations in bone strength and fracture occurrence. For example, fractures occur even in older adults with BMD measurements in the non-osteoporotic range (Cranney et al. 2007), and very small improvements in BMD (~4%) have been reported in patients who have taken

osteoporosis-related medication, which do not fully explain the substantial reduction in fracture risk (~50%) (Cummings et al. 2002). Therefore, there are other factors that contribute to skeletal health.

What is Bone?

Bone is a composite material composed of a mineral phase, an organic phase, and an aqueous phase. Bone mineral comprises approximately 50–70% of bone material by mass (approximately 45% by volume) and is believed to be in the form of highly substituted hydroxyapatite. The major non-mineral constituent of bone is osteoid, which is synthesized and secreted by osteoblasts during bone formation. It constitutes approximately 45% of bone tissue volume. Osteoid, which is 85–90% type 1 collagen, provides toughness to bone (Zioupos 2001; Fantner et al. 2004). Osteoid acts as a template for bone mineralization, by providing nucleation sites for crystal formation and subsequent growth (Boskey 1998). The hydroxyapatite aggregates into nanoscale mineral structures in the gap zones within the fibrils and surrounding the collagen fibrils (McNally et al. 2012). Mineralization stiffens osteoid, providing important biomechanical functionality.

Enzymatic Collagen Cross-Links

Collagen molecules are linked together by action of enzymes (lysyl oxidases [LOX]), which convert hydroxylysine and lysine residues in the telopeptide domains of the type 1 collagen molecules into hydroxyallysine and allysine, respectively (Jepsen et al. 1997; Uzawa et al. 1999; Robbins and Bailey 1977). The pattern of formation of cross-links also depends on lysine hydroxylation by lysyl hydroxylases. These two enzymes are responsible for the formation of immature divalent cross-links: dehydro-dihydroxylysinonorleucine (deH-DHLNL), dehydro-hydroxylysinonorleucine (deH-HLNL), and dehydro-lysinonorleucine. Immature cross-links undergo condensation reactions to form trivalent mature cross-links: pyridinoline (PYD; a.k.a hydroxylysylpyridinoline), deoxypyridinoline (DPD; a.k.a.lysylpyridinoline), pyrrololine (PYL; a.k.a. hydroxylysyl-pyrrole), and deoxypyrrololine (DPL; a.k.a. lysyl-pyrrole) (Saito and Marumo 2015; Eyre et al. 1984). Generally, the immature cross-links outnumber mature pyridinoline cross-links by 2–4 times and are believed to strongly contribute to bone toughness (Saito and Marumo 2010). For the purposes of this chapter, we will refer to the LOX cross-link types as immature and mature LOX cross-links or enzymatic cross-links.

There are various factors affecting LOX cross-linking. For example, vitamin B6 (pyridoxal phosphate) and copper-dependent tyrosyl-lysine quinone are important cofactors for LOX (Opsahl et al. 1982; Masse et al. 1996), and LOX-mediated cross-linking is inhibited in cases of vitamin B6 and copper deficiencies (Opsahl et al. 1982; Masse et al. 1996). It can also be inhibited by β-amino-propionitrile (Oxlund et al. 1995). These inhibitions result in a condition termed lathyrism, known for weak collagenous tissues, including weak, brittle bone (Oxlund et al. 1995). LOX expression is also affected by transforming growth factor beta (TGF-β), tumor

necrosis factor alpha (TNF-α), fibroblast growth factor (FGF), prostaglandin E_2, connective tissue growth factor (CTGF), insulin-like growth factor 1 (IGF-1), estrogen, and vitamin D3 (1,25-dihydroxyvitamin D3) (Saito and Marumo 2015). Homocysteine also inhibits LOX-mediated cross-linking by negatively regulating LOX expression and also by capping allysine and hydroxyallysine sites, presumably with its amine group reacting with the aldehyde of these cross-linking precursors (Saito and Marumo 2015).

Nonenzymatic Collagen Cross-Links

Pentosidine, shown in Fig. 1, is a divalent covalent cross-link that forms between collagen molecules in their helical domains (Biemel et al. 2001; Sell et al. 1991). It belongs to a family of spontaneously forming glycation-dependent chemical entities called advanced glycation end products (AGEs) (Dyer et al. 1991b). Pentosidine is a divalent cross-link in the sense that it cross-links between sites on two adjacent collagen molecules, just as immature LOX-catalyzed enzymatic cross-links are divalent. Unlike the immature LOX cross-links, pentosidine bridges between a lysine residue and an arginine residue rather than a lysine and a hydroxylysine (Biemel et al. 2001; Sell et al. 1991). The formation of pentosidine is believed to occur by the Maillard reaction (Dyer et al. 1991a). Briefly, an aldose or reactive carbonyl metabolite reacts with an ε-amine group on a lysine, forming a Schiff's base through the process of glycation (Dyer et al. 1991a). Through Amadori rearrangements and a late oxidation step, the pentosidine structure is formed (Dyer et al. 1991a). Notably, pentosidine is most readily formed in vitro by ribose and formation requires first glycation and subsequent oxidation (Grandhee and Monnier 1991).

Similar to the formation of mature enzymatic cross-links, DPD and PYD, pentosidine is stable against low pH, high temperatures, and irradiation. It is naturally fluorescent, making it relatively easy to isolate and detect using acid hydrolysis and fluorescence detection, respectively (Bank et al. 1997; Saito

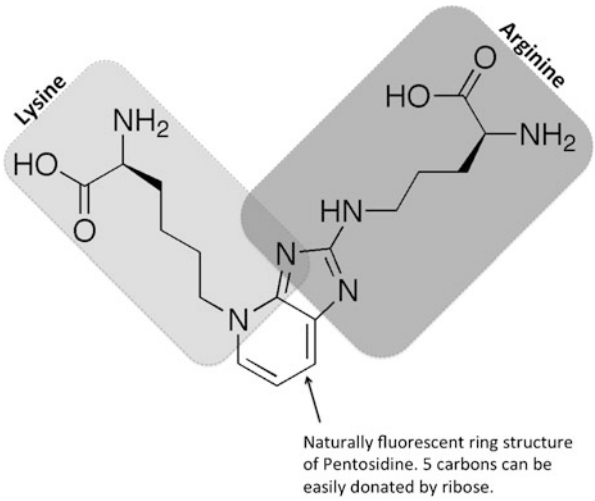

Fig. 1 The chemical structure of pentosidine

et al. 1997). Over the last few decades, multiple high-performance liquid chromatography (HPLC) (Bank et al. 1997; Saito et al. 1997) and enzyme-linked immunosorbent assay (ELISA) methods have been established for its detection in serum, urine, and other tissues (Takahashi et al. 1996; Izuhara et al. 1999). Pentosidine can be detected in bone using its characteristic excitation and emission wavelengths of 335 and 385 nm, respectively (Bank et al. 1997; Saito et al. 1997). Relatively simple reversed-phase HPLC methods combine pentosidine quantification with pyridinoline cross-link quantification (Bank et al. 1997), and more comprehensive techniques using ion exchange columns and post-column derivatization include immature LOX cross-links (Saito et al. 1997). Recently, higher performance techniques have been established using mass spectrometry (Gineyts et al. 2010a). Both enzymatic and nonenzymatic cross-links in bone contribute to overall bone strength and therefore can be described as important bone qualities.

The *Bone Quality* Framework

According to a consensus reached by experts at the National Institutes of Health Bone Quality Conference, *bone quality* is an umbrella term for characteristics of bone at the tissue level that influence bone's resistance to failure or fracture and properties that influence the unpredicted portion of fracture risk in assessment tools relying on BMD (Fyhrie 2005). It is a rather vaguely defined concept. Figure 2

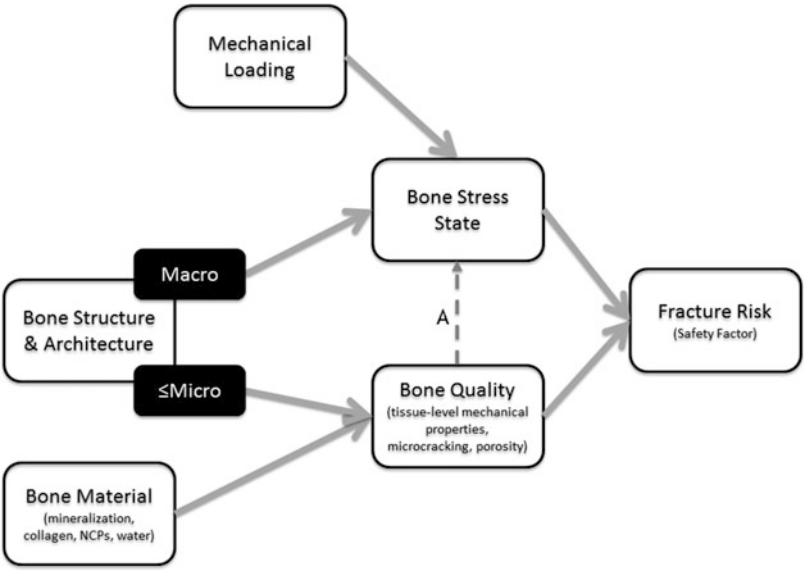

Fig. 2 The bone quality paradigm extended to determining fracture risk. The *dashed arrow* marked with *A* indicates that determinants of bone quality such as micro-cracking and porosity can affect the tissue-level stress state locally

displays the concept of bone quality and specific factors that influence the propensity of a bone to fracture. In basic terms, bone quality considers bone as a material (i.e., at the tissue level) that has nanoscale, microscopic, and macroscopic characteristics (anatomy, geometry) that influence mechanical properties and resistance to fracture. Bone size, shape, trabecular bone microarchitecture, and cortical bone thickness affect fracture propensity (Boonen et al. 1995; Faulkner et al. 1993; Gordon et al. 1998; Parfitt et al. 1983; Zebaze et al. 2010). In addition, material tissue-level influences (bone qualities) such as the accumulation or removal of micro-cracks, porosity, mineralization and crystallinity, collagen denaturation/degradation, and covalent cross-links between collagen molecules also affect bone strength (Zioupos et al. 1999; Mashiba et al. 2000; Paschalis et al. 1997; Saito et al. 2006c).

Modifications in bone material quality, specifically the type and amount of type 1 collagen cross-links, affect fracture propensity (Saito and Marumo 2010). Ex vivo studies have shown that when the amount of mature enzymatic cross-links is reduced, bone strength decreases (Oxlund et al. 1996). This is because type 1 collagen is responsible for providing strength, ductility, and toughness to bone (Garnero 2015). Toughness is defined as the maximum amount of work (often normalized to volume and reported as a strain energy density) a structure or material can absorb before failure (Bouxsein et al. 2007). These concepts are important for overall bone strength because when a load is applied to bone that exceeds bone strength (a measure of maximum load or stress that a structure or material can resist before succumbing to ultimate failure) and its ability to absorb energy, fracture will occur. This phenomenon can be visually depicted using the stress–strain curve, shown in Fig. 3. The yield point on the stress–strain curve is an approximation of the elastic limit of bone. When a bone is stressed beyond the elastic limit, irreversible, permanent damage occurs in the form of micro-cracking (Zioupos et al. 2008).

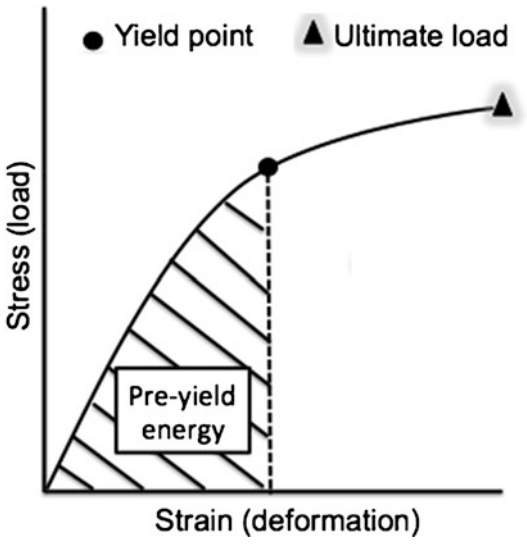

Fig. 3 An example of stress–strain (load-deformation) curve. Similar curves are generated during mechanical testing of bone as part of bone quality assessment

Under normal physiological loading of the skeleton, mechanical stress states exceeding the elastic limit are rare. However, this can occur on a very localized level around defects and pre-existing cracks, termed stress concentrators. Given a sufficient number of loading cycles, micro-cracks can develop and some grow into larger critically sized cracks that propagate into frank fractures (O'Brien et al. 2003). Deficiencies in bone quality are known to elevate fracture risk by lowering the bone tissues' inherent ability to resist crack growth initiation and propagation (fracture toughness) (Ritchie 2010). Because crack growth initiation and propagation are highly localized phenomena, local bone quality is important. Pre-existing cracks also reduce the strength and toughness of a bone. Given that bone contains many defects (multi-scale porosity) and micro-cracks under normal conditions and these increase in number and concentration in many bone-affecting diseases, it is necessary to consider bone's inherent resistance to crack growth initiation and propagation under slow monotonic, dynamic, and cyclical, more physiological loading conditions. In brief, fracture risk is a function of bone quality and the stress state of the bone. The stress state is a function of both the mechanical loading experienced by the bone in question and its structure. Fracture is a localized phenomenon and its progression depends on local bone quality and local stress state. These two interact where local increases in porosity and/or micro-cracking affect the local stress state.

Bone Biomarkers

Bone turnover markers provide insight into the pathophysiology of osteoporosis and disease-related bone loss, as levels reflect the metabolic activity of osteoblasts and osteoclasts (Bonde et al. 1996; Garnero et al. 2008). While the use of bone turnover markers is not recommended for the diagnosis of osteoporosis (Papaioannou et al. 2010), bone turnover markers can help in understanding the pathophysiology of bone diseases (Szulc and Delmas 2008). For example, studies in adults with type 2 diabetes have shown that diabetes is associated with lower bone turnover (Oz et al. 2006), which may contribute to bone fragility, as low bone turnover may allow microdamage to accumulate in bone and lead to reduced strength (Mashiba et al. 2000). Conversely, higher rates of bone turnover have been reported in postmenopausal women (Garnero et al. 1996b; Recker et al. 2004; Ebeling et al. 1996), which is also related to fracture due to loss of bone mass and volume and therefore whole bone strength (Garnero et al. 1996a; Sornay-Rendu et al. 2005). Therefore, both elevated and suppressed rates of bone turnover can predispose an individual to fracture, but the mechanisms are not fully elucidated.

Biomarkers for Bone Resorption and Formation

Various national and international organizations (International Osteoporosis Foundation, International Federation of Clinical Chemistry and Laboratory Medicine, National Bone Health Alliance) have recommended the use of two biomarkers,

serum procollagen type 1 N-terminal propeptide (s-P1NP) and C-terminal cross-linking telopeptide of type 1 collagen (s-CTX), as *gold standard* reference for bone formation and resorption, respectively (Brown et al. 2009; Vasikaran et al. 2011). Other bone turnover markers have been identified (such as osteocalcin, pyridinoline, deoxypyridinoline, bone-specific alkaline phosphatase, NTX, etc.); however, s-CTX and s-P1NP are superior due to availability and automation of standardized assays and reference standards, abundance of good quality data linking these markers to fracture risk, low analytic variability, and established factors influencing specimen stability and variability (i.e., circadian rhythm, diet and exercise, etc.) (Vasikaran et al. 2011).

Serum CTX and Serum P1NP

Serum CTX is a measurement of cross-linked C-terminal telopeptides of type 1 collagen that are released into circulation during bone resorption (Fig. 4). These cross-links contribute to the mechanical properties of bone, as they are involved in the formation of mature pyridinium enzymatic cross-links in bone (Oxlund et al. 1995). CTX can also be measured in the urine, but it is more advantageous to measure s-CTX due to better reproducibility, and urinary CTX must be corrected to urinary creatinine to control for variability in urine concentration (Chubb 2012). Just as s-CTX is an indicator of bone resorption, P1NP is an indicator of bone formation. Procollagen is secreted by osteoblasts during bone formation and is cleaved in the process, sending P1NP into circulation (Fig. 4). Large prospective cohort studies have demonstrated that both CTX and P1NP levels are associated with fracture risk. For example, in a subsample of participants from the EPIDOS study (n = 115 fracture cases, n = 293 control), participants with serum CTX values above the premenopausal reference range were 86% more likely to experience a hip fracture (hazard ratio [HR] 1.86, 95% confidence interval [CI] 1.01–3.76) (Chapurlat

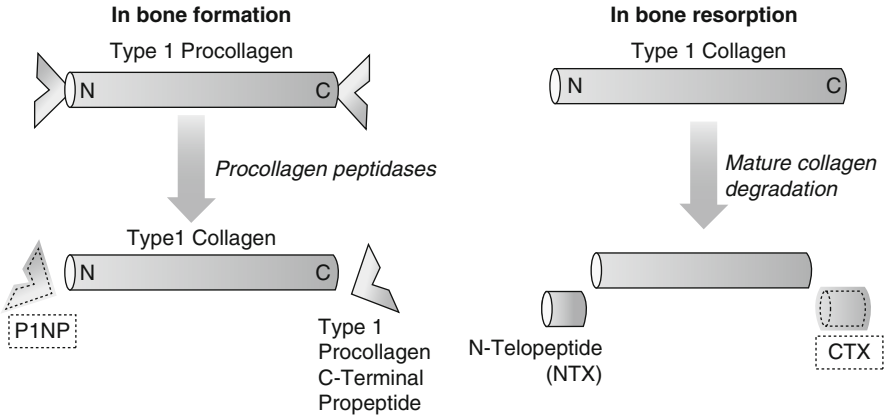

Fig. 4 The generation of procollagen type 1 N propeptide (*P1NP*) fragment from procollagen molecule during bone formation, and C-terminal cross-linking telopeptide of type 1 collagen (*CTX*) from type 1 collagen molecule during bone resorption

et al. 2000). In a larger study over a longer period of time (9 years of follow-up), Ivaska and colleagues reported a higher risk of any osteoporotic fracture (HR 1.13, 95% CI 1.01–1.27) and vertebral fracture (HR 1.32, 95% CI 1.05–1.67) with higher s-CTX levels (Ivaska et al. 2010). In addition, after adjustment for structural bone quality characteristics (femoral neck BMD, cortical thickness, and porosity), the risk of fracture was 30% higher in postmenopausal women in the Tromsø Study with higher levels of s-P1NP (Shigdel et al. 2015). Elevated bone turnover is believed to be disadvantageous to bone strength and a contributor to the development of osteoporosis in postmenopausal women. Treatment with antiresorptive medication (i.e., bisphosphonates or denosumab) reduces bone turnover, improves BMD, and reduces fracture risk in postmenopausal women (Eastell et al. 2011a, b).

Pentosidine in Bone, Serum, and Urine

Pentosidine and other AGEs accumulate in bone and low turnover tissues (such as cartilage) (Verzijl et al. 2003). The accumulation of pentosidine in bone is attributed in part to aging, as Odetti and colleagues reported a positive correlation between age and cortical bone pentosidine content ($r = 0.610$, $p < 0.001$) (Odetti et al. 2005). However, in a study conducted by Saito et al., the rate of increase of pentosidine content in cortical bone was not constant, suggesting that the formation and accumulation of pentosidine in bone are driven by other factors (Saito et al. 1997). In addition to age and time, other factors such as bone turnover rate (low vs. high bone turnover), oxidative stress, and glycation contribute to pentosidine accumulation in bone (Saito and Marumo 2010, 2015; Allen et al. 2015). Understanding this phenomenon in the context of human aging and disease is not trivial due to varying levels of oxidative stress and glycemia in different diseases, such as aging, type 2 diabetes, and chronic kidney disease.

Pentosidine in Urine and Serum as a Biomarker for Poor Bone Quality and Elevated Fracture Risk

Just as CTX is liberated from bone during bone resorption by osteoclasts, pentosidine is also released into circulation when collagen is broken down. Takahashi and colleagues established a methodology using column-switching high-performance liquid chromatography (HPLC) that yielded low inter-assay and intra-assay variability (coefficient of variation <6%) for serum and urine pentosidine (Takahashi et al. 1996). They also demonstrated that serum and urine pentosidine levels are highly related ($r = 0.797$, $p < 0.01$). Yoshihara et al. also used HPLC to determine an age-related increase in serum and urine pentosidine in older study participants (Yoshihara et al. 1998). Though used less often than HPLC for detecting systemic pentosidine, an ELISA method has also been developed, which yields results that are related to serum pentosidine measured by the HPLC method (Sanaka et al. 2002).

As pentosidine can accumulate in skeletal tissue, the amount of pentosidine in serum and urine is believed to be reflective of the rate of bone turnover. Demonstrating that systemic pentosidine is reflective of overall bone turnover, Schwartz et al. reported a positive relationship between urine pentosidine and s-P1NP and s-CTX (Schwartz et al. 2009). In addition, Hein and colleagues reported higher levels of serum pentosidine in patients with osteoporosis and *higher bone turnover* compared to patients with osteoporosis and *lower or normal bone turnover* (Hein et al. 2003). These results suggest that as bone is turned over, traditional bone turnover markers such as s-CTX and s-P1NP are released into circulation and pentosidine is also released into circulation. However, the clinical relevance of higher levels of systemic pentosidine is not fully understood and confounded by different factors. Table 1 provides an overview of clinical studies examining the relationship between systemic pentosidine (urine or serum) and fracture risk.

Appraisal of Studies: Systemic Pentosidine and Fracture Risk

The majority of studies reviewed in Table 1 show a positive relationship between serum or urine pentosidine and fracture risk. When measured in urine, pentosidine was associated with a 3–33% increase in incident fracture risk in Japanese peri- or postmenopausal women (Tanaka et al. 2011; Shiraki et al. 2008, 2011). The risk associated with an increase in urinary pentosidine was even higher for patients with type 2 diabetes, as Schwartz and colleagues reported a 42% higher risk for incident clinical fracture with each 1 standard deviation increase in log pentosidine (Schwartz et al. 2009). There were fewer studies that assessed pentosidine in serum, but for the two studies reviewed, the relationship was also positive (Yamamoto et al. 2008; Neumann et al. 2014), but only in patients with type 1 diabetes and females with type 2 diabetes. Only two studies reported no significant relationships between pentosidine and fracture risk (Gineyts et al. 2010b; Schwartz et al. 2009). Of note, these were the only studies that included a subset of participants without other diseases, such as type 2 diabetes (Schwartz et al. 2009), or examined participants that were predominantly Caucasian (Gineyts et al. 2010b). Evidently, there is heterogeneity in the results. This warrants discussion of factors that may be influencing the results such as bone turnover rate, presence of disease, ethnicity, and sex.

Bone turnover rate may influence systemic pentosidine levels and should be accounted for in statistical analyses. As pentosidine is released into urine and serum during bone turnover, it may be that elevated systemic pentosidine is indicating elevated bone turnover. For example, in patients with type 1 diabetes who typically have elevated bone turnover (Starup-Linde et al. 2016), it is not surprising that systemic pentosidine, which is released into circulation during bone turnover, is related to prevalent fracture (Neumann et al. 2014). This is similar to the findings reported by Tanaka (Tanaka et al. 2011) and Shiraki (Shiraki et al. 2008) who studied Japanese postmenopausal women, where bone turnover is generally elevated compared to premenopausal women. Therefore, in order to better understand whether pentosidine independently predicts fracture and provides more information about bone metabolism than s-CTX and s-P1NP, multivariate models should adjust for s-CTX or s-P1NP. Unfortunately, none of the studies reviewed made these

Table 1 Review of clinical studies assessing serum or urine pentosidine and bone-related outcomes

First author, year	Study design	Study population	Method	Fracture outcome	Expression of risk	Covariates included in regression model
Pentosidine in urine						
Shiraki et al. 2011	Prospective cohort	Japanese postmenopausal women with osteoporosis + starting on a bisphosphonate (alendronate or risedronate) (N = 251)	HPLC	Incident vertebral fracture	HR 1.03 (1.00–1.05), p = 0.039 For 5 pmol pentosidine/mg Cr	Baseline age, lumbar spine BMD, prevalent osteoporotic fracture, baseline urinary pentosidine, baseline plasma homocysteine
Tanaka et al. 2011	Prospective cohort	Japanese postmenopausal women (N = 765)	HPLC	Incident vertebral fracture *and* long bone fracture	HR 1.17 (1.04–1.33), p = 0.010 For 1SD increase in pentosidine	NTX, BAP, age, body weight, diabetes, lumbar spine BMD, prior fracture, back pain
				Incident vertebral fracture	HR 1.15 (1.01–1.31), p = 0.038 For 1SD increase in pentosidine	
Gineyts et al. 2010b	Prospective cohort	French postmenopausal women in OFELY cohort (N = 396)	HPLC	Incident clinical fracture	HR 1.23 (0.54–2.82), p = 0.62 For 1 log pentosidine increase	Age, prevalent fracture, hip BMD T-score
Schwartz et al. 2009	Prospective cohort	HABC participants with and without T2D (N = 928)	HPLC	Incident clinical fracture	For participants with T2D: HR 1.42 (1.10–1.83), p = 0.007 For 1SD increase in log pentosidine	Age, race, gender, smoking status, baseline BMD, baseline weight, weight loss of 5+ pounds in year before baseline, cystatin-C, A1C, and use of supplements (vitamin D, calcium), oral steroids, osteoporosis drugs

(*continued*)

Table 1 (continued)

First author, year	Study design	Study population	Method	Fracture outcome	Expression of risk	Covariates included in regression model
					For participants without T2D: HR 1.08 (0.79–1.49), $p = 0.630$ For 1SD increase in log pentosidine	(bisphosphonates, calcitonin, raloxifene), thiazide diuretics, statins, oral estrogen In models with T2D participants: insulin, metformin, sulfonylureas, thiazolidinediones, oral hypoglycemic medications
				Baseline vertebral fracture	For participants with T2D: OR 5.93 (2.08–16.94), $p = 0.001$ For 1SD increase in log pentosidine For participants without T2D: OR 0.74 (0.30–1.83), $p = 0.519$ For 1SD increase in log pentosidine	
Shiraki et al. 2008	Prospective cohort	Nagano Cohort, peri- and postmenopausal and women, no osteoporosis treatment (N = 432)	HPLC	Incident vertebral fracture	HR 1.33 (1.01–1.76), $p = 0.04$ For highest quartile of pentosidine compared to other quartiles	Baseline age, lumbar spine BMD, number of prevalent vertebral fractures

Pentosidine in serum						
Neumann et al. 2014	Cross-sectional	Men and premenopausal women with T1D (N = 128)	HPLC	Prevalent fracture	OR 1.017 (1.004–1.029), $p = 0.008$ For each increase in pmol pentosidine/mL	BMD, esRAGE, CML, age, BMI, family history of osteoporosis, smoking status, vitamin D deficiency
Yamamoto et al. 2008	Cross-sectional	Japanese men and postmenopausal women with type 2 diabetes (N = 153)	ELISA	Prevalent vertebral fracture	In females: OR 2.50 (1.09–5.73), $p = 0.030$ For each SD increase in μg pentosidine/mL In males: OR 0.79 (0.41–1.52), $p = 0.475$ For each SD increase in μg pentosidine/mL	Age, body weight, height, HbA1c, estimated GFR, duration of diabetes, duration of postmenopausal state, retinopathy or neuropathy, history of using insulin or pioglitazone, smoking status, alcohol intake, prevalent nonvertebral fractures, lumbar spine BMD

Abbreviations: *T2D* type 2 diabetes, *T1D* type 1 diabetes, *HPLC* high-performance liquid chromatography, *ELISA* enzyme-linked immunosorbent assay, *RR* relative risk, *HR* hazard ratio, *OR* odds ratio, *BAP* serum bone-specific alkaline phosphatase, *u-NTX* urinary N-terminal telopeptide of type 1 collagen
Results are expressed as ratios risk ratios (*RR*), hazard ratios (*HR*), odds ratios (*OR*), and 95% confidence intervals

adjustments using s-CTX and s-P1NP, but Tanaka et al. forced urinary NTX and bone-specific alkaline phosphatase (BAP) into their model. After adjusting for these markers of bone turnover, age, body weight, diabetes status, lumbar spine BMD, prior fracture, and back pain, each 1 standard deviation increase in urinary pentosidine was associated with a 17% increase in incident vertebral fracture and long bone fracture in Japanese postmenopausal women (Tanaka et al. 2011). However, a limitation of this study is that the authors did not collect the *gold standard* bone turnover markers (s-CTX and s-P1NP) and it was not clear whether the independent variable (pentosidine) was normalized to urinary creatinine in the regression model, which is typically done to account for variability in urine concentration (Chubb 2012). With a lack of large studies accounting for bone turnover (using s-CTX and s-P1NP), the role that pentosidine *independently* plays in predicting fracture is not fully understood.

Another factor to consider in the relationship between pentosidine and fracture risk is the presence of other diseases that affect bone metabolism. For example, it is well established that bone turnover is suppressed in patients with longer-standing (i.e., greater than 5 years) type 2 diabetes (Garcia-Martin et al. 2012; Oz et al. 2006; Gerdhem et al. 2005). In the study conducted by Schwartz and colleagues, there was no difference in urinary pentosidine between participants with type 2 diabetes and controls without type 2 diabetes (Schwartz et al. 2009). But, the authors report that pentosidine content is linked to fracture risk in participants with type 2 diabetes and not in participants without diabetes. In this case, it is possible that the link between systemic pentosidine and fracture is indicating bone pathology due to other factors like impaired bone mineralization or bone microarchitecture, which were not assessed in the study (Pritchard et al. 2012, 2013). In addition, if bone turnover were adjusted for in the final analysis, it could rule out that suppressed bone turnover was linking pentosidine to fracture risk.

Regarding ethnicity and sex, most studies reported a significant positive relationship between systemic pentosidine and fracture risk in Japanese participants, but not in Caucasian participants (without type 2 diabetes) (Schwartz et al. 2009) or French participants (Gineyts et al. 2010b). Therefore, this suggests that the relationships may be influenced by ethnicity and other lifestyle factors linked to culture and physical environment, including physical activity and diet. The role that these factors play in the relationship between systemic pentosidine and fracture risk needs to be further elucidated in large, population-based prospective studies.

Finally, given the heterogeneity in expression of results, it is difficult to compare the value of the associations reported in each study. There is no standardized approach to reporting systemic pentosidine as the independent variable. Some studies report associations between fracture and log-transformed pentosidine, pentosidine normalized to creatinine, and the highest quartile of pentosidine. These inconsistencies in reporting make it difficult to draw clear conclusions about the added value (beyond assessing established clinical risk factors and gold standard bone turnover markers) of measuring systemic pentosidine in clinical studies.

Bone-Specific Pentosidine as a Biomarker for Poor Bone Quality and Elevated Fracture Risk

The assessment of urine and serum pentosidine is advantageous for large population-based clinical studies, as the fluids can be easily obtained and assessment methods for pentosidine have been established. However, pentosidine can be released from a number of different tissues, in addition to bone (i.e., cartilage), making it questionable as to whether systemic pentosidine is truly reflecting bone-specific pentosidine. Although Odetti and colleagues reported a correlation between serum pentosidine and cortical bone pentosidine, the relationship was modest at best ($r = 0.248$, $p < 0.05$) (Odetti et al. 2005). In addition, there was no relationship between trabecular bone pentosidine and serum pentosidine (Odetti et al. 2005). Therefore, pentosidine in urine and serum has questionable value toward assessing actual bone quality. Assessing bone-specific pentosidine may address the third criterion for a good biomarker: "the biomarker's appearance must be associated as specifically as possible with quantifiable damage of a particular tissue" (NIH 2001), if the association between pentosidine and damage can be clarified. Furthermore, bone-specific pentosidine may be more powerful than systemic pentosidine because fracture is a site-specific, localized phenomenon that depends on local bone quality and stress state. For example, in a rare study using biopsied tissue, Saito et al. reported that in patients with intracapsular hip fractures, the bone at the fracture site contained altered cross-linking including elevated pentosidine content by two- to fivefold in the older and younger osteons, respectively (Saito et al. 2006a). However, obtaining bone biopsies is a barrier to further understanding the link between bone-specific pentosidine and fracture risk. Table 2 provides an overview of studies examining the relationship between bone pentosidine, bone collagen, and mechanical properties of bone.

Appraisal of Studies: Bone Pentosidine and Mechanical Properties

In vitro studies have provided some insight into the impact of bone pentosidine on bone strength and toughness. Pentosidine levels and modifications to other cross-links, including LOX cross-link maturation, can be experimentally altered in bone samples by incubating bone in solutions of ribose (Vashishth et al. 2001; Willett et al. 2013; Viguet-Carrin et al. 2008). After incubation for weeks, cortical and trabecular bone samples subjected to ribation had increased pentosidine content and reduced ability to deform and absorb strain energy before fracture (Vashishth et al. 2001; Willett et al. 2013; Viguet-Carrin et al. 2008; Tang et al. 2007, 2008; Tang and Vashishth 2010). This was attributed to an over-stiffening of the collagen phase that in turn suppressed the bone's ability to deform before fracture (accommodate strain after the elastic limit) (Vashishth et al. 2001; Willett et al. 2013). Such experimental studies were paralleled by other studies of human and animal bone, which are reviewed in Table 2. Most of the studies listed in Table 2 reported negative correlations between mechanical properties and pentosidine content in cortical and trabecular bone. For example, in human cortical bone harvested from the tibia and femur from older adults, pentosidine content was negatively correlated with

Table 2 Studies in human cadaveric bone and animal bone showing the relationships between collagen characteristics and mechanical properties

First author, year	Independent variable	Dependent variable	Result
Human cortical bone			
Nyman et al. 2007	Bone pentosidine content (tibia)	Energy dissipation (toughness)	r in the low to high -0.6 range
	Bone collagen content	Energy dissipation (toughness)	r = 0.5–0.6
Wang et al. 2002b	Bone collagen strength	Bone work-to-fracture (toughness)	r = 0.54
	Bone collagen work-to-fracture	Bone work-to-fracture (toughness)	r = 0.54
	Bone collagen pentosidine	Bone work-to-fracture (toughness) *Also strength and KIc (transverse)	r = −0.66
Zioupos et al. 1999	Collagen connectivity (contraction rate)	Work-to-fracture Fracture toughness (J_{Ic})	r = 0.83 r = 0.59 (age adjusted) r = 0.86 r = 0.76 (age adjusted)
Human trabecular bone			
Viguet-Carrin et al. 2006	Log (pen conc.)	Log (failure load) Log (work-to-fracture)	r = −0.39 r = −0.49 (aBMD adjusted)
Hernandez et al. 2005	Bone pentosidine *Also declining pyr and deoxy-pyr with age but no increase trend in pen?	Tensile ductility of trabeculae	r2 = 0.09 (r negative)
Banse et al. 2002	Mature enzymatic cross-links (PYD/DPD)	Compressive strength	r = 0.4
Karim and Vashishth 2012	PYD/DPD	Toughness	r = 0.49 (p = 0.06)
	Pentosidine	Strain to failure	r = 0.44 (p < 0.05)
Follet et al. 2011	Collagen cross-links and more (microdamage, mineralization)	Compressive strength	No correlations detected

(*continued*)

Table 2 (continued)

First author, year	Independent variable	Dependent variable	Result
Animal cortical bone			
Wang et al. 2000 (aging in baboons)	Bone denatured collagen content	Work-to-fracture Fracture toughness (K_{Ic})	$r = -0.492$ $r = -0.362$ ($p = 0.06$)
Saito et al. 2011b (glucocorticoid-treated rats)	Bone collagen immature LOX cross-links	Toughness (energy to fracture)	$r = 0.405$
Oxlund et al. 1995 (β-amino-propionitrile-treated female rat femora)	Bone collagen mature LOX cross-links	Cortical bone strength and toughness	Negative
Animal trabecular bone			
Saito et al. 2015 (Female cynomolgus monkeys)	Immature LOX cross-links	Ultimate load Energy to failure	$r_S = 0.211$ $r_S = 0.314$
	Pentosidine	Ultimate load Energy to failure	$r_S = -0.373$ $r_S = -0.356$

Abbreviations: *r* Pearson's correlation coefficient, r_S Spearman's correlation coefficient, *aBMD* areal bone mineral density (measured by DXA), * draws attention to the note that follows it

measures of toughness (such as work-to-fracture) (Nyman et al. 2007; Wang et al. 2002b). In vertebrae, the results are more heterogeneous. Vignet-Carrin et al. detected negative correlations between bone pentosidine and trabecular bone failure load or work-to-fracture in a regression model that also accounted for bone content (areal BMD [aBMD]) (Viguet-Carrin et al. 2010). Hernandez et al. reported a negative correlation between trabeculae ductility and pentosidine content (Hernandez et al. 2005), but as shown in Table 2, other studies have failed to detect relationships (Follet et al. 2011).

Presumably, due to the multiple in vitro models and ex vivo studies reviewed, the idea of a causal mechanism in which pentosidine is responsible for suppressing bone toughness and degrading bone quality has become a relatively popular idea. However, correlation is not causation and the in vitro models do not completely model all of the changes occurring in bone with aging and disease. Therefore, what bone-specific pentosidine actually indicates remains unclear.

Appraisal of Studies: Bone Pentosidine and Mechanical Properties, Aging

Table 2 reviews studies in humans and animals that investigate the impact of aging and other diseases on changes in bone collagen, its cross-linking, and its bone mechanical properties. Studies by Wang et al. demonstrate age-correlated losses in bone collagen

content, strength, and toughness with increases in denatured collagen content (Wang et al. 2001, 2002a, b, 2003). Bone collagen content, strength, and toughness all correlated with degraded bone mechanical properties. There was also a significant negative relationship between denatured collagen and cortical bone toughness ($r = -0.492$, $p < 0.05$) (Wang et al. 2000). Furthermore, Zioupos et al. measured the net connectivity of cortical bone collagen over a large range of ages using thermomechanical testing and reported a strong negative correlation between age and connectivity (Zioupos et al. 1999). When collagen connectivity was tested against cortical bone work-to-fracture and elastic–plastic fracture toughness (J_{Ic}; highly dependent on the bones ability to deform post-yield), strong positive correlations were found (Zioupos et al. 1999). This demonstrates the importance of the overall connectivity and nativity of the bone collagen toward bone mechanical properties and quality.

With aging, the concentrations of enzymatic cross-links (both immature and mature) in human cortical bone have not been found to vary significantly after skeletal maturity (Saito et al. 1997; Zioupos et al. 1999). Losses in bone collagen strength and toughness and positive correlations between net connectivity and bone toughness are not consistent with the idea that pentosidine as a cross-link, which should increase collagen connectivity and strength, causes reduced bone strength and toughness (Willett et al. 2014). The ribation model does not include these other important changes (denaturation, loss of connectivity). In fact, the strength and stiffness of the bone collagen (Vashishth et al. 2001), as well as the net connectivity (Willett et al. 2013), have all been found to increase in the ribose model. Willett et al. detected a weak positive correlation between pentosidine content and work-to-fracture in the ribation model using bovine cortical bone (Willett et al. 2013). In an in vitro model of fetal bovine cortical bone allowed to mature without ribose, both of the pyridinolines increased along with pentosidine content (Garnero et al. 2006). Furthermore, the authors reported positive correlations between all three of these cross-link contents and measures of post-yield toughness in compression (Garnero et al. 2006). Therefore, other changes, such as collagen degradation and alteration in LOX cross-linking, are probably more important than pentosidine content; however, a study clearly demonstrating this using comprehensive characterization of the human bone constituents and relevant mechanical properties has not yet been reported.

Appraisal of Studies: Bone Pentosidine and Mechanical Properties, Bone Turnover Diseases

In disease states with abnormal turnover rates, the role of pentosidine is even more unclear. In diseases and animal models of diseases, with high bone turnover, such as postmenopausal osteoporosis and high turnover chronic kidney disease bone and mineral disorder, multiple studies demonstrate that in addition to elevated pentosidine, the LOX-dependent enzymatic cross-links are disrupted (refer to Table 3). Decreases in the immature, mature, or both types of LOX cross-links have been reported (refer to Table 3). It is well established that the strength and toughness of bone are positively correlated with enzymatic cross-link content (Oxlund et al. 1995; Saito et al. 2006b; McNerny et al. 2015). In models of LOX

inhibition using β-amino-propionitrile, strength and toughness are reduced (Oxlund et al. 1995; McNerny et al. 2015; Paschalis et al. 2011). Therefore, to determine if pentosidine should be indicted for causing poor bone quality and elevated fracture risk in high turnover disease, conceivably the absolute changes in the total amount of all cross-links should be studied. To this point, the magnitude of pentosidine increase measured in high turnover disease specimens is approximately one to two orders of magnitude smaller than the amount of the enzymatic cross-links lost relative to healthy controls in various studies (Willett et al. 2014). Therefore, it is difficult to comprehend how pentosidine could have a significant role in the face of a net cross-linking deficit that is at least an order of magnitude larger. As mentioned above, enzymatically derived cross-links are critical to bone achieving its strength and toughness. Certainly, there is no evidence that a pentosidine cross-link is ten times more effective than either an immature or mature enzymatic cross-link.

In low turnover diseases, such as types 1 and 2 diabetes and low turnover chronic kidney disease bone and mineral disorder, results are mixed and data from human tissues is sparse. A study in WBN/Kob rats, which is an animal model of type 2 diabetes, revealed decreased LOX cross-linking and elevated bone pentosidine compared to control Wistar rats (Saito et al. 2006c). Notably, the ratio of pentosidine to total LOX cross-link content was more strongly positively associated with energy absorption, stiffness, elastic modulus, and maximum load than total LOX cross-link content or pentosidine alone (Saito et al. 2006b). However, this study was limited as other changes in bone quality (i.e., microarchitecture, cortical porosity) were not accounted for, and the diabetic rats did not have lower bone turnover, which is characteristic of adults with longer-standing type 2 diabetes (Garcia-Martin et al. 2012; Oz et al. 2006; Gerdhem et al. 2005). Only one published study has investigated pentosidine content in bone in human adults with type 2 diabetes. Oren and colleagues reported 32% higher levels of pentosidine content in bone from male total knee replacement patients with type 2 diabetes compared to controls (Oren et al. 2011). In another unpublished study, Pritchard and colleagues found no significant difference in bone pentosidine content in total hip replacement patients over age 65 years with type 2 diabetes compared to controls without diabetes (author's unpublished work). Interestingly, iliac crest biopsies from type-1 diabetics with various fragility fractures were found to contain greater levels of pentosidine in the trabecular bone along with higher mineralization compared to nondiabetic non-fracture controls (Farlay et al. 2016). These differences were not detected in the cortical bone. Mature enzymatic cross-links were not detectably different and immature cross-links were not measured. Diabetic non-fracture controls had intermediate but not statistically separable levels of trabecular pentosidine and mineralization. Unfortunately, these specimens lacked fracture site specificity. Inconsistencies in results from these studies may be due to differences in the age, type, and location of bone sampled (cortical vs. trabecular bone), bone turnover rate, and diabetes-related medications.

Animal models of type 1 diabetes show increased bone pentosidine with *increased* deoxypyridinoline cross-links (Silva et al. 2009). Similarly, in a rat model of chronic kidney disease, the low turnover model demonstrated greatly

Table 3 Changes in collagen cross-linking due to aging and disease in human bone and animal bone

First author, year	LOX cross-links in controls (mol/mol)	LOX cross-links in disease state or model (mol/mol)	Pentosidine in controls (mol/mol ×10^−3)	Pentosidine in disease state or model (mol/mol ×10^−3)	Tissue + disease
Human cortical bone					
High turnover condition					
Bailey et al. 1992		−20% to −40% immature cross-links			Human femoral neck with osteoporosis
Saito et al. 2006a, b	Low density young osteons	−10% of total cross-links	4.7 ± 1.8	19.1 ± 12.5 (+406%)	Human intracapsular hip fractures
	High density older osteons		7.7 ± 2.4	15.8 ± 10.63 (+205%)	
Normal or low turnover condition					
Wang et al. 2002b		No change in mature cross-links detected		0.44 ± 0.21 (19–49y) 0.90 ± 0.23 (5–69y) 1.39 ± 0.29 (>70y)	Human femur – no separation of sexes (aging study; no bone-affecting conditions)
Li et al. 2003		No change in mature cross-links detected		0.416 ± 0.189 (19–45y) 0.927 ± 0.259 (45–69y) 1.39 ± 0.307 (> = 70y)	Human femur – no separation of sexes (aging study; no bone-affecting conditions)
Nyman et al. 2007		No change in mature cross-links detected		0.316 ± 0.111 (M + F, 50s) 0.537 ± 0.101 (M + F, 70–90y)	Human tibia (aging study; no bone-affecting conditions)

Saito et al. 1997	Mature cross-links increase up to ~40y	Immature and mature cross-links plateaued after 40y		<0.01 (0–10y) 0.02 (40–50y) 0.045–0.05 (~70–80y)	Human diaphysial femur from males (accidental deaths)
Karim et al. 2013			Linear increase w age	r = 0.717 ($p < 0.001$)	Human femur
				No correlation w age	Human tibia
Human trabecular bone					
High turnover					
Oxlund et al. 1996		−24% to −30% immature cross-links			Human vertebral trabeculae (osteoporosis)
Mitome et al. 2011	Immature/mature ratio 2.62 ± 0.67	Same ratio 3.41 ± 1.41 [a]Due to decrease in mature xls		Tenfold increase (~40 ± 20)	CKD iliac crest biopsies
Low or normal turnover					
Karim et al. 2013				r = .280 w age	Human femur trabecular bone
				r = 0.385 w age (much higher concentrations than in femur)	Human tibia trabecular bone
Animal cortical bone					
Oxlund et al. 1995		−45% mature cross-links			β-Amino-propionitrile-treated female rat femora
Saito et al. 2011b	1.626 ± 0.086 immature	1.341 ± 0.102 immature (−17.5%)	na	na	Glucocorticoid-treated rats (femoral diaphysis)
Saito et al. 2010	0.633 ± 0.059	0.480 ± 0.028 (−24%) total	0.172 ± 0.063	0.188 ± 0.070 (+9.3%) 0.342 ± 0.090[a] (+98.8%)	Female rabbits (OVX; high turnover)

(*continued*)

Table 3 (continued)

First author, year	LOX cross-links in controls (mol/mol)	LOX cross-links in disease state or model (mol/mol)	Pentosidine in controls (mol/mol ×10^−3)	Pentosidine in disease state or model (mol/mol ×10^−3)	Tissue + disease
Saito et al. (2006c)		−36% approximately		+221% approximately	Male WBN/Kob rats (diabetes type II model) at 18 months old and B6 deficient
Nojiri et al. 2011		−20%		+33%	Sod1−/− mice
Allen et al. 2015		−20% mature cross-links		+71%	Male Cy/+ rats (high turnover)
		DPD +24%		+71%	Male Cy/+ rats (low turnover)
Animal trabecular bone					
Saito et al. 2011a	1.069 ± 0.283	0.867 ± 0.206 (−18.9%) total cross-links	1.128 ± 0.387	1.816 ± 1.094 (+61%)	Female cynomolgus monkeys (OVX; high turnover)
Saito et al. 2015	0.908 ± 0.082 immature 0.145 ± 0.023 mature	0.774 ± 0.080 immature 0.118 ± 0.024 mature	1.141 ± 0.201	1.763 ± 0.490 (+54.5%)	Female cynomolgus monkeys (OVX; high turnover)

[a]Indicates methionine-rich diet inducing hyperhomocysteinemia
Abbreviations: *OVX* ovariectomy

increased pentosidine content along with an *increase* in deoxypyridinoline (Allen et al. 2015). This is consistent with the theory that reduced bone turnover rate suppresses the liberation of enzymatic and nonenzymatic cross-links from bone, therefore promoting higher bone cross-link levels in disease states with low bone turnover.

What Does In Situ Bone-Specific Pentosidine Actually Indicate?

In general, elevated bone-specific pentosidine correlates with degraded bone quality in multiple disease states, including both high and low turnover conditions. Whether pentosidine plays a causal role in degrading bone mechanical properties and therefore increasing fracture risk remains to be further elucidated. In fact, there is growing consensus in the literature that pentosidine is a result of and therefore an indicator of oxidative stress and the resulting damage rather than a causal factor in degrading bone quality via over-cross-linking (Saito and Marumo 2010, 2015; Willett et al. 2014; Allen et al. 2015). As reviewed in section "Nonenzymatic Collagen Cross-links," pentosidine requires oxidation for its formation. Furthermore, elevated oxidative stress plays an important role in aging, diabetes, osteoporosis, chronic kidney disease, and many human diseases where inflammation is elevated including hyperhomocysteinemia and perhaps inflammatory bowel disease (Saito and Marumo 2015).

A theory promoted by Saito and others in multiple recent publications explains graded bone quality in osteoporosis, diabetes, chronic kidney disease, and potentially other diseases in terms of bone collagen modifications determined by elevated oxidative stress (Saito and Marumo 2010, 2015; Willett et al. 2014; Allen et al. 2015). It is proposed that oxidative stress inhibits LOX activity and therefore enzymatic cross-link formation, and this facilitates AGE formation (including pentosidine) by eliminating competition for (hydroxyl)lysine sites (Saito and Marumo 2010, 2015; Willett et al. 2014; Allen et al. 2015). Given that the LOX cofactor vitamin B6 is an antioxidant, its contribution to LOX catalysis may be inhibited as it acts as an antioxidant instead (Sendur et al. 2009; Muthusami et al. 2005; Maggio et al. 2003; Hamada et al. 2009; Grune et al. 2013). As further support for the important role of oxidative stress, impaired enzymatic collagen cross-linking and elevated pentosidine content were reported in a mouse model having elevated cytoplasmic oxidative stress (Nojiri et al. 2011). Knockout of cytoplasmic copper/zinc superoxide dismutase resulted in a form of low turnover osteoporosis, with a 20% decrease in total enzymatic cross-links (−0.1 mol/mol collagen) and a 30% increase (+0.05 mmol/mol collagen) in pentosidine (Nojiri et al. 2011). Importantly, treatment with vitamin C (a powerful antioxidant) provided partial protection against these degradations. A study of human femoral neck fractures by Saito et al. is also consistent with this idea (Saito et al. 2006a). Compared to non-fracture controls, they reported greater pentosidine in the bone of the fracture cases, especially in the younger osteon, and less total LOX cross-linking in both young and old osteons, but most significantly in the older osteons. Furthermore, the fracture cases had slightly

higher plasma homocysteine levels and much lower plasma B6 levels (Saito et al. 2006a).

Free radicals play a key role in oxidative stress and have the ability to fracture/scission protein chains (Grune et al. 2013). This is another means by which collagen network connectivity and potential denatured collagen can occur. Decreased collagen network connectivity and increased denatured collagen content are associated with degraded human cortical bone mechanical properties (Zioupos et al. 1999; Wang et al. 2002b). Some studies have also noted disordered mineralization (Saito et al. 2006a, c; Mitome et al. 2011) in association with pentosidine and fractures. This could be the result of altered templating of the mineralization process due to disrupted enzymatic cross-linking, imbalance in amine to carboxyl or carbonyl groups (Ehrlich et al. 2010; Grune et al. 2013), collagen fragmentation and denaturation (Zioupos et al. 1999; Wang et al. 2002b), or some other forms of oxidative damage.

Pentosidine is often proposed as a surrogate biomarker for total AGE content. It is often proposed that total AGE cross-linking content could reach the same levels as the LOX-mediated cross-links and that this might be sufficient to suppress bone ductility and toughness, particularly under low turnover conditions (Saito and Marumo 2015). This would parallel what occurs in the in vitro ribose model (Willett et al. 2013; Vashishth et al. 2001). However, no other AGE cross-links have been detected or measured in bone to date. The most compelling candidate AGE cross-link is glucosepane (Sell et al. 2005; Biemel et al. 2002). Glucosepane has a similar chemical structure and mechanism of formation to pentosidine and, in skin, it is abundant (Sell et al. 2005; Biemel et al. 2002). Unfortunately, it is neither acid stable nor fluorescent, and therefore isolation and detection are more challenging (Sell et al. 2005; Biemel et al. 2002). Glucosepane has not been detected in bone.

It is becoming clear that pentosidine accumulates in states of increased oxidative stress (inflammation), independent of bone turnover rate, and at this time, it is best considered as an indicator of degraded bone quality (a surrogate biomarker) rather than a causal factor. It is unlikely that pentosidine independently plays a *causal* role due to its very low concentrations in bone (Willett et al. 2014). Clearly, much more research is required, particularly in terms of bone quality studies of specimens from human disease populations rather than animal models of disease. Comprehensive bone quality assessment including bone collagen chemistry, bone turnover markers, relevant mechanical testing and quantification of oxidative stress, and associated collagen modifications is required to fully understand what pentosidine indicates. In addition, consideration should be given to other disease-related changes in bone quality, such as bone microarchitecture and cortical porosity. As pentosidine is thus far the only AGE cross-link that has been detected in bone, important next steps include seeking methods to analytically measure glucosepane and other AGE cross-links in bone and to test correlations between pentosidine and these other cross-links in order to determine if pentosidine can act as a surrogate marker for all AGE cross-links in bone.

Potential Applications to Prognosis, Other Diseases, or Conditions

The prognostic value of bone-related turnover markers has received significant attention over the last decade or so. Some suggest that s-CTX and s-P1NP, and to a lesser extent other bone turnover markers, may have value in predicting fracture risk, rate of bone loss and response to therapy, and understanding secondary osteoporosis (Vasikaran et al. 2011). However, where bone-specific and systemic pentosidine are concerned, the concept of pentosidine as a biomarker for bone fragility and bone quality is in its infancy. For example, much more work is required to understand and account for sources of variation and error (reliability and validity), especially pre-analytical issues like intraindividual variation and interindividual differences. Meaningful differences (least significant change) will also need to be determined. Currently changes on the order to 27–36% for CTX levels are considered clinically meaningful (Chubb 2012).

Multiple serum and urine CTX studies and NTX studies demonstrate that pentosidine has some power to predict fracture (reviewed in Table 1). This power to predict fracture could be improved by normalizing to bone turnover marker levels, particularly s-CTX, thus providing a means of measuring the amount of pentosidine being liberated per unit of collagen being resorbed. This could provide an indirect measurement of bone pentosidine level and a surrogate for systemic bone quality. Unfortunately, no such studies have been conducted, and the systemic nature of the biomarker means a lack of specificity to known or potential fracture sites. In addition, only one study has examined bone and systemic pentosidine and reported only a modest correlation between levels (Odetti et al. 2005). This is a clear direction for future studies.

Based on the preceding review, one can reason that bone-specific pentosidine should have significant higher value than traditional bone turnover makers in the diagnosis of poor bone quality and prognosis of fracture due to its decoupling from turnover and greater specificity to bone tissue. Challenges with bone-specific pentosidine include the invasiveness of sampling a patient's bone at clinically meaningful sites (i.e., femoral neck, vertebrae) and, on top of that, bone microarchitecture varies by location. Given that there are inconsistencies between pentosidine content measured in trabecular versus cortical bone, it will be difficult to control for the sampling site to reach a consensus about pentosidine's independent contribution to bone quality.

The differences in turnover rate between diseases (low vs. high) mean that bone-specific pentosidine should not be used alone as a biomarker for poor bone quality. For the assessment of fracture risk, there is insufficient evidence to use bone-specific or systemic pentosidine with the World Health Organization-endorsed FRAX^{TM} fracture risk assessment tool. Further studies of bone-specific pentosidine (both cross-sectional and prospective) are needed and warranted to determine its diagnostic and prognostic value. Certainly, many more studies of human disease populations with appropriate controls are required. This would be aided immensely by the

development of means for less or noninvasive and yet comprehensive assessment of site-specific bone quality, including bone collagen and its modifications including LOX- and AGE-mediated cross-link but also mineralization, porosity, and relevant mechanical properties.

Conclusion

Pentosidine has potential as a biomarker for poor bone quality and elevated fracture risk, but neither systemic pentosidine nor bone-specific pentosidine meet all three criteria for a good biomarker (NIH 2001). Serum and urine pentosidine alone provides a measure of bone resorption. By adjusting for a validated bone resorption biomarker such as s-CTX, this could become a useful tool to estimate systemic bone quality. Bone-specific pentosidine is clearly associated with poor bone quality, which can lead to elevated susceptibility to fracture. It must be augmented with quantification of as many other collagen cross-links as possible and ideally other measures of bone quality such as mineralization and microarchitecture. Currently, sampling of bone-specific pentosidine for diagnosis or prognosis is much too invasive. For real value in fracture risk assessment, site-specific measurements are required because the determinants of fracture risk (bone quality, stress state) are site dependent.

Summary Points

- The risk of skeletal fractures is higher in people with many bone-affecting different diseases, such as osteoporosis, type 1 and type 2 diabetes, and chronic kidney disease.
- Fractures are associated with a loss of independence, reduction in quality of life, and increased risk of mortality.
- Bone mineral density (BMD) is measured by dual x-ray absorptiometry (DXA) to assess bone health and is used to predict fractures; however, there are many other factors that contribute to overall fracture risk.
- In addition to structural factors that contribute to fracture risk, the material composition of bone is important, for which type 1 collagen cross-linking plays an important role.
- Pentosidine is a nonenzymatic glycation end product which accumulates in tissues, including bone, in people, and in animal models with high blood glucose and oxidative stress.
- Pentosidine can be measured in various tissues and fluids, including in bone, urine, and serum.
- Pentosidine is liberated from bone and other tissues during tissue turnover and therefore is positively related to other markers of bone turnover, such as serum procollagen type 1 N propeptide of type 1 collagen (s-P1NP) and serum C-terminal cross-linking telopeptide of type 1 collagen (s-CTX).

- Pentosidine measured in urine and serum is positively related to incident clinical fracture in most studies, but the findings from the majority of clinical studies may be confounded by baseline bone turnover, disease status, ethnicity, and sex.
- When measured in bone samples (in situ), pentosidine is negatively related to overall bone strength and/or toughness, suggesting that pentosidine accumulation is linked to poor bone quality.
- In order for pentosidine to be considered a biomarker for fracture risk, more research is needed in the areas of controlling for turnover rate and other pre-analytical sources of variation in the case of serum and urine pentosidine and, for in situ bone-specific pentosidine, methods for noninvasive but comprehensive site-specific bone quality assessment.

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Vitamin D: Biological Significance and Diagnosis of Mild Deficiency

17

Enrico Carmina

Contents

Abstract

Activated vitamin D has a main role in bone metabolism by increasing intestinal calcium absorption and kidney calcium resorption but also by activating both bone formation and resorption. This last effect may be mainly indirect by modulating PTH secretion. In mild forms of vitamin D deficiency, the increase in PTH secretion is probably the main factor determining bone loss.

In diagnosis of vitamin D deficiency, the establishment of a circulating vitamin D (mono or di-hydroxylated vitamin D) cutoff is particularly important but has been difficult because differences in used criteria. Based on PTH circulating levels and femoral neck bone density, a mono-hydroxylated vitamin D cutoff of 25 ng/ml may be the best criterion for distinguishing a mild vitamin D deficiency.

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Vitamin D action is not limited to bone metabolism but involves modulation of immune function, stimulation of insulin, and other hormone secretion and inhibition of cell proliferation. Epidemiological studies have correlated low vitamin D levels to increased prevalence of some forms of cancer (mainly colon cancer but also breast and prostate cancer), type II diabetes, some autoimmune disorders, and cardiovascular diseases. However, in all these conditions, with a few exceptions, trials using high doses of vitamin D have been unsuccessful. The possibility that in nonskeletal diseases, different criteria for determining a vitamin D deficiency should be used is discussed.

Keywords

Vitamin D • Bone metabolism • Osteoporosis colon cancer • Breast cancer • Prostate cancer • Diabetes • Autoimmune diseases • Cardiovascular diseases

List of Abbreviations

Activated vitamin D	$1,25(OH)_2$-vitamin D
PTH	Parathyroid hormone
VDR	Vitamin D receptor
CaM	Calmodulin
BBMI	Brush border myosin I
CaBP	Calbindin
TRPV6	Calcium channel 6
CaSR	Calcium sensing receptor
RIA	Radioimmunoassay
DEXA	Dual-energy X-ray absorptiometry
DC	Dendritic cells
Th cells	T helper cells

Key Facts of Vitamin D

- Vitamin D controls mineral metabolism and the bone homeostasis by increasing calcium availability, by regulating PTH secretion, and by direct effects on bone formation and resorption
- Diagnosis of mild vitamin D deficiency requires circulating levels of 25-OH-vitamin D lower than 25 ng/ml
- Vitamin D has many nonskeletal effects that depend on its ability to reduce cell proliferation, to regulate immune cells, and to stimulate the production of some hormones
- Vitamin D normal levels across the life span may be important for preventing colon cancer and some autoimmune diseases and for reducing the appearance or the progression of breast and prostate cancer, type II diabetes, and cardiovascular diseases
- Long-term administration of vitamin D in at risk populations may be important in reducing the incidence of some cancers and many chronic diseases

Definition of Words and Terms

Rickets	A condition of reduced mineralization of child bone
Osteomalacia	A condition of reduced mineralization of adult bone
Osteoporosis	A condition of increased risk of bone fractures
T helper cells	A lymphocyte cell type that has an important role in the immune system by suppressing or regulating immune responses.
Cutoff	The blood levels of a circulating substance that distinguishes between low and normal levels
Trial	A controlled study involving the administration of a biologic substance compared to the administration of a placebo to a group of patients.
Long-term follow-up	A study in which the studied subjects are controlled for several years to understand the long-term effects of a natural condition or of a therapy

Introduction

A mild deficiency of vitamin D represents the most common cause of altered mineral metabolism and may have important consequences not only on bone quality but also on metabolism and cardiovascular function. In this review, we will present the biological role of vitamin D on mineral metabolism and on extraskeletal tissues, and then we will discuss the way to make diagnosis of mild vitamin D deficiency.

Vitamin D Synthesis and Biological Actions

Activated ($1,25(OH)_2$-vitamin D) vitamin D and parathyroid hormone (PTH) represent the most important regulators of calcium (and phosphate) metabolism (De Luca 2004).

Vitamin D (that includes both ergocalciferol or vitamin D_2 and cholecalciferol or vitamin D_3) is formed in the epidermidis by the effects of ultraviolet rays but to be active needs to be hydroxylated (25OH-vitamin D) in the liver and then in the kidney ($1,25(OH)_2$-vitamin D). 25OH-vitamin D is the principal circulating form of vitamin D, and most assays of vitamin D measure this compound but the biological activity is determined by the $1,25(OH)_2$-vitamin D (activated vitamin D).

Activated vitamin D links to specific receptors that are present in many tissues (Carlberg and Campbell 2013). The main receptor (vitamin D receptor (VDR)) is a nuclear receptor (part of the family of the receptors for steroid hormones, thyroid hormones, and retinoids). Inside the nucleus, VDR heterodimerizes with the receptor of retinoid X (RXR) and the complex VDR/RXR is essential for stabilizing the

binding with vitamin D and producing maximal biological effects. The complex activated vitamin D–VDR/RRX produces a number of different gene transcriptions depending on the interested cell. Activated vitamin D produces also nongenomic effects, such as calcium transportation across cell membranes, that are mediated by different receptors, probably located in the cell membrane, that have not been well characterized.

Vitamin D Role in Mineral Metabolism

That includes activation of intestinal calcium absorption, promotion of bone formation (probably by regulating the expression of several bone growth factors) and resorption, kidney calcium resorption, and inhibition of PTH production (by direct effects on parathyroid cells and indirectly by increasing calcium blood levels).

The most known effect of activated vitamin D is to increase intestinal calcium absorption (Bikle 1990; Wasserman and Fullmer 1995; Hoenderop et al. 2005). Calcium enters the microvillus of the intestinal epithelial cell through TRPV6 calcium channel and then binds to a specific protein, calmodulin (CaM) that is itself bound to brush border myosin I (BBMI). The calcium/CaM complex moves into the terminal web where the calcium is picked up by another specific protein, calbindin (CaBP), and transported through the cytoplasm inside endocytic vesicles. At the basolateral membrane, the calcium is pumped out of the cell by the Ca-ATPase. Activated vitamin D enhances intestinal calcium absorption by inducing most of the mechanisms involved in the microvillus active intestinal calcium transport (TRPV6, CaBP, Ca-ATPase, and the amount of CaM bound to BBMI in the brush border).

Activated vitamin D increases calcium resorption also in kidney with a mechanism that is similar to that found in intestinal microvillus (Friedman and Gesek 1995; Biber et al. 2013). In fact, the molecules critical for calcium reabsorption in the distal tubule appear to be the VDR, calbindin, TRPV5, and the Ca-ATPase.

More difficult has been to determinate whether activated vitamin D has also a role in bone metabolism (Underwood and De Luca 1984; Suda et al. 1992; Takeda et al. 1999; Panda et al. 2004). VDR is found in osteoblasts, and activated vitamin D promotes the differentiation of osteoblasts and increases the production of proteins such as alkaline phosphatase and osteocalcin that are markers of bone formation. Activated vitamin D also increases the production of RANKL so activating the formation of osteoclasts. Patients with vitamin D deficiency present an increase of several bone factors that are linked to bone resorption and formation. However, the rickets resulting from vitamin D deficiency or VDR mutations can be corrected by supplying adequate amounts of calcium and phosphate, and it suggests that the direct vitamin D effect on bone is relatively modest.

Probably, more important for bone metabolism are the indirect effects of vitamin D. In particular, part of the skeletal phenotype in vitamin D deficiency is due to the hyperparathyroidism that develops in the vitamin D deficient state. The relationships

between activated vitamin D availability and PTH secretion are complex (Demay et al. 1992; Liu et al. 1996; Hawa et al. 1996). PTH stimulates the production of 1, 25 $(OH)_2$-vitamin D and in turn 1, 25$(OH)_2$-vitamin D inhibits the production of PTH. This seems to be a direct effect of activated vitamin D on PTH producing cells because within the promoter of the PTH gene is a region that binds the VDR and mediates the suppression of the PTH promoter by 1,25$(OH)_2$-vitamin D. However, calcium alters the ability of activated vitamin D to regulate PTH gene expression. Calcium is a potent inhibitor of PTH production and secretion, acting through the calcium sensing receptor (CaSR) on the plasma membrane of the parathyroid cell. 1, 25$(OH)_2$-Vitamin D induces the CaSR in the parathyroid gland making it more sensitive to calcium.

Figure 1 schematizes the effect of activated vitamin D on bone formation and mineralization.

In states of severe vitamin D deficiency, the reduction of calcium availability has the main effect on bone inducing bone demineralization and, as consequence of it, patients develop rickets if children and osteomalacia if adults.

States of mild vitamin D deficiency increase bone turnover and bone loss determining a condition of osteoporosis. It is probable that the effects of mild vitamin D deficiency on bone are mainly mediated by a direct effect on parathyroid cells with a consequent PTH increase. In fact, in these patients, serum calcium is normal, while circulating PTH is moderately increased and it has been used to monitor the vitamin D deficiency.

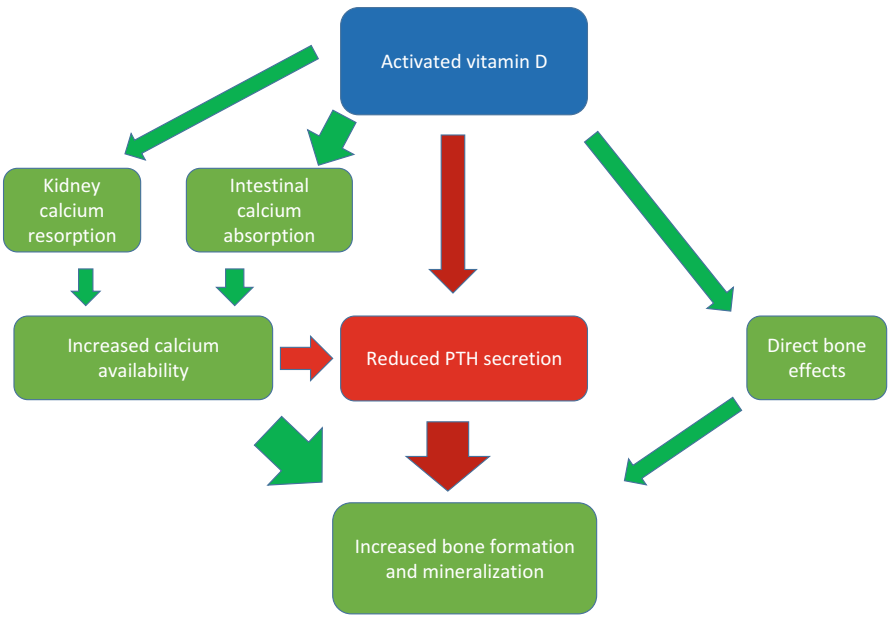

Fig. 1 Effects of activated vitamin D on bone formation and mineralization

Diagnosis of Mild Vitamin D Deficiency

A crucial point for protecting individuals from the consequences of vitamin D deficiency, and at the same time avoiding unneeded therapies is to establish the correct way to make the diagnosis of mild vitamin D deficiency. On this respect, the assay techniques for circulating mono-hydroxylated (25-OH) and di-hydroxylated (1,25-OH) vitamin D have progressed from competitive protein binding assay to radioimmunoassay (RIAs) that utilize both I (125) and chemiluminescent reporters (Carmina et al. 2014). These methods have shown to be very useful in the screening of osteoporotic women for underlying vitamin D deficiency and to be reliable indicators of vitamin D status. Generally 25-OH-vitamin D measurement is a good marker of vitamin D status, while 1, 25-vitamin D assay is needed when kidney function is impaired.

While the assessment of severe vitamin D deficiency is easy, the diagnosis of the much more common mild vitamin D deficiency remains controversial. In fact, completely different threshold values for mild vitamin D deficiency have been suggested. In particular, the American Institute of Medicine has suggested that serum 25-hydroxyvitamin D values lower than 20 ng/ml indicate vitamin D deficiency (Institute of Medicine 2011; Ross et al. 2011), while guidelines of Endocrine Society have indicated that all 25-OH-D values lower than 30 ng/ml are low (Holick et al. 2011). This disagreement depends on different ways to determine vitamin D deficiency, but it has great consequences on clinical decisions. The American Institute of Medicine indicated a threshold value of 20 ng/ml because it corresponded to 2 SD above the median calculated needs of vitamin D. At the contrary, other organizations, in putting the threshold values at 30 ng/ml, used criteria linked to circulating PTH changes. While it is clear that increases in PTH circulating values may reflect bone effect of mild vitamin D deficiency, it may determine an over diagnosis of vitamin D deficiency because small changes of PTH may not have a clinical impact on bone metabolism.

The establishment of a threshold value may also be based on changes of markers of bone resorption (and formation) (Eastell and Hannon 2011) and on bone density. Several biochemical markers of bone turnover are measurable and are widely used for assessing bone formation and resorption. Markers of bone formation include serum bone alkaline phosphatase, total osteocalcin, and the procollagen type I N-terminal propeptide assay. Among the various markers of bone resorption, serum C-terminal cross-linked telopeptides are the most sensitive and specific. In osteoporosis-treatment studies, markers of bone turnover appear even more strongly associated with fracture risk reduction than bone mineral density (BMD).

In a large population study in Southern Italy, we have determined the possible threshold for vitamin D deficiency plotting serum 25OH values against bone t-score values by DEXA plus PTH and bone marker blood levels (Napoli et al. 2014). While the interpretation of the data was complicated by the fact that the studied population was formed by women of postmenopausal age (therefore including also an important component of women having increased bone resorption), our study showed that a

Table 1 Differences in bone t-score by dual X-ray densitometry, bone alkaline phosphatase (*BAP*), collagen telopeptide (*CTX*), and PTH values in a large group of postmenopausal women depending on possible cutoffs for vitamin D deficiency (Modified from Napoli et al. 2014)

	Lumbar T score	Neck femoral T score	BAP	CTX	PTH
20 ng/ml cutoff	NO	YES	NO	YES	YES
25 ng/ml cutoff	NO	YES	NO	YES	YES
30 ng/ml cutoff	NO	NO	NO	YES	NO

threshold value of 25 ng/ml is the best for making diagnosis of mild vitamin D deficiency.

In fact, in our study, using the different cutoffs of 20, 25, and 30 ng/ml of monohydroxylated vitamin D, while no differences in bone markers were noted at any cutoff, a statistically significant decrease in femoral neck t-score and a statistically significant increase in PTH levels were observed when using the 25OHD cutoff of 25 ng/mL but not for 30 ng/ml 25OHD cutoff. The changes in neck femoral bone and PTH were similar using 20 and 25 ng/ml cutoff (Napoli et al. 2014). In Table 1, the results of this study are schematized.

This suggests that a status of vitamin D deficiency exists in women having vitamin D lower than 20 or 25 ng/mL, while the level of 30 ng/mL may be too high. Our data are consistent with the finding of the National Health and Nutrition Survey (NHANES) III where the risk of hip fracture was significantly reduced among participants with 25OHD levels greater than 25 ng/mL compared with those who had lower concentrations (Looker and Mussolino 2008).

In conclusion, it is very difficult to establish in a sure way the threshold for diagnosing mild vitamin D deficiency, but a cutoff of 25 ng/ml seems reasonable and correspondent to the main known effects of vitamin d mild deficiency on bone metabolism.

Vitamin D Effects on Nonskeletal Tissues

While the importance of vitamin D on calcium metabolism and bone maintenance is well proven, in recent years a large debate has occurred regarding possible important extra-skeletal effects of activated vitamin D (Rosen et al. 2012; Cipriani et al. 2015). In fact, vitamin D receptors have been found in most tissues (Rosen et al. 2012; Lee et al. 1994; Bikle 2012), and many studies have shown that activated vitamin D may influence many biological function including cell differentiation and proliferation in many tissues, immune system responses, and some hormone secretions (Carlberg and Campbell 2013; Rosen et al. 2012).

One of the main nonskeletal biologic functions of activated vitamin D is the regulation of immune function. Nuclear receptors for vitamin D (VDR) have been found in many cells of the immune system including macrophages, dendritic cells, and activated T and B lymphocytes (van Etten and Mathieu 2005). In

general, activated vitamin D enhances the innate immune response, whereas it inhibits the adaptive immune response by reducing T cell proliferation, shifting the balance of T cell differentiation from the Th1 and Th17 pathways to Th2 and Treg pathways, and inhibiting the maturation of dendritic cells (DC) important for antigen presentation. Because autoimmune diseases are characterized by excessive Th17 activation, normal availability of activated vitamin D may be essential for preventive excessive inflammatory responses and avoiding the onset of autoimmune diseases (Froicu et al. 2003: van Etten and Mathieu 2005: Adorini and Penna 2008). While vitamin D analogs have shown the ability to improve some disorders like psoriasis, studies in most autoimmune disorders have produced inconclusive results.

It has been suggested that activated vitamin D may protect against insurgence or progression of some cancers by stimulating several inhibitors of cell proliferation. Epidemiologic studies have shown a negative correlation between sun exposure and vitamin D availability and a number of cancers but mainly cancers of the colon, breast, and prostate (Yin et al. 2010; Touvier et al. 2011; Tretli et al. 2012; Maalmi et al. 2014; Wang et al. 2014; Xu et al. 2014; Jacobs et al. 2016). The preventive effect of vitamin D seems particularly important for colon cancer, but activated vitamin D may also reduce the progression and/or the mortality of breast and prostate cancers, too (Jacobs et al. 2016). However, the results of several trials with high doses of vitamin D have been disappointing because no improvement in patients affected by different forms of cancer was observed (Jacobs et al. 2016).

Activated vitamin D may stimulate some hormone secretion. In particular, it may enhance insulin secretion and protect pancreatic beta cells against cytokine-mediated destruction (Lee et al. 1994; Kadowaki and Norman 1985; Both VDR and calbindin are found in pancreatic beta cells. Epidemiological studies have shown that low vitamin D levels are associated to increased risk for type 1 and type 2 diabetes mellitus (Forouhi et al. 2008; Bojesen and Nordestgaard 2013) rising the hope that vitamin D supplementation may reduce the prevalence or the clinical expression of the different forms of diabetes. However, results of randomized controlled trials with vitamin D in patients having type II diabetes have been disappointing because no improvement of the disorder was observed (Avenell et al. 2009; de Boer et al. 2008; Nakashima et al. 2016).

Finally, a deficiency of activated vitamin D has been involved in cardiovascular diseases (Wang et al. 2010; Pittas et al. 2010). Mechanisms related to a better control or a lower prevalence of hypertension have been suggested (Forman et al. 2008), but activation of inflammatory processes and increased atherogenic processes may be also involved. However, as for other disorders that have been linked to vitamin D deficiency, the results of several trials of vitamin D supplementation have been negative, and in some cases the possibility that high doses of vitamin D could be harmful has been raised (Chowdhury et al. 2014; Bjelakovic et al. 2014)

Figure 2 schematizes the effect of activated vitamin D on nonskeletal tissues.

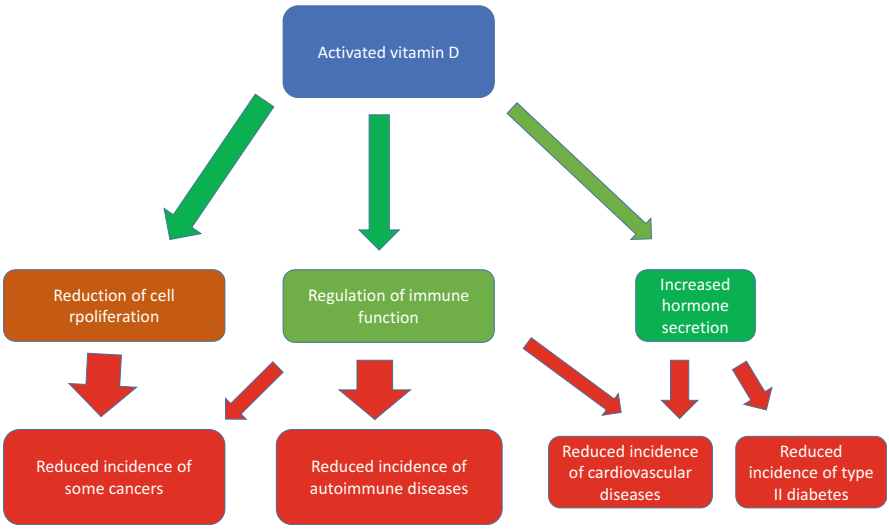

Fig. 2 Effects of activated vitamin D on nonskeletal tissues

Diagnosing Mild Vitamin D Deficiency in Chronic Diseases

Because of the disappointing results of most trials using vitamin D supplementation in chronic diseases, some experts have become convinced that low vitamin D is just a marker of some chronic conditions and not a causal factor.

While it remains a possibility, the quality of many studies has been low and more and better planned studies are needed to understand whether is the role, if any, of vitamin D in these chronic diseases.

It is probable that unsatisfactory results depend on the use of vitamin D in patients who already developed diseases. Most of the nonskeletal effects of activated vitamin D regard the partial inhibition of processes that, if activated, in genetically predisposed individuals, may determine the development of cancers, immune and chronic diseases. It is possible that administration of vitamin D is more effective in preventing than in curing these disorders. Long-term follow-up of populations treated with vitamin D is needed to clarify this possible preventive effect.

Another crucial point is to establish what vitamin D levels should be considered low. Using too high threshold values, while opening the way to a more generalized use of vitamin D supplement, may include in trial studies a too much heterogeneous population so reducing the possibilities of getting correct information. In addition, because vitamin D has a main effect on bone metabolism and other effects on many different tissues, it cannot be excluded that extra-skeletal effects of vitamin D deficiency may require different quantities of vitamin D. If so, it is probable that bone metabolism is sensitive to minor deficiencies of vitamin D, while other tissues

Table 2 Possible cutoffs for vitamin D deficiency in skeletal and extra-skeletal diseases

	Cutoff for vitamin D deficiency
Skeletal diseases	<25 ng/ml
Extra-skeletal chronic diseases	<20 ng/ml

may be affected only when more severe deficiency is present. Using parameters related to mineral metabolism may be incorrect and determine the false conclusion that, in chronic nonskeletal diseases, vitamin D supplementation is not useful. Maybe, until new parameters related to extra-skeletal vitamin D effects are found, trials of vitamin supplementation in chronic diseases should be directed only to patients presenting severe vitamin deficiency or at least lower threshold values as the 20 ng/ml cutoff.

In Table 2, the possible cutoffs for establishing vitamin D deficiency in skeletal and nonskeletal tissues are shown.

Potential Applications

Activated vitamin D has widespread effects that are not limited to mineral metabolism but also to the regulation of the function of different tissues. The consequences of vitamin D deficiency may be rickets, osteomalacia, or osteoporosis but also the appearance or the worsening of chronic diseases like autoimmune diseases, diabetes, cardiovascular disorders, and some forms of cancer, in particular colon cancer.

Vitamin D administration may have an important role in preventing not only classic bone diseases but also colon cancer and some chronic diseases and should be directed to all individuals bearing low circulating 25OH-vitamin D levels. Screening general population for mild vitamin D deficiency may be needed.

Summary Points

- Vitamin D is formed by the effect of ultraviolet exposure but requires two hydroxylations (in liver and kidney) to be activated and be able to determine biologic effects.
- Biologic effects require the link of activated vitamin D with specific receptors that are present in a large number of tissues.
- The main receptor (VDR) is a nuclear receptor and the complex activated vitamin D – VDR activates multiple gene transcriptions. Other receptors may be found in cell membrane and may determine non genomic effects like calcium transportation across cell membrane.
- Activated vitamin D is one of the main regulators of mineral metabolism and bone homeostasis by increasing active calcium absorption in intestinal microvilli and in kidney. These effects are mainly mediated by the link to VDR because require synthesis of multiple proteins.

- Activated vitamin D may also directly increase bone formation and reduces bone resorption but these effects are mainly mediated by its effect on reducing PTH secretion.
- In diagnosis of mild vitamin D deficiency, measurement of 25OH-vitamin D is generally used unless kidney altered function is present.
- Different cutoffs of 25OH vitamin D have been suggested, but a cutoff of 25 ng/ml seems the most reasonable and corresponds to initial bone damage.
- Activated vitamin D has many extra-skeletal actions and in particular may regulate cell proliferation, immune function, and some hormone production.
- Epidemiologic studies have shown a negative correlation between vitamin D availability or levels and several chronic diseases including cancer of the colon, type II diabetes, some autoimmune and cardiovascular diseases. However, in these disorders, trials with vitamin D administration have been disappointing.
- Unsatisfactory results with vitamin D trials in chronic diseases may depend on the use of too high cutoffs and therefore of unselected population but probably depend mainly on the use of this substance in subjects who already developed diseases. Long-term preventive administration of vitamin D in general population may be needed.

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Hip Fracture Risk Is Strongly Related to Circulating Levels of the Advanced Glycation End Product Carboxy-Methyl Lysine (CML)

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Contents

Abstract

Advanced glycation end products (AGEs) are markers of oxidative stress, the process whereby the body is unable to neutralize the effects of oxygen radicals generated during the process of metabolism. Oxidative stress is believed to underlie (in part) the biological process of aging. In this chapter we describe how one particular AGE – carboxymethyl-lysine (CML) – is related to hip fracture risk in a cohort of over 3,000 older adults (mean age 78 years).

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Hip fractures are an age-related disorder with a high degree of morbidity and mortality and are costly to national health care systems.

The study found that for every one standard deviation increase in serum CML level the hazard ratio for hip fracture increased 27% (HR 1.27, 95% CI 1.16–1.40; $p < 0.001$). With adjustment for risk factors strongly associated with osteoporotic fractures (advanced age, low body mass index, white or Asian race, and low bone mineral density), as well as other factors (such as kidney function, alcohol use, and energy consumption), the risk remained significant (HR 1.17, 95% CI 1.05–1.31; $p = 0.006$).

It is concluded that serum CML levels are strongly associated with hip fracture risk independent of other risk factors for hip fracture. These findings suggest that serum CML levels may be a useful tool for gauging hip fracture risk.

Keywords

Hip fracture • Bone quality • Carboxymethyl-lysine (CML) • Oxidative stress • Advanced glycation end products (AGEs)

List of Abbreviations

AGEs	Advanced glycation end products
BMD	Bone mineral density
BMI	Body mass index
CEL	Nε-carboxyethyl-lysine
CHS	Cardiovascular Health Study
CI	Confidence interval
CML	Carboxymethyl-lysine
DM	Diabetes mellitus
DXA	DEXA
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
HR	Hazard ratio
ICD-9	International Classification of Diseases
IL1	Interleukin 1
IL6	Interleukin 6
IQ	Interquartile
NF kB	Nuclear factor kappa B
RAGE	Receptor for advanced glycation end product
TNF-alpha	Tumor necrosis factor alpha

Key Facts

Carboxymethyl-lysine Levels as an Indicator of Hip Fracture Risk

- Hip fractures are a large health care problem among older adults.
- The identification of people at risk for hip fractures is imperfect and requires refinement beyond standard risk factors such as age (the older one is the higher

the risk), sex (women have more fractures than men), bone thickness (the thicker the bone the lower the risk), and race (African Americans have less fractures than whites).
- This study presents evidence that a chemical product that increases in the blood as a person ages is strongly associated with the risk of a hip fracture. This chemical product, called an advanced glycation end product, is also strongly related to the aging process.
- This finding offers the possibility of developing this factor as a risk factor for hip fractures.

Definition of Words and Terms

Advanced glycation end products	These substances stem from glycation reactions, which refer to the addition of a carbohydrate to a protein without the involvement of an enzyme. Glucose can bind with proteins in a process called glycation, making cells stiffer, less pliable, and more open to damage and premature aging. Oxidative radicals drive these reactions.
Bone mineral density	Is a term referring to the amount of mineral matter per square centimeter of bone. It is an indirect measure of bone strength.
Carboxymethyl-lysine	Is one type of AGE which is often used as a marker of total AGE levels.
Osteoporosis	A bone disorder in which bone density and architecture are disrupted leading to an increased risk of fracture.
Oxidative stress	Is the imbalance between the production of oxygen radicals and the ability of a cell to detoxify the effects of these products. Toxic effects occur through the production of peroxides and free radicals that damage proteins, lipids, and DNA.

Introduction

Fragility fractures of the hip are a large and growing societal problem. It is estimated that in 2010 there were 258,000 such fractures in the USA, costing the health care system nearly three billion dollars. By 2030, the number of hip fractures is projected to reach 289,000, an increase of 12% (Online document, Centers for Disease Control 2015).

Bone mineral density (BMD) testing is a widely used and effective tool for assessing fracture risk. Yet, BMD only explains a part of fracture risk (Compston

2009), and it cannot identify which individuals will or will not sustain a fracture (Health Quality Ontario 2006; Marshall et al. 1996). Alternative risk markers which are able to supplement the information gained from BMD testing could be useful to refine risk prediction. Such a marker should preferably reflect the pathophysiological mechanisms that lead to osteoporosis, a leading cause of fractures; should be related to aging, since age is *the* predominant risk factor for hip fractures; and should be independent of BMD, reflecting bone quality independent of bone thickness.

In the following chapter, the association of the advanced glycation end product (AGE) carboxymethyl-lysine (CML) with hip fracture risk is presented. First, what CML is and how AGEs reflect oxidative stress and the aging process is discussed. Next, how oxidative stress leads to bone disease is discussed. Last, the results of a study showing a prospective association between serum CML levels and hip fracture risk is presented with a discussion of the implications of such a finding.

Background

It is hypothesized that the primary factor underlying the biological process of "aging" is the overproduction of oxygen radicals in the mitochondria (Kong et al. 2014). This process derives from an imbalance of pro- and anti-oxidant stresses inside the body's cells. According to this hypothesis, senescence-related disorders are brought about by the accumulation of multiple injuries due to oxidative stress. These injuries include peroxidation of membrane fatty acid chains, modification of DNA (including base alterations, single-strand breaks, and DNA-protein cross-links), and carbonylation of proteins. With regard to the latter, all amino acid residues of proteins are susceptible to oxidation. These reactions lead to formation of protein-protein cross-linkages and oxidation of the protein backbone, resulting in protein structural distortion.

AGEs are markers of ambient oxidative and carbonyl stress of proteins (Vistoli et al. 2013). AGEs are complex molecules that result from glycation reactions, the addition of a carbohydrate to a protein without the involvement of an enzyme (Prasad and Dhar 2014). AGEs include modifications of arginine residues on proteins by glyoxal and methylglyoxal; lysine adducts such as Nε-carboxymethyl-lysine (CML) and Nε-carboxyethyl-lysine (CEL); and pentosidine.

Glycation products alter bone physiologic processes in three ways:

1. AGEs cross-link proteins, making them less soluble to digestion and removal. This is especially so in bone, whose main component is long-lived collagen (Saito et al. 2014; Sanguineti et al. 2014). These alterations make for a less stable secondary and tertiary protein structure and for a more fragile bone that contains more "old" bone than usual. It also leads to reduced tensile flexibility and a greater susceptibility to fracture.
2. The binding of AGEs to their receptor – RAGE – triggers chemical reactions resulting in activation of the nuclear factor kappa B (NF kB) gene (Ramasamy

et al. 2005; Hein 2006). This gene in turn leads to the release of cytokines, adhesion molecules, and inflammation factors (e.g., TNF-alpha, IL-1, and IL-6) by bone cells with AGE receptors. In bone, an increased concentration of AGEs is negatively associated with bone density and mineralization (Sanguineti et al. 2014). Addition of AGEs to cultures of osteoblastic cells leads to lower than normal collagen and osteocalcin production (Hein 2006; Li et al. 2012). RAGE activation inhibits osteoblast proliferation and adhesion of osteoblasts to bone matrix (Odetti et al. 2005). Conversely, RAGE activation enhances osteoclast maturation (Zhou et al. 2006). IL-6 and TNF-alpha promote osteoclast differentiation and bone resorption (Sanguineti et al. 2014). Such effects lead to a loss of equilibrium between bone formation and bone absorption (Odetti et al. 2005).

3. Another mechanism by which AGEs may lead to fragility fractures is through microvascular disease. This mechanism of action was first proposed in 1980 (Weintroub et al. 1980), but it has not gained general acceptance. AGEs are strongly associated with several forms of microvascular disease. These include proteinuric renal disease (Chilelli et al. 2013); retinal eye disease (Kandarakis et al. 2014); and degenerative brain diseases (Jomova et al. 2010). AGEs deposit in all three microvascular beds, consistent with a pathogenic role. Recently, it has been shown that albuminuria, retinal eye disease, and brain white matter disease are each associated with hip fracture risk, providing population-based evidence that microvascular disease may have a direct role (Bůžková et al. 2014). Since these microvascular diseases are associated with AGE levels, it follows that AGEs may be associated with hip fracture risk. The demonstration that the osseous microvasculature contains specialized endothelial cells that control bone cell formation and whose decline leads to decreased bone mass makes this hypothesis an attractive possibility (Kusumbe et al. 2014).

Prior Cross-Sectional Studies of Oxidative Stress, AGEs, and Bone

If the oxidative hypothesis of aging as a risk factor for the development of fragile bones is correct, then markers of oxidative stress should be associated with fracture risk. Several clinical studies indirectly suggest that such is the case. One line of evidence comes from food frequency questionnaire surveys that have estimated the dietary intake of antioxidants. These studies suggest that consumption of foods high in antioxidants and vitamins B and E protect against fractures (Zhang et al. 2006; Sun et al. 2014; Holvik et al. 2014). Cross-sectional and case control studies have related high levels of F2-isoprostanes (prostaglandin-like compounds formed in vivo via a nonenzymatic mechanism involving the free radical-initiated peroxidation of arachidonic acid) in the urine, or low levels of antioxidant enzymes in serum, with lower bone mineral density and higher risk of osteoporosis, respectively. A prospective analysis from the Nurses' Health Study found that higher levels of fluorescent oxidation products (a global measure of oxidation from proteins, lipids, and DNA) predicted hip fractures over a median of ~20 years follow-up (Yang et al. 2014).

With regard to AGEs and fracture risk, several studies have relied upon cadaveric bone specimens and tissue AGE levels giving equivocal results (reviewed by Vashisth 2009). These types of study are time-consuming, expensive, too invasive for clinical use, and impractical as a measure of risk. Other studies have used urine levels of the AGE pentosidine. The results have been inconsistent and primarily focused upon vertebral fractures. One study showed no independent association (Gineyts et al. 2010), others showed positive associations (Shiraki et al. 2008; Tanaka et al. 2011), another showed a positive effect in women but not in men (Yamamoto et al. 2008), and yet another, an association in people with diabetes but not in people without diabetes (Schwartz et al. 2009). There are no prospective studies of AGEs associated with the development of osteoporosis and/or hip fractures. Were levels of an AGE found to be associated with fracture risk it would: (1) serve to validate the oxidative stress theory of aging and of osteoporosis, and (2) potentially prove to be a valuable marker of risk categorization. The results of such a study are now presented.

Prospective Association of AGEs with Hip Fractures

In the study, the association of levels of serum Nε-carboxymethyl-lysine (CML) with hip fracture risk was examined (Barzilay et al. 2014). CML is the major AGE epitope recognized by antibodies prepared against AGE proteins. It is brought about by the reaction of the sugar glyoxal with lysine (Jono et al. 2004). It reflects primarily the glycoxidation of proteins. CML is also formed from the peroxidation of polyunsaturated fatty acids and therefore may be a product of lipo-oxidation (Loidl-Stahlhofen et al. 1994). Both oxidation and glycation are needed for its formation. CML accumulation has been shown to parallel AGE formation overall. Polyclonal antibodies to proteins containing AGEs recognize CML. In addition, CML appears to be the dominant component of AGEs; on average, 30% of lysines present on a protein are incorporated into CML after glycation (Reddy et al. 1995).

A competitive enzyme-linked immunosorbent assay (ELISA) (AGE-CML ELISA, Microcoat, Penzberg, Germany) was used for this study (Boehm et al. 2004). This assay has been validated and shows no cross-reactivity with other compounds (Zhang et al. 2005). The minimum level of detectability of the assay is 5 ng/ml, which is below the concentration found in human studies. Both the intra- and inter-assay coefficients of variation were $<5\%$. Importantly, this assay is relatively easy to perform and does not require specialized equipment.

For this study, data from the Cardiovascular Health Study (CHS) was used. The CHS is a prospective, observational population-based cohort study of 5,888 adults $\geq$65 years in four US communities (Fried et al. 1991). Two cohorts were recruited. In the original cohort, 5,201 eligible men and women were enrolled during 1989–1990. In the second recruitment, during 1992–1993, an additional 687 predominantly black men and women were enrolled. Clinic examinations were performed at study baseline and at annual visits through 1998–1999 and again in 2005–2006. Participants were contacted by telephone annually between exams, and

twice per year during 2000–2004 and 2007, when no clinic examinations occurred. All participants signed informed consent upon study entry.

The cohort for the study was from the 1996 to 1997 examination. At that point, 1,180 of the 5,888 participants had died, and 296 were lost to follow-up or refused further visits. Of the remaining 4,412 participants, 3,373 had an adequate blood sample available from the 1996 to 1997 visit which remained frozen at −80 °C until assayed. Also during the 1996–1997 visit, information was gathered regarding covariates that affect the risk of hip fractures. As a measure of renal function, cystatin C levels (mg/L) were measured from stored samples. Estimated glomerular filtration rate (eGFR) was calculated based on cystatin C levels. Information regarding smoking history, medication use, history of falling in the preceding year, history of diabetes (DM), amount of energy (kcal) expended per week based on the Minnesota Leisure Time Activity Questionnaire, and alcohol use were obtained. Weight, blood pressure, waist circumference, height, grip strength, and the time needed to walk 15 ft were measured. Frailty was defined using a phenotype that requires ≥3 of the following criteria to be present: unintended weight loss >10 lbs in the prior year; self-reported exhaustion most of the time; physical activity in the lowest 20% of CHS cohort; weakness as measured by the lowest 20% of grip strength in the CHS cohort; and slowness of walking (lowest 20% in each sex) (Barzilay et al. 2007). Those with one or two criteria were considered "prefrail," a syndrome with increased risk for the development of frailty. Random urine specimens were used to measure urinary albumin to creatinine ratio; albuminuria was available for 2,972 participants.

A subset of the cohort ($n = 1{,}315$) underwent bone mineral density (BMD) scanning 1–2 years before blood was drawn to measure for serum CML levels. BMD was measured by DEXA (QDR 2000 or 2000±; Hologic Inc, Bedford, MA). All scans were completed using the array beam mode. Scans were read blindly at the University of California, San Francisco reading center with Hologic software version 7.10. The coefficient of variation for total hip BMD was <0.75%.

Data on hip fractures was obtained through patient report and confirmed from hospital discharge codes. Incident hip fracture was identified using International Classification of Diseases, Ninth Revision (ICD-9) codes from hospitalization records from the time of the 1996 to 1997 visit through June 30, 2008. CHS prospectively gathers all hospitalization data, including discharge summaries, from participants every 6 months. To ensure completeness of hospitalization records, data were checked against US government administrative data files (Medicare claims data) to identify any hospitalizations that were not reported by the participant. Hip fracture was defined as ICD-9 code of 820.xx. Admissions for pathologic fractures (ICD-9 code 773.1x) and motor vehicle accidents (E810.xx- E825.xx) were excluded.

The median value of CML was 584 ng/ml (interquartile range, 498, 703 ng/ml) (Fig. 1). Factors crudely associated with increasing levels of CML were older age, decreased eGFR, frailty, higher systolic blood pressure, a greater number of falls in the preceding year, and prevalent CVD. All of these are disorders of "aging." Higher serum CML levels were associated with lower body mass index (BMI) and C-reactive protein levels. Of interest, the prevalence of diabetes did not differ by quartile of CML.

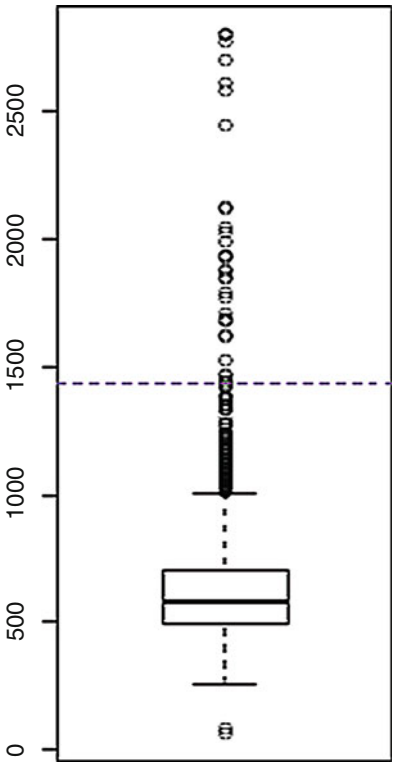

Fig. 1 Box plot of median and interquartile ranges of CML levels in the Cardiovascular Health Study. The 99th percentile is the *dotted line*

Over a median follow-up period of 9.22 years (IQ range, 5.12, 11.42 years), 348 hip fractures were documented during 27,409 person-years of follow-up. Survival free of hip fracture was greatest among individuals with the lowest CML levels, similar among those in the intermediate quartiles, and least among participants with the highest levels (log-rank $p < 0.001$; Fig. 2). The unadjusted HR for hip fracture risk for a one standard deviation increment in CML levels (189 ng/ml) was 1.27 (1.15, 1.40) (Fig. 3). Adjustment for demographic factors had little impact on the HR (1.25 [1.13, 1.38]). Additional adjustment for factors associated with osteoporosis risk only moderately attenuated the HR of hip fracture with serum CML levels (HR 1.18 [1.06, 1.31]). Further adjustment for falling – in the causal pathway for fractures – did not attenuate the association of CML level change with hip fracture risk (HR 1.17 [1.05, 1.31], $p = 0.006$). The association of serum CML levels with hip fracture risk differed little even after exclusion of individuals taking calcium supplements or a bisphosphonate (HR adjusted for Model 2 covariates 1.19 [1.04, 1.35] $p = 0.009$).

When the cohort was stratified by gender, men and women had similar HR for hip fracture in association with CML in the fully adjusted model. There were also no differences in HR between participants with or without diabetes.

Finally, the association of serum CML levels with total hip bone density was examined. Among those undergoing DXA scanning, the mean total hip bone mineral density was 0.83 mg calcium/cm^2 $\pm$ 0.18 (median 0.82 [interquartile range, 0.70,

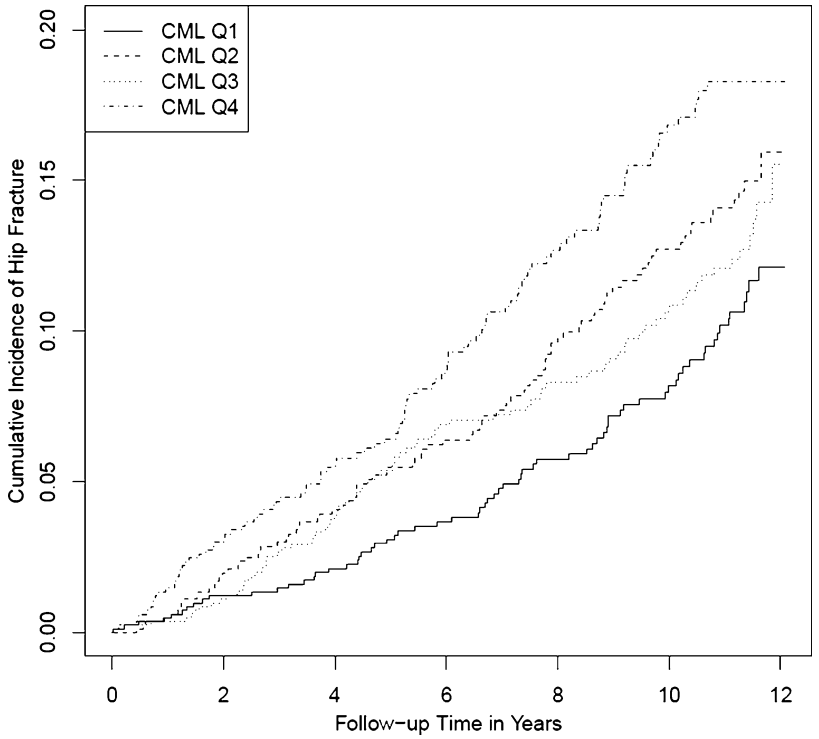

Fig. 2 Cumulative incidence of hip fractures by quartiles of CML levels from the Cardiovascular Health Study

0.95]) and the mean T score was −1.4 + 1.42. Osteoporosis (T score < −2.5) was present in 23.3% of individuals. The correlation between hip bone density and CML levels was small ($r = -0.073$; $p = .01$). In adjusted linear regression models, the association of CML levels with hip BMD was modest and not statistically significant (decrease in BMD per SD increase −0.007; SE 0.004; $p = 0.07$) (Fig. 4). When this sub-cohort was examined prospectively, a one standard deviation increase in CML (189 ng/ml) was significantly associated with an increased risk of hip fracture independent of other risk factors for hip fracture such as age and total hip BMD (Table 1).

Implications of Findings and Potential Applications

These findings have two important implications. First, it is demonstrated that a marker of oxidative stress is prospectively associated with the risk of hip fracture. The association is independent of traditional risk factors for osteoporosis such as age, sex, renal function, diabetes, and smoking. More importantly, the association is independent of bone mineral density (BMD). This latter fact is one of the requisites for a useful new marker, since BMD testing is already widespread in clinical

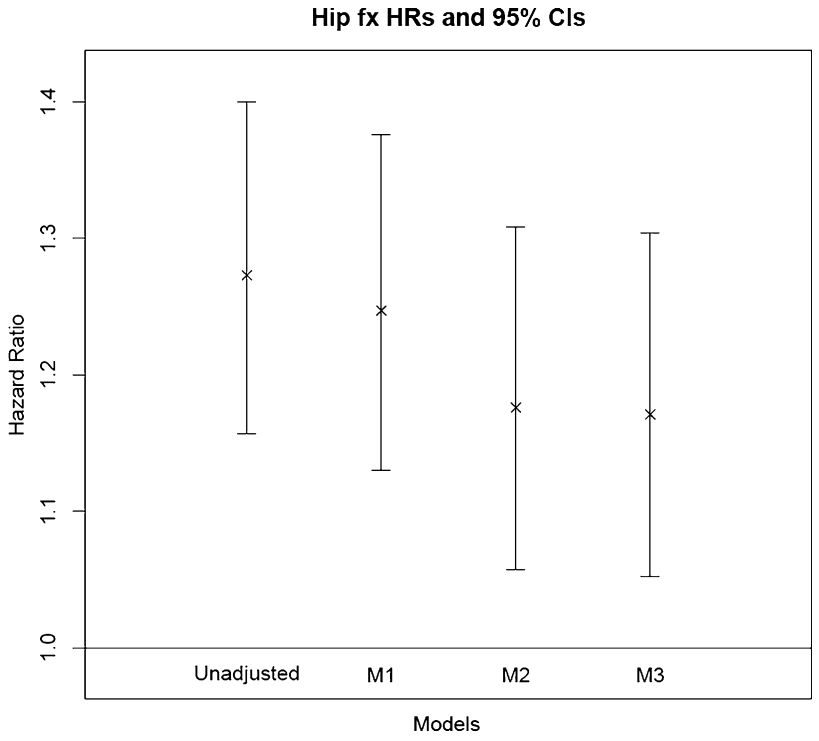

Fig. 3 The hazard ratios of hip fracture per one standard deviation increase of CML level (189 ng/ml) adjusted for factors that are associated with hip fracture risk. *Model 1*: adjusts for gender, age, race/ethnicity, and clinic site. *Model 2*: further adjusts for prevalent coronary heart disease, smoking, BMI, alcohol use, level of physical activity, and baseline eGFR. *Model 3*: further adjusts for a history of falls

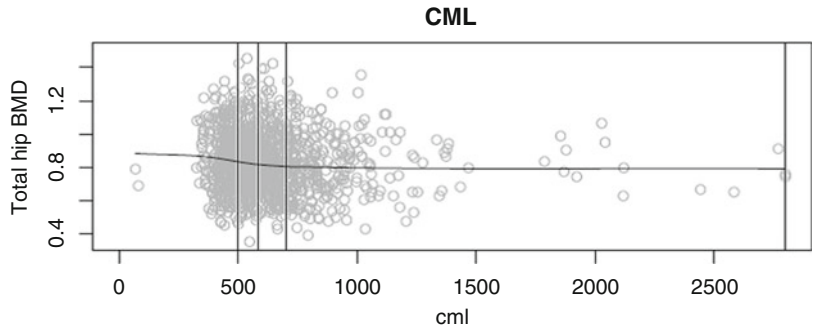

Fig. 4 The association of hip bone mineral density with CML levels in participants from the Cardiovascular Health Study

Table 1 Hazard ratios of hip fracture by an increase of 1 standard deviation (189 ng/ml) of CML level in participants with BMD scanning sequentially adjusted for factors associated with hip fracture, including total hip BMD

	HR	95% CI	*P* value
CML (per SD)	**1.25**	**1.02, 1.52**	**0.03**
Male sex	**2.74**	**1.56, 4.85**	**<0.001**
Age (years)	**1.13**	**1.04, 1.23**	**0.005**
Black race versus White	**0.31**	**0.11, 0.85**	**0.024**
Pittsburgh, Pennsylvania versus Davis, California	1.21	0.77, 1.91	0.41
Former smoker versus current	0.48	0.23, 1.04	0.063
Never smoker versus current	**0.45**	**0.21, 0.98**	**0.044**
1– 7 alcoholic drinks per week versus none	0.71	0.43, 1.15	0.16
Seven or more alcoholic drinks per week versus none	1.41	0.74, 2.70	0.30
BMI per one unit (kg/m^2) increase	1.007	0.95, 1.07	0.81
Prevalent CHD	1.003	0.60, 1.69	0.99
Kcal expended/week (per 1000 kcal)	0.954	0.804, 1.131	0.59
eGFR per ml/minute/1.73m2	**0.99**	**0.97, 0.99**	**0.041**
Fall	1.13	0.66, 1.94	0.66
Prefrail versus no frailty	0.82	0.50, 1.35	0.43
Frail versus no frailty	1.18	0.55, 2.53	0.66
Hip BMD per mg/cm squared	**0.993**	**0.991, 0.995**	**<0.001**

findings. This latter finding suggests that serum CML levels may be a marker of bone quality, i.e., bone strength that is independent of its thickness. Of further interest is the fact that CML levels had only a modest association with sex, BMI, race, and age in the cohort. These are factors that are consistently related to osteoporosis and hip fracture risk. Such a weak degree of association suggests that serum CML levels may be a truly independent marker of fracture risk. Last serum CML levels rise with increasing age (Viteri et al. 2004) – thereby fulfilling an essential requirement for it to be a risk factor for hip fracture risk. However, because CML has not been widely measured, these findings require corroboration.

Second, the present findings have implications for people with type 2 diabetes. It is estimated that up to 20–25% of adults over age 65 years – the age at which hip fractures typically occur – have type 2 diabetes (Online document, American Diabetes Association 2015). Prospective studies have demonstrated that individuals with type 2 diabetes have an increased risk of osteoporotic fractures, particularly of the hip, despite having higher BMD than people without diabetes (Vestergaard 2007). The risk is approximately 20% higher compared to people without diabetes (Lipscombe et al. 2007). The present finding – that a marker of oxidative stress impacts bone quality – may help to explain the paradoxical increase in hip fracture risk despite preserved or increased BMD.

At this point, there is not a direct method for reducing AGEs that might be used as a therapeutic agent to reduce hip fracture risk, especially in those with elevated

serum levels of CML. Although at least one agent has been tested in early clinical studies (Zieman et al. 2007), none has yet advanced to more promising Phase III trials. Nonetheless, there may be lifestyle interventions that could reduce CML levels if the present promising studies are confirmed. For example, cooking has an important impact on the generation of AGEs in food (Uribarri et al. 2010), and dietary AGEs accumulate in key organs in animal models (Roncero-Ramos et al. 2014). These results point to potential interventions to improve bone quality based on AGE measurement in the future.

Conclusion

A strong relationship between serum CML levels and hip fracture risk has been demonstrated in this study. This is the first population-based evidence that AGEs may have role in deteriorating bone quality with age. The results require confirmation and ideally would show statistical evidence that CML helps to discriminate between individuals who will and will not go on to develop a hip fracture above and beyond traditional risk factors. Equally, intervention trials that test whether reducing AGEs can improve bone quality are still needed. Until then, it can only be said that CML offers the tantalizing possibility of improving risk stratification and prevention of hip fracture.

Summary Points

- Oxidative stress – the overproduction of oxygen radicals in the mitochondria – is believed to play a key role in the aging process.
- Oxidative stress can lead to qualitative changes in bone strength that may increase the risk of hip fracture, an age-related disorder.
- The advanced glycation end product carboxymethyl-lysine (CML) is formed through oxidative stress and is a marker of oxidative stress.
- In this summary chapter it is demonstrated that hip fracture risk increases in older adults with increasing serum CML levels. Importantly, this increased risk is independent of age, sex, body mass index, race, and bone mineral density. These latter factors are the main determinants that have been identified to date for hip fractures.
- The results suggest that serum CML levels may be a useful marker for hip fracture risk in older adults.

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Tartrate-Resistant Acid Phosphatase as a Biomarker of Bone Remodeling

19

Divya Vohora and Bushra Parveen

Contents

Abstract

Tartrate-resistant acid phosphatase (TRACP) is an enzyme abundantly expressed in tissues such as the bone, spleen, liver, lungs, and skin. It has two isoforms, namely, TRACP 5a and TRACP 5b. TRACP 5a is specific for the placenta and

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lungs, whereas TRACP 5b is exclusive to the bone, spleen, thymus, kidney, colon, and brain. This chapter discusses the physiological functions as well as the pathological conditions associated with TRACP. While TRACP 5a is a prognostic marker for chronic inflammation, TRCAP 5b is a marker of osteoclast number. Plethora of studies revealed its importance in treatment monitoring, owing to absence of diurnal fluctuations, food effect, and accumulation in renal and liver impairment. Through this work, an effort has been made to collate the scientific information on the role and mechanism of TRACP in bone resorption and its advantages over other bone markers.

Keywords

Tartrate-resistant acid phosphatase • TRACP 5a • TRACP 5b • Bone resorption • Osteoclast number

List of Abbreviations

AOD2	Type II autosomal dominant osteopetrosis
AP	Acid phosphatase
BMPs	Bone morphogenetic protein
Cbfa1	Core-binding factor alpha 1
CRP	C-reactive protein
CTX	Collagen-type 1 cross-linked C-telopeptide
FSD	Functional secretory domain
FSD	Functional secretory domain
GLP-2	Glucagon-like peptide 2
HRT	Hormone replacement therapy
IGF-1	Insulin-like growth factor 1
IgM	Immunoglobulin M
IL-6	Interleukin 6
kD	Kilo Dalton
NF-kB	Nuclear factor kappa B
NTX	Amino-terminal collagen cross-link
OA	Osteoarthritis
ODF	Osteoclast differentiation factor
OPGL	Osteoprotegerin ligand
PAP	Plant acid phosphatase
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RANKL	Receptor-activated nuclear factor kappa ligand
ROS	Reactive oxygen species
TGF-β	Tumor growth factor β
TRACP	Tartrate-resistant acid phosphatase
TRANCE	Tumor necrosis factor-related activation-induced cytokine

Key Facts

1. In the mid-1990s, researchers partially purified a bovine splenic phosphatase with an acidic pH and a purple or violet color. About 20 years later, in 1974, Schlosnagle and colleagues were able to purify a basic, progesterone-induced, intrauterine glycoprotein with a purple color from uterine fluids of pigs, which had acid phosphatase activity. Later on, this enzyme was named uteroferrin, because of the proposed role in iron transport from maternal to fetal circulation.
2. Protein phosphatase differs essentially from the TRACP family by having a water molecule instead of the tyrosine residue coordinating the ferric iron, which accounts for the loss of purple color.
3. TRACP 5b is secreted from osteoclasts in the enzymatically active form and gets activated upon losing its iron content. It is finally degraded to fragments that are eventually cleared from the circulation through the liver. Therefore, it is the secreted TRACP 5b that accurately describes the amount of intact TRACP 5b enzyme molecules that have been freshly released from osteoclasts.
4. It has been estimated that only approximately 10% of TRACP circulates as intact enzymatically active molecules in human blood, while the remaining approximately 90% circulates as inactive fragments. On the other hand, approximately 87% of TRACP molecules in human blood circulate as the non-osteoclastic form TRACP 5a, while only approximately 13% circulate as the osteoclast-derived TRACP 5b. Therefore, a diagnostic TRACP-method describing bone resorption should measure only enzymatically active TRACP 5b, without measuring enzymatically active TRACP 5a or interfering inactive fragments.

Definitions

Apoptosis	Series of biochemical events that induces programmed cell death.
Cytokines	The cell signaling molecules that assists in immune response by establishing cell-to-cell communication and directs the movement of cells toward infection or inflammation.
Enzymes	The macromolecular biocatalysts that speed up the biochemical reactions and act on substrates to convert them into products.
Gaucher's disease	A genetic disorder characterized by accumulation of fatty substances "sphingolipids" in different cells and organs.
GLP-2	Gastrointestinal hormone secreted from intestinal endocrine cells that improves epithelial barrier function.
Hairy cell leukemia	Hairy cell leukemia is rare type, slow-growing cancer of white blood cells, wherein, the bone marrow produces excess of white blood cells or "lymphocytes" and appears hairy-like under microscope.

Hemochromatosis	Abnormal accumulation of iron in the parenchymal tissues leading to organ toxicity.
Integrins	Heterodimeric transmembrane cell adhesion receptors that attach a cell to the extracellular matrix.
Metastases	Spread of cancer cells from one part to another in the whole living system.
Orchidectomy	Removal of one or both testicles surgically.
Osteoblast	Bone cells with single nuclei involved in bone formation.
Osteoclast	Multinucleated bone cells associated with resorption of bone.
Osteoclast number	The mean number of osteoclasts per square millimeter of total tissue area.
Osteoclastoma	The giant cell tumor of the bone characterized by the presence of osteoclast-like multinucleated giant cells. It is usually benign in nature.
Osteopetrosis	Rare inherited clinical syndrome characterized by failure of osteoclasts to resorb bone. Also known as marble bone disease or Albers-Schönberg disease.
Osteoporosis	Bony disease characterized by brittle, fragile, and reduced strength of the bone.
Paget's disease	Abnormal breakdown of the bone in specific parts of the skeleton followed by formation of bone which are comparatively weak and fragile.
Remodeling	The turnover of bone involving coordinated processes of bone formation and resorption.

Introduction

Tartrate-resistant acid phosphatase (TRACP) *is a* glycosylated monomeric metalloprotein enzyme that belongs to the family of enzymes acid phosphatases [3.1.3.2]. Acid phosphatases are a class of phosphatase enzyme [EC 3.1.3] that belongs to esterase-hydrolase [EC 3.1] category of hydrolase enzyme (Moss 2015). The enzyme classification is summarized in Figure 1. Tartrate-resistant acid phosphatase (TRACP) was earlier abbreviated as TRAP, but after the recommendation from the nomenclature committee, "C" was added to TRAP to differentiate it from acid phosphatases (AP) (Delmas et al. 2000). TRACP is also referred to as acid phosphatase 5, purple acid phosphates, or uteroferrin. The name type-5 acid phosphates is derived from the studies of Li and coworkers, where they found seven distinct isomers of acid phosphatase from human leucocytes as type 0, 1, 2, 3a, 3b, 4, and 5 in order of their electrophoretic mobility toward cathode (Li et al. 1970a, b), and only the most cathodal band 5 was resistant to tartrate. TRACP was named as purple acid phosphatase due to the unique purple color of the enzyme and uteroferrin when TRACP is purified from the uterine fluids of pigs (Ek-Rylander et al. 1997).

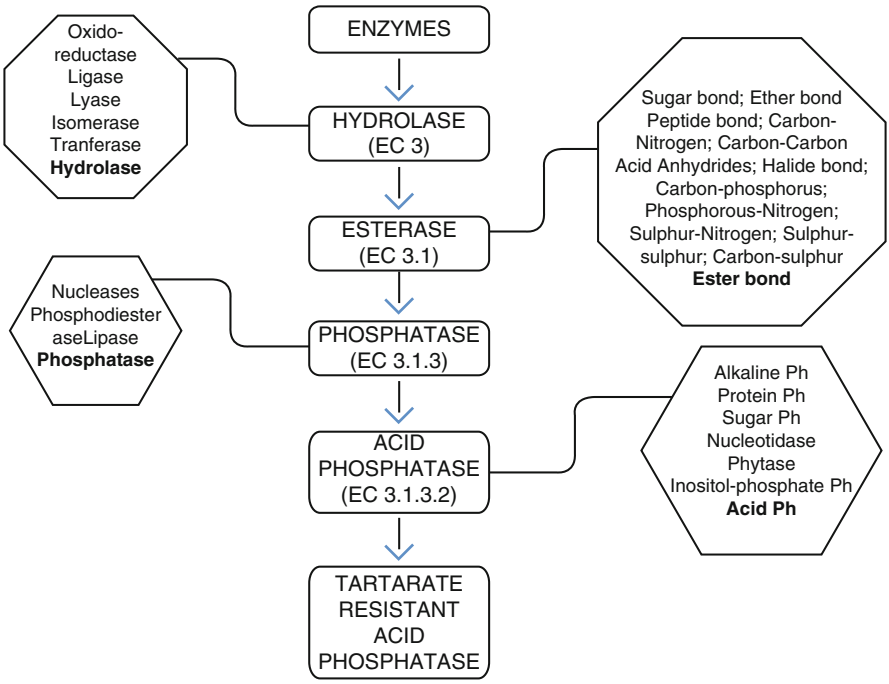

Fig. 1 Hierarchical position of TRACP in enzyme classification

Structure

TRACP isolated from mature human consists of 304 amino acids sequence. The relative molecular weight of unglycosylated TRACP is 34,193 (Klabunde et al. 1995). The amino acid sequence contains two potential *N*-glycosylation sites (Asn95 and Asn126), seven residues coordinating two Fe atoms (Asp12, Asp50, Tyr53, Asn89, His184, His219, and His221), and a Cys(X)5 Arg catalytic motif (Cys198-Arg204) essential for phosphoester hydrolysis (Fagerlund 2009). Acid phosphatases are also found in nonmammalian species such as bacteria, fungi, parasites, and plants, and most of them share structural similarities with mammalian acid phosphatase enzyme (Araujo and Vihko 2013; Fig. 1).

The molecular weight of human TRACP is 34 kD whereas of plant PAPs is 110 kD. Despite differences in plant and humans' sequence homology, their core structure is similar to large extent due to presence of seven metal coordinating amino acids in the catalytic residues. The catalytic site has a dinuclear metallic center consisting of divalent-trivalent ions. The trivalent ion is Fe^{+3}, whereas the divalent ion is Fe^{+2} in mammals and Zn^{+2} or Mn^{+2} in plants. And the purple color arises due to charge transfer from tyrosine to Fe^{+3} (Klabunde et al. 1995). TRACP is the only metallo-enzyme of this family which requires a metal-binding site for its catalytic activity (Araujo and Vihko 2013).

The human sequence reveals that a disulfide bond is located between two cysteine residues (Cys140 and Cys198). Similar structure is observed in uteroferrin, rat bone TRACP, and human recombinant TRACP. The repression loop formed by residues 140–165 has an important role in regulating enzymatic activity as it is susceptible to proteolytic hydrolysis. Two sites (Asn95 and Asn126) are present for N-linked glycosylation, but studies show glycosylation only at Asn95. However, rat recombinant TRACP expressed in *Spodoptera frugiperda* 9 (Sf9) cells were glycosylated at both potential sites (Wang et al. 2005).

Expression and Location

TRACP is abundantly expressed in various tissues as a minor enzyme for acid phosphatase activity like in the liver, the epithelial linings of the gastrointestinal tract, tongue and oropharynx, lung, dental papillae, thymus, and skin. However, the highest expression is found in osteoclasts and macrophages (Hayman et al. 2000a, b). TRACP is an established marker of osteoclast. The enzyme is also reported in osteoblasts and osteocytes, lying in close vicinity to osteoclasts (Solberg et al. 2014). Its function in these cells is debatable. One theory states that the osteoclastic TRACP is endocytosed by the osteoblasts or osteocytes for inactivation (Perez-Amodio et al. 2006). But endogenous synthesis of the enzyme is found in osteoblasts and osteocytes, lying closer to the surface of osteoclasts. This suggests that TRACP may take part in the mechanisms directing osteoclastic bone resorption (Solberg et al. 2014). TRACP has also been shown to be expressed by alveolar macrophages in the lung and Kupffer cells in the liver (Efstratiadis and Moss 1985; Yaziji et al. 1995). Histochemical studies in rats suggest that TRACP/PAP has also been expressed in several parts of peripheral and central nervous system. TRACP activity was demonstrated in the nerve cells of olfactory bulb in rats (Lang et al. 2001).

TRACP is found to be elevated in pathological conditions such as hairy cell leukemia (Yam et al. 1971), Gaucher's disease (Robinson and Glew 1980), osteoclastoma (Hayman et al. 1989), Paget's disease (Torres et al. 1991), hyperparathyroidism (Stepan et al. 1983), and in inflammatory conditions such as rheumatoid arthritis (Janckila et al. 2002). Serum concentration of TRACP is increased in patients of breast and ovarian cancer with bone diseases (Capellar et al. 2003). Furthermore, TRACP expression has been found in tissues of breast and ovarian cancer and in malignant melanoma (Hoing et al. 2006; Adams et al. 2007).

Studies have demonstrated the subcellular location of TRACP in the cytoplasm of osteoclast, packed in vesicles, vacuoles, and/or small dense granules of mitochondria (Clark et al. 1989). It is reported that TRACP is present intracellularly only and not in resorption lacunae or ruffled borders (Halleen et al. 1999a), whereas other revealed that TRACP is present in intracellular lysosome-like vesicles but more concentrated in resorption lacunae or ruffled borders (Reinholt et al. 1990). In some studies, TRACP has been demonstrated in osteoblastic lysosomes and in the Golgi complex, including lamellae, vesicles, and vacuoles (Yamamoto and Nagai 1998).

Table 1 Difference between TRACP 5a and TRACP 5b isoforms

Property	TRACP 5a	TRACP 5b
Structure	Single polypeptide	Two subunit
Distribution in circulation	90%	10%
Molecular weight	35kD	16 + 23 kD
Sugar chain	High mannose type, contains sialic acid	Multiantennary complex type
Specific activity	Low, ~200 U/mg	High, ~2000 U/mg
Optimum pH	5.0–5.2	5.8
Tissue expression	Lung and placenta	Bone, spleen, liver, kidney, thymus, colon, and brain
Source	Macrophages Dendritic cells	Osteoclasts
Loop peptide	Intact	Cleaved
Purpose	Marker of inflammatory conditions	Marker of bone resorption

Isoforms

TPACP exists in two isoforms, TRACP 5a and TRACP 5b, of which TRACP 5b is specific for osteoclast function (Minkin 1982), while TRACP 5a is expressed by macrophages. Structurally, TRACP 5a differs from 5b due to an additional sialic acid chain in the former and single polypeptide nature. The additional sialic acid chain confers different electrophoretic mobility to both forms (Fohr et al. 2003). Although they are the products of same gene, the difference arises due to posttranslational processing, which imparts them different enzymatic activity, pH optimum, and substrate preference. The 5b form exhibits approximately 5.5-fold higher specific activity than the 5a form (Lam et al. 1981). The pH optimum is 5.0–5.2 for TRACP 5a and 5.8 for TRACP 5b (Janckila et al. 2001). As repression loop is responsible for enzymatic activity, hence proteolytic excision of the loop is responsible for different activities and altered pH optimum (Funhoff et al. 2001; Janckila et al. 2005). Traditional assay methods were mostly colorimetric and did not differentiate the isoforms 5a and 5b, while specific immunoassays are now developed which are specific for the isoforms (Halleen et al. 1999a). The differences between two isoforms are summarized in Table 1.

Physiological Functions of TRACP

Acid Phosphatase Enzymatic Activity

In an acidic environment, TRACP catalyzes hydrolysis of nucleoside tri- and diphosphates, phosphoesters (4-nitrophenyl phosphate, α-naphtyl phosphate, and phosphotyrosine), and phosphoanhydrides (pyrophosphate), but aliphatic

phosphates and aliphatic phosphoesters are not hydrolyzed efficiently (Llyod and Mason 1996). The acid phosphatase activity of the enzyme is inhibited competitively by inorganic phosphate and noncompetitively by fluoride, copper, and zinc (Janckila et al. 1992). However, complete deactivation of the enzyme occurs by oxidizing agents such as hydrogen peroxide and strong reducing agent, dithionite (Schlosnagle et al. 1974; Fagerlund 2009). While β-mercaptoethanol and dithiothreitol act by reducing the disulfide bond, ascorbate reduces the redox-active ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) with change in color from purple to pink. Hydrogen peroxide acts by converting the Fe^{2+} to Fe^{3+}, whereas dithionite deactivates the enzyme irreversibly by removing the iron center, resulting in a colorless form (Funhoff et al. 2005; Ljusberg et al. 1999). The repression loop is the major regulator of AcP activity of the enzyme. And an increase in activity is observed when the peptide loop is excised (Ljusberg et al. 1999; Fagerlund 2009).

Reactive Oxygen Species Generation

Kajia et al. (2002) studied the acid phosphatase and ROS generating activity of TRACP at pH optimum of 4.5 and 6.5, respectively (Kajia et al. 2002). For ROS generation, neutral environment is most favorable. The ROS generation is facilitated by redox-active iron through the Fenton reaction (Hayman and Cox 1994). As the enzyme is in a mixed valent form (Fe^{3+}–Fe^{2+}), Fe^{2+} reacts with hydrogen peroxide to produce Fe^{3+} and hydroxyl radical (OH·) (Eq. 1). The reduced forms (pink) of uteroferrin and bovine spleen phosphatase acted as better catalysts than their oxidized (purple) forms (Sibille et al. 1987). The Fe^{3+} produced further reacts with hydrogen peroxide, regenerating the Fe^{2+} and forming a superoxide anion (O_2·) (Eq. 2). This sequence of reactions continues as long as hydrogen peroxide is available (Halleen et al. 2003):

$$\mathrm{TRACP-Fe^{+2}} + \mathrm{H_2O_2} \rightarrow \mathrm{TRACP-Fe^{+3}} + \mathrm{OH^{\cdot}} + \mathrm{OH} \quad (1)$$

$$\mathrm{TRACP-Fe^{+3}(purple)} + \mathrm{O_2^{-}} \rightarrow \mathrm{TRACP-Fe^{+2}(pink)} + \mathrm{O_2} \quad (2)$$

Osteoclastic Bone Resorption

Bone growth and remodeling are normal physiological processes that progress as a result of coupled actions between osteoblasts that synthesize new bone material and osteoclasts which are responsible for breaking down existing bone material. Equilibrium is maintained between resorption and formation so that old bone is continuously replaced by new tissue and adapts to mechanical load and strain. The remodeling phenomenon was first defined by Frost, in 1990 (Hadjidakis and Androulakis 2006). Disruption in the remodeling process may result in

various skeletal abnormalities such as osteoporosis, Paget's disease, hyperparathyroidism, hyperthyroidism, osteomalacia, and osteopetrosis (Kini and Nandeesh 2012).

The association between osteoclasts and the enzyme TRACP has been shown in several studies. Ample amounts of evidence from in vitro, in vivo, and clinical studies indicate that TRACP is implicated in the bone resorption process (Bull et al. 2002). The in vitro osteoclast-cultured experiments show the development of resorption lacunae and accumulation of TRACP. The addition of primary osteoblasts or osteoblastic cell lines to the osteoclast-like multinucleated cells increase the number of resorption pits. This indicates that cell-to-cell interaction between osteoblast and osteoclast facilitates osteoclastic function (Phan et al. 2004). In vivo studies on TRACP knockout mice show development of mild osteopetrosis (Hayman et al. 1997; Hayman and Cox 2003). A higher serum concentration of TRACP is observed in people with skeletal disease than in normal control subject (Halleen et al. 1999b). A direct relationship is observed between osteoclast facilitated bone resorption and appearance of TRACP in the circulation. This suggests that serum TRACP can be regarded as a biomarker for the clinical diagnosis of bone resorption and for quantitatively monitoring the rate and progression of metabolic bone disorders (Bull et al. 2002; Rico and Villa 1993).

Mechanism of Bone Remodeling

Osteoclasts originate from the hematopoietic stem cells of mononuclear lineage. These cells undergo differentiation followed by migration to the site of bone resorption, possibly under the influence of cytokines (Hadjidakis and Androulakis 2006). A premyeloid precursor can differentiate into a macrophage, dendritic cell, or osteoclast, as all three have a common differentiation pathway, depending upon their exposures to different ligands. Exposure to receptor activator of NF-kB ligand RANKL, also called tumor necrosis factor-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), or osteoclast differentiation factor (ODF), differentiates PML into osteoclast (Kong et al. 1999).

There are several modifications that take place to ease the resorption process including formation of a ruffled border, a sealing zone, and a functional secretory domain (FSD) (Kalervo et al. 2000). First and foremost, there is formation of sealing zone upon arrival of osteoclasts at the resorption site facilitated by integrin αVβ3, expressed by resorbing osteoclasts (Teitelbaum 2000). Integrins are molecules that enable attachment between two entities, in this case, the bone matrix and the osteoclast. Although four different types of integrins are expressed on osteoclast surface, namely, αvβ3, αvβ5, α2β1, and αvβ1 (Nesbitt et al. 1993), it is integrin αVβ3 that has received much attention. The attachment usually occurs by structures called podosomes, which are dynamic and helps in movement of osteoclast across the bone surface (Hadjidakis and Androulakis 2006). As osteoclast begins to resorb and attaches itself to the bone matrix, the ruffled border develops within the sealing zone and is encircled by it. These specific membrane-domain adaptations form a specialized resorbing organelle, which is made from the fusion between numerous intracellular acidic vesicles and plasma membrane facing bone matrix (Bull et al. 2002).

At an initial stage of bone resorption, the mineral framework of bone consisting of hydroxyapatite is first dissolved. Bone demineralization involves acidification of the

isolated extracellular microenvironment. The source of protons is the cytoplasmic carbonic anhydrase II enzyme, which is synthesized in large amounts within the osteoclasts. The protons are released across the ruffled membrane in the resorption lacunae by a vacuolar H^+-adenosine triphosphatase (H^+-ATPase) pump located in the ruffled border membrane and intracellular vacuoles (Li et al. 1999). This results in a low pH of around 2.5–3.0 in the osteoclastic resorption space (Silver et al. 1988). After dissolution of mineral contents of bone, the organic matrix is still intact. The second step in bone resorption proceeds with degradation of bony organic matrix. This is catalyzed by proteolytic enzymes such as cathepsin K, a proteinase, and matrix metalloprotease 9 (collagenase B), synthesized by osteoclasts, and secreted into the sealed resorption zone (Bull et al. 2002). This results in the formation of a Howslip's lacuna into the bone.

The free-organic and nonorganic fragments of the bone matrix liberated from the bone in the resorption lacunae are endocytosed across the ruffled border through a transcytotic vesicular pathway into the osteoclast cell. These vesicles then fuse with a specialized region of the basal membrane called the FSD and release the contents into the extracellular space (Nesbitt and Horton 1997).

How TRACP Facilitates Bone Resorption

TRACP is shown to play role in bone resorption both extracellularly and intracellularly. The extracellular accumulation of TRACP in the bone matrix near ruffled border of resorbing osteoclast suggests the extracellular role for TRACP in bone resorption. As TRACP is a nonspecific phosphatase, it has been shown in vitro that osteopontin, bone sialoprotein, and osteonectin are TRACP substrates. It acts as a phosphoprotein phosphatase, by catalyzing the release of phosphate groups from these protein molecules (Ek-Rylander et al. 1994). Osteopontin, a non-collagenous protein, and sialoprotein both are unable to attach to osteoclast when dephosphorylated. Osteopontin is highly expressed at the bone surface opposite the sealing zone of resorbing osteoclasts and is essential for resorption to take place (Sodek et al. 2000). It is hypothesized that TRACP is secreted in the ruffled border, acts on osteopontin to dephosphorylate them, and thus allows bone resorption to occur. Osteopontin enables the binding of osteoclast to the bone surface by binding with integrins (Chellaiah et al. 2003). But when TRACP removes phosphate group from osteopontin, the adhesion of osteoclasts to the bone gets disrupted, therefore enabling osteoclast migration and further resorption (Ek-Rylander et al. 1994). Pyrophosphates also act as substrates for TRACP. As bone matrix is rich in pyrophosphate, which is an inhibitor of bone resorption. TRACP hydrolyzes pyrophosphate and liberates them from bone matrix, hence enabling bone resorption (Bull et al. 2002).

TRACP has also been found to act intracellularly in the endocytosed vesicles along with the fragmentation products of bone matrix. TRACP catalyzes the generation of ROS because TRACP in its native reduced form has an Fe(III)-Fe(II) active site, with the ferrous ion acting as an electron donor (Steinbeck et al. 1994). TRACP-generated ROS can participate in bone matrix degradation intracellularly in transcytotic vesicles. This also explains why the concentration of TRACP increases in the circulation during bone resorption. The enzyme is present in the transcytotic

vesicles to help dispose of the products of bone breakdown. It along with fragments of bone matrix is released into the extracellular environment as an active enzyme by exocytosis at the FSD. TRACP then subsequently leaks into the circulation through the interstitial fluid (Halleen et al. 1999b).

Fate of TRAP

TRACP when secreted is exposed to physiological conditions of the body fluids. It has a tendency to bind and form complex with a high molecular weight molecule in serum, namely, α_2 macroglobulin (Brehme et al. 1999). Therefore, α_2 macroglobulin acts as a carrier molecule for TRACP that facilitates its clearance from areas of bone resorption followed by circulation. Degradation of the enzyme starts with the disruption of structure. The fragments generated from these events are metabolized by the liver and/or removed in the urine (Bull et al. 2002).

TRACP in Osteoblast Regulation

The differentiation of osteoblasts is regulated by numerous local factors, e.g., TGF-β, BMPs, hedgehogs, and the transcription factor core-binding factor α-1, Cbfa 1. IGF-1 is a regulator of bone mineral density. RANKL, an essential cytokine for the formation and activation of osteoclasts, is expressed on the surface of osteoblasts. Hayman and colleagues performed preliminary studies on osteoblasts from TRACP −/− mice which proposed that TRACP has a role in osteoblast regulation. There was an increase in the production of alkaline phosphatase which correlated with the increase in mineralization in the TRACP −/− mice. Conventional PCR demonstrated there was an increase in the expression of cbfa1 and osteocalcin in TRAP −/− osteoblasts (Hayman 2008). Other investigators have suggested that osteoclast-derived TRACP may regulate bone formation and may play a role in the coupling of bone formation to resorption. Sheu and colleagues have previously suggested that osteoclast-derived TRACP may function as a potential factor to stimulate osteoblast differentiation and activity (Sheu et al. 2003). However, in contrast to the reports from knockout mice study, Sheu and colleagues suggested that TRACP is a positive regulator of osteoblast activity. Orthovanadate, an inhibitor of TRACP, has been reported to stimulate the development of the differentiated osteoblast phenotype (Johnson and Henderson 1997). This supports that TRACP is a negative regulator of osteoblast activity. Cell culture studies suggest that osteoblasts endocytose osteoclastic TRACP for inactivation. (Solberg et al. 2014) reported that increased osteoclast activity was not associated with the expression of TRACP on osteoblasts or osteocytes and that increased TRACP in these cells is due to increased synthesis (Solberg et al. 2014).

TRACP in Bone Pathologies

Osteoporosis is a condition characterized by the loss of the normal density of bone, resulting in fragile bone. Osteoporosis leads to abnormally porous bone

characterized by bone fragility and increased fracture risk. TRACP-5b is used for evaluation of osteoclastic resorption activity.

Increased levels of TRACP have been observed in conditions with enhanced bone resorption, such as healthy postmenopausal women and individuals with osteopenia, osteoporosis, or Paget's disease. Also children have slightly elevated TRACP levels which are further elevated in those with vitamin D deficiency. Bone metastases in all cancer types have significantly higher serum levels of TRACP. Elevated serum levels of TRACP are also reported in multiple myeloma patients and reflect the progression of the disease, which makes TRACP as a predictive tool for bone disease in multiple myeloma (Chao et al. 2010). TRACP however not only detects high bone turnover but also predicts future bone loss or fracture risk. The pathological conditions associated with TRACP are summarized in Table 2.

Clinical Utility of TRACP

TRACP 5a: A Marker of Chronic Inflammation

TRACP 5a is the principal isoform secreted by macrophages and dendritic cells in vitro (Janckila et al. 2002). A monoclonal antibody to recombinant human TRACP 5a, mab220, was developed and was found to stain macrophages and inflammatory cells specifically. This suggests that TRACP 5a is a specific marker of chronic inflammation (Janckila et al. 2008). Elevated serum TRACP 5a levels have been reported in patients with hemochromatosis and rheumatoid arthritis (RA) but not in osteoarthritis (Janckila et al. 2002). Serum TRACP 5a is elevated in approximately one-third of RA patients (Chao et al. 2005). TRACP 5a activity is found to be significantly correlated with IgM rheumatoid factors (Janckila et al. 2008) and C-reactive protein, an acute-phase protein marker of inflammation but not with NTx and ICTP (Janckila et al. 2002). TRACP 5a can also be regarded as a marker of disease severity as TRACP 5a protein, and IgM rheumatoid factors are shown to be significantly higher in RA patients with nodules than those without (Janckila et al. 2008). Studies suggest that TRACP 5a protein amount could be considered a prognostic marker for the degree of systemic inflammation and macrophage burden in RA. Since TRACP 5a levels showed show no correlation to TRACP 5b or other markers of bone metabolism in RA, it further asserts that the two isoforms have different secretory origins (Janckila et al. 2008).

TRACP 5b: A Marker of Osteoclast Number

TRACP 5b is the principal isoform secreted by osteoclasts (Janckila et al. 2002). Several studies suggest that secreted TRACP 5b is a marker of osteoclast number or bone resorption rate. Increased TRACP 5b activity has also been observed in

Table 2 TRACP in pathological states

Reference	Study population	Findings on TRACP
Rosenbrock et al. 2002	Healthy premenopausal, perimenopausal, and early postmenopausal women	TRACP is significantly increased in the transition period from peri- to postmenopause
Halleen et al. 2002	*Osteoporosis patients*	Serum TRACP 5b *activity was elevated*
Halleen et al. 2001	303 individuals Control populations: Healthy premenopausal women, healthy postmenopausal women, and breast cancer (BC) patients without evidence of bone metastases (BC−) Patients with bone disease: primary vertebral osteoporosis, osteopenia, active Paget's disease of bone, and BC patients with overt bone metastases (BC+)	Serum TRACP 5b is greatly increased in patients with bone diseases. The most pronounced changes were observed in patients with BC+ and osteoporosis.
Capeller et al. 2003	192 samples from patients with breast cancer with and without bone metastases and in 53 healthy pre- and postmenopausal women	Serum TRAP 5b levels are elevated in patients with bone metastases and breast cancer
Koizumi et al. 2003	75 cancer patients with and 201 cancer patients without skeletal metastasis	TRAP 5b increased in patients with a small bone metastatic burden
Lyubimova et al. 2004	Patients with breast cancer and prostate cancer having bone metastases. Healthy donors and patients without skeletal injuries	In patients with breast cancer, the diagnostic sensitivity and specificity of TRAP 5b as a marker of skeletal metastases were 82 and 87%, respectively. In patients with prostate cancer, these indexes were 71 and 83.4%, respectively
Chao et al. 2004	30 early breast cancer patients without bone metastasis and in 30 aged-matched breast cancer patients with bone metastasis. Another 60 normal volunteers were recruited as controls	TRACP 5b activity can be considered as a surrogate indicator of bone metastasis in breast cancer patients. Serum TRACP 5b activity correlated well with BAP activity in breast cancer patients with bone metastasis, but not in normal individuals or in patients without bone metastasis
Jung et al. 2004	117 prostate cancer patients (pN0M0, $n = 39$; pN1M0, $n = 34$; M1, $n = 44$), 35 healthy men, and 35 patients with benign prostatic hyperplasia	Logistic regression analysis resulted in a model with OPG and TRACP as variables that predicted bone metastasis with an overall correct classification of 93%
Salminen et al. 2005	130 prostate cancer patients; bone metastases +, $n = 25$) and without bone metastases -, $n = 105$ skeletal metastases. Among them 64 were treated with and 66 were not treated with androgen deprivation (AD)	TRACP 5b is regarded to have a role in the diagnoses of skeletal changes in prostate cancer with a focus on treatment-related skeletal changes

(*continued*)

Table 2 (continued)

Reference	Study population	Findings on TRACP
Gerdhem et al. 2004	1040 randomly recruited 75-year-old women	S-TRACP5b was higher in women with fractures especially in clinical vertebral fracture *but not elevated in hip fractures suggesting that it predicts fractures of trabecular bone*
Takahashi et al. 2000	24 sera each from healthy, end-stage renal disease (ESRD), and rheumatoid arthritis (RA) subjects	*TRACP* isoform 5b was found to be elevated in ESRD only. TRACP isoform 5a was normal in both ESRD and RA
Terpos et al. 2003a	121 patients with multiple myeloma (MM), 63 of them were on pamidronate administration	*TRACP 5b was elevated in MM patients as compared to control. However, the treatment with pamidronate reduced the TRACP 5b levels*
Terpos et al. 2004	51 patients (35 M/16 F) of MM underwent ASCT in the follow-up period of 12 months	*At baseline TRACP 5b was increased. ASCT showed decline in the levels of TRACP*
Terpos et al. 2003b	Forty-three patients with newly diagnosed MM	*Patients with MM reported elevated TRACP 5b levels at baseline as compared to controls*
Terpos et al. 2003c	Patients with MM, stage II or III, were randomly assigned to receive either pamidronate 90 mg (group I, 23 patients) or ibandronate 4 mg (group II, 21 patients	TRACP-5b showed significant reduction in the pamidronate group from the second month of treatment and in the ibandronate group from the sixth month
Avbersek-Luznik et al. 2005	80 HD patients and 50 age-matched controls	Mean serum levels of TRACP 5b increased in hemodialysis patients
Chu et al. 2003	14 chronically dialyzed patients and 6 healthy control subjects	Serum TRACP levels correlate well with histological indices of osteoclasts and may serve as a specific marker for osteoclastic activity in patients with renal bone disease
Reichel et al. 2003	141 unselected hemodialysis patients	Serum TRACP 5b is elevated in patients with end-stage renal disease. The serum levels correlate strongly with other biomarkers as well as strong associations between intact PTH and biochemical bone markers were observed

TRACP tartrate-resistant acid phosphatase, *BC* breast cancer, *BC+* breast cancer positive, *BC−* breast cancer negative, HD hemodialysis, *MM* multiple myeloma, *PTH* parathyroid hormone, *BAP* bone alkaline phosphatase, *ESRD* end-stage renal disease, *RA* rheumatoid arthritis, *OPG* osteoprotegerin, *ASCT* autologous stem cell transplant

patients affected by osteopetrosis Albers-Schönberg disease or type II autosomal dominant osteopetrosis (ADO2) (Alatalo et al. 2004), a disease resulting from impaired bone resorption. Higher TRACP 5b levels have also been reported in

osteopetrotic rat strains (Alatalo et al. 2003a). Several pathological conditions such as osteoporosis, hyperparathyroidism, Gaucher's disease, and some cancers also involve altered serum TRACP 5b activity suggestive of increased bone resorption (Capeller et al. 2003; Chao et al. 2010; Mose et al. 2003). Serum TRACP 5b is strongly correlated to osteoclast number in human osteoclast cultures and ovariectomized rats (Rissanen et al. 2008). Additionally, it has been shown that TRACP 5b values can predict future fracture risk (Gerdhem et al. 2004). However, conditions involving increased bone resorption are usually associated with an increase in osteoclast number, explaining the good performance of TRACP 5b as a marker of bone resorption in many clinical studies.

Monitoring Antiresorptive Treatment

Monitoring antiresorptive treatment is one of the most accepted uses of bone turnover markers. TRACP along with degradation products of collagen CTx and NTx provide in-depth study of resorption changes in bone. It is recommended to record a baseline value of the markers for each patient individually before initiation of treatment. Marker measurements should be repeated at sufficient intervals, for example, after 3-month treatment, and then be compared with the baseline value. However, the treatment may prove to be ineffective if there appears an increase in the marker value from the baseline. It is beneficial to use bone markers in treatment monitoring because it educates the patient that the treatment is working, which in turn enhances their compliance to the treatment (Halleen et al. 2006). The level and activity of TRACP 5b and markers of the collagen fragmentation products for monitoring antiresorptive treatment has been studied with alendronate, clodronate, risedronate, pamidronate, clodronate, ibandronate, bisphosphonate, hormone replacement therapy (HRT), and raloxifene as given in Table 3.

Advantages Over Other Markers

Some of the major limitations of most of the serum markers of bone resorption include their high diurnal variability and the effects of feeding and renal function on the values obtained (Woitge et al. 1999). TRACP has an edge over other markers in these aspects. It is not affected by diurnal variability as osteoclast number doesn't change overnight (in 24 h) (Hannon et al. 2004). To avoid feeding effect, it is required that serum samples must be collected after overnight fasting. Recently it was reported that diurnal variability and the effect of feeding occur due to the gastrointestinal hormone glucagon-like peptide-2 (GLP-2) that varies osteoclast activity (Henriksen et al. 2005). Since GLP-2 has no effect on osteoclast number, it should not affect serum TRACP 5b levels. TRACP 5b activity is not affected by renal function as it is cleared from the blood circulation through liver. During hepatic dysfunction, the fragments of inactive TRACP get accumulated in the serum, and the active enzyme can be measured without interference (Halleen et al. 2001).

Table 3 Summary of studies showing TRACP and its correlation with other bone markers during antiresoptive treatment

Drug treatment	Effect on TRACP	Correlation with other markers	Reference
Estrogen (HRT) for 6 months	Decreased	Correlated significantly with S-P1NP, S-P1CP, and U-DPD	Halleen et al. 2002
Raloxifene for 12 weeks	Decreased (10%)	CTX, NTx, BALP, and osteocalcin decreased after treatment	Hansdottir et al. 2004
Alendronate for 12 months	Decreased	All bone markers including CTx, P1NP, osteocalcin, S-BALP, U-DPD decreased after treatment. S-CTX and P1NP were best correlated with TRACP 5b. Osteocalcin was not significantly correlated	Nenonen et al. 2005
Clodronate for 2 years	Decreased (−18%)	U-NTx and P1NP were decreased more than TRACP	Välimäki and Taheta 2005
Pamidronate	Decreased	Strong correlation with NTx. No effect on OPG	Terpos et al. 2003c

TRACP tartrate-resistant acid phosphates, *NTx* amino-terminal collagen cross-links, *CTx* collagen type 1 cross-linked C-telopeptide, *P1NP* total procollagen type 1 amino-terminal propeptide, *OPG osteoprotegerin, U-DPD urinary* deoxypyridinoline, *BALP* bone-specific alkaline phosphatase

Potential Applications to Prognosis, Other Diseases, or Conditions

Metastasis of cancer to bone is considered as the terminal event. TRACP 5b is the biochemical bone marker of osteoclast activity which finds wide applicability in prognosis and treatment management of various cancers including the breast, prostate, lung, kidney, and multiple myeloma, with high incidence of metastasis (Chao et al. 2004). Studies have shown that metastasis of cancer to bone interferes with bone metabolism, and hence TRACP 5b is induced significantly and elevated serum levels are reported.

TRACP is a nonspecific indicator of lipid storage, and hence elevated levels may indicate lipid accumulation in Gaucher's disease, which is an autosomal disorder of the enzyme glucocerebrosidase that leads to accumulation of glucocerebroside in the monocyte or macrophage cells. TRACP is not a direct diagnostic marker but aids in monitoring disease progression and response to treatment.

TRACP-positive cells, i.e., dendritic and macrophage cells, have been reported in the synovial intima of osteoarthritis (OA). These cells are actively involved in the cytokine synthesis as well as joint destruction, progressing to OA. Hence, the level of TRACP in synovial fluid and serum is elevated in OA and can be used as a biomarker for early detection of cartilage destruction and OA (Lee et al. 2008).

TRACP 5a has been also looked upon in potential to its role in cardiovascular diseases. A study reveals that only TRACP5a, logCRP, and cholesterol are

elevated in symptomatic patients. And serum TRACP5a correlated with age, logCRP, logIL-6, and log-triglycerides in symptomatic patients. Moreover TRACP 5a was regarded as a more specific predictor of myocardial infarction than CRP (Janckila et al. 2011).

Summary Points

1. Tartarate-resistant acid phosphatase (TRACP) *is a* glycosylated monomeric metalloprotein enzyme that belongs to the family of enzymes acid phosphatases, consisting of 304 amino acid sequence.
2. TRACP is abundantly distributed and expressed in tissues like the bone, spleen, liver, the linings of the gastrointestinal tract, lung, thymus, and skin and has two isoforms: TRACP 5a expressed by macrophages and inflammatory cells and TRACP 5b exclusively expressed by osteoclasts.
3. Different pH conditions assign different function to TRACP: phosphatase activity or ROS generation.
4. The role of TRACP in bone resorption is reflected through various in vitro, in vivo, and clinical studies.
5. TRACP gets accumulated in the bone matrix immediately next to the ruffled border of resorbing osteoclasts and acts on substrates.
6. Altered TRACP levels are found in conditions such as osteoporosis, postmenopausal women, Paget's disease, Gaucher's disease, and bone metastasis of tumors.
7. TRACP has potentials to be clinically used as a marker of osteoclast number and to monitor antiresorptive therapy.

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Parathyroid Hormone (PTH) and the Relationship Between PTH and Bone Health: Structure, Physiology, Actions, and Ethnicity

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Abstract

Parathyroid hormone (PTH) regulates calcium homeostasis by coordinating the hypercalcemic actions of the skeleton, intestine, and kidney. The actions of PTH on this organ axis serve to maintain circulating calcium concentrations in order to support critical functions such as nerve conduction and muscle contraction. At the level of bone tissue, PTH exhibits both anabolic and catabolic actions, making a single PTH result challenging to interpret. The anabolic or catabolic function of PTH on bone is determined by both the dose and periodicity of exposure. Therefore, the measurement of additional biomarkers to include vitamin D metabolites, markers of bone turnover and osteokines to include receptor activator of nuclear factor kappa-B ligand, osteoprotegerin, and sclerostin, is critical when interpreting the effect of PTH on bone tissue. Additionally, race, sex, age, dietary, and lifestyle factors impact PTH concentrations and must be considered. This chapter will discuss the roles of PTH on each of its target organs, biomarkers of PTH action, regulators of PTH secretion, and interpretation of PTH results gleaned from bone studies.

Keywords

Parathyroid hormone • Bone • Turnover • Calcium • Vitamin D • RANKL • OPG • Sclerostin

List of Abbreviations

$1,25(OH)_2D_3$	1,25-Dihyrdoxyvitamin D_3
AMP	Adenosine-3′,5′-monophosphate
BMC	Bone mineral content
BMD	Bone mineral density
CaSR	Calcium-sensing receptor
DKK1	Dickkopf 1
FGF23	Fibroblast growth factor 23
Fzd	Frizzled
LRP5/6	LDL-receptor-related protein 5 or 6
NaPi2a/c	Sodium-phosphate cotransporters a or c
OPG	Osteoprotegerin
PKC	Phospholipase/protein kinase C
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
PTHR1	PTH receptor 1
RANKL	Receptor activator of nuclear factor kappa-B ligand
SNP	Single nucleotide polymorphism
TRPV5/6	Transient receptor potential cation channel subfamily V 5 or 6

Key Facts of PTH

- PTH maintains blood calcium and phosphorus levels within a very narrow range by coordinating the actions of the skeleton, intestine, and kidney.
- In adults, elevated PTH is associated with increased rates of bone breakdown and formation and thus results in weaker bones.
- Paradoxically, PTH is also important for the building of bone, and so it is important to interpret PTH values in the context of other factors including age, race, diet, and physical activity.
- Blacks/African Americans have higher PTH values compared to whites but also have better bone mineral density and lower fracture risk.
- Maintaining adequate vitamin D and calcium intake is essential for stabilizing PTH levels and support of bone health.

Definitions of Words and Terms

Bone turnover	The coupled process of osteoclastic bone resorption followed by bone formation by osteoblasts.
Cells of osteoblastic lineage	Mesenchymal progenitor cells that progressively differentiate to preosteoblasts then osteoblasts and finally to osteocytes.
Phosphatonin	An effector that acts on the kidney to inhibit renal phosphorus reabsorption.

Introduction: PTH Structure and Physiology/Systemic Biology

Parathyroid hormone (PTH) is a peptide hormone that functions as a master regulator of calcium metabolism and coordinates the actions of a three organ axis in order to maintain circulating calcium within a narrow concentration range. For a detailed description of assays for detection of PTH, see Chap. 6, "► Parathyroid Hormone (PTH) Assays and Applications to Bone Disease: Overview on Methodology" (Arya and Sachdeva 2016). Bone cells, absorptive intestinal epithelial cells, and reabsorptive renal cells are responsible for coordinating the hypercalcemic actions of PTH (Fig. 1 and Table 1). At the level of bone tissue, PTH increases bone turnover directly via signaling in osteoblasts and osteocytes (Figs. 2 and 3) and indirectly through osteoblast or osteocyte recruitment and activation of osteoclasts. The secretion and synthesis of PTH by parathyroid chief cells are regulated by the plasma membrane-bound calcium-sensing receptor (CaSR). In normocalcemic conditions,

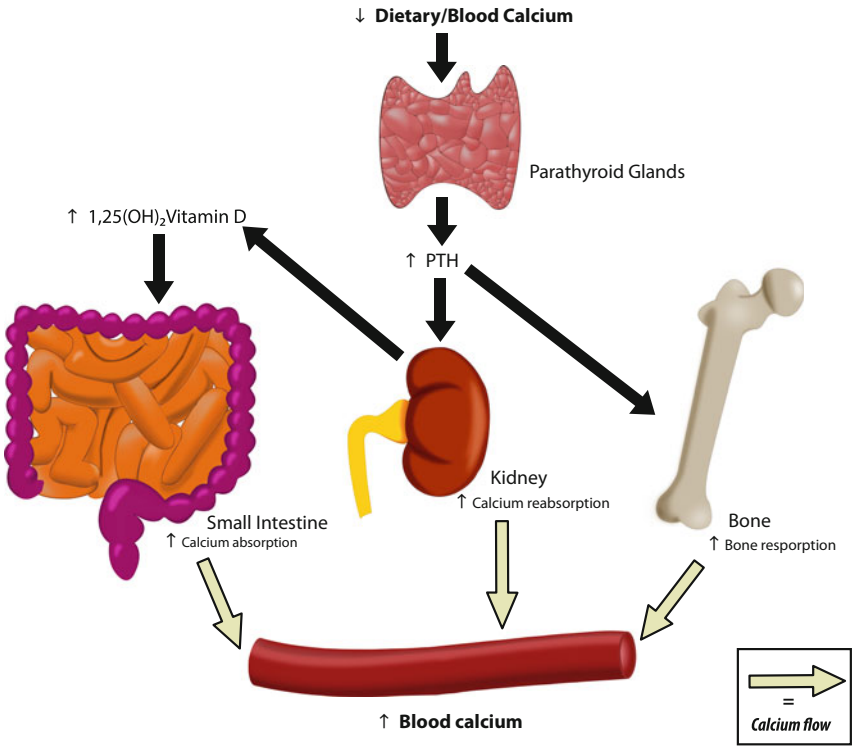

Fig. 1 Three organ axis controlling calcium homeostasis

Table 1 Cell-specific effects of PTH signaling

Cell type	Effects of PTH signaling
Intestinal absorptive epithelial cells	Increased Ca absorption: indirect due to 1,25 $(OH)_2D$ induced expression of calcium transport proteins
Osteoblasts	Increased FGF23 expression: secondary to increased 1,25$(OH)_2D$ Increased RANKL expression
Osteoclasts	Increased differentiation of precursor cells as well as the function of mature osteoclasts: indirect through osteoblast and osteocyte regulation of the RANKL-OPG-RANK pathway
Osteocytes	Increased FGF23 expression: secondary to increased 1,25$(OH)_2D$ Increased RANKL expression Decreased sclerostin expression
Renal thick ascending limb of the loop of Henle and the distal convoluted tubule	Increased reabsorption of calcium Decreased reabsorption of phosphorus
Renal proximal tubule	Stimulation of the 25$(OH)D_3$-1α hydroxylase [1α (OH)ase] which activates vitamin D Decreased reabsorption of phosphorus

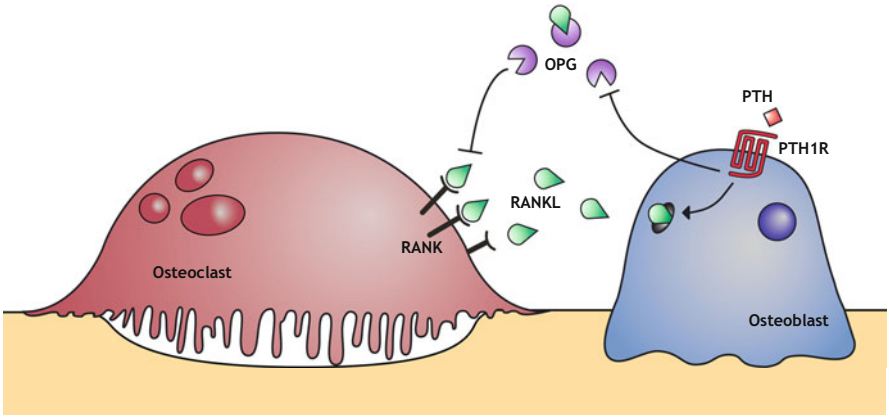

Fig. 2 PTH signaling in osteoblasts controls osteoclastic bone resorption through the RANKL-OPG-RANK signaling pathway

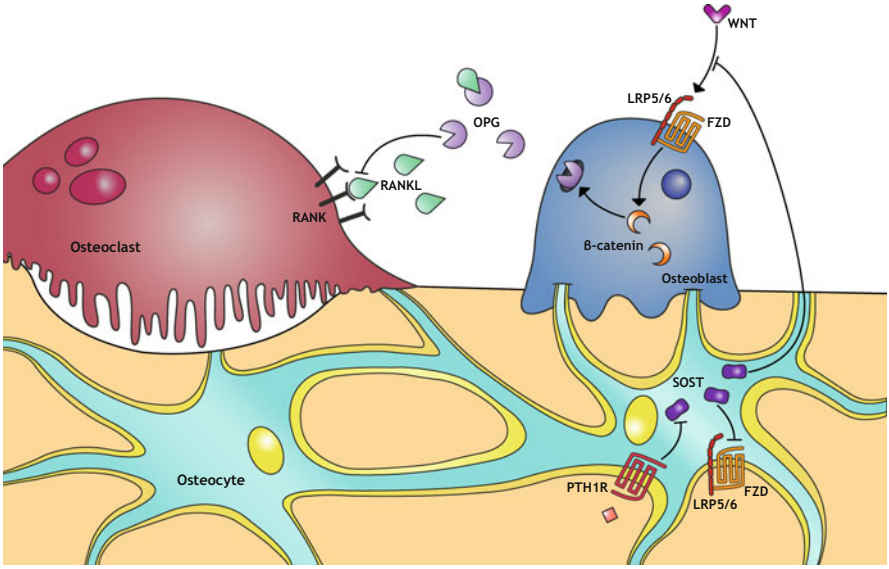

Fig. 3 PTH signaling in osteocytes controls Wnt signaling through downregulation of the osteocyte derived Wnt inhibitor sclerostin

the extracellular venus flytrap domain or ligand binding domain of the CaSR is occupied and PTH secretion is inhibited. Conversely, small, but physiologically relevant, decreases in circulating ionized calcium (iCa) stimulate PTH secretion. The CaSR-mediated PTH secretion can occur quickly after changes are sensed (DIAZ et al. 1998). As the half-life of PTH is 2–3 min, PTH is therefore able to rapidly

regulate circulating iCa concentrations. If hypocalcemia continues over several minutes up to an hour, intracellular PTH degradation is reduced. For prolonged hypocalcemia over several hours or a few days, PTH gene transcription is increased, and parathyroid cell proliferation is enhanced if hypocalcemia persists for several days, weeks, or longer. The iCa-PTH response curve is a steep inverse sigmoidal shape, thus very small decreases in iCa (1.2–1.24 mM) cause a disproportionately large increase in circulating PTH (DIAZ et al. 1998). The set point of iCa concentration along this curve and the slope of the curve surrounding this set point control the iCa-PTH relationship.

PTH Actions in the Kidney

In the kidney, PTH signaling results in increased tubular calcium reabsorption, decreased phosphate reabsorption, and enhanced activation of 25(OH)D to its endocrine form, $1,25(OH)_2D_3$. Each of these actions serves to regulate circulating calcium and phosphate concentrations, and bone metabolism. While the majority of calcium is reabsorbed in the proximal tubule (~90%), the effect of PTH on tubular calcium reabsorption is manifested in the distal tubule. In this way, PTH serves to refine calcium reabsorption and urinary calcium excretion. The actions of PTH on the distal tubule can be estimated by measuring urinary nephrogenous cyclic adenosine-3′,5′-monophosphate (cAMP), particularly in patients with primary hyperparathyroidism, as cAMP is increased in response to PTH signaling and released in the urine (Babka et al. 1976). PTH also regulates renal reabsorption of phosphate at both the proximal and distal tubule where PTH signaling increases urinary phosphate excretion. In the proximal tubule, this regulation occurs through PTH inhibition of sodium-dependent phosphate reabsorption by reductions in brush-border membrane expression of sodium-phosphate cotransporters, NaPi2a and NaPi2c (Kido et al. 2013). In sum, the actions of PTH signaling in the kidney decrease urinary calcium and increase urinary phosphate excretion.

PTH also stimulates the activity of the renal 25-hydroxyvitamin D-1–αhydroxylase in order to increase circulating $1,25(OH)_2D_3$ concentrations. $1,25(OH)_2D_3$ then further increases circulating calcium levels by increasing intestinal calcium absorption and bone resorption and also directly suppresses PTH through negative feedback inhibition at the level of the parathyroid gland (Fig. 1).

PTH Actions in the Intestine

The major impact of PTH on intestinal calcium handling is indirect through $1,25(OH)_2D_3$-induced expression of calcium transport proteins (Table 1) (Fleet and Schoch 2010). A direct effect of PTH also likely exists as the PTH receptor (PTHR1) is expressed in the basolateral membrane of small intestinal absorptive cells (Gentili et al. 2003). Furthermore, PTH is able to stimulate calcium uptake

in vitro in both chick duodenal loops as well as isolated rat enterocytes (Nemere and Norman 1986; Picotto et al. 1997). Thus, in vitro evidence supports a direct role for PTH in regulating intestinal calcium absorption, but this role has been difficult to study in vivo as PTH stimulates $1,25(OH)_2D_3$ expression which independently increases calcium absorption, making it challenging to isolate the effects of PTH alone in vivo. The mechanism by which $1,25(OH)_2D_3$ enhances intestinal absorption of calcium is through increased expression of calcium transport proteins (Fleet and Schoch 2010). Vitamin D stimulates both the transcellular facilitated diffusion pathway as well as the paracellular pathway of calcium absorption. Several calcium transport proteins contain a vitamin D response element whereby the vitamin D-vitamin D receptor (VDR) complex is able to bind and enhance gene expression. Of these genes, the transcellular facilitated diffusion proteins, transient receptor potential cation channel subfamily V (TRPV5/6), calbindin D9K, and sodium calcium exchanger, and the paracellular pathway calcium-permissive proteins, claudin-2 and claudin-12, are increased in response to $1,25(OH)_2D_3$. Consistent with the role for vitamin D in regulating intestinal calcium absorption, VDR-receptor knockout mice exhibit reduced intestinal calcium absorption during normal calcium intakes, elevated PTH and $1,25(OH)_2D_3$, and osteomalacia. However, a high calcium diet is able to normalize circulating calcium and prevent osteomalacia indicating that vitamin D-independent transport mechanisms also exist in the intestine (Song et al. 2003).

PTH Actions in Bone

While the hypercalcemic actions of PTH involve increased bone resorption, paradoxically, PTH also controls bone anabolism. The dose and periodicity of PTH exposure determine whether the catabolic or anabolic pathway predominates, with continuous PTH exposure favoring bone catabolism and intermittent PTH favoring bone anabolism (Fig. 4). The dose and periodicity of PTH differentially stimulate intracellular signaling pathways which result in different patterns of gene expression or the expression of the same genes in a sustained or acute manner. Some of the differential effects of PTH signaling in bone can be explained by the recruitment of bone-resorbing osteoclast cells by bone-forming cells of the osteoblastic lineage. PTH binds to the membrane-bound PTH1R which is expressed in cells of the osteoblast lineage including osteocytes, but not osteoclasts. The PTHR1 is a G-protein-coupled receptor with intracellular signaling through $G\alpha_s$ and $G\alpha_q$ resulting in the activation of the adenylyl cyclase/cyclic AMP and phospholipase/protein kinase C (PKC) cascades, respectively (Gardella et al. 1995; Kousteni and Bilezikian 2008; Potts 2005). As detailed below, the result of these signaling cascades includes increased receptor activator of nuclear factor kappa-B ligand (RANKL) production and decreased OPG production thus resulting in a higher RANKL:OPG ratio which favors osteoclastogenesis. The actions of continuous PTH exposure on osteoclast function are thus indirect as PTHR1 is not expressed in osteoclasts.

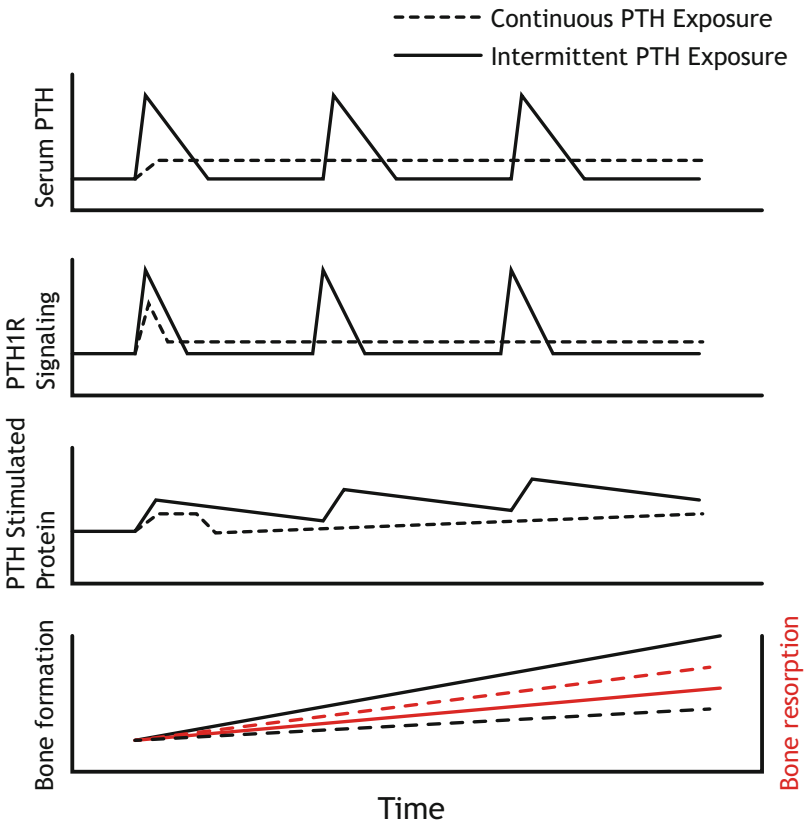

Fig. 4 The dose and periodicity of PTH exposure determine whether the catabolic or anabolic pathway predominates (Reprinted from Qin et al. (2004), Copyright (2004), with permission from Elsevier)

Biomarkers of PTH Action in Bone: Interpreting PTH Changes

RANKL:OPG

The actions of PTH on bone are complex and often involve direct communication between several bone cell types. The RANKL-OPG-RANK pathway is a critical pathway by which PTH regulates bone resorption (Fig. 2). PTH signaling in cells of the osteoblast lineage induces expression of RANKL. RANKL expression along with the presence of another osteoblast secreted factor, macrophage colony-stimulating factor, induces differentiation of osteoclast precursor cells as well as the function of mature osteoclasts, thus resulting in increased osteoclastic bone resorption and calcium and phosphorus release from bone matrix. The regulation of osteoclast function through the RANK pathway is further refined by an osteoblast

secreted "decoy" RANK receptor ligand, osteoprotegerin (OPG). PTH inhibits the expression of OPG, thus increasing the RANKL:OPG ratio allowing more RANKL-RANK binding and subsequent osteoclastogenesis and bone resorption. In addition to osteoblasts, RANKL is produced by osteocytes (Nakashima et al. 2011; Xiong et al. 2011, 2014), and in murine models, this cell type may be the dominant source of RANKL.

In light of the dual actions of PTH on the skeleton, measurement of circulating RANKL, OPG and calculation of the RANKL:OPG ratio can provide a physiologic readout of PTH action on bone. Both of these biomarkers may be measured using commercially available enzyme-linked immunoabsorbent assays. Increases in this ratio indicate net bone resorption and decreases indicate net bone formation. In a cross-sectional analysis, circulating OPG levels were also associated with the risk of hip fracture in postmenopausal women in the Women's Health Initiative. In this nested case–control study, LaCroix et al. (2013) evaluated OPG levels in 400 incident hip fracture cases and 400 matched controls without hip fracture and found that OPG levels were associated with an almost twofold increased risk of hip fracture. Changes in RANKL:OPG can also be useful in predicting changes in BMD. For example, recombinant leptin replacement therapy decreases PTH and RANKL:OPG and restores BMD in females with hypothalamic amenorrhea (Foo et al. 2014). The RANKL:OPG ratio is also correlated with bone resorption markers as well as femoral bone loss, at least in patients with primary hyperparathyroidism in which circulating PTH is elevated (Nakchbandi et al. 2008). In addition, continuous pharmacological PTH infusion results in elevations in circulating RANKL and reductions in OPG (Ma et al. 2001). Thus circulating RANKL and OPG can be useful biomarkers of PTH action at the level of bone tissue in order to determine whether the catabolic or anabolic actions of the hormone predominate as they are associated with both bone density and fracture risk.

Further support for the clinical significance of RANKL signaling has been demonstrated through pharmacological inhibition of RANKL by the potent antiresorptive agent denosumab (Amgen, Thousand Oaks, CA, USA). Denosumab is a human monoclonal antibody against RANKL which causes reductions in bone resorption markers, increased BMD and bone strength (McClung et al. 2006; Lewiecki et al. 2007), as well as reduced risk of new vertebral, hip, and nonvertebral fractures in osteoporotic postmenopausal women (Cummings et al. 2009). Interestingly, the beneficial effects of RANKL inhibition may be greater and/or more sustained than bisphosphonates, another class of antiresorptive agents approved for osteoporosis treatment. Consistent with this notion, the long-term follow-up arm of the denosumab trial found increased cortical thickening and bone mass and increased cortical strength in women receiving denosumab. These effects were likely due to sustained reductions in cortical porosity and continued modeling-based formation compared to those receiving bisphosphonates (Papapoulos et al. 2012).

Several studies in humans have detected effects of nutrition and/or energy status and physical activity on the RANKL:OPG ratio, supporting a role for these biomarkers in determining effects of dietary and/or physical activity interventions on bone metabolism (Hooshmand et al. 2014; Josse et al. 2012; Gaffney-Stomberg

et al. 2014; Bergstrom et al. 2012; Banfi et al. 2012). For example, dietary supplementation with dried plum has been shown to improve BMD. In a recent study aimed at elucidating the mechanism for this effect, Hooshmand et al. found that 1 year of dried plum intake attenuated increases in RANKL and increased OPG compared to the dried apple control (Hooshmand et al. 2014). In overweight and obese premenopausal women, high dietary protein and dairy intake resulted in decreased PTH and increased OPG:RANKL after a 16-week weight loss exercise program compared to those in the weight loss program who received adequate protein with medium or low intake of dairy (Josse et al. 2012). As this study did not match dairy or calcium intake between the groups, it is not possible to conclude whether the observed effects were due to dietary protein, dairy, calcium intake, or some combination of these factors. However, this study supports a role for RANKL and OPG as biomarkers of PTH action on skeletal tissue. Similarly, in a recently completed randomized, double-blind, placebo-controlled trial of calcium and vitamin D supplementation on bone health in young adults undergoing initial military training, Gaffney-Stomberg and colleagues found that PTH was stabilized and RANKL:OPG decreased in those consuming supplemental calcium and vitamin D (Gaffney-Stomberg et al. 2014). This study also demonstrated improved volumetric BMD and cortical bone mineral content and thickness of the tibia in the supplemented group, as measured by peripheral quantitative computed tomography (pQCT), further supporting the relationship between changes in RANKL:OPG and bone tissue.

While studies of combined nutrition and exercise interventions (with or without weight loss) more consistently result in changes in PTH and RANKL:OPG, studies evaluating the effects of various exercise interventions alone result in less consistent findings. For example, a one-year exercise program consisting of walking and aerobic training sessions resulted in increased OPG in postmenopausal women compared to sedentary controls (Bergstrom et al. 2012), while acute exercise did not modify circulating RANKL or OPG, at least in elite trained individuals (Banfi et al. 2012).

Sclerostin

Recent studies have clarified the role of osteocytes in regulating bone modeling and remodeling. In addition to their role in RANK pathway control of bone turnover, osteocytes also secrete soluble inhibitors of canonical Wnt/β-catenin signaling, the dominant signaling pathway controlling osteoblast differentiation, proliferation, activity, and survival (Fig. 3) (Costa et al. 2014; Baron and Rawadi 2007). Wnt/β-catenin signaling occurs through Wnt binding to its membrane-bound coreceptor complex, frizzled (Fzd) and LDL-receptor-related protein 5 or 6 (LRP5 or LRP6). Wnt ligand binding results in the release of the transcription factor, β-catenin, from a cytosolic destruction complex thus allowing β-catenin to translocate to the nucleus and induce osteoblastogenic gene expression (Burgers and

Williams 2013). Sclerostin, which is predominantly secreted by osteocytes, is an inhibitor of canonical Wnt/β-catenin signaling. Circulating sclerostin can be detected using commercially available ELISA assays, but the results obtained vary greatly between assays (Durosier et al. 2013). Sclerostin exerts its inhibitory effects on canonical Wnt/β-catenin signaling by binding to the extracellular domain of the Fzd/LRP5-6 coreceptor thus preventing Wnt ligand binding. In the absence of Wnt binding, or the presence of inhibitor binding, β-catenin remains bound by its destruction complex in the cytosol and thus bone formation is reduced.

PTH signaling in osteocytes inhibits sclerostin production, and consistent with this, serum sclerostin is negatively correlated with PTH (Bellido et al. 2013; Costa et al. 2011), and PTH infusion reduces circulating sclerostin (Drake et al. 2010). Perhaps the best characterized relationship between sclerostin, PTH, and bone is in the context of microgravity or unloading-induced bone loss. Under these conditions circulating sclerostin is increased and PTH decreased. The increase in sclerostin is believed to be responsible at least in part for the rapid reduction in BMD that occurs with unloading (Spatz et al. 2012). In further support of this, treatment with sclerostin-neutralizing antibodies is currently underway in phase III human trials as a treatment for osteoporosis (Shah et al. 2015). In phase I and II trials, two humanized monoclonal antibodies, romosozumab (AMG 785, Amgen Inc., Thousand Oaks, CA, USA) and blosozumab (LY2541546, Eli Lilly and Company, Indianapolis, IN, USA) have been found to transiently increase bone formation markers, chronically suppress markers of bone resorption, and increase BMD at several clinically relevant sites including lumbar spine, total hip, and femoral neck (McClung et al. 2014; Mccolm et al. 2014).

However, outside of unloading, cross-sectional studies in children and adults report both positive and negative associations between circulating sclerostin and BMD limiting its utility as a biomarker under normal gravity conditions. These discrepant findings may be due to differences in sex, age, habitual activity, hormonal status, and nutrition, all of which affect sclerostin levels. For example, sclerostin levels are known to increase with age and to be inversely associated with free estrogen index in postmenopausal women (Mirza et al. 2010). In a recent study comparing adolescent athletes and nonathletes, sclerostin was positively associated with lumbar spine BMD in both amenorrheic and eumenorrheic athletes but negatively associated with lumbar spine BMD in nonactive controls (Fazeli et al. 2013). Obesity is known to be detrimental to bone geometry, quality, and strength relative to body weight, particularly in nonweight-bearing sites. In a study comparing associations between bone strength and geometry and circulating PTH, sclerostin, and bone turnover markers in obese and normal weight children, obese children had higher PTH and bone turnover and lower sclerostin. PTH was also negatively correlated with measures of bone strength (Radetti et al. 2014). Thus, in the case where PTH and bone turnover are elevated, PTH-induced bone resorption may exert the predominant effect on bone metabolism. Interestingly, in patients with anorexia nervosa (AN), the negative correlation between PTH and sclerostin is interrupted as patients with AN exhibit increased PTH, sclerostin, and bone resorption markers

whereas bone formation markers, insulin-like growth factor 1 and the Wnt/β-catenin inhibitor Dickkopf 1 (DKK1), are all decreased (Maimoun et al. 2014). These findings suggest that energy status may impact the relationship between sclerostin and PTH.

In addition, sex differences in the association between PTH and sclerostin have been reported. For example, daily supplemental calcium and vitamin D provided to men and women (aged ≥65 years) for 2 years resulted in increased circulating sclerostin in men, but not women, even after controlling for season of measurement, baseline physical activity levels, baseline serum sclerostin, and total body bone mineral content (BMC) (Dawson-Hughes et al. 2014). In contrast, supplemental calcium and vitamin D resulted in increased iCa and reduced PTH in both men and women. Importantly, some of the inconsistent findings reported in the literature can be explained by differences in the sclerostin fragments that are detected by various immunoassays (Durosier et al. 2013) resulting in wide differences in detected circulating sclerostin levels which necessarily affect associations with calcitropic hormones, including PTH. As sclerostin is secreted by osteocytes, the most prevalent cell type in bone, some studies support adjustment for total body BMC which may alter these relationships (Durosier et al. 2013). While sclerostin can be positively or inversely associated with BMD, bone turnover, and PTH depending on the factors described above, most (Ardawi et al. 2012, 2013; Arasu et al. 2012) but not all (Garnero et al. 2013) studies show a positive relationship between circulating sclerostin and fracture risk at least in postmenopausal women. In sum, interpretation of PTH levels should be made within the context of physiological state, other markers of bone turnover, and effectors of PTH activity such as OPG:RANKL, sclerostin, and $1,25(OH)_2D_3$.

Known Regulators of PTH Synthesis, Secretion, and Activity

Vitamin D

Several factors regulate circulating PTH levels in addition to iCa. These factors include circulating 25-hydroxyvitamin D_3 (25(OH)D) and $1,25(OH)_2D_3$. When setting the Dietary Reference Intake (DRI) for vitamin D, the Institute of Medicine included suppression of PTH as one indicator of vitamin D adequacy (2010). There is a known inverse relationship between PTH and 25(OH)D and both biomarkers exhibit seasonal variation with peak 25(OH)D concentrations observed in June-July and the nadir in February-March north of the equator (Harris and Dawson-Hughes 1998). PTH controls the conversion of 25(OH)D to its active hormone form, $1,25(OH)_2D_3$, which exerts hypercalcemic actions on several target tissues as described above. Conversely, $1,25(OH)_2D_3$ exerts negative feedback inhibition on the parathyroid chief cells through the VDR, independent of circulating calcium levels. This negative feedback inhibition of PTH secretion is due to VDR-dependent inhibition of PTH and CaSR transcription (Demay et al. 1992; Kumar and Thompson 2011).

Phosphorus and FGF23

Circulating phosphorus affects PTH secretion both directly and indirectly. While reductions in circulating iCa potently stimulate PTH secretion, increased serum phosphorus induces PTH secretion. Some of the stimulatory effect of phosphorus on PTH secretion is indirect due to binding of circulating calcium by phosphorus, thereby reducing free calcium concentrations and CaSR activation on the parathyroid chief cells. However, phosphorus is also known to directly stimulate PTH secretion and synthesis by stabilizing PTH mRNA (Moallem et al. 1998). As yet another indirect method of regulation, increased circulating phosphorus affects PTH by stimulating osteoblast/osteocyte secretion of a phosphatonin known as fibroblast growth factor 23 (FGF23) (Fukumoto 2008). The primary role of FGF23 is to reduce serum phosphate levels by inhibiting renal phosphate reabsorption. FGF23 increases urinary excretion of phosphate through downregulation of the sodium-phosphate cotransporters, NaPi2a and NaPi2c, similar to hyperphosphaturic renal actions of PTH. In a negative feedback loop, FGF23 directly suppresses PTH secretion by the parathyroid chief cells (Krajisnik et al. 2007). Given the ability of high circulating phosphorus to induce secondary hyperparathyroidism, the effect of increased phosphorous consumption on bone health is an area of research interest, particularly as consumption of inorganic phosphates has increased due to their use as food preservatives. While circulating phosphorus levels affect FGF23 production, whether high intake of phosphorus, or a low dietary calcium:phosphorus ratio, is a risk factor for reduced BMD and increased fracture risk is currently debated as recently reviewed by Calvo and Tucker (Calvo and Tucker 2013).

In addition to the FGF23-PTH axis, there is evidence that FGF23 participates in two other endocrine loops that affect bone: the FGF23-vitamin D axis and the FGF23-insulin axis. Extensive discussion of these axes is beyond the scope of this chapter, but briefly, in the FGF23-vitamin D axis, $1,25(OH)_2D_3$ directly induces FGF23 expression in bone cells in a VDR-dependent manner. FGF23 then inhibits $1,25(OH)_2D_3$ production by the kidney (Liu et al. 2006; Kolek et al. 2005). As phosphate is a critical substrate in energy metabolism and insulin signaling increases peripheral muscle phosphate uptake, it is possible that FGF23 may participate in energy metabolism, potentially participating in the controversial bone-pancreas endocrine pathway (Clemens and Karsenty 2011). The evidence for a role of FGF23 in energy metabolism is more limited as reviewed elsewhere (Quarles 2012).

Race Differences in the PTH-1-alphahydroxylase-vitamin D Axis

Pronounced biological race differences exist for both PTH and 25(OH)D, as blacks/African Americans exhibit lower circulating 25(OH)D and higher circulating PTH and $1,25(OH)_2D_3$ compared to whites (Gutierrez et al. 2011). Consistent with this, the 25(OH)D threshold at which PTH increases is lower in blacks/African

Americans (37 nmol/L) compared to whites (59 nmol/L) as reported by Aloia and colleagues in a study of females aged 20–80 years (Aloia et al. 2010). Despite elevated PTH levels, black females have lower bone turnover, higher fractional calcium absorption, higher calcium retention, lower bone loss, and lower fracture risk compared to white females. This favorable calcium economy is due at least in part to skeletal resistance to PTH in blacks compared to whites (Cosman et al. 1997). Consistent with this, Bischoff-Ferrari (2004) reported lower sensitivity of BMD to vitamin D status in black women (Bischoff-Ferrari et al. 2004). These race differences in bone, calcium, and vitamin D metabolism are also observed in adolescents (Bell et al. 1993; Bryant et al. 2003). Bryant et al. reported that black female adolescents have greater calcium retention, bone formation rates, fractional calcium absorption, and lower urinary calcium excretion compared to whites (Bryant et al. 2003). Interestingly, when dietary calcium and race are used to predict calcium absorption and retention, adding circulating $1,25(OH)_2D_3$ to the model does not further explain variation in these parameters (Weaver et al. 2008). In sum, race differences in calcium economy explain the greater BMD and lower fracture rates observed in blacks compared to whites. The underlying mechanisms for these differences in bone, calcium, and vitamin D metabolism are not yet fully elucidated.

In a provoking investigation, Powe and colleagues reported that when vitamin D binding protein (VDBP) levels were measured and bioavailable 25(OH)D calculated, bioavailable 25(OH)D (defined as the fraction not bound to VDBP) was not lower in blacks/African Americans compared to whites (Powe et al. 2013). This result is in contrast to prior reports by other groups (Winters et al. 2009; M'Buyamba-Kabangu et al. 1987) which did not document race differences in circulating VDBP and may be due to variability in affinity for VDBP between the different antibodies used in commercially available kits (Bouillon et al. 1980). The VDBP gene contains several known single nucleotide polymorphisms (SNPs) which result in differences in the VDBP protein product that may impact affinity for various antibodies used in commercially available ELISA kits. To add to this complexity, there are known race differences in the prevalence of various VDBP SNPs as the GC1F variant is most common in blacks/African Americans and the GC1S variant is most common in European populations (Chun 2012). The GC1F variant results in VDBP with the lowest affinity for vitamin D as well as potentially lower affinity for the antibody used by Powe et al. (Engelman et al. 2008; Constans et al. 1985). Thus, whether the low VDBP levels reported by Powe and colleagues were due to limitation in assay detection has not been fully addressed.

Potential Applications

Measurement of circulating PTH is a useful indicator of calcium metabolism and bone turnover but needs to be interpreted in the context of additional biomarkers due to both catabolic and anabolic effects of PTH on the skeleton. To this end, markers of

bone formation and resorption, vitamin D metabolites, RANKL, OPG, and sclerostin are useful biomarkers of bone health. When interpreting PTH it is also important to consider other factors affecting this hormone including age, sex, race, diet, and physical activity. In general, in adults, higher PTH is associated with elevated bone turnover with resorption outpacing formation resulting in temporal weakness of bone matrix. Adequate dietary calcium and vitamin D status are inversely associated with PTH and are necessary in order to prevent secondary hyperparathyroidism and support bone health.

Summary Points

- PTH is a master regulator of calcium homeostasis and exerts this function by coordinating the actions of a three organ axis: bone, intestine, and kidney.
- The actions of PTH on bone are complex and include both anabolic and catabolic pathways depending on dose and periodicity of PTH exposure.
- Measurement of additional biomarkers including vitamin D metabolites, RANKL, OPG, sclerostin, and bone turnover markers is necessary to interpret PTH action in research studies.
- PTH biology differs by race, age, and sex and these factors need to be considered when interpreting PTH concentrations.
- Dietary factors including vitamin D, calcium, and possibly phosphorus intake affect PTH secretion.

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Part III

Genetic, Histological, Physical, and Imaging Methods

Biomarker Genes in Autosomal Dominant Osteopetrosis Type II (ADO II)

21

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Contents

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Abstract

Type II autosomal dominant osteopetrosis (ADO II) is a rare genetic disease characterized by an increase in bone mass. This pathology is caused by osteoclast impairment due, in 60% of cases, to *CLCN7* heterozygous mutations. ADO II patients present specific X-ray features, but until recently the disease lacked biological markers. It has been demonstrated that elevated serum tartrate-resistant acid phosphatase (TRAP) could be a good marker. In addition, microarray analysis and various validation experiments comparing gene expression levels in osteoclasts from ADO II patients and healthy donors have demonstrated that *ITGB5* expression is increased in the former and that PFR1, SERPINE2, and WARS expression are all decreased in ADO II osteoclasts. Of these new biological markers, two are described for the first time in osteoclasts.

Keywords

Osteopetrosis • Osteoclasts • CLCN7 • Microarray • Biological markers

List of Abbreviations

ADO II	Autosomal dominant osteopetrosis type II
CBS	Cystathionine βsynthase
CLCN7	Chloride channel 7
ECM	Extracellular matrix
M-CSF	Macrophage colony-stimulating factor
TRAP	Tartrate-resistant acid phosphatase
v-ATPase	Vacuolar ATPase proton pump
WARS	Tryptophanyl-tRNA synthetase

Key Facts of ADO II

- The bone is composed of a mineralized extracellular matrix (ECM).
- This mineralized ECM is synthesized by osteoblasts and resorbed by osteoclasts.
- A perfectly tuned balance exists between bone formation and bone resorption.
- Imbalance of these processes leads to disease.
- Type II autosomal dominant osteopetrosis (ADO II), also known as the Albers-Schönberg disease, was first described in 1904.
- ADO II is caused by osteoclast impairment leading to increased bone mass.
- ADO II patients have an increased fracture risk, but the disease is not life threatening.
- More than 30 heterozygous *CLCN7* mutations have been described as leading to ADO II, but they account for only 60% of all known ADO II cases.
- CLC7 is a chloride/proton exchanger needed for bone resorption. When mutated, CLC7 function is impaired, leading to altered acidification.
- ADO II has 66% penetrance and its phenotype can be highly variable. There is no genotype/phenotype correlation.

Definition of Words and Terms

Integrin	A type of protein that mediates cell adherence to the extracellular matrix. Integrins are always composed of an α subunit and a β subunit. In humans, 18 different α subunits and 8 different β subunits have been described, and there are 24 known associations between different α and β subunits. Integrins αvβ3 and αvβ5 are involved in mediating osteoclast attachment to bone matrix.
Microarray	Molecular biology technology allowing the analysis of a great number of genes all at once (e.g., up to 48,000 genes).
Osteoclast	Large, multinucleated, specialized bone cell responsible for bone resorption. Facilitates both the degradation of the mineral bone matrix and the digestion of the proteic bone matrix.
Osteopetrosis	Heritable, rare genetic disease characterized by osteoclast impairment.
Perforin	A protein specialized in pore formation in the plasma membrane of the target cell in order to trigger the death of this cell.
Transcription	Molecular process by which a gene is expressed in the form of a messenger RNA which will be translated into protein.
Transfer RNA	A protein is a precise assembly of amino acids. Each amino acid corresponds to a nucleic acid base triplet (codon) specified in the messenger RNA. The transfer RNA mediates recognition of the specific codon by the appropriate amino acid and its incorporation at the correct position of the nascent polypeptide.

Introduction

Healthy bone is characterized by a fine balance between bone formation by osteoblasts and bone resorption by osteoclasts. Perturbation of this equilibrium results in bone disease.

Human osteopetrosis was first described by Albers-Schönberg in 1904 (Albers-Schonberg 1904). Osteopetrosis is a rare, heritable disease characterized by an abnormal increase in bone mass due to osteoclast impairment (de Vernejoul and Kornak 2010).

Two main clinical forms exist and differ in their modes of inheritance. Autosomal recessive osteopetrosis (ARO), also known as malignant infantile osteopetrosis, is almost invariably lethal in early childhood when untreated (for review see (Sobacchi et al. 2013)). Different recessive mutations have been identified as causing ARO: for example, mutations of the genes *CAII* and *TCIRG1* lead to impaired osteoclast acid secretion; mutations of *CLCN7* and *OSTM1*, coding for physically interacting proteins, cause impairment of bone resorption by osteoclasts. The bones of such ARO patients are characterized by an increase in osteoclast number, albeit the cells are functionally impaired. This type of ARO is termed osteoclast-rich ARO. More

recently, new cases of ARO caused by mutation of two additional genes, *TNSF11* (encoding RANKL) and *TNFRSF11A* (encoding RANK), have been described. Bones from patients bearing homozygous mutations of *TNSF11* or *TNFRSF11A* are characterized by a total absence of osteoclasts. This type of ARO is referred to as osteoclast-poor ARO. The second main form is autosomal dominant osteopetrosis (ADO, also known as Albers-Schönberg disease; for review see (Bollerslev et al. 2013)). ADO is more benign than ARO and has an adult onset.

Osteoclasts

The osteopetroses are caused by failure of osteoclast differentiation or function, and mutations in at least 10 genes (Table 1) have been identified as giving rise to osteopetrotic development in humans.

Osteoclasts are highly specialized cells responsible for degradation of the bone organic matrix and its demineralization (Cappariello et al. 2014; Charles and Aliprantis 2014). This degradation is essential to bone remodeling and mineral homeostasis. Osteoclasts are multinucleated cells (three or more nuclei) formed by fusion of cells derived from hematopoietic precursors shared by macrophages, monocytes, dendritic cells, and osteoclasts. Under the influence of factors secreted by osteoblasts and/or stromal cells present in the bone microenvironment, these precursors differentiate into osteoclasts (Fig. 1).

As osteoclasts share a common origin with hematopoietic cells, it was not surprising to observe that mutations of the genes NEMO (Roberts et al. 2010),

Table 1 List of the gene mutations causing osteopetroses. The table lists the different types of osteopetrosis (*ADO II* autosomal dominant osteopetrosis type II (also called Albers-Schonberg disease), *ARO* autosomal recessive osteopetrosis, *IARO* intermediate autosomal recessive osteopetrosis), the mutated genes causing the disease, and the proteins encoded by the genes

Osteopetrosis form	Genetic transmission	Gene	Protein
ARO	**Autosomal recessive**	*TCIRG1*	α3 subunit V-ATPase
		CLCN7	Chloride channel *7*
		OSTM1	Osteopetrosis associated transmembrane protein
		PLEICH111	Pleckstrin homology domain containing family M. member 1
		SNX10	Sorting nexin 10
		TNFSF11	Receptor activator for nuclear factor kB ligand
		TNERSF11A	Receptor activator for nuclear factor kB
ARO with RTA	**Autosomal Recessive**	*0411*	Carbonic anhydrase II
ADO	**Autosomal dominant**	*CLCN7*	Chloride channel 7
		TCIRG1	α3 subunit V-ATPase

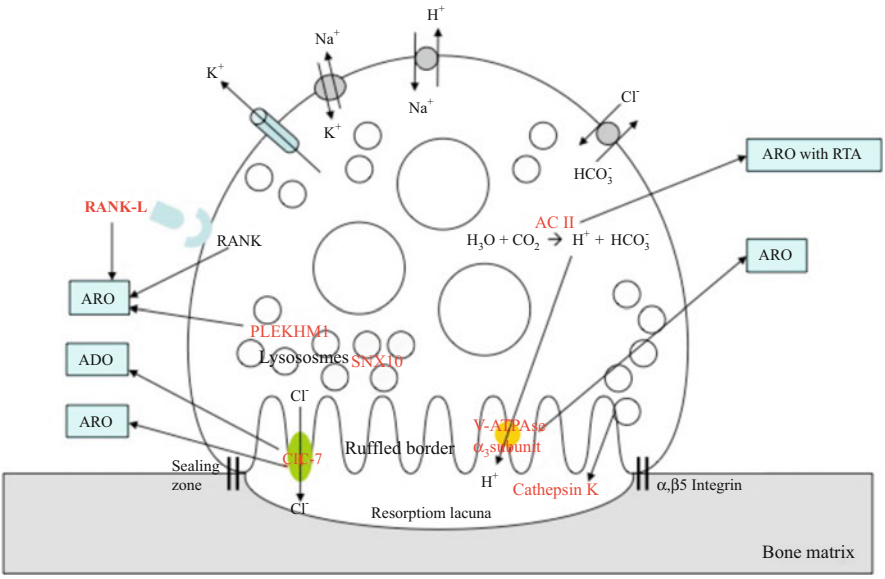

Fig. 1 Representation of a mature osteoclast showing the principal molecules whose mutation leads to development of osteopetrosis. An osteoclast is a large multinucleated cell specialized in bone resorption. The cell is highly polarized, with an apical pole where the principal ion channel pumps are localized and a very specific basal pole composed of a ruffled border which increases the membrane surface in contact with the bone matrix. To perform bone resorption, the osteoclast must be tightly attached to the bone matrix, notably via the αvβ5 integrin, which allows the formation of a sealing zone that defines the resorption lacuna where bone matrix demineralization takes place followed by proteolysis. For demineralization to occur, the lacuna needs to be an acidic environment, which is achieved by secretion of chloride ions and protons into the lacuna mediated by CLC7 and v-ATPase, respectively. The production of chloride and protons is made possible through the activity of carbonic anhydrase II (*CAII*). Osteoclasts also possess significant lysosomal activity. The illustration depicts the proteins which when mutated cause one form or another of osteopetrosis (*ADO II* autosomal dominant osteopetrosis type II, *ARO* autosomal recessive osteopetrosis, *ARO* with *RTA* autosomal recessive osteopetrosis with renal tubular acidosis)

CalDAG-GEF1 (Kilic and Etzioni 2009) and kindlin-3 (Malinin et al. 2009) (involved in blood cell function) can give rise to different autosomal recessive osteopetroses associated with an immune system defect. Osteoclast differentiation requires signaling via RANKL and macrophage colony-stimulating factor (M-CSF). Although patients with an M-CSF mutation have yet to be identified, op/op mice, which do not express functional M-CSF, do not produce osteoclasts and exhibit an osteopetrotic phenotype (Wiktor-Jedrzejczak 1991). However, osteopetrosis caused by RANKL mutations and several osteopetrotic families with a RANK mutation have been described (Guerrini et al. 2008; Lo Iacono et al. 2013). A lack of osteoclast differentiation associated with these mutations is responsible for rare cases of osteoclast-poor ARO, characterized by a total absence of mature osteoclasts (Sobacchi et al. 2013).

Terminally differentiated osteoclasts are able to degrade extracellular bone matrix thanks to the action of specific enzymes. To resorb bone matrix, the osteoclast must be perfectly polarized with a ruffled border and a sealing zone. These two elements allow the creation of a resorption lacuna into which protons are actively secreted in order to acidify the lacuna and allow dissolution of the bone matrix hydroxyapatite (Cappariello et al. 2014; Charles and Aliprantis 2014). Most of the osteoclast-rich osteopetroses are due to mutations in genes coding for proteins involved in this lacuna acidification system. The acid secretion process involves two main molecules that facilitate ionic transport: vacuolar ATPase proton pump (v-ATPase) and chloride channel 7 (ClC7; encoded by *CLCN7*) (Cappariello et al. 2014; Charles and Aliprantis 2014). Homozygous mutations in the α3 subunit of v-ATPase (encoded by the *TCIRG1* gene) and in the *CLCN7* gene trigger severe osteopetrosis, both in humans and mice. *TCIRG1* mutations are responsible for more than 50% of ARO cases, underlining the importance of this proton pump in osteoclastic activity (Sobacchi et al. 2001, 2013). Interestingly, dominant-negative mutations of *CLCN7* are responsible for development of ADO II (de Vernejoul et al. 1993; Bollerslev et al. 2013).

The protons and chloride ions secreted for lacuna acidification need to be continuously replaced intracellularly in order to avoid osteoclast alkalinization. This is accomplished by carbonic anhydrase II (CAII) and anion exchangers (Cappariello et al. 2014; Charles and Aliprantis 2014). In view of the importance of CAII in kidney function, it is not surprising that *CAII* mutation triggers development of ARO associated with renal tubular acidosis (de Vernejoul et al. 1993; Batlle and Haque 2012).

The collagenous bone matrix is dissolved by two groups of enzymes: matrix metalloproteases and lysosomal cathepsins. Cathepsin K especially was identified as a key enzyme in this osteoclastic function (Cappariello et al. 2014; Charles and Aliprantis 2014). This enzyme is secreted in the resorption lacuna and degrades type I collagen in an acidic environment. Inhibiting cathepsin K prevents bone matrix degradation (Yamashita and Dodds 2000), and inactivation of cathepsin K in mice gives rise to an osteopetrotic phenotype (Saftig et al. 2000). In humans, homozygous mutations of the gene encoding cathepsin K lead to pycnodysostosis, a rare skeletal dysplasia associated with bone abnormalities (Motyckova and Fisher 2002).

The acquisition and maintenance of osteoclast membrane polarity require complex systems of vesicle trafficking and continuous cytoskeletal renewal. One of the proteins involved in these processes is PLEKHM1 (Pleckstrin homology domain containing family M with runt domain member 1) (McEwan and Dikic 2015). This protein plays a crucial role in the acidification and trafficking of the intracellular vesicles (Cappariello et al. 2014; Charles and Aliprantis 2014). *PLEKHM1* homozygous mutations have been associated with ARO development (Van Wesenbeeck et al. 2007; Del Fattore et al. 2008b). SNX10 (sorting nexin 10) regulates endosomal homeostasis and seems to physically interact with v-ATPase and participate in its proper targeting to the ruffled border (Pangrazio et al. 2013). *SNX10* homozygous mutations have been associated with ARO onset (Aker et al. 2012; Pangrazio et al. 2013).

Despite these molecular advances, the mutations identified so far explain only 70% of the osteopetrotic cases, and so the search for the mutations responsible for the remaining 30% is still ongoing. Understanding of osteopetrosis pathophysiology, as for many other diseases, benefits immensely from available mouse models. Moreover, many genetic defects which express an osteopetrotic phenotype in mice have not yet been observed in humans, making the human homologues of those mutated murine genes good candidates for future studies.

CLCN7

CLCN7 is a member of a large gene family encoding chloride channels. *CLCN7* is the most broadly expressed member, and its protein product, chloride channel 7 (CLC7), is localized in the membranes of late endosomes and lysosomes as well as the ruffled membrane of osteoclasts (Zhao et al. 2009). CLC7 is composed of 803 amino acids, specifying 12 transmembrane domains, where the catalytic domain is localized, and cytoplasmic N- and C-terminal extremities (Fig. 2). CLC7 also has two cytoplasmic CBS (cystathionine βsynthase (Dutzler 2006)) domains that are situated in the C-terminal part of the polypeptide. These domains are supposed to be regulatory domains of CLC7 even though it is not clear how they function. The functional protein is a homodimeric complex which provides the chloride conductance required for efficient proton pumping into the osteoclast-ruffled membrane (Wang et al. 2012). CLC7 is a voltage-gated $2Cl^-/1H^+$ exchanger that couples Cl^- influx with H^+ efflux (Leisle et al. 2011). More than 50 *CLCN7* mutations have been

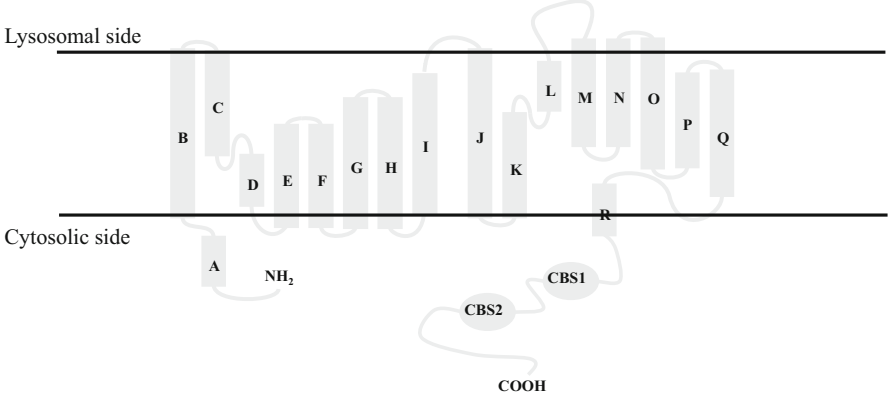

Fig. 2 CLC7 organization. CLC7 is a voltage-gated $2Cl^-/1H^+$ exchanger that couples Cl^- influx with H^+ efflux (Leisle et al. 2011). CLC7 is inserted into lysosomal and ruffled membranes via its 12 transmembrane domains, where the catalytic domain is nested (Dutzler 2006). The N- and C-terminal extremities are cytoplasmic. In addition, the C-terminal part harbors two regulatory domains called CBS, for cystathionine βsynthase (Dutzler 2006). The lysosomal and cytosolic sides are also represented (Adapted from (Dutzler et al. 2002; Bollerslev et al. 2013; Coudert et al. 2014))

reported in association with osteopetrosis. Interestingly, some when homozygous lead to ARO, and others when heterozygous lead to ADO II. To date, 34 *CLCN7* heterozygous mutations have been linked to ADO II (Table 2). They are mainly missense mutations, but three small deletions have also been described (Cleiren et al. 2001; Li et al. 2014). It seems that these mutations preserve the overall topology of the chloride channel and its 12 transmembrane domains (Fig. 2). They are mainly localized in the transmembrane region and on the cytosolic side of the protein (Bollerslev et al. 2013). In addition, as CLC7 functions as a homodimer, these mutations might have a dominant-negative effect (Cleiren et al. 2001; Bollerslev et al. 2013). Moreover, CLC7 needs a β subunit for its stability, and Ostm1 plays this role. Although neither the precise site of interaction with CLC7 nor the precise role of Ostm1 is clear, Ostm1 mutations are known to lead to osteopetrosis (Chalhoub et al. 2003; Lange et al. 2006).

ADO II

ADO II is commonly called benign osteopetrosis; however, 60–80% of the patients showing radiological signs of ADO II also have clinical signs (de Vernejoul et al. 1993). The pathology prevalence has been estimated at 5 per 100,000. ADO II is characterized by an increase in bone mass. However, the bones are denser but more fragile (Bollerslev et al. 2013).

ADO II clinical and radiological signs occur quite late in childhood or during the teenage years, although earlier occurrences have been reported (Bollerslev et al. 2013). ADO II radiological images show bones with highly characteristic features. Osteosclerosis predominates at the vertebral level, with the so-called sandwich vertebrae feature (Fig. 3; Coudert et al. 2014), which is a diagnostic criterion for ADO II. Most patients present an endobone (known also as “bone in bone”) aspect, observed mainly in the iliac bones but sometimes in other epiphyses (Coudert et al. 2014). An increase in cranial bone density can also occur. The main ADO II complications concern the skeleton. Bone fractures, both traumatic and nontraumatic, occur in 80% of patients, with a mean of three fractures per patient (de Vernejoul et al. 1993). Some patients can have more than ten fractures. The femur is the most fractured bone in this pathology, but fractures can occur on any long bone and even involve the posterior arch of the vertebrae, often inducing a spondylolisthesis. Scoliosis is not uncommon. Hip arthritis is frequent (in 50% of cases) and could be due to excessive stiffness of the subchondral bone. Arthritis can occur in other locations as well. Mandibular osteomyelitis (reminiscent of the side effect observed when bisphosphonates are administrated intravenously at high doses) is often associated with dental abscess or carious cavity. Cranial nerve compressions caused by osteosclerosis are rare. Auditory or visual impairment occurs in less than 5% of affected patients. Orthopedic treatment is often necessary to treat fractures and arthritis. Orthopedic treatment of fractures can be technically difficult, and post surgery complications such as strengthening delay, infection, and

Table 2 The 34 *CLCN7* mutations causing ADO II. The table lists all the *CLCN7* mutations implicated so far in ADO II. Shown are the mutations at the protein level, with the amino acid (3- and 1-letter codes) changes; the mutation at the RNA level with the location of the nucleotide change; the exon localization of the mutation out of the 25 *CLCN7* exons; and the reference that described the mutation

Polypeptide change	Nucleotide change	Exon, type of mutation	References
A299V (p.Ala299Val)			(Zheng et al. 2014)
A316F (p.Ala316Phe)			(Bollerslev et al. 2013)
A316G (p.Ala316Gly)	20247 C > G	Exon 11, missense	(Wang et al. 2012)
A542V (p.Arg542Val)	1625 C > T	Exon 17, missense	(Zheng et al. 2015)
A784FS (p. Arg784GlyfsX29)	2460delA	Exon 25, frameshift	(Li et al. 2014)
A788D (p.Ala788Asp)	2401C > A	Exon 25, missense	(Letizia et al. 2004)
E313K (p.Glu313Lys)	937G > A	Exon 11, missense	(Wang et al. 2012)
E798FS (p. Glu798GlyfsX129)	2392-/G	Exon 25, frameshift	(Zhang et al. 2009)
F318L (p.Phe318Leu)	954C > G		(Pangrazio et al. 2010)
F758L (p.Phe758Leu)	2274C > A		(Pangrazio et al. 2010)
G215R (p.Gly215Arg)	681G > A	Exon 7, missense	(Cleiren et al. 2001)
G677V (p.Gly677Val)	2068G > T	Exon 22, missense	(Frattini et al. 2003)
G725D (p.Gly725Asp)		Exon 24, missense	(Coudert et al. 2014)
G741R (p.Gly741Arg)	2221 G > C	Exon 24, missense	(Wang et al. 2012)
G765B			(Cleiren et al. 2001)
L213F (p.Leu213Phe)	675C > T	Exon 7, missense	(Waguespack et al. 2003)
L490F (p.Leu490Phe)	1506C > T		(Frattini et al. 2003)
P249L (p.Prol249Leu)	784		(Guerrini et al. 2008)
R286Q (p.Arg286Glu)	895G > A	Exon 10, missense	(Aker et al. 2012)
R286W (p.Arg286Trp)	894C > T	Exon 10, missense	(Guerrini et al. 2008)
R409W (p.Arg409Trp)	1225 C > T	Exon 15, missense	(Bollerslev et al. 2013)
R674Q (p.Arg674Glu)	2021G > A	Exon 22, missense	(Campos-Xavier et al. 2005)
R743W (p.Arg743Trp)	2227 C > T	Exon 24, missense	(Wang et al. 2012)
R762L (p.Arg762Leu)	2323G > T	Exon 24, missense	(Waguespack et al. 2003)
R762Q (p.Arg762Glu)	1577G > A		(Pangrazio et al. 2010)
R767W (p.Arg767Trp)	2337C > T	Exon 24, missense	(Pangrazio et al. 2010)
S290F (p.Ser290Phe)	868 C > T	Exon 10, missense	(Wang et al. 2012)
V289L (p.Val289Leu)	865 G > C	Exon 10, missense	(Zheng et al. 2015)
W127G (p.Trp127Gly)	15091 T > G	Exon 5, missense	(Wang et al. 2012)
W179X	574G > A		(Frattini et al. 2003)
W319R (p.Trp319Arg)			(Zheng et al. 2014)
Y99C (p.Tyr99Cys)	296A > G	Exon 4, missense	(Del Fattore et al. 2006)
ΔL688			(Cleiren et al. 2001)
	2423delAG	Exon 24, deletion	(Cleiren et al. 2001)

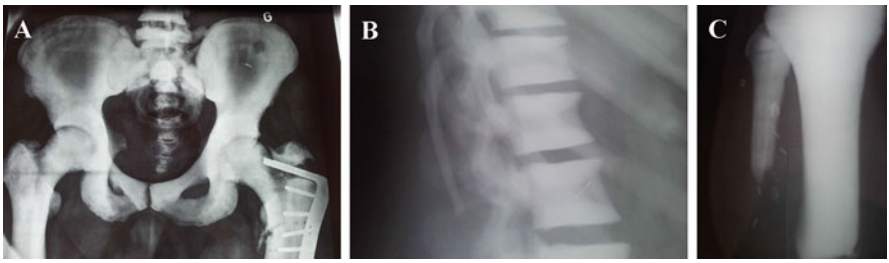

Fig. 3 Typical ADO II X-ray features. (a) A sclerotic vertebral plate, also called "sandwich vertebra." (**b**) Endobone observed on the iliac bone, also called "bone in bone." (**c**) A typical Erlenmeyer femoral diaphysis

pseudarthrosis are frequent (50% of cases) due to bone stiffness (de Vernejoul et al. 1993).

CLC7 loss of function caused by an inactivating mutation of one of the alleles of the *CLCN7* gene is responsible of ADO II (Bollerslev et al. 2013). *CLCN7* homozygous mutations are responsible for a few cases of ARO, as previously stated, whereas heterozygous mutations give rise to most cases of Albers-Schönberg disease (Bollerslev et al. 2013). However, *CLCN7* mutations explain only 70% of ADO II cases (Del Fattore et al. 2006, 2008a; Bollerslev et al. 2013), and the causative mutations of the other 30% remain to be found, although a heterozygous mutation in *TCIRG1* was recently reported to be involved in an ADO II case (Wada et al. 2013). According to the literature, penetrance is between 60% and 90% (Benichou et al. 2000; Cleiren et al. 2001). Clinical severity is highly variable, even within the same family (Bollerslev et al. 2013). Studies (notably by Econs's group) have demonstrated that 1/3 of subjects with a *CLCN7* mutation are clinically normal with neither biochemical nor radiological evidence of the disease. In addition, the disease severity in the remaining 2/3 is highly variable (Waguespack et al. 2007). It is not unusual to observe both unaffected gene carriers and severely affected individuals in the same family. In addition, there is no genotype/phenotype correlation (Chu et al. 2005, 2006). The mechanism by which mutation carriers manifest the disease (or not) is likely to involve both environmental and genetic factors. For example, polymorphism of the "healthy" *CLCN7* allele might be at play, as could a segregating genetic background or genetic modifiers. Indeed, Chu et al. suggested that a modifier gene (or genes) is located at the 9q21-22 locus (Chu et al. 2005).

Most of the ADO II patients in a recently described French cohort (Coudert et al. 2014) had a milder phenotype compared to the abovementioned ADO II patients studied by Waguespack et al. (2007). Still, only 1/3 of these newer patients were asymptomatic and most of them presented fractures (Benichou et al. 2000). Although the French cohort included several patients from the same family, it was not large enough and the phenotypes did not contrast enough to allow investigation of differences in gene expression that might explain the genotype/phenotype variability (Coudert et al. 2014).

Systemic Bone Markers of ADO II

ADO II is characterized by increased bone density due to impaired osteoclast function. This osteosclerosis is visible on X-ray images (Fig. 3), and such patients are also obviously characterized by a high Z-score (Fig. 4a). Some analyses of bone serum markers have been conducted in ADO II patients (Del Fattore et al. 2006; Coudert et al. 2014). The serum level of tartrate-resistant acid phosphatase (TRAP),which reflects osteoclast number, appears to be a useful marker of ADO II, according to the literature (Henriksen et al. 2004; Del Fattore et al. 2006; Fig. 4b). These data are in agreement with observations made on ADO II bone biopsies showing an increase in both the size and number of osteoclasts, which make ADO II an osteoclast-rich osteopetrosis. In contrast, CrossLaps peptide (CTX), which reflects the amount of bone resorbed, was not increased. Interestingly, for the first time for this disease, two biological markers of bone formation, bone alkaline phosphatase and procollagen type 1 N propeptide (P1NP), can be measured (Fig. 4c). However, their levels are not reduced, as would be expected when bone resorption is reduced. Some patients had high levels of both markers. These observations support the hypothesis that osteoclasts incapable of resorption can transmit a signal to osteoblasts so as to enhance their differentiation (Karsdal et al. 2007). This is the basis of the theory that inhibiting osteoclast activity, and not their number, could be used to treat osteoporosis, as it would decrease bone resorption without decreasing bone formation (Schaller et al. 2004; Karsdal et al. 2007; Baron 2012).

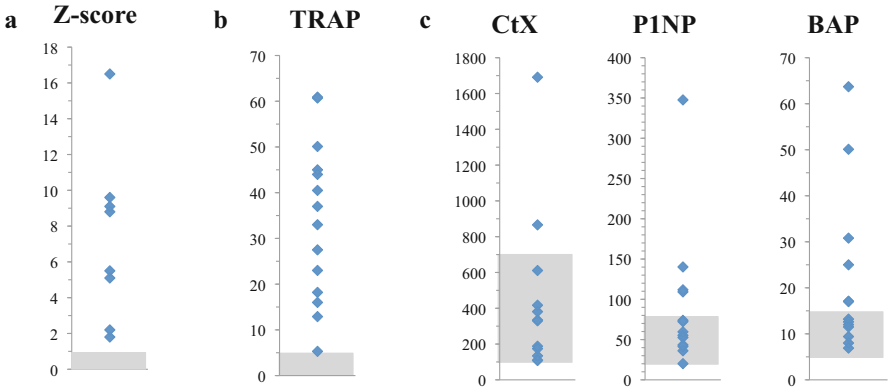

Fig. 4 **Systemic markers. (a)** Femoral Z-scores measured in osteopetrotic patients. (**b**) Tartrate-resistant acid phosphatase (*TRAP*) serum level measured in osteopetrotic patients. Studies revealed its consistency as a marker of ADO II disease. (**c**) CrossLaps (*CTX*), bone alkaline phosphatase (*BAP*), and procollagen type 1 N propeptide (*P1NP*) serum levels measured in osteopetrotic patients. Studies revealed their lack of consistency as markers of ADO II. The *gray* areas represent the normal ranges

Osteopetrotic Osteoclasts

In situ, osteopetrotic osteoclasts are larger and more numerous compared to osteoclasts in normal bone. But, independently of their number, osteopetrotic osteoclasts have insufficient resorbing activity, resulting in brittle, woven bone and filled medullary space (Chu et al. 2006). However, it has been demonstrated that, ex vivo, osteoclasts from osteopetrotic patients are not different from osteoclasts from healthy donors in terms of morphology, size, number, rate of differentiation, and TRAP activity. Even the levels of expression of osteoclast marker genes are comparable (Coudert et al. 2014). However, and as expected, osteopetrotic osteoclasts resorb less bone than those of healthy donors (Chu et al. 2006). Chu et al. suggested in 2006 that ADO II status could be determined through properties intrinsic to the osteoclasts and that studies directed toward understanding the mechanism of variable penetrance in ADO II should focus on osteoclast-specific factors (Chu et al. 2006). In accordance with this suggestion and in order to gain insights into the nature of osteopetrotic osteoclasts, a microarray analysis was carried out using RNA extracted from osteoclasts differentiated from peripheral blood mononuclear cells (PBMCs) obtained from 15 osteopetrotic patients and 31 healthy donors, both males and females. The ADO II patients carried six different *CLCN7* mutations (in red in the Table 2; for details see (Coudert et al. 2014)) and expressed a mild form of the disease. The microarray analysis and the follow-up molecular confirmation experiments allowed four ADO II genetic markers to be defined, involving both new and previously known pathways of osteoclast biology (Coudert et al. 2014).

ADO II Genetic Markers

PBMCs purified from osteopetrotic patients and healthy donors were cultured for 14 days in the presence of M-CSF and RANKL (Coudert et al. 2014). The PBMCs from patients with ADO II differentiated into osteoclasts in a similar way as those from healthy donors, in accordance with other studies (Chu et al. 2006). ADO II osteoclast resorption activity was not assessed in the study, but it was previously shown to be reduced (Henriksen et al. 2005; Chu et al. 2006; Del Fattore et al. 2006), with (Henriksen et al. 2005) or without (Chu et al. 2006) decreased osteoclast acidification. RNA was extracted from these cultures and a microarray analysis performed. Gene profiling had not been previously investigated in ADO II osteoclasts, and several genes were found to be differentially expressed. Although microarray is a powerful technology, working with such a rare disease and a limited number of patients blunts the statistical power. However, from the many genes whose expression was modified between the osteopetrotic and healthy donor osteoclasts, 18 were selected for further study because they showed the greatest variation in expression (for details see (Coudert et al. 2014)). Of these 18 potential ADO II genetic markers, the expression of seven was confirmed by qPCR: ITGB5, PRF1, SERPIN2, WARS, CES1, GBP4 and UCHL1 (Fig. 5). Western blotting and immunofluorescence confirmed modified expression at the protein level for four of the

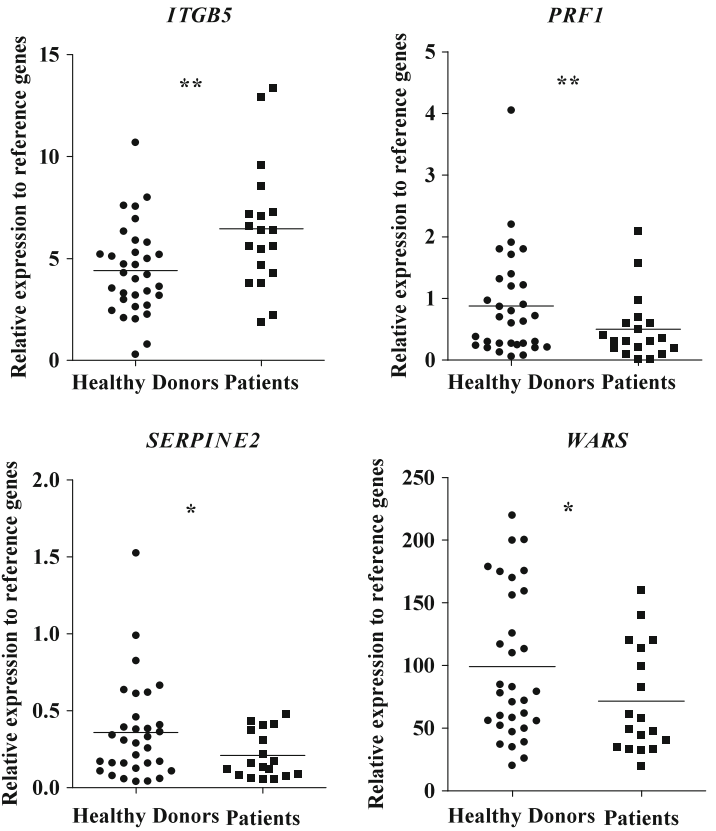

Fig. 5 The four ADO II biomarkers validated by qPCR. The expression levels of *ITGB5*, *PRF1*, *Serpine2*, and *WARS* in 15 osteopetrotic patients compared to 31 healthy donors. Each *dot* represents the relative expression of the considered gene for an individual. *Horizontal lines* represent the mean

candidates. Unfortunately, none of these seven genes maps to the chromosome 9 locus where a potential modifier gene lies (Chu et al. 2005; Coudert et al. 2014).

Of the seven genes validated by qPCR, five of them (*CES1, UCHL1, PRF1, WARS,* and *GBP4*) were not previously known to be expressed in osteoclasts. The integrin αvβ5 (encoded by *ITGB5*) was previously shown to be more highly expressed in immature osteoclasts than in fully differentiated osteoclasts (Inoue et al. 1998). However, αvβ5 cannot substitute for αvβ3 when the latter is absent in cytokine-stimulated osteoclast precursors (McHugh et al. 2000). The other gene already described in osteoclasts encodes SERPINE2, a serine protease inhibitor also known as nexin-1 or plasminogen activator inhibitor type 1 (Yang et al. 1997). Subsequently, SERPINE2 was demonstrated not to be involved in osteoclast differentiation but to be important for non-collagenous protein degradation of non-mineralized matrix (Daci et al. 1999).

ITGB5, one of the four genes defined as an ADO II genetic biomarker (Coudert et al. 2014), was reported to be negatively regulated during osteoclast differentiation in mice (Inoue et al. 2000). Furthermore, the absence of expression of β5 integrin in female mice increases osteoclast activity (Lane et al. 2005). In the French cohort of osteopetrotic patients described above, osteoclast differentiation was unchanged, at least in terms of the number of multinucleated and TRAP-positive cells and the expression of osteoclast-specific genes such as Cathepsin K, TRAP, and RANK [32]. The elevated expression of β5 integrin observed in osteoclasts of osteopetrotic patients could have a role in osteoclast motility (Teitelbaum 2011). Osteoclast motility has previously been reported to be modified in patients with osteopetrosis, both in patients with *CLCN7* mutations (Del Fattore et al. 2006) and in a single patient with an unknown mutation whose osteoclasts exhibited increased expression of β5 integrin (Blair et al. 2009).

No changes in expression of other β integrins (amount or localization) were observed, precluding any alteration of general integrin function (Coudert et al. 2014). Similarly, the expression profiles of other genes involved in the integrin pathway were unchanged in both the microarray and qPCR analyses of the French cohort (Coudert et al. 2014).

Perforin1 is a 66 kDa protein that polymerizes to form a transmembrane pore-like structure in lipid bilayers (Pipkin and Lieberman 2007; Pipkin et al. 2010) of the plasma membrane of target cells (Zhou 2010; Voskoboinik et al. 2015). This channel is dependent on Ca^{2+} and delivers granzymes into the cytosol of target cells; it is the only known delivery molecule for granzymes (Pipkin and Lieberman 2007; Pipkin et al. 2010). Perforin1 is less broadly expressed than most granzymes (Chowdhury and Lieberman 2008) and might thus serve as a defining marker of cells with the capacity to kill (Pipkin et al. 2010). Interestingly, perforin1 in mammals is encoded by the single-copy *PRF1* gene, and there is a high degree of conservation among perforin homologues from different species (Hoves et al. 2010). However, in contrast to the uniqueness of perforin1, a variety of granzymes, the specific inducers of target-cell apoptosis, exists in the genome (Hoves et al. 2010). Moreover, the repertoire of granzymes is highly variable in mammals (Hoves et al. 2010). Granzymes do not cause the death of target cells if they are transferred into cells without perforin, suggesting that perforin must do more than simply mediate the entrance of granzymes into cells (Kagi et al. 1994; Zhou 2010). In addition, the cell-death mechanism mediated by perforin is not fully understood, and two scenarios have been put forward to explain it (Hoves et al. 2010; Zhou 2010). The first scenario, also the simplest, postulates that the contents of secretory granules are released into the immunological synapse, followed by perforin1 pore formation in the target-cell plasma membrane to facilitate the direct flow of proapoptotic granzymes into the cytosol, where they cleave endogenous intracellular substrates (Hoves et al. 2010; Voskoboinik et al. 2010; Zhou 2010). The alternative hypothesis proposes that perforin1 and granzymes are confined to endocytic vesicles inside the target cells, which perforin1 then lyses from the inside and thus delivers granzymes into the cytosol (Hoves et al. 2010; Voskoboinik et al. 2010; Zhou 2010). The secretory granules are a subtype of lysosomes on the basis of their acidic pH and possession of

some classic lysosomal membrane proteins (e.g., H+-ATPase) (Hoves et al. 2010). Some proteins are unique to secretory granules such as perforin1 and granzymes (Hoves et al. 2010). However, it is not clear if a specialized pathway exists for targeting perforin1 and granzymes exclusively to secretory granules or if generic lysosomal trafficking pathways deliver a proportion of granzymes and perforin1 to the granules, with the rest delivered to conventional lysosomes (Voskoboinik et al. 2010). It is important to also note that at the acidic pH of granules, perforin1 is incapable of activity due to its inability to bind to calcium ions. That expression of perforin1 (and granzymes, too, but without modification between the two types of osteoclasts – see supplemental data in (Coudert et al. 2014)) has been demonstrated in osteoclasts raises two questions. Are osteoclasts, in fact, more closely related to immune cells than what is currently accepted, which could explain the presence of putative secretory granules, but what is the target in this case? Or is perforin expressed in cells (osteoclasts) other than cytotoxic immune cells? In addition, the fact that the perforin1 expression is modified in osteopetrotic osteoclasts is consistent with the idea that these osteoclasts have some kind of lysosome impairment, more precisely a problem of lysosome acidification.

SERPINE2 is also known as protease inhibitor or protease nexin I. Serpine2 is part of the serine protease inhibitor superfamily which includes proteins that share a precise three-dimensional structure but modest sequence identity (Huntington 2006). In vertebrates, six subgroups of SERPINES exist (van Gent et al. 2003). SERPINES are the most abundant inhibitors in humans (Huntington 2006). They are involved in various processes such as blood coagulation, fibrinolysis, programmed cell death, development, and inflammation (van Gent et al. 2003). SERPINE2 is a thrombin and urokinase inhibitor that shares several features with antithrombin III, an abundant plasma thrombin inhibitor. Serpine2 is highly expressed and developmentally regulated in the nervous system (Carter et al. 1995). SERPINE2 expression in osteoclasts was first demonstrated by Yang et al. using murine cells (Yang et al. 1997). As part of the plasminogen system, serpine2 was suggested to be involved in the removal of non-collagenous proteins present in the unmineralized bone matrix, but not required in osteoclast differentiation or for the resorption of mineralized matrix (Daci et al. 1999). SERPINE2 was identified as a candidate susceptibility gene for chronic obstructive pulmonary disease (Demeo et al. 2006). SERPINE2 was also shown to be involved in the modulation of fear conditioning and extinction (Meins et al. 2010). It was shown that low density lipoprotein receptor-related proteins (LRPs) are the principal clearance receptors for SERPINES and SERPINE-protease complexes (Herz and Strickland 2001), and Serpine2 is able to interact with several different LRPs when complexed or not with its proteinase target (Jensen et al. 2009). SERPINE2 is expressed on the surface of monocytes and monocyte-derived macrophages, and this expression is increased during infection (Mansilla et al. 2008). SERPINE2 is a secreted protein that binds to the extracellular matrix (Ghanipour et al. 2009) and is inhibited by collagen type IV but not by collagen type I (Olszewski et al. 2006). SERPINE2 is also involved in the regulation of dense-core secretory granules, where peptide hormones are stored, by preventing the degradation of granule proteins (Dutzler et al. 2002). ADO II is, paradoxically, an

osteoclast-rich form of osteopetrosis, as though more osteoclasts (albeit defective ones) are produced to try to compensate the phenotype. Likewise, it could be hypothesized that reduced expression of SERPINE2 serves the same goal. Indeed, TRAP, which participates in bone matrix protein degradation, is cleaved by serine proteases (such as trypsin) and cysteine proteases (such cathepsin K), resulting in its increased enzymatic activity. As SERPINE2 is as serine protease inhibitor, it could be hypothesized that, by decreasing SERPINE2 expression, the action of trypsin is increased in order to increase the TRAP activity. However, ultimately this does not work because acidification of the resorption lacuna is impaired by the *CLCN7* mutation.

WARS or tryptophanyl-tRNA synthetase is an aminoacyl-tRNA synthetase and thus an essential component of protein synthesis (Hersh et al. 2006). Indeed, WARS catalyzes the aminoacylation of tRNA (Trp) with tryptophan so that the amino acid is available for protein synthesis. As expected, aminoacyl-tRNA synthetases are ubiquitous and highly conserved, given their fundamental role in protein translation (Hersh et al. 2006). However, studies published during the last decade have suggested additional roles for these synthetases, roles unrelated to protein synthesis. These additional activities such as cytokine-like activities were first demonstrated for the tyrosyl-tRNA synthetase (Wakasugi and Schimmel 1999) and then for WARS. A naturally occurring truncated form of WARS was shown to be an effective inhibitor of angiogenesis (Wakasugi et al. 2002). In addition, WARS is the only aminoacyl-tRNA synthetase whose expression is regulated by interferon-γ (Shaw et al. 1999). In recent studies, it was even demonstrated that, in addition to being differentially expressed in colorectal cancer, reduced expression of WARS can be correlated to a higher rate of recurrence of the cancer (Ghanipour et al. 2009). Finally, it has been previously shown that monocytes purified from blood express the WARS mRNA, and this expression increases as these cells mature into macrophages (Krause et al. 1996). Unfortunately, the fact that WARS expression is regulated according to the differentiation state or pathological status of monocytes/macrophages and osteoclasts does not provide a clue to its function.

The main questions raised by the results presented here are: How does a defective chloride channel, namely, CLC7, have an effect on the transcriptional regulation of genes? Does acidification, either inside lysosomes or in the resorption lacuna, impact the transcriptional regulation in osteoclasts?

Potential Applications to Prognosis, Other Diseases, or Conditions

ADO II Biomarkers as Therapeutic Targets

Recent advances in high-throughput genomics have spurred progress in disease biomarker research, irrespective of the prevalence of the disease or the organ affected. Although rare and highly variable in phenotype, ADO II is a disease still

in need of biomarkers. The recent discovery of four genetic biomarkers of ADO II (*ITGB5, WARS, PRF1, SERPIN2*) (Coudert et al. 2014) opens a path to the discovery of new drugs for the treatment of this disease. A potential drawback is that none of these four biomarkers is expressed exclusively in osteoclasts, but this is also a limitation of many current therapies. On the other hand, the observed reduction of perforin1 expression in ADO II highlights the emerging field of osteoimmunity, a quite young subdiscipline in the bone research field linking it to immunology and adding growing evidence of the interdependence of these two areas of biology.

ADO II as a Model to Understand Osteoclast Differentiation and Function, Leading to the Discovery of New Targets in Other Diseases Involving Osteoclast Impairment or Hyperactivity

The finding of altered expression of *ITGB5* in ADO II is essentially new, but *ITGB5* was already a known actor in osteoclast biology. The identification of a potential role for WARS in osteoclast function and ADO II pathology is a novel finding, although tempered because it should not be surprising to find this protein in any cell type, given its general role in protein translation.

Concerning SERPINE2 and ADO II, the situation falls somewhere in between those for ITGB5 and WARS. Expression of SERPINE2 has previously been described in osteoclasts but with a very precise and peculiar function. It would be of interest to dig deeper to specify this role more fully and to identify the interactions SERPINE2 has with other proteins. Moreover, it is a story involving vesicles and intracellular transport, as osteoclasts are highly vesicular cells. More light needs to be cast on this vesicular compartment to fully understand the functioning of osteoclasts.

Perforin1 is another story entirely. This protein was supposed to be per se a marker of cytotoxic immune cells. The potential involvement of this protein first in osteoclasts and second in ADO II is interesting and definitely needs to be pursued. How osteoclasts express and then regulate the expression of *PRF1* is puzzling. Are osteoclasts able to target any cells through the granzyme/perforin pathway? If so, for what purpose? Or has PRF1 another function not yet described that is required for osteoclast life and/or function? These questions need answers.

Another very interesting question is how mutation of *CLCN7*, encoding a chloride ion transporter, able to influence the transcription of such different proteins. Does the altered acidification state in the resorption lacuna affect gene transcription? Studies need to be conducted on this aspect.

Investigating these new leads could bring new insights in the life and death of osteoclasts. Osteoclasts are of course specialized cells, specialized bone cells. But they also are the focus of considerable attention in the treatment of very common diseases such as osteoporosis, one of the great public health problems of our time. Finally, even if osteoclast function seems to be well understood, more knowledge is needed to be able to better target them and ultimately better treat diseases caused by osteoclast dysfunction.

Summary Points

- This chapter focuses on type II autosomal dominant osteopetrosis (ADO II).
- ADO II patients have increased bone mass associated with bone fragility.
- ADO II is caused by osteoclast impairment.
- Heterozygous *CLCN7* mutations cause ADO II.
- ADO II has specific radiographic features.
- Microarray analysis revealed new biomarkers validated by other analyses.
- Some biomarkers (*ITGB5* and *WARS*) belong to already known pathways.
- Some biomarkers (*PRF1* and *SERPINE2*) belong to new pathways in osteoclasts.
- These new ADO II biomarkers give new insights into osteoclast function.

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Analysis of Integrin Alpha$_2$Beta$_1$ ($\alpha_2\beta_1$) Expression as a Biomarker of Skeletal Metastasis

22

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Abstract

Skeletal metastasis is a frequent and debilitating end product of tumor progression affecting roughly a third of cancer patients within the USA. Tumor growth within the bone uncouples normal bone remodeling which leads to a net loss or gain of bone. These cancer-induced bone changes compromise the integrity of the skeleton and may cause pathologic fracture, severe pain, nerve compression, and metabolic imbalances. Therefore, to improve patient outcome, the identification of tumor cell markers with which to accurately predict skeletal metastasis potential is needed. The integrin family of adhesion molecules is a class of cell surface receptors that facilitate cell attachment to the extracellular matrix and promote cancer metastasis. The present chapter is focused on current literature evidence supporting that integrin $\alpha_2\beta_1$ is both a mediator and predictive marker of metastasis, particularly metastasis to the skeleton.

Keywords

Prostate cancer • Skeleton • Metastasis • Integrin • Collagen

List of Abbreviations

ALDH	Aldehyde dehydrogenase
$\alpha_2\beta_1$	Integrin alpha$_2$beta$_1$
BPH	Benign prostatic hyperplasia
BrCa	Breast cancer
Col I	Collagen type I
DTC	Disseminated tumor cell
FAK	Focal adhesion kinase
GAS6	Growth arrest-specific 6
GFOGER	Glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine
IHC	Immunohistochemistry
ILK	Integrin-linked kinase
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
mRNA	Messenger ribonucleic acid
Neu	Neuroblastoma-derived oncogene homology
PCa	Prostate cancer
PI3-K	Phosphatidylinositol 3 kinase
PSA	Prostate-specific antigen
Rac	Ras-related C3 botulinum toxin substrate
RGD	Arginine-glycine-aspartic acid
Rho GTPase	Ras homology guanosine-5′-triphosphatase
SCID	Severe combined immunodeficiency
shRNA	Small hairpin ribonucleic acid

Src	Rous sarcoma virus homology proto-oncogene tyrosine kinase
TGFβ	Transforming growth factor beta
TMA	Tissue microarray
uPAR	Urokinase-type plasminogen activator receptor
VCAM-1	Vascular cell adhesion molecule-1
VLA-2	Very late antigen-2

Key Facts of Skeletal Metastasis

- Skeletal metastases are a frequent and debilitating end product of the progression of multiple solid tumors including melanoma and cancers of the prostate, breast, kidney, thyroid, and lung.
- Tumor growth within the bone can uncouple normal bone remodeling leading to net bone destruction (osteolysis) or bone formation (osteogenesis).
- Cancer-induced bone changes compromise the integrity of the skeleton and can produce severe pain, pathologic facture, nerve compression, and paralysis.
- Growth of cancer within the bone can cause calcium and phosphate imbalances which may lead to neurologic problems and the formation of kidney stones.
- The bone marrow microenvironment can support the growth of tumor cells and protect against the effect of chemotherapeutic drugs.

Definitions of Words and Terms

Extracellular matrix	A collection of proteins that serve as a substrate for cell attachment.
Integrin	Integrins are a family of receptors located on the cell surface that mediate the attachment of cells to extracellular matrix proteins.
Metastasis	The movement of tumor cells from the site of origin to a distant organ, typically via blood or lymphatics vessels.
Premetastatic niche	The premetastatic niche is an emerging concept in tumor biology where hematopoietic-derived cells arrive within a target organ and alter the environment to promote metastasis by regulating the homing, attachment, and growth of tumor cells.
Tumor dormancy	Tumor dormancy describes the retention of tumor cells within an organ site that do not grow but retain the capacity to form metastases at a later point in time.

Introduction

Cancer metastasis is a very complex biological process consisting of numerous sequential and interdependent steps. Many of these steps are characterized by interactions between the tumor cell and host tissues including endothelial cells and

extracellular matrix proteins. The cell surface receptors that mediate these interactions are therefore well positioned to regulate the metastatic process at multiple points. The integrin family is a class of transmembrane adhesion molecules that mediate cellular attachment to extracellular matrix proteins. The term integrin was proposed by Tamkun et al. in 1986 to denote the role of an "integral membrane complex involved in the transmembrane association between the extracellular matrix and the cytoskeleton" (Tamkun et al. 1986). Direct evidence that integrins participate in metastasis was published 3 years later by Tressler and colleagues who showed that blocking integrins with arginine-glycine-aspartic acid (RGD) peptides reduced the attachment of highly metastatic large cell lymphoma cells to liver endothelial cells compared to low metastatic parental cells (Tressler et al. 1989). These data suggested that integrins not only play a role in organ specificity (by directing adhesion to target organ microvessel endothelial cells) but also may serve as diagnostic and prognostic markers of metastasis. Research conducted during the intervening 26 years has provided both preclinical and clinical evidence that integrins contribute to metastasis and has established the family as viable a therapeutic target for the treatment of malignant disease. One integrin in particular, integrin alpha$_2$beta$_1$ ($\alpha_2\beta_1$/VLA-2/CD49b-CD29), has been shown to regulate metastasis, specifically metastasis to the skeleton.

Integrin $\alpha_2\beta_1$

The integrin family is a class of transmembrane glycoproteins composed of noncovalently linked α and β subunits. Each αβ heterodimer mediates attachment to a specific set of extracellular matrix proteins whose binding is highly dependent by divalent cations. Integrin $\alpha_2\beta_1$ is a high affinity receptor for collagen type I (Kern et al. 1993). The integrin binds to collagen I through the specific amino acid sequence GFOGER (Gly-Phe-HPro-Gly-Glu-Arg) (Knight et al. 2000) when present in the native triple-helical conformation of the type I collagen fibril.

The integrin β subunit of integrin $\alpha_2\beta_1$ can interact with numerous intracellular signaling molecules including focal adhesion kinase (FAK), integrin-linked kinase, and the non-receptor tyrosine kinase Src (reviewed in (Miranti and Brugge 2002)). Induction of these molecules through β_1 integrin can mediate cellular proliferation and motility through the activation of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3 kinase (PI3-K), or RhoA GTPase (Longhurst and Jennings 1998; Slack-Davis and Parsons 2004). The cytoplasmic region of the α_2 integrin subunit can also promote RhoA activation and cell spreading suggesting that both integrin subunits contribute to outside-in signal transduction (Keely et al. 1998; Evers et al. 2000; Price and Collard 2001). We have shown that collagen I binding to $\alpha_2\beta_1$ activates the Rho family member RhoC GTPase (Hall et al. 2008) resulting in prostate cancer (PCa) cell invasion and migration. Thus, integrin $\alpha_2\beta_1$ can activate multiple signal transduction pathways which may support tumor cell invasion and metastasis (Fig. 1).

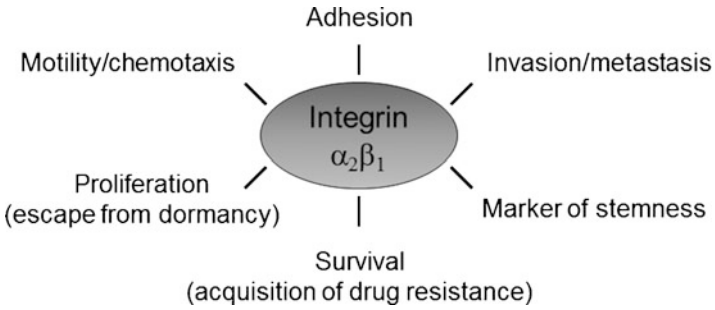

Fig. 1 Biological properties influenced by integrin $\alpha_2\beta_1$ in cancer cells. Preclinical data has shown that integrin $\alpha_2\beta_1$ can influence the properties of cancer cells, adhesion, motility, invasion, survival, and proliferation, as well as serve as a marker of stemness

Integrin $\alpha_2\beta_1$ as a Marker of Cancer Stem/Tumor-Initiating Cells

Characterization of cell surface markers with which to identify cancer cells and predict those cells that will ultimately metastasize will facilitate early intervention within cancer patients. Of particular interest is the identification of biomarkers of cancer stem cells or tumor-initiating cell populations. Numerous studies have established that the expression of CD133, CD44, and aldehyde dehydrogenase 1 (ALDH1) are enriched within stem cell populations isolated from patient tumors and cancer cell lines (reviewed in (Islam et al. 2015)). There is also evidence to suggest that the expression of integrin $\alpha_2\beta_1$ is a marker of cancer stem/tumor-initiating cells. Integrin $\alpha_2\beta_1$ is enriched in stem cell populations of colorectal cancer where it regulates lineage commitment and clonogenicity (Kirkland 2009). In human prostate cancer (PCa), the high surface expression of integrin $\alpha_2\beta_1$ (as determined by rapid adhesion to collagen) was shown to be a marker of prostate cancer stemness (Collins et al. 2001, 2005; Maitland et al. 2011). Specifically, integrin $\alpha_2\beta_1$ was found to be expressed on primitive cells with a basal cell phenotype that were capable of self-renewal and tumor initiation within immunocompromised mice with as few as 100 cells (Maitland et al. 2011). Collectively, these data support that integrin $\alpha_2\beta_1$ is a potential marker of cancer stem/tumor-initiating cells and therefore may be an effective cancer biomarker.

Integrin $\alpha_2\beta_1$ as a Cancer Biomarker

For the present discussion, a biomarker is defined as a biological molecule associated with cancer that has either diagnostic and/or prognostic value within the disease in question. Examples may include BRCA1 in breast cancer and prostate-specific antigen (PSA) in PCa. Based on current literature evidence, we would argue that no integrin has risen to the level equal to either of the above examples. However,

there is evidence that the expression (or lack of expression) of a particular integrin is frequently associated with cancer and may contribute to tumor development or progression. Prominent examples might include integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_4\beta_1$ which have been investigational targets in clinical trials (ClinicalTrials.gov). In the context of the present chapter focused on integrin $\alpha_2\beta_1$, there are clinical data to support that integrin $\alpha_2\beta_1$ is a biomarker in select human cancers.

The protein expression of $\alpha_2\beta_1$ integrin has been evaluated by immunohistochemistry in human tissue taken from patients with gastric cancer, thyroid cancer, renal cell cancer, and PCa (Anastassiou et al. 1995; Dahlman et al. 1998; Ura et al. 1998; Matsuoka et al. 2000; Sottnik et al. 2013). The gene expression of the α_2 subunit has also been evaluated in clinical cases of human melanoma (Vuoristo et al. 2007). Collectively these data show that integrin $\alpha_2\beta_1$ is a marker of malignant progression within these diseases. For example, $\alpha_2\beta_1$ expression was found to be increased in peritoneal metastases versus primary lesions of human gastric cancer (Matsuoka et al. 2000). Its expression was further associated with lymph node and liver metastases and was shown to be an independent factor related to liver metastasis of gastric cancer (Ura et al. 1998). In human thyroid cancer, integrin $\alpha_2\beta_1$ was exclusively expressed in anaplastic carcinomas, an undifferentiated and highly aggressive form of this disease, relative to all other thyroid tissues examined (Dahlman et al. 1998), and in renal cell carcinoma, the expression of $\alpha_2\beta_1$ was absent in primary lesions but was expressed in metastatic tissues (Anastassiou et al. 1995). In human melanoma, the high mRNA expression of the α_2 subunit was associated with poorer overall survival (Vuoristo et al. 2007) providing further evidence that $\alpha_2\beta_1$ is a marker of malignant progression in human disease. The expression of $\alpha_2\beta_1$ has also been evaluated in clinical case of human breast cancer but within this disease its expression appears to be lost with progression (Zutter et al. 1990). A detailed analysis of this interesting dichotomy follows later within this chapter.

Integrin $\alpha_2\beta_1$ as a Marker of PCa Skeletal Metastasis

As introduced above, integrin $\alpha_2\beta_1$ is a potential marker of PCa stem/tumor-initiating cells. Consistent with these data, two studies of putative stem cell markers in human PCa patients showed that the percent of $\alpha_2\beta_1$ positive cells in the primary tumor and in bone marrow aspirates was associated with the progression of bone metastasis and the occurrence of bone metastasis-free survival (Colombel et al. 2012; Ricci et al. 2013). We recently published the analysis of $\alpha_2\beta_1$ protein expression in tissues from men who died of PCa (Sottnik et al. 2013). We report that integrin $\alpha_2\beta_1$ expression was increased in PCa skeletal metastases compared to either PCa primary lesions or soft tissue metastases of the liver or lymph node (Sottnik et al. 2013). Analysis of $\alpha_2\beta_1$ staining intensity alone revealed that bone metastases had the highest percent of strong staining samples compared to soft tissue metastases, particularly lymph node metastases. Quantitatively, lymph node metastases had odds of weaker staining 2.3 times that of bone metastases [Wald confidence limits

(1.02, 5.22), p-value, 0.042] consistent with the hypothesis that integrin $\alpha_2\beta_1$ is a marker of PCa skeletal metastasis (Sottnik et al. 2013).

Our analysis further revealed that $\alpha_2\beta_1$ protein was highly expressed within normal and benign prostatic hyperplasia samples to a level equivalent to skeletal metastases (Sottnik et al. 2013). The data showed that $\alpha_2\beta_1$ expression was decreased in primary PCa lesions compared to normal and benign tissues and was further diminished in PCa soft tissue metastases of the lymph node (Sottnik et al. 2013). The expression of $\alpha_2\beta_1$ within skeletal metastases was increased relative to PCa primary lesions and soft tissue metastases back to levels found in nonneoplastic prostate tissue (Sottnik et al. 2013). These data suggest that $\alpha_2\beta_1$ expression is biphasic during PCa progression (Eaton et al. 2010) and may be a predictive biomarker of skeletal metastasis.

Role of Collagen Type I/Integrin $\alpha_2\beta_1$ Activity in Tumor Progression and Metastasis

A permissive microenvironment is required to promote tumor development and progression (reviewed in (Langley and Fidler 2011)). For example, the presence of a fibrotic, reactive stroma in primary lesions was shown to be associated with shortened biochemical-free recurrence in men with PCa (Ayala et al. 2003). In human breast cancer, women with high breast density, characterized by enriched stromal collagen I, were at increased risk for local recurrence (Park et al. 2009) where patients with fibrotic foci of the breast had a higher risk of developing bone and lymph node metastasis (Hasebe et al. 2002). In preclinical models, the overexpression of collagen I in MMTV transgenic mice accelerated mammary tumor initiation and progression compared to MMTV wild-type mice, whereas the induction of fibrosis in the lung through the expression of TGFβ_1 was found to enhance the experimental metastasis of indolent mammary tumor cells (Provenzano et al. 2008; Barkan et al. 2010). Collectively, the data show that a collagen-rich, fibrotic environment contributes to tumor initiation and metastasis.

The receptors for type I collagen include integrin ($\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_{11}\beta_1$) and non-integrin receptors (discoidin domain receptors 1 and 2, glycoprotein VI, leukocyte-associated IG-like receptor-1, and the mannose receptor family); however, the most common cell surface receptors for collagen I are integrins, particularly $\alpha_2\beta_1$ (reviewed in (Felding-Habermann 2003; Leitinger and Hohenester 2007)). In addition to being a potential cancer biomarker, integrin $\alpha_2\beta_1$ may have a mechanistic role in tumor invasion and metastasis. For example, we and others have shown that integrin $\alpha_2\beta_1$ directs the in vitro attachment to and migration toward collagen I in multiple cancer types including PCa, ovarian carcinoma, pancreatic adenocarcinoma, rhabdomyosarcoma, and osteosarcoma (Chan et al. 1991; Vihinen et al. 1996; Fishman et al. 1998; Grzesiak and Bouvet 2006; Hall et al. 2006a; Van Slambrouck et al. 2009). Consistent with these observations, the overexpression of α_2 integrin accelerated the experimental metastasis of melanoma and rhabdomyosarcoma cells (Chan et al. 1991; Yoshimura et al. 2009), whereas $\alpha_2\beta_1$ blockade

using neutralizing antibodies reduced tumor dissemination to the liver or lymph node in animal models of melanoma, gastric cancer, and colon cancer (Yamaguchi et al. 2000; van der Bij et al. 2008; Yoshimura et al. 2009). Collectively, these preclinical data support that collagen type I/integrin $\alpha_2\beta_1$ activity is a potential mediator of tumor progression and metastasis.

Role of Type I Collagen/Integrin $\alpha_2\beta_1$ Activity in Skeletal Metastasis of Human PCa

The most frequent distant metastases formed by malignant PCa are within the skeleton (Hall et al. 2006b). The skeleton is often the first site of PCa distant metastasis and the first site of recurrence following androgen deprivation therapy. At autopsy, greater than 80% of all men who die of PCa will be found to have metastatic disease within the skeleton (Bubendorf et al. 2000; Smith et al. 2011) particularly in the pelvis, femur, and vertebral body. The molecular mechanisms that mediate the preferential metastasis of PCa cells to the skeleton are not well defined. Adhesion to bone-specific factors may facilitate the selective metastasis of PCa cells to the skeleton. Identification of these factors may reveal important clues about the mechanism of skeletal metastasis as well as provide new targets for the prevention of skeletal metastasis.

Collagen type I is a protein factor that is expressed at high levels in the tendon, dermis, and bone. It is the most abundant protein within the bone making up over 90% of the total protein within this site (Buckwalter et al. 1996). Published data support a mechanistic role for collagen I/integrin $\alpha_2\beta_1$ in the metastasis of PCa cells to the skeleton. For example, antibodies to $\alpha_2\beta_1$ were found to block the attachment of PC-3 PCa cells to the bone matrix (Kostenuik et al. 1996), whereas treatment with transforming growth factor β_1 (Kostenuik et al. 1997) or osteoblast-conditioned medium (Knerr et al. 2004) enhanced $\alpha_2\beta_1$ synthesis and adhesion to collagen I. We have demonstrated that the ability to bind collagen I is a characteristic of PCa cells isolated from bone versus soft tissue metastases (Hall et al. 2006a).

To examine the relationship between collagen I adhesion and bone metastatic potential within PCa cells, a collagen I binding variant of human LNCaP PCa cells was derived through serial passage on collagen I (Hall et al. 2006a). These cells, $LNCaP_{col}$, displayed a 51% increase in the surface expression of $\alpha_2\beta_1$, bound tightly to collagen I, and were stimulated to invade through collagen I in a $\alpha_2\beta_1$ integrin-dependent manner (Hall et al. 2006a). When injected directly into the bone, the collagen I binding $LNCaP_{col}$ cells had an increased ability to form osseous lesions compared non-collagen binding LNCaP cells (Hall et al. 2006a). These data demonstrated that an enhanced ability to bind to collagen I can promote PCa establishment within the bone and may provide a possible explanation for the preferential metastasis of PCa to the skeleton.

To test whether collagen I/$\alpha_2\beta_1$ activity contributes to PCa skeletal metastasis, α_2 integrin was selectively knocked down in collagen binding $LNCaP_{col}$ cells through the stable expression of small hairpin RNA molecules (shRNA) specific to α_2

integrin. Transduction with a shRNA molecule that targets position 1528 on α_2 integrin reduced α_2 RNA and protein expression 2.4-fold and 31%, respectively, compared to a non-targeting control shRNA-transduced cells without affecting the expression of integrins α_1, α_3, α_5, α_6, or $\alpha_v\beta_3$ (Sottnik et al. 2013). However, α_2 shRNA cells were found to have a corresponding 26% decrease in the protein expression of β_1 integrin indicating that PCa cells coordinate the expression of $\alpha_2\beta_1$ at the cell surface (Sottnik et al. 2013). This modest reduction in $\alpha_2\beta_1$ integrin expression suppressed the ability of knockdown cells to bind to or migrate toward collagen I indicating that α_2 knockdown led to $\alpha_2\beta_1$ loss of function (Sottnik et al. 2013). Upon direct injection into the tibiae, α_2 integrin knockdown cells displayed a 3.2-fold or 69% decrease in tumor burden compared to control shRNA-transduced cells (Sottnik et al. 2013). Overexpression of α_2 integrin in parental LNCaP cells did not significantly increase intraosseous tumor burden compared to vector control cells (Sottnik et al. 2013) demonstrating that $\alpha_2\beta_1$ expression was necessary for the formation of PCa bone lesions but was not itself sufficient to confer this ability to low-tumorigenic LNCaP cells.

To investigate whether the effect of α_2 knockdown was specific to the bone, α_2 integrin knockdown cells or control cells were injected into the left cardiac ventricle of SCID mice to allow systemic delivery of tumor cells (Sottnik et al. 2013). The data show that the intracardiac injection of control shRNA cells produced metastases within the liver and/or mandible (bone) in 73% (8/11) of the mice (Sottnik et al. 2013). In contrast, mice injected with α_2 knockdown cells developed overt metastases at these sites in only 9% (1/11) of the mice, $p = 0.0075$, compared to control shRNA-injected animals (Sottnik et al. 2013). Consistent with the results obtained following intratibial injection, mice injected with α_2 knockdown cells had reduced tumor burden in both the liver and skeleton compared to control shRNA-injected mice, $p < 0.15$ (Sottnik et al. 2013). Collectively, the data suggest that collagen I/$\alpha_2\beta_1$ integrin promotes the metastatic growth of PCa and other cancer cells in collagen I-rich organs such as the liver and skeleton.

$\alpha_2\beta_1$ as a Metastasis Suppressor in BrCa

A general theme of the data reviewed within this chapter support that integrin $\alpha_2\beta_1$ is both a cancer biomarker and a mediator of metastasis, specifically skeletal metastasis. A possible exception to this theme is human BrCa. The preponderance of clinical data support that the expression integrin $\alpha_2\beta_1$ is in fact lost in poorly differentiated BrCa lesions compared to normal breast tissues (Zutter et al. 1990). The enforced expression of the α_2 integrin subunit significantly reduced the ectopic growth of murine mammary tumor cells supporting that $\alpha_2\beta_1$ is a bona fide tumor suppressor (Zutter et al. 1995). Similarly, the formation of spontaneous mammary tumors occurred at a faster rate and metastasized to the lung with greater frequency in α_2 integrin-deficient MMTV-Neu mice compared to control WT-MMTV-Neu mice (Ramirez et al. 2011) confirming that mammary tumor growth was unimpeded in mice that lacked α_2 integrin. A possible mechanism for this effect may be due to

increased neoangiogenesis in α_2 integrin-deficient mice through expression of tumor-derived placental growth factor as was described in a murine melanoma model (Zhang et al. 2008). These data are consistent with the hypothesis that $\alpha_2\beta_1$ is a potential tumor and/or metastasis suppressor in both murine and human BrCa.

That said, one of the most widely used models to study BrCa metastasis, human MDA-MB-231 cells, is highly metastatic in immunocompromised mice, and collagen type I/integrin $\alpha_2\beta_1$ activity has been shown to promote MDA-MB-231 cell metastasis, particularly to the bone (Lundström et al. 1998; Ibaragi et al. 2011; Dudley et al. 2014). For example, a functional blocking antibody 4C3 that targets the α-I domain of the α_2 integrin subunit was shown to prevent postextravasation growth in vivo and reduce experimental bone metastasis in the MDA-MB-231 model (Dudley et al. 2014). Whether these results are specific to the MDA-MB-231 cell line or a general feature of BrCa remains to be determined. Recently, it was shown in human melanoma cells that integrin $\alpha_2\beta_1$ associated with E-cadherin to promote cell/cell interactions but promoted cell/matrix interactions and invasion when associated with N-cadherin. It is therefore probable that the nature of the molecules co-localized with the integrin will ultimately determine whether $\alpha_2\beta_1$ promotes or inhibits metastasis (Siret et al. 2015).

Mechanisms of Integrin $\alpha_2\beta_1$-Directed Metastasis

The signal transduction pathways activated following integrin ligation are well characterized (for review, see (Miranti et al. 2002; Slack-Davis and Parsons 2004; Seguin et al. 2015)); however, the molecular events activated by integrin $\alpha_2\beta_1$ within tumor cells have only more recently been described. For examples, the extracellular matrix protein βig-h3 was found to interact with integrin $\alpha_2\beta_1$ to mediate the experimental metastasis of osteosarcoma cells to the lung following activation of the PI3-K pathway (Guo et al. 2014). Collagen I binding to integrin $\alpha_2\beta_1$ in MDA-MB-231 BrCa cells was shown to stimulate the production of MMP13 through FAK and p38 MAPK activation to promote osteolysis and bone metastasis (Ibaragi et al. 2011). Similarly, Van Slambrouck et al. demonstrated that α_2 integrin promoted the invasiveness of PCa cells through the expression of MMPs 2 and 9 via a signaling cascade involving FAK/SRC/paxillian/Rac/JNK (Van Slambrouck et al. 2009). We have published that collagen I binding via integrin $\alpha_2\beta_1$ results in the activation of RhoC GTPase which leads to PCa cell migration and invasion (Hall et al. 2006a; Hall et al. 2008).

RhoC GTPase is one of the three Rho isoforms that belong to the Ras superfamily of small GTP-binding proteins (Malliri and Collard 2003; Wheeler and Ridley 2004; Jaffe and Hall 2005). The members of this family cycles between an inactive GDP-bound and active GTP-bound state which stimulate downstream effector proteins (Olson 1996; Geyer and Wittinghofer 1997). The Rho GTPases participate in the formation of contractile actin/myosin filaments and therefore act as molecular switches involved in all aspects of cell morphogenesis and cellular motility. Due to their involvement in motility, Rho GTPases have been implicated in tumor progression and metastasis. RhoC in particular was shown to participate in the metastasis of

several cancers including breast, pancreas, and melanoma (Suwa et al. 1998; Clark et al. 2000; van Golen et al. 2002). We previously demonstrated that RhoC was expressed in bone metastatic PC-3 PCa cells and was responsible for the invasive capabilities of these cells (Yao et al. 2005). We have further shown that RhoC GTPase is activated upon integrin $\alpha_2\beta_1$ engagement and regulates the collagen I-mediated invasion of PCa cells (Hall et al. 2008). The activation of RhoC invasive programs following collagen I binding suggests a mechanism for the preferential metastasis of PCa cells to the skeleton where collagen I is in abundance.

In addition to activating signal transduction pathways that promote motility, such as PI3-K, SRC, and RhoC, collagen I/integrin $\alpha_2\beta_1$ may promote tumor survival and the acquisition of drug resistance. Interestingly, but perhaps not unexpectedly, the signal transduction pathways that regulate survival share significant overlap with the pathways that promote motility. For example, in head and neck squamous cell carcinoma, collagen I conferred resistant to low-dose paclitaxel through a mechanism involving PI3-K/AKT activation (Cohen et al. 2013). In T-cell acute lymphoblastic leukemia, collagen binding via $\alpha_2\beta_1$ promoted the survival of cells in the absence of growth factors and protected against doxorubicin-induced apoptosis (Naci et al. 2012; Naci and Aoudjit 2014). The survival in this model was mediated through integrin-dependent activation of MAPK1 although both MAPK1 and p38 MAPK were found to promote collagen-stimulated migration (Naci and Aoudjit 2014). Viewed together, collagen I/integrin $\alpha_2\beta_1$ can activate multiple signal transduction pathways to promote tumor survival, invasion, and metastasis.

Role for $\alpha_2\beta_1$ in the Acquisition of Tumor Dormancy and Escape to Progressive Growth

An unanswered question in the management of men with PCa is that patients frequently will develop metastatic disease years to decades after successful treatment of their primary cancer. Each year, approximately 40,000 men who "should" have been cured of their cancer following surgery or radiation therapy will develop metastatic disease, most frequently within the skeleton, often years after removal of the primary lesion (Chen and Pienta 2011). A recent community-based study of men who experienced biochemical recurrence, as measured by rising PSA, following radical prostatectomy showed that 24% of patients recurred during a 2-year period (Greene et al. 2004). Analysis of preoperative bone marrow aspirates from PCa patients found that disseminated tumor cells (DTCs) were present in the bone marrow in 72% of men prior to surgery and in 45% of patients who remained disease-free >5 years post-surgery (Morgan et al. 2009). These data suggest that tumor cell dissemination to the bone marrow occurs early in PCa development and that these cells can remain dormant many years post-surgery. Dormant DTCs are refractory to therapy and may be the source of overt skeletal metastases (Naumov et al. 2002).

Tumor dormancy can result from either cellular dormancy, where individual tumor cells are induced to exit the cell cycle and become quiescent, or from tumor mass dormancy, where balanced rates of cellular growth and death lead to no net changes in

tumor formation (reviewed in (Aguirre-Ghiso 2007)). In both cases, dormancy is influenced by interactions of the cancer cell with the tumor microenvironment. Recent data has shown that both cell/cell and cell/matrix interactions within the microenvironment help determine whether a DTC will become dormant or grow progressively. For example, the binding of human PCa cells to annexin II on osteoblasts within the bone marrow was shown to induce growth arrest-specific 6 (GAS6) expression leading to PCa cell dormancy (Shiozawa et al. 2010). Similarly, low attachment to fibronectin resulting from decreased expression of the urokinase-type plasminogen activator receptor (uPAR) and integrin $\alpha_5\beta_1$ led to protracted dormancy of hepatocellular carcinoma cells through increased activity of p38 MAP kinase (MAPK14) (Aguirre-Ghiso et al. 2001). Escape from dormancy in these models was mediated through the reverse interactions, i.e., reduced annexin II binding and increased uPAR/$\alpha_5\beta_1$ expression. Recently, indolent breast cancer skeletal micrometastases were shown to escape dormancy and grow progressively through the increased expression of vascular cell adhesion molecule-1 (VCAM-1) (Lu et al. 2011). Dormant breast cancer cells that over time acquired VCAM-1 expression were able to engage integrin $\alpha_4\beta_1$ on osteoclasts to promote osteoclastogenesis and skeletal metastasis. Blocking VCAM-1/$\alpha_4\beta_1$ binding in vivo decreased osteoclastogenesis and reduced the formation of skeletal metastases. Taken together, these studies demonstrate that physical interactions of the tumor cell with its microenvironment regulate both the acquisition of dormancy and the escape to progressive growth in multiple cancers.

A specific role for integrin $\alpha_2\beta_1$, versus $\alpha_4\beta_1$ or $\alpha_5\beta_1$, in tumor dormancy or escape has not been described. However, a mechanistic role for collagen type I in the proliferation of quiescent or dormant tumor cells has been reported. As described above, fibrotic tumor microenvironments are associated with a higher risk of developing distant metastases. Barkan and colleagues demonstrated that induction of fibrosis in the lung through the expression of TGFβ_1 enhanced the experimental metastasis of indolent mammary tumor cells (Barkan et al. 2010). Within their model, indolent D2.0R mammary cancer cells disseminated to the lung following I.V. injection but failed to proliferate due to cell cycle arrest (Barkan et al. 2008). However, when injected into mice whose lungs were made fibrotic through the expression of TGFβ_1, D2.0R were stimulated to grow progressively (Barkan et al. 2010). Using a three-dimensional culture system to model dormancy, the mechanism for the increased proliferation was shown to occur through β1-integrin activation of SRC and FAK, leading to an MAPK-dependent myosin light chain phosphorylation by myosin light chain kinase (Barkan et al. 2010). These data support that a collagen-rich, fibrotic environment can contribute to the escape to progressive growth of dormant tumor cells.

Integrins and the Formation of a Premetastatic Niche

We have reviewed in this chapter data which support that collagen-rich microenvironments both promote metastasis and regulate tumor dormancy. It is possible that these processes are linked and may converge at the formation of a

premetastatic niche (Kaplan et al. 2005). The premetastatic niche is an emerging concept in tumor biology where hematopoietic-derived cells arrive within a target organ parenchyma and alter the microenvironment to promote metastasis by regulating the homing, attachment, and growth of circulating tumor cells. It is believed that the removal of the premetastatic niche may predispose to tumor dormancy (Kaplan et al. 2006). In the first paper on the topic, fibronectin bound by integrin $\alpha_4\beta_1$ on VGFR1$^+$, CD11b$^+$ hematopoietic progenitor cells facilitated the spontaneous metastasis of Lewis lung and B16 murine tumor cells from the skin to the lung (Kaplan et al. 2006). A recent report showed that fibrocytes, which are a bone marrow-derived mesenchymal progenitor cell that produce collagen type I, increased the metastasis of B16-F10 murine melanoma cells by recruiting Ly-6C$^+$ monocytes to the lung to form a premetastatic niche (van Deventer et al. 2013). In the context of the bone, hypoxia-induced lysyl oxidase was shown to stimulate osteoclastogenesis which led to the formation of focal premetastatic lesions that serve as a platform for circulating BrCa cells to form overt bone metastases (Erler et al. 2009; Cox et al. 2015). Based on these data, it is attractive to postulate that collagen type I both attracts hematopoietic progenitor cells to sites of future metastasis and serves as the ligand that promotes tumor cell attachment and growth (presumable through integrin $\alpha_2\beta_1$) resulting in the formation of an overt metastasis.

Therapeutic Targeting Integrin $\alpha_2\beta_1$ in Cancer

Inhibitors that block integrin binding to extracellular matrix are well characterized. Many integrins bind a tripeptide motif containing arginine-glycine-aspartic acid (RGD). Both linear peptide and cyclic RGDs, such as cilengitide, have shown modest efficacy in clinical trials for the treatment of solid tumors including PCa and therefore support the use of integrins as viable therapeutic targets (Goel et al. 2008; Bradley et al. 2011). As integrin $\alpha_2\beta_1$ binds collagen I through a peptide sequence other than RGD, these inhibitors would be ineffective against $\alpha_2\beta_1$ (Knight et al. 2000). However specific antagonists to $\alpha_2\beta_1$ are available and may be of use to treat tumor progression. Generally, these compounds fall into three classes: biologics, such as chondroadherin and antibodies including vatelizumab (GBR 500) and 4C3 (Attinger et al. 2011; Dudley et al. 2014); disintegrins, including rhodocetin (Eble et al. 2009) and viperistatin analogs vimocin and vidapin (Momic et al. 2014); and small molecule inhibitors such as E7820, a sulfonamide derivate with antiangiogenic activity shown to reduce $\alpha_2\beta_1$ integrin expression (Mita et al. 2011). The results for 4C3 in an experimental BrCa skeletal metastasis model were discussed previously within this chapter (Dudley et al. 2014). Vatelizumab is in clinical trials in patients with multiple sclerosis but was reported to reduce the establishment of PCa metastases in animal models (Attinger et al. 2011). The disintegrins with $\alpha_2\beta_1$ activity were isolated from the venom of snakes such as the Malayan pit viper *Calloselasma rhodostoma*. Shown to block $\alpha_2\beta_1$ activity in vitro, molecules in this class may

prove useful to treat $\alpha_2\beta_1$-dependent cancers. E7820 is in clinical trials in patients with colorectal cancer (ClinicalTrials.gov). Unique in that it does not target $\alpha_2\beta_1$ activity directly but rather leads to decreased mRNA expression of $\alpha_2\beta_1$ in platelets, endothelial cells, and tumor cells, E7820 could quickly be evaluated in preclinical animal models of skeletal metastasis to support broader translational studies in men who have experienced disease progression. Viewed together, integrin $\alpha_2\beta_1$ may be a viable therapeutic target for the treatment of metastasis within multiple diseases.

Conclusion

Skeletal metastasis is a frequent and debilitating end product of tumor progression within a number of solid tumors. Although the precise mechanisms that lead to the formation of these lesions are unclear, there is increasing evidence that integrins, particularly integrin $\alpha_2\beta_1$, may mediate the selective metastasis of tumor cells to the skeleton. In the case of PCa, integrin $\alpha_2\beta_1$ may promote skeletal metastasis at multiple steps. Integrin $\alpha_2\beta_1$ appears to be a prognostic marker of PCa osseous metastasis as it is a putative marker of PCa stem cells whose expression within primary PCa lesions is associated with progression of skeletal metastases. Thorough interaction with collagen type I within the bone, integrin $\alpha_2\beta_1$ may regulate PCa cell adhesion and motility as well as the transition from dormancy to progressive growth. We believe that remodeling within the bone releases collagen I fragments into the circulation that serve as chemotactic factors for PCa cell homing to the skeleton (Fig. 2). Once within the bone microenvironment, PCa cells strongly adhere to collagen type I through the high surface expression of the integrin $\alpha_2\beta_1$. Integrin

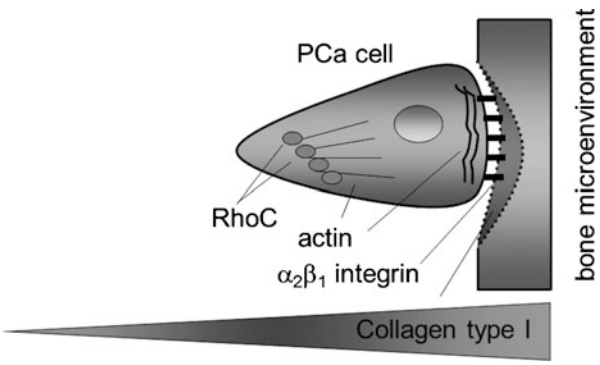

Fig. 2 Model for the role of collagen type I/integrin $\alpha_2\beta_1$ in PCa skeletal metastasis. Bone remodeling releases type I collagen fragments into the circulation that serve as chemotactic factors for PCa cell homing to the bone. Once within the bone microenvironment, PCa cells strongly adhere to the collagen type I matrix through surface expression of the integrin $\alpha_2\beta_1$. $\alpha_2\beta_1$ ligation leads to activation of RhoC GTPase that promotes invasion into the bone to produce a clinically relevant metastasis

$\alpha_2\beta_1$ ligation leads to the activation of motility programs including RhoC GTPase which promote the extravasation of PCa cells into the bone and the formation of an osseous metastasis. As a result, integrin $\alpha_2\beta_1$ may be a useful therapeutic target and therefore has the significant potential to prolong the lives of PCa patients through the prevention and treatment of PCa skeletal metastases.

Potential Applications to Prognosis, Other Diseases, or Conditions

The potential application of $\alpha_2\beta_1$ to prognosis, other diseases, or conditions has been discussed throughout this chapter, but key points are summarized here. Extensive analyses have established that integrin $\alpha_2\beta_1$ is a regulator of tumor invasion and metastasis. Within PCa, integrin $\alpha_2\beta_1$ is a marker of PCa stem cells and may promote the selective metastasis of PCa cells to the skeleton (see section "Integrin α2β1 as a Marker of PCa Skeletal Metastasis"). In diseases such as melanoma, gastric cancer, and colon cancer, integrin $\alpha_2\beta_1$ expression is associated with metastasis and appears to promote dissemination to visceral sites rich in collagen I such as the liver (see section "Role of Collagen Type I/Integrin α2β1 Activity in Tumor Progression and Metastasis"). However in BrCa, it is the loss of integrin $\alpha_2\beta_1$ expression which is associated with metastasis (see section "α2β1 as a Metastasis Suppressor in BrCa"). These data support that integrin $\alpha_2\beta_1$ expression is a potential prognostic marker of metastasis, particularly skeletal metastasis, and suggest that modulating $\alpha_2\beta_1$ expression or activity may be a viable and effective treatment for the prevention of metastasis in multiple solid tumors (see section "Therapeutic Targeting Integrin α2β1 in Cancer"). As specific antagonists of integrin $\alpha_2\beta_1$ are in clinical development, the therapeutic benefit of $\alpha_2\beta_1$ blockade is worthy of additional study.

Summary Points

- This chapter focuses on integrin $\alpha_2\beta_1$, a receptor for type I collagen on tumor cells, as a biomarker of skeletal metastasis.
- The expression of integrin $\alpha_2\beta_1$ within patient tissues is associated with metastasis in multiple cancer types.
- Integrin $\alpha_2\beta_1$ is also a marker of PCa stem cells whose elevated expression within primary lesions is associated with progression to skeletal metastasis in PCa patients.
- $\alpha_2\beta_1$ integrin has a mechanistic role in metastasis as evidenced by preclinical data which show that α_2 integrin overexpression accelerated experimental metastasis, whereas $\alpha_2\beta_1$ blockade reduced tumor dissemination to collagen-rich sites such as the liver or bone.
- Outside-in signal transduction through integrin $\alpha_2\beta_1$ may regulate metastasis by promoting cellular motility and/or the acquisition of drug resistance through the activation of PI3-K or MAPK pathways.

- Fibrotic tumor microenvironments rich in collagen I are a risk factor for the development of skeletal metastasis in certain cancers.
- Collagen type I may regulate cellular dormancy and metastasis by promoting the proliferation of quiescent tumor cells.

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Panoramic Radiomorphometric Indices of Mandible: Biomarker for Osteoporosis

23

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Contents

Abstract

Regressive changes occur in all the tissues of the body involving the bone, and osteoporosis is one of such change that is seldom diagnosed before the occurrence of fracture. Osteoporosis most of the time manifests in the maxillofacial region involving jaw bones. Radiographic bone density can be assessed from simple

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radiographs in two main ways: by taking linear measurements (morphometric analysis) or by measuring the optical density and comparing it with a step wedge (densitometric analysis). It has been proposed that osteopenia observed on panoramic radiographs could be used as a referral criterion for bone mineral density (BMD) assessment.

Panoramic radiography has been an important component of dental diagnostic radiology for over 40 years. It is a technique that produces an image of the teeth and jaws on a single film. Dental panoramic radiography accounts for 32% of all medical radiologic examinations in the United Kingdom, with approximately two million panoramic radiographs taken annually in National Health Service dental practices in England and Wales alone. So the incidental findings detected on panoramic radiographs of these patients can be used for the identification of women who have no awareness of their low BMD and who could benefit from BMD testing. Qualitative and quantitative panoramic indices, including the mandibular cortical index (MCI), mental index (MI), or panoramic mandibular index (PMI), have also been used for panoramic radiographs to assess the bone quality and to observe signs of resumption and osteoporosis.

Keywords

Osteoporosis • Panoramic radiographs • Radiomorphometric indices • Bone mineral density • Mandibular cortical index • Mental index • Panoramic mandibular index

List of Abbreviations

AI	Antegonial index
BMD	Bone mineral density
DPA	Dual photon absorptiometry
DXA	Dual-energy X-ray absorptiometry
GI	Gonial index
MCI	Mandibular cortical index
MI/MCW	Mental index/mandibular cortical width
PMI	Panoramic mandibular index
QCT	Quantitative computed tomography
RANKL	Receptor activator of NF-kB ligand
SPA	Single photon absorptiometry

Key Facts of Radiomorphometric Index

- The prevalence of osteoporosis based on reduced hip bone density was estimated at 4% of women 50–59 years of age compared to 44% of women 80 years of age and older. It is estimated that over 200 million people worldwide have osteoporosis.
- The number of hip fracture is likely to increase from 1.7 million in 1990 to a projected 6.3 million in 2050 worldwide.

- Various techniques have been used to detect the osteoporotic changes, namely, microradiography, single photon absorptiometry (SPA), dual photon absorptiometry (DPA), quantitative computed tomography (QCT), dual-energy X-ray absorptiometry (DXA), and panoramic radiomorphometric indices.
- Among these techniques, the panoramic radiomorphometric index is a simple, inexpensive, and noninvasive technique which can detect the osteoporotic changes before the onset of clinical symptoms.
- Panoramic radiograph is a standard radiograph used by dentists that show the entire maxilla and mandible.
- Over two million panoramic radiographs are taken in England and Wales alone annually to visualize maxillofacial area by the dental practitioners.
- These panoramic radiographs can detect the osteoporotic changes in patients who are unaware of the disease, and radiomorphometric indices are performed on these radiographs.
- On confirmation of the diagnosis, these patients can be referred to respective physician and also use the strong negative predictive value which can be used to exclude patients from unnecessary DXA screening.
- These panoramic mandibular indices can detect the osteoporotic changes at an early stage in an asymptomatic individual and can take prophylactic measures to prevent bone fractures.

Definitions of Words and Terms

Antegonial index	the measurement of cortical width in the region anterior to the Gonion
Bone mineral density (BMD)	the standard gauge used to diagnose osteoporosis. A measure of bone mass in mg/cm^2 at a specific site
Gonial index	the thickness of the mandibular angular cortex
Mandibular cortical index	the appearance of the lower border cortex of the mandible distal to the mental foramen as viewed on panoramic radiograph
Mandibular cortical width/mental index	the measure of the mandibular cortex in the region of mental foramen
Osteopenia	a denotation of reduced BMD that is not as severe as osteoporosis
Osteoporosis	a term used to define decreased bone mass per unit volume of anatomical bone
Panoramic mandibular index	a measure of mandibular osteoporosis representing the ratio of the thickness of the mandibular cortex to the distance between the superior/inferior borders of

	the mental foramen and mandibular cortex
Panoramic radiograph	a standard radiograph used by dentists that show the entire maxilla and mandible
Radiomorphometric indices	measurements made on the panoramic radiographs

Introduction

"Skeleton in the closet" meaning that bone is static once formed is a misconception. The skeleton is a metabolically active organ that undergoes continuous remodeling throughout life (Raisz 1998). Bones change with development and aging in terms of structure (Boskey and Coleman 2010). The adult skeleton is continuously remodeled through life by the coordinated activities of osteoclasts and osteoblasts (Almeida and O'Brien 2013). Osteoclasts derive from the cells of hematopoietic lineage and are responsible for bone resorption, whereas osteoblasts originate from the mesenchymal lineage and are responsible for bone formation. Osteoblast differentiation is regulated by Wnt/β-catenin pathway. Receptor activator of NF-kB ligand (RANKL) pathway along with hormones and inflammatory cytokines differentiate osteoclasts to bring about resorption (Rachner et al. 2011).

Bone Remodeling

Bone remodeling is the restructuring of existing bone which is in constant resorption and formation. In the balanced phenomenon, about 5% of the cortical bone and 20% of the trabecular bone are renewed per year. In the early adult life, the balance is positive, that is, there is more bone deposition (Hernandez-Gil et al. 2006). With aging, the amount of bone resorbed by osteoclasts is not fully restored with bone deposited by osteoblasts and this imbalance leads to loss of bone mass and strength (Almeida and O'Brien 2013).

Estrogen plays a pivotal role in maintaining positive balance in bone remodeling by supporting osteoblasts and preventing bone resorption by suppressing osteoclast formation, stimulating osteoclast apoptosis, and counteracting oxidative stress (Cervellati et al. 2014) In men, both testosterone and estrogen are important for maintaining bone health (Das and Crockett 2013). Bone remodeling is regulated by various factors such as genetic, mechanical, vascular, nutritional, hormonal, and local factors (Hernandez-Gil et al. 2006). Peak bone mass is achieved during early adulthood but varies between men and women. In most women, bone mass reaches its peak in the third decade of life and declines thereafter (Ramesh et al. 2011).

There are three distinct phases of changes of bone mass which include:

1. Attainment of peak bone mass by linear and radial growth
2. Slow, age-dependent phase of bone loss around 40 years both in cortical and trabecular bones
3. The third process, which occurs in women, is the transient accelerated postmenopausal bone loss due to estrogen deficiency (Riggs 1991).

Both men and women start losing bone in their 40s. Women experience a rapid phase of loss during the first 5–10 years after menopause, due to loss of estrogen. In men, this phase is obscure, since there is only a slow and progressive decline in sex steroid production. Hence, the loss of bone in men is linear and slower (Manolagas 2000). This loss of bone in both men and women lead to a more porous bone leading to a condition called osteoporosis.

Osteoporosis

Osteoporosis, the Greek word for porous bone, is the term used to define decreased bone mass per unit volume of anatomical bone. It is the most common metabolic disorder of old age in humans (Manologas and Parfitt 2010). Osteoporosis is also known as "silent disease." It is not a disease of aging but it is only age-associated. Osteoporosis is defined as "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk" (Kanis et al. 2008; Ramesh et al. 2011; Faienza et al. 2013).

Osteoporotic bone, by definition, is bone that fractures easily. Both trabecular and cortices are often thinner, the mineral content per area of tissue is often increased, and the mean crystal size, collagen maturity and carbonate contents are increased relative to those of age-matched controls (Boskey and Coleman 2010). According to National Health and Nutrition Examination Survey (NHANES) data, the prevalence of osteoporosis based on reduced hip bone density was estimated at 4% in women 50–59 years of age compared to 44% of women 80 years of age and older (Vondracek and Linnebur 2009). It is estimated that over 200 million people worldwide have osteoporosis (Reginster and Bulet 2006).

The risk factors for osteoporosis are hormonal variations, family history, insufficient calcium intake, lack of moderate physical activity, and toxic habits, such as smoking and excessive consumption of alcohol or medications, especially glucocorticoids (Lopez-Lopez et al. 2011). Osteoporosis is divided into localized and generalized based on the number of bones involved (Tarvade and Daokar 2014).

Generalized osteoporosis is further classified as follows:

1. Primary osteoporosis
   (a) Idiopathic juvenile osteoporosis
   (b) Idiopathic osteoporosis in young adults

(c) Involutional osteoporosis
  (i) Type I (postmenopausal) osteoporosis
  (ii) Type II (age-related) osteoporosis
2. Secondary osteoporosis (Riggs 1991)

Primary osteoporosis is characterized by absence of systemic diseases. Idiopathic juvenile osteoporosis and idiopathic osteoporosis in young adults are self-limiting disorders. Postmenopausal osteoporosis affects women within 15–20 years after menopause. Age-related osteoporosis occurs in both men and women aged 70 years and above.

Secondary osteoporosis occurs as a result of a number of systemic diseases, such as hypogonadism, hyperthyroidism, malabsorption syndrome, chronic obstructive jaundice, anorexia nervosa, multiple myeloma, osteogenesis imperfect, etc., and due to drug intake.

Osteoporosis is frequently asymptomatic and often undetected until a fracture occurs. The common clinical features include back pain, loss of height, spinal deformity, and fractures of vertebrae, hips, wrists, and other bones. Early diagnosis is very important to prevent pathological fractures which have severe health and economic consequences.

The diagnosis of osteoporosis includes:

1. General medical examination to assess severity and to exclude secondary diseases
2. Radiographs of spinal column to diagnose vertebral fractures
3. Bone densitometry (Riggs 1991)

Bone mineral density is the amount of bone mass per unit volume (volumetric density) or per unit area (areal density) and both can be measured in vivo (Kanis et al. 2013). Bone mineral density testing is a vital component in the diagnosis and management of osteoporosis (Cosman et al. 2014). Bone mineral density is measured using the following techniques: dual-energy X-ray absorptiometry (DXA), dual photon absorptiometry, single photon absorptiometry, quantitative computed tomography, quantitative ultrasound, digital X-ray radiogrammetry, and most simplest being panoramic radiography (Bajaj et al. 2010; Link 2012; Kanis et al. 2013).

The gold standard procedure for the assessment of bone mineral density is DXA in the vertebrae, femoral neck, and forearms. The diagnosis is described as T-score or Z-score which are units of standard deviation (Kanis et al. 2013). According to WHO criteria, if T-score is less than −2.5 standard deviations of young healthy population then it is diagnosed as osteoporosis (Lopez-Lopez et al. 2011) But, DXA is impossible to use in all situations due to limitations in availability and economy. All other advanced techniques are not cost-effective for screening older populations to detect osteoporosis (Ramesh et al. 2011).

Alveolar bone has been used to detect osteoporosis as the cortical height and mineral density seems to follow a similar pattern as seen in the general mineral status of the skeleton. DXA measurements and densitometric analysis of jaw bones were attempted by many clinicians. Again, because of lack of feasibility, an easier

alternative was being searched. So, clinicians measured various parameters on the panoramic radiographs of jaw bone measurements of panoramic radiographs in attempt to diagnose osteoporosis as it was highly economical. Radiomorphometric analysis have now become markers for osteoporosis.

The oral cavity and jaws are radiographically examined more often than any other part of the human body. Panoramic radiograph could be useful as a simple screening method in the diagnosis of osteoporosis and can provide valuable information on the quality of the jaw bone. Osteoporosis can be diagnosed by observing tooth loss, thinning of the mandibular inferior cortex, and changes in the morphology of the endosteal margin of the cortex and of the spongy bone of the jaws (Devlin and Horner 2008).

Dental panoramic radiography is the most frequently performed radiograph for the diagnosis of diseases affecting dento-maxillofacial region. Particularly, women, who have no awareness on their low BMDs, would be benefited by the incidental findings found in dental panoramic radiographs (Taguchi et al. 2004). Klemetti and Kolmakow (1997) have validated the choice of morphology of mandibular cortex on panoramic radiograph in the assessment of bone quality rather than the trabecular bone or alveolar process.

Radiographic Features of Osteoporosis in Skeleton

In the skeleton, the cardinal radiographic features of osteoporosis include generalized osteopenia, thinning and accentuation of the bone cortices, and accentuation of primary and loss of secondary trabeculation (Wowern 1986). Subservient radiological features include spontaneous, atraumatic fractures involving spine, wrist, hip, or ribs, basilar invagination in the skull, and granular appearance of the bone in the skull.

Radiographic Features of Osteoporosis in Jaw Bone

The characteristic radiological features which are manifested in jaws include relative radiolucency of both the jaws with reduced definition of the cortices. An in vitro study by Wowern et al. showed that the normal dentate jaws of older individuals had relatively thin porous cortical bone lamellae with endosteal demineralization, and these age-related cortical changes were more common in females than males (Bras et al. 1982).

Bulgarelli et al. (2002) conducted a review regarding the radiographic signs of osteoporosis which included alveolar bone resorption and reduction in cortical mandibular bone thickness. They concluded that panoramic radiograph can be used as an important tool to provide indications for the diagnosis of osteoporosis (Bulgarelli et al. 2002).

So, the physician must suspect of the risk of osteoporosis, when the following signals in the panoramic X-ray in the jaws (Watanabe et al. 2008) are seen:

- Class II or III by Klemetti or presence of bubbles in the inferior mandibular cortex
- Decrease in width of the inferior mandibular cortex

- Disorganized trabecular pattern like decrease in number and reduced connectivity
- Emphasized contrast between mandibular ramus/body and structures of reinforcement, as the oblique line
- Emphasized radiolucency in the jaws

Finally, dentists who use orthopantomographs may play a vital role in screening patients with osteoporosis, mainly postmenopausal women; this is because it is most often advised as a part of routine investigation and as it is also less expensive than DEXA scan. Mandibular cortical index can reliably be used as a diagnostic tool for screening patients with osteoporosis. Identification of the signs of osteoporosis in dental panoramic radiographs is crucial in the diagnosis of this condition (Dagistan et al. 2010).

Evidence Supporting Panoramic Radiographs to Screen for Osteoporosis

A retrospective investigation was carried out to determine the strength of association of spinal bone density and the density of selected mandibular sites as determined from panoramic radiographs. Panoramic films of known low bone density and high bone density in women between the ages of 50 and 75 were evaluated. According to this study, blinded observers should be able to differentiate between persons of high and low bone density using panoramic radiographs (Mohammad et al. 1996).

Another study by Taguchi et al. (1995) was conducted to assess the relationship between oral signs and osteoporosis and the possibility of the same to use as an indicator of osteoporosis. They considered thoracic spine fracture as demonstrated on lateral chest radiographs as an osteoporotic sign. Oral signs which were included were the number of teeth present, mandibular cortical width, alveolar bone resorption, and the morphologic classification of the inferior cortex on panoramic radiographs. The number of teeth present (N) was highly significant in relation to the probability of thoracic spine fracture. They derived the probability equation for the presence of thoracic spine fracture: probability value $= 1/(1 + \text{e-z})$, $Z = 18.68\text{-}0.29$ age -0.27 N. If the probability value is > 0.5, it suggests the possibility of thoracic spine fracture. Finally, they concluded that this equation combined with panoramic radiographic findings could serve as a simple and useful diagnostic tool for dentists to assess the possibility of latent osteoporosis (Taguchi et al. 1995).

Evidence Against Using Panoramic Radiographs to Screen for Osteoporosis

Mohajery and Brooks (1992) conducted a study to determine whether radiographic changes could be detected in the mandible in patients with mild-to-moderate postmenopausal osteoporosis and whether these changes could be used as a diagnostic tool to differentiate normal from osteoporotic patients (Mohajery and Brooks 1992). Subjects were classified as either osteoporotic or normal based on the bone density

measurements of the lumbar spine and femoral neck, as determined by dual photon absorptiometry. Using panoramic and periapical radiographs, mandibular bone density measurements were made. The thickness of the cortex at the angle of the mandible, sinus floor, and lamina dura of the tooth socket was also measured. They concluded that there was no significant difference in any of the mandibular measurements between the normal and osteoporotic subjects and there was no correlation between skeletal and mandibular bone measurements. Women with mild-to-moderate osteoporosis could not be distinguished from women with normal bone density.

In an another study, panoramic mandibular index was used to find out the correlation between bone mineral densities of the femoral neck, the lumbar area, and the trabecular and cortical parts of the mandible in a group of postmenopausal women. Using quantitative computed tomography of the mandible and dual-energy X-ray absorptiometry of the femoral neck and lumbar area, the bone mineral density values were measured. Linear correlation of the panoramic mandibular index with all bone mineral density values was weak. However, the low and high index subgroup means were clearly dependent on the bone mineral density variables. The authors concluded that in spite of significant differences in PMI between osteoporotic subjects and controls, panoramic assessment cannot be advocated as an indicator for assessment of osteoporosis (Klemetti et al. 1993).

Although radiomorphometric indices are regarded as an ancillary method for the diagnosis of osteoporosis on panoramic radiographs, they should be a routine procedure in dental examination, and dentists may be able to refer postmenopausal women younger than 65 years for bone densitometry on the basis of incidental findings on dental panoramic radiographs.

Panoramic Radiomorphometric Indices

These indices are measurements made on the panoramic radiographs, which can evaluate bone loss that can predict a possible loss of bone mineral density. This is also known as panoramic radiomorphometric indices that have been developed to assess mandibular cortical shape and width either qualitatively or quantitatively (Leite et al. 2010; Lopez-Lopez et al. 2011).

Some of the qualitative and quantitative indices are the mandibular cortical index (MCI), mental index (MI), and panoramic mandibular index (PMI). These have been used for panoramic radiographs to assess the bone quality and to observe signs of resorption and osteoporosis (Asha et al. 2014; Gulsahi et al. 2008).

Mandibular Cortical Index

Mandibular cortical thickness (MCI) is one of the most established indices for qualitative assessment of inferior mandibular cortex. This index describes the porosity of the mandible and is related to the mandibular bone mineral density.

This index was first devised by Klemetti, so it is also referred to as Klemetti index (Devlin et al. 2007).

Mandibular cortical index (MCI) has been developed to assess osteoporosis in the cortical area of the mandible using panoramic radiographs. The appearance of the MCI on the panoramic radiographs is assessed by observing the inferior cortex of the mandible at the antegonial notch site distal to mental foramen bilaterally and is categorized into one of the three groups according to the following criteria given by Klemetti et al.:

1. C1: The endosteal margin of the cortex is even, regular, and sharp on both sides of the mandible (Fig. 1).
2. C2: The endosteal margin appears to have semilunar defects or has resorptive cavities with cortical endosteal residues one to three layers thick on one or both sides (Fig. 2).
3. C3: The endosteal margin consists of numerous (>3) thick cortical endosteal residues and is clearly porous (Yasar and Akgunlu 2008; Cakur 2009; Fig. 3).

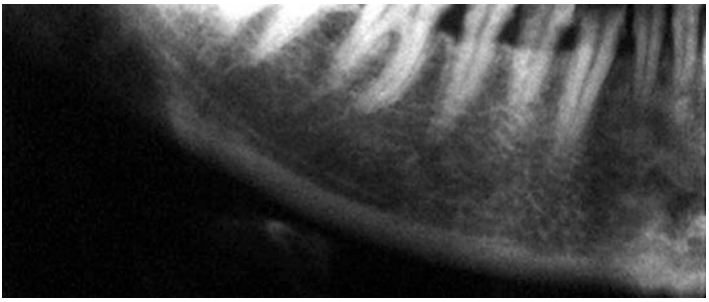


Fig. 1 MCI category C1 – endosteal margin of the cortex is sharp and even on both sides of the mandible

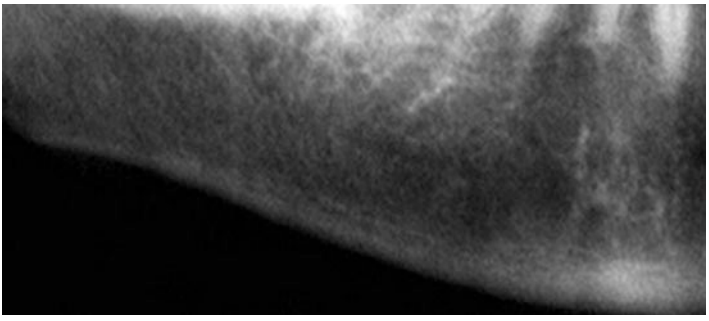


Fig. 2 MCI category C2 – endosteal margin with semilunar defects (resorption cavities) and cortical residues 1–3 layers thick on one or both sides

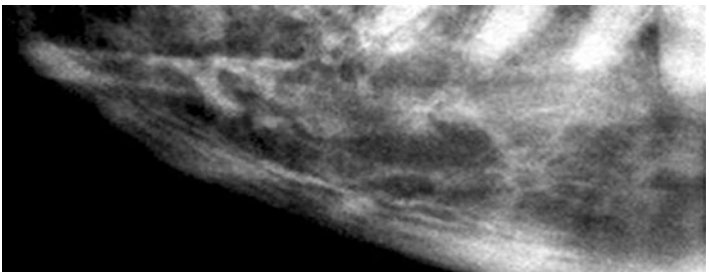


Fig. 3 MCI category C3 – endosteal margin appearing clearly porous with thick cortical residues

This is a simple three-graded classification method used to identify individuals either with a low skeletal bone mineral density (BMD) or at high risk of osteoporosis in contrast to DXA. The validity of MCI that is based on the thickness and patterns of intracortical resorption of the mandibular cortex has demonstrated that such assessments may be useful in screening for osteopenia/osteoporosis compared with bone mineral density at the lumbar spine as measured by dual-energy X-ray absorptiometry (Gulsahi et al. 2008).

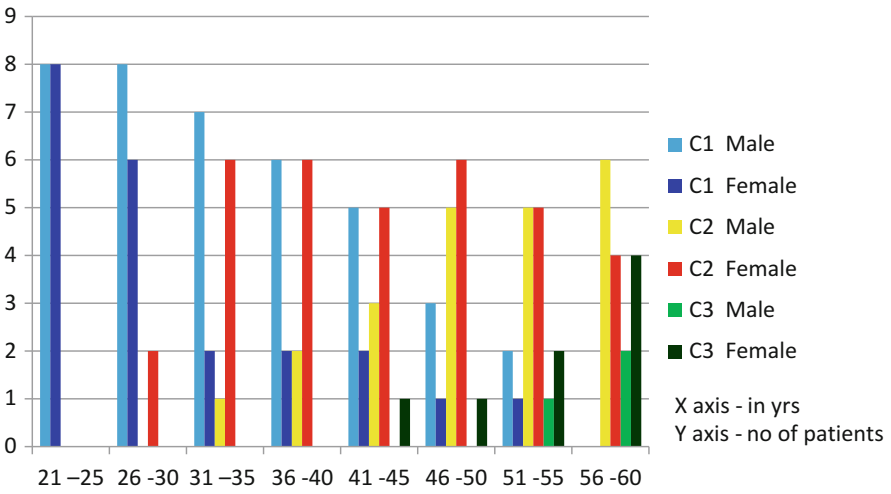
The prevalence of porosity in the mandibular cortex increases with age, and MCI is significantly related to mandibular BMD. There is also a significant relationship between MCI and lumbar spine BMD as measured with quantitative computed tomography (Horner et al. 2007). In the original work by Klemetti et al., MCI was found to be highly repeatable in their study with interobserver agreement of 98%; Taguchi et al. achieved a kappa index of 0.86 and an overall agreement of 92% for intraobserver performance (Ledgerton et al. 1999).

Klemetti and Kolmakow, Taguchi et al., and Bollen et al. have demonstrated that MCI is a useful indicator of skeletal BMD, the risk of osteoporotic fracture of bone turnover in postmenopausal women. Knezovic-Zlataric et al. also reported that the age-related distribution of MCI showed an age-related increase in the number of individuals with C3 cortex appearance and a significantly higher incidence of women with C3 cortex appearance in an older age group presumably reflecting age-related bone loss.

Zlataric and Celebic have reported that patients with lower BMD values in the mandible had a far more porous cortical layer of the inferior border of mandible. Horner and Devlin have reported that both MCI and bone quality index were significantly correlated with BMD of the body of mandible as measured by DXA (Cakur et al. 2009).

A study conducted by Govindraju and Chandra (2014) showed that the C1 was the most detected category followed by C2 and C3 being the least (Graph 1).

MCI is an objective index. According to several studies, MCI has been proposed as a method for identifying patients with poor bone quality in primary dental care settings as it requires no measurements or calculations but depends on visual assessments. For any method to be practically useful, it must be reproducible. In various studies, different levels of intra- and interobserver agreement results have



Graph 1 Comparison of MCI in males and females. MCI with the C1 in the younger age group and C3 in the older age group especially more in females

been reported for MCI. In some studies, it has been shown that even among expert observers, there were limitations in the reproducibility particularly in interobserver agreement in MCI evaluation which is the major drawback or a serious problem for a method to be used clinically. The results might have varied because of different sample sizes and differences in the ability of observers with regard to accurate diagnosis (Yasar and Akgunlu 2008; Cakur et al. 2009; Taguchi 2010).

In MCI determinations, caution should be exercised in at least three situations:

1. When the trabecular bone tail is connected to the inferior cortex of the mandible. This type of cortex usually seen in young adults with normal skeletal BMD might be misdiagnosed as an eroded cortex.
2. In cases with markedly thinned smooth cortex. As this type of cortex is smooth, it may be diagnosed as a normal cortex. However, markedly thinned smooth cortex is not seen in young adults with normal skeletal BMD. Careful observation of this type of cortex usually reveals severe endosteal cortical residue, and this is the final feature of a severely eroded cortex.
3. In cases where the hyoid bone projected on panoramic radiographs conceals the cortical shape of the mandible (Taguchi 2010).

As reported by Taguchi et al., mandibular cortical shape which is defined as C2 has greater range of appearances radiographically than C1 and C3 because early cases can sometimes be defined as C1 by some observers and also some late cases of C2 can be defined as C3 by other observers (Yasar and Akgunlu 2008).

It may be stated that the modification of MCI by dividing C2 into early and late C2 changes would improve the classification because the main discrepancy occurs in

those who lay on the border of two categories C1/C2 or C2/C3 (Ledgerton et al. 1999; Zlataric et al. 2001).

Mandibular Cortical Width (MCW)/Mental Index (MI)

It is the mandibular cortical thickness at the mental foramen. This is measured using the technique described by Ledgerton et al., "A line is traced which passes perpendicular to the tangent to the lower border of mandible and through the center of mental foramen. Cortical thickness in the region of interest is measured on the right and left sides of the mandible because different sides of the mandible may be influenced by different occlusal forces and thus could have the asymmetric sign in the topographic anatomy, after the measurements, the mean values of the obtained findings will be calculated" (Jagelaviciene 2010; Fig. 4).

Studies have shown significant relationship between the MCW and bone mineral density. The MCW increases rapidly in the second decade in women, while it increases gradually from the second to third decades in men. Since occlusal function due to the permanent teeth is established during the second decade, this might affect the formation of the cortical bone of the mandible locally and individually. The maturation of the entire skeleton in young adult women, including the jaws, is influenced by several factors, such as the age of menarche, menstruation cycle, nutritional intake, and physical activity. Menopause is the major contributing factor in postmenopausal women contributing to the rapid decrease in the BMD of the skeleton including the jaws (Taguchi et al. 2011).

Dutra et al. observed higher values for MCW in men. Knezovic-Zlataric et al. have shown that the values begin to decrease sharply in women as compared to men. Devlin and Horner reported that a cortical width of 3 mm is the most appropriate threshold for referral for bone densitometry. White et al. believe that it is more appropriate to post the threshold in the mid 4 mm range.

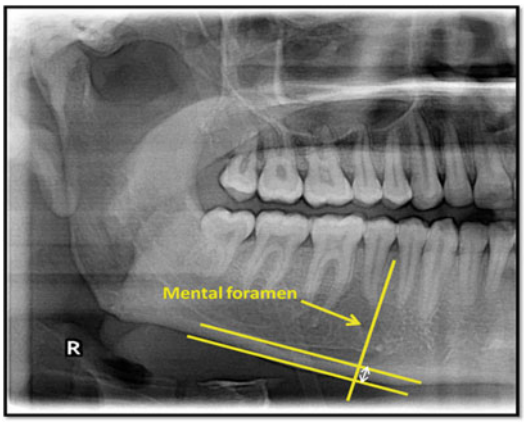
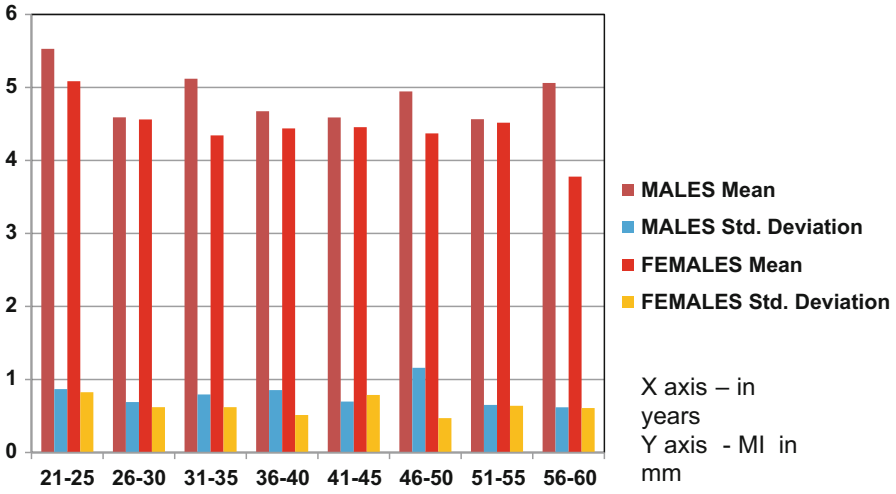


Fig. 4 Mental index/ mandibular cortical width



Graph 2 Comparison of MI values in males and females. Smaller MI values among older females when compared to those among males of the same age group

A study by Govindraju and Chandra (2014) has proved that MI values were smaller among older females when compared to those among males of the same age group (Graph 2).

Klemetti et al. reported that a threshold of 4 mm is optimal, but not sufficient by itself for excellent classification of subjects (Hastar et al. 2011). Klemetti et al. calculated sensitivity and specificity of MI in the diagnosis of an "osteoporosis risk" group and reported a sensitivity of 0.37 and a specificity of 0.85 when a diagnostic threshold of a 4 mm MI was used.

A mental cortical width of less than or equal to 3 mm would provide a sensitivity of 20% (95% CI = 10.4–33) and a specificity of 100% in diagnosing reduced skeletal bone density and a sensitivity of 25.9% (95% CI =11.2–46.3) and specificity of 93.6% (95% CI = 82.4–98.6) in diagnosing osteoporosis. Using a threshold mental cortical width of 3 mm, we calculate from the data in Ledgerton et al. that 2% of 50-year-old patients would be found to require further investigation and referral, increasing to 19% of 60-year-old and 35% of 70-year-old women (Devlin and Honer 2002).

Almost 90% of women with cortical width <3.0 mm had low BMD, and 60% of women with cortical width <3.0 mm had osteoporosis; however, there were no significant differences between prevalence rates of women with low BMD or osteoporosis in the intermediate risk group of cortical width (3.2–4.2 mm) and background prevalence rates. This suggests that women in the high-risk group, defined by thin cortical width (<3.0 mm), should be identified. Devlin and Horner reported that a diagnostic threshold for cortical width of 3 mm (or less) was suggested as the most appropriate threshold for referral for bone densitometry in 74 Caucasian women (Taguchi et al. 2006).

The mental index is the only quantitative index that has moderate correlation with hip and lumbar BMDs with relatively high intra- and interobserver agreement. The most accurate indices for predicting women with osteoporosis (T-score ≤ -2.5) and those who need treatment (T-score ≤ -2.0) are the mental index and the qualitative measures, mandibular cortical index and visual estimation of cortical width, (Leite et al. 2010).

In a study, Kribbs observed that MI is smaller in the osteoporosis group than in healthy individuals. Studies have shown that MI decreases with increasing age. It is lower in women than in men and is lower among female patients with osteoporosis compared with healthy female individuals (Dagistan and Bilge 2010). Mandibular cortical width may not reflect increased bone turnover after menopause but rather peak bone mass in younger age. Horner et al. has reported that the thinning of the mandibular cortex in a normal perimenopausal female is associated with low skeletal BMD (Taguchi et al. 2003).

Panoramic Mandibular Index (PMI)

This is a radiomorphometric index which was presented by Benson et al. in 1991. It is partly based on the Wical and Swoope method which is a theory of the correlation of the residual ridge resorption with mandibular height below the inferior edge of the mental foramen. They suggested that despite the alveolar bone resorption seen above the foramen, the distance from the foramen to the inferior border of mandible remains relatively constant through life. Thus, the PMI provides a measure of mandibular cortical thickness for normal mandibular size, and it could be used for the evaluation of local bone loss in dental practice. In this index, multiple measurements are made and divided by each other, so these could show some intraobserver and interobserver differences; therefore, the repeatability, reproducibility, and precision should be evaluated (Gungor et al. 2006; Figs. 5 and 6).

The measurements for PMI will be made according to the following criteria:

1. A line is drawn which is passed perpendicular to the tangent to the lower border of the mandible and through the center of the mental foramen.
2. Measurements will be made along this line of cortical width, the distance between the lower border of the mandible and the inferior margin and superior margin of the mental foramen.

Step 1 was described by Ledgerton et al. and step 2 was described by Benson et al. who introduced the index.

The superior PMI and inferior PMI are calculated as:

Superior PMI: cortex thickness/distance from superior margin of mental foramen to inferior border of mandible

Inferior PMI: cortex thickness/distance from inferior margin of mental foramen to inferior border of mandible

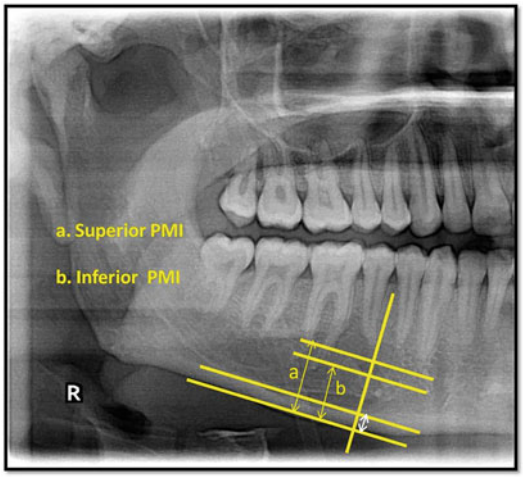


Fig. 5 Panoramic mandibular index

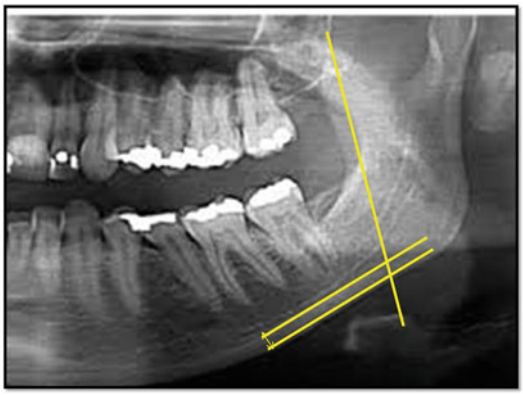
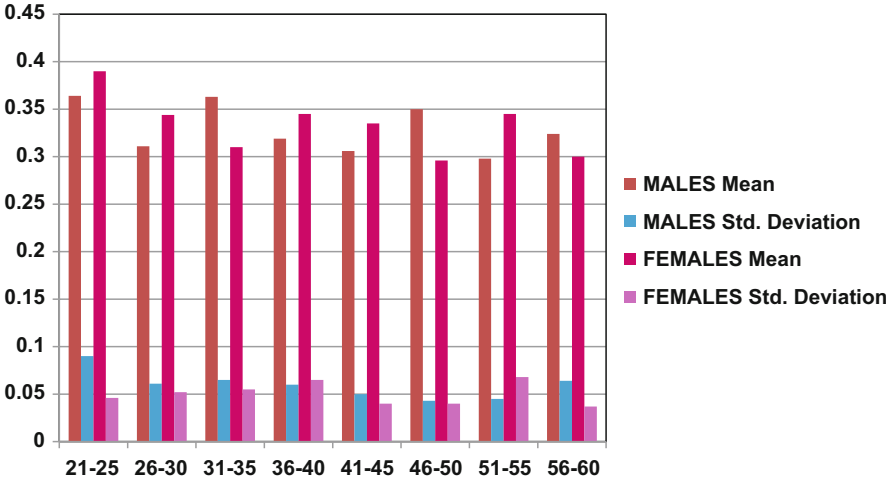


Fig. 6 Antegonial index

When the superior border of the inferior mandibular cortex is ill defined on the radiographs, the smallest width of compact cortical bone lying below the mental foramen is measured (Gungor et al. 2006).

Precise determination of the inner and outer margin of the cortical bone together with rigorous vertical linear measurements is a prerequisite for the calculation of panoramic radiomorphometric indices. A study by Govindraju and Chandra (2014) showed a significant difference was seen between males and females for PMI values where females showed a significance of 0.013 ($p < 0.05$) (Graph 3).

Kribbs et al., Klemetti et al., Taguchi et al., and Bollen et al. described that the thickness of the lower border of the mandible tends to be reduced in subjects with osteoporosis (Jagelaviciene et al. 2010). Benson et al. observed that mandibular cortical thickness decreased with age, and the amount of resorption was higher among women and among white individuals when compared to black individuals.



Graph 3 Comparison of PMI values in males and females. Smaller PMI values among females compared to males

In a study by Horner and Devlin, the PMI values measured in females with osteoporosis on panoramic radiographs were compared with mandibular bone values measured with DXA, and a significant relationship was found between the two. They concluded that PMI could be used as an indicator of mandibular bone density. Studies measuring PMI values have found that the values decrease with increasing age are smaller among white individuals than among black individuals and among women compared with men. They are also smaller in female patients with osteoporosis than in healthy female individuals which was found to be statistically significant (Dagistan and Bilge 2010).

Benson et al., Klemetti et al., and Horner and Devlin concluded that PMI had no distinct advantages over MI as a tool for mandibular BMD measurement (Jagelaviciene et al. 2010). It has been recently proved that the measurements are reliable if they do not cross the midline and the use of indices with the relation of the two vertical dimensions is reliable (Zlataric et al. 2001). In many studies, MCI and PMI were significantly correlated with mandibular BMD and showed moderate sensitivity and specificity for diagnosis of mandibular BMD (Horner and Devlin 1998).

One major advantage of PMI over MI is that its method of calculation takes account of differences in magnification associated with different panoramic equipment. Thus, unlike the other linear indices, it is possible to make a direct comparison of absolute figures with other published studies (Ledgerton et al. 1999). Studies have shown that there was a relationship between the quality of the cortex and the quantitative index of the mandible. When the quality of the cortex is low, the value of the MCW decreases. Zlataric et al. demonstrated that the patients with severe erosion of the cortex had significantly lower BMD values in comparison to other patients (Khatoonabad et al. 2011).

Recent work has used computer-active shape modeling software to automatically search for the lower border of the mandible on panoramic radiographs and measure the cortical width. By using this computer technology, reproducibility has significantly improved (Devlin and Horner 2008).

Antegonial Index (AI)

Antegonial index was described by Ledgerton et al. (1999) as a measurement of cortical width in the region anterior to the gonion at a point identified by extending a line of "best fit" on the anterior border of the ascending ramus down to the lower border of the mandible (Kribbs et al. 1983). Antegonial index (AI) is a linear radiomorphometric index that measured the mandibular cortical thickness on the line perpendicular to the mandibular cortex at the intersection with the tangent line to the anterior border of the ramus (normal value $>$ 3.2 mm) (Fig. 7).

Studies on antegonial index have showed that, in relation to age, gender, and dental status, the measured changes were inversely proportional. It was also suggested that reduction in the thickness of the cortex in the gonial region among elderly women might be used as a predictor for osteoporosis (Dubravka and Asja 2005; Knezovic´ Zlataric´ et al. 2002).

AI was found not to be useful because of problems associated with repeatability and the precision (Taguchi 2010).

Gonial Index (GI)

Gonial index is the mandibular cortical thickness measured on the bisectrix of the angle between the tangent lines to the posterior border of the branch and the bottom of the mandible having a normal value of $>$1.2. This index was first described by

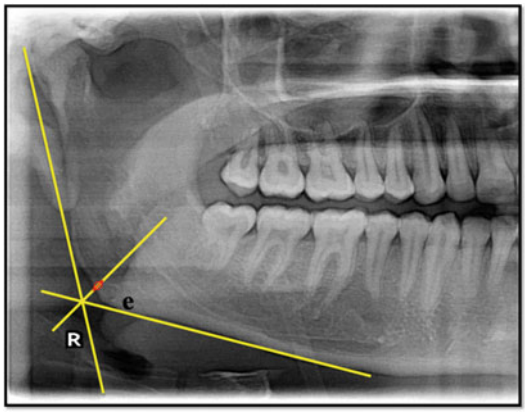


Fig. 7 Gonial index

Bras et al. in 1982 as the thickness of the mandibular angular cortex. Ledgerton et al. in 1999 named this index as gonial index and described as effective screening tool for identifying undetected osteoporosis in postmenopausal women (Taguchi 2010). GI demonstrates a negative correlation with age. Gonial cortical bone demonstrates a very gradual thinning with age until the sixth decade and then the value drops quite sharply. Brass et al. studied the thickness of the cortex at the gonial region and found no marked differences in males with age and with the dental status. The precision of measurements at the gonial region can be very poor due to the relatively small dimensions of cortical width in this region. Many studies have shown that gonial index is not used for detecting osteoporosis in elderly individuals for the following reasons.

First, the measurement error in GI will markedly influence the result, because the GI is small. Second, unstable horizontal magnification on panoramic radiographs will influence the results. Third, the site of measurement of GI is not clear. Finally, as the masseter and medial pterygoid muscles attach to the mandibular angle, occlusal function may influence the GI measurements (Taguchi 2010).

The dentists should both consider this as an opportunity to screen patients and refer those with positive findings for further assessment for the potential diagnosis of osteopenia/osteoporosis and also use the strong negative predictive value as a possibility for excluding large populations from unnecessary DXA screening by informing both the patient and physician of already available data from panoramic dental radiographs (Halling et al. 2005).

Measurements from panoramic dental radiographs can only be used to detect patients at high risk of osteoporosis and do not provide a definitive diagnosis. Although many studies have demonstrated a relationship between mandibular measurements (such as MCI) and vertical bone density, the method lacks diagnostic accuracy. It therefore does not seem justified to subject patients to a panoramic radiograph for the purposes of an osteoporosis screening but rather to provide additional information about osteoporosis risk to patients having this radiograph taken for other purposes (Devlin and Horner 2008).

Potential Application to Prognosis, Other Diseases, or Conditions

Increase in the elderly population worldwide will cause a dramatic rise in osteoporotic fractures. Osteoporosis is a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue which lead to enhanced bone fragility and an increase in fracture risk. Although radiomorphometric indices are regarded as an ancillary method for the diagnosis of osteoporosis on panoramic radiographs, they should be a routine procedure in dental examination. These indices can predict low bone mineral density on panoramic radiographs. MCI is an objective index. According to several studies, MCI has been proposed as a method for identifying patients with poor bone quality in primary dental care settings as it requires no measurements or calculations but depends on visual assessments. The mental index is the only quantitative index that has moderate correlation with hip and

lumbar BMDs with relatively high intra- and interobserver agreement. PMI provides a measure of mandibular cortical thickness for normal mandibular size, and it could be used for the evaluation of local bone loss in dental practice. Studies on antegonial index and gonial index involving elderly women have showed a reduction in the thickness of the cortex in the gonial region that can be used as a biomarker in identifying undetected osteoporosis.

Dentists should refer those with positive findings for further assessment for the potential diagnosis of osteopenia or osteoporosis and also use the strong negative predictive value as possibility for excluding large populations from unnecessary DXA screening.

Summary Points

- This chapter focuses on the biomarker which aids in diagnosing the undetected osteoporosis using panoramic radiographs.
- Osteoporosis is termed as a "silent disease" before the onset of fracture.
- Early identification of this condition can prevent the mortality and morbidity of the affected patients and can improve the overall prognosis.
- Qualitative and quantitative indices, including the mandibular cortical index (MCI), mental index (MI), and panoramic mandibular index (PMI), are useful in detecting these osteoporotic changes that are manifested in jaws.
- Dental panoramic radiography accounts for 32% (two million panoramic radiographs) of all medical radiologic examinations in the United Kingdom, taken annually in National Health Service dental practices in England and Wales alone and the most commonly advised screening radiograph worldwide.
- So the incidental findings detected on panoramic radiographs of these patients can be used for the identification of women who have no awareness of their low BMD and who could benefit from BMD testing.

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Subchondral Bone Features and Mechanical Properties as Biomarkers of Osteoarthritis

24

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Abstract

Osteoarthritis (OA) is a painful, debilitating disease most characterized by cartilage degeneration at joint surfaces. In addition to cartilage degeneration, OA is marked by bony changes including attrition, osteophyte formation, cyst presence, bone marrow lesions, altered shape, as well as altered density and mechanical properties of underlying subchondral bone. As subchondral bone is densely innervated, it may be a plausible site of debilitating pain associated with OA. However, the role of subchondral bone in OA pathogenesis and pain remains unclear. Medical imaging offers the ability to quantitatively characterize and monitor subchondral bone properties in vivo in people living with OA and investigate changes in relation to clinical OA symptoms. Incorporating medical imaging data with computational finite element modeling enables study of bone mechanical properties in the OA affected joint. These imaged-based biomarkers have potential to elucidate the mechanism underpinning OA pain and clarify the role of mechanical loading in OA pathogenesis. By characterizing and monitoring subchondral bone features and mechanical properties, image-based biomarkers provide unique, noninvasive avenues to improve our understanding of OA initiation, progress, and treatment. This chapter will summarize recent evidence of associations between subchondral bone features and mechanical properties as biomarkers of OA onset, progression, and pain initiation.

Keywords

Osteoarthritis • Subchondral bone • Imaging • MRI • CT • BMD • BML

List of Abbreviations

2D	Two dimensional
3D	Three dimensional
BMC	Bone mineral content (g)
BMD	Bone mineral density (g/cm^2 with DXA, g/cm^3 with QCT)
CT	Computed tomography
DXA	Dual energy x-ray absorptiometry, same as DEXA
E	Elastic modulus or Young's modulus (MPa)
FE	Finite element
FSA	Fractal signature analysis
HU	Hounsfield unit
MRI	Magnetic resonance imaging
OA	Osteoarthritis

pQCT	Peripheral Quantitative computed tomography
QCT	Quantitative computed tomography
RA	Rheumatoid arthritis
ROI	Region of interest

Key Facts of Osteoarthritis and Subchondral Bone

- Osteoarthritis is a painful, debilitating disease primarily characterized by cartilage degeneration.
- There is no cure for osteoarthritis, the exact cause is unknown, and the disease is usually not detected until after significant cartilage damage. Although pain is the dominant symptom, the source of pain is poorly understood.
- In addition to cartilage degeneration, osteoarthritis is marked by morphological and mechanical alterations to underlying subchondral bone.
- Different constituents of subchondral bone (subchondral cortical, subchondral trabecular, and epiphyseal trabecular bone), which differ in location and depth from the subchondral surface, appear to be affected differently by osteoarthritis.
- Although osteoarthritic subchondral bone is generally thought to be highly stiff (i.e., sclerotic = hardened), in the majority of cases the elastic modulus of diseased subchondral bone is lower or similar to normal subchondral bone.
- It is unclear whether subchondral bone alterations occur pre- or post-cartilage degeneration, but evidence indicates that specific morphological and mechanical changes in subchondral bone potentially initiate and accelerate the osteoarthritis disease process.
- Cartilage is aneural and insensate whereas subchondral bone is densely innervated; thus, subchondral bone is a likely site of pain generation associated with osteoarthritis.

Definitions of Words and Terms

Areal bone mineral density	Measure of bone mass per unit image area (g/cm^2, acquired using DXA)
Attrition	Refers to flattening or depression of the subchondral bone surface
Bone marrow lesion	Irregular high-signal intensity areas within the subchondral region observed using magnetic resonance imaging
Bone mineral density	Measure of bone mass per unit volume (g/cm^3, acquired using QCT)
Bone volume fraction	Measure of bone volume per unit volume; expressed as a percentage (%)
Elastic modulus	Describes intrinsic material properties of bone itself, independent of specimen geometry or size; defined via the slope of a stress–strain curve; also referred to

	as material stiffness, modulus of elasticity, or Young's modulus (MPa)
Material density	Measure of bone mass per unit bone volume (g/cm^3)
Osteophyte	Bony projection that forms along the periphery of joints, also known as bone spurs
Sclerosis	Generally referred to as a stiffening or hardening of a structure
Subchondral bone	Bone below (sub) cartilage (chondral)
Surface stiffness	Describes bone's response to deflection directly at the subchondral bone surface, dependent upon both material stiffness properties and physical size of the structure being tested; defined via the slope of a load-deformation curve; also referred to as structural stiffness

Introduction

Osteoarthritis (OA) is a painful, debilitating disease most notably characterized by cartilage degeneration at joint surfaces. There is no cure for OA and the disease is usually not detected until after significant cartilage damage. The exact cause is unknown but is thought to be related to malalignment and/or mechanical overloading. Although pain is a dominant symptom (McAlindon et al. 1992), the source of OA-related pain is poorly understood.

In addition to cartilage degeneration, OA is marked by bony changes including attrition, osteophyte formation, cyst presence, bone marrow lesions (BML), altered shape, as well as altered morphology and mechanical properties of underlying subchondral bone. Importantly, subchondral bone is densely innervated (Bjurholm et al. 1988) and may be a plausible site of debilitating pain associated with OA (cartilage, in contrast, is aneural and insensate (Dye and Vaupel 1994)). Accordingly, these bony changes have strong potential to serve as image-based biomarkers for early disease detection and for improving our understanding of OA pathogenesis and pain initiation. This information is important as it could assist with monitoring treatments aimed at preventing or delaying OA onset and progression.

Medical imaging offers the ability to quantitatively characterize these bone biomarkers in vivo in people living with OA, with longitudinal assessments and relations to clinical symptoms. Also, as bone can be viewed and assessed using multiple clinical imaging modalities, including magnetic resonance imaging (MRI), radiography or x-rays, dual-energy x-ray absorptiometry (DXA), and computed tomography (CT), it is possible to evaluate links between biomarkers potentially overlooked when limited to a single imaging modality. By incorporating computational finite element (FE) modeling with medical imaging, it is also

possible to study bone's mechanical properties and response to loading in people living with OA.

This chapter is focused on current evidence of bone biomarkers of OA pathogenesis and pain initiation, with a specific focus on subchondral bone features and mechanical properties assessed noninvasively using imaging and computational modeling.

Subchondral Bone

At the joint, the long bone end (epiphysis) is covered with articular cartilage and directly underneath cartilage is a bony zone known as subchondral bone. Subchondral bone includes various layers of bony tissues that can be separated into layers, including:

- Calcified cartilage, a thin partially mineralized tissue interconnected with subchondral cortical bone which begins at the tidemark (Fig. 1)
- Subchondral cortical bone (also known as subchondral plate or endplate), a thin highly mineralized layer of bone (Figs. 1 and 2)
- Subchondral trabecular bone, a spongy cancellous bone which is attached to and supports overlying subchondral cortical bone (Figs. 1 and 2)

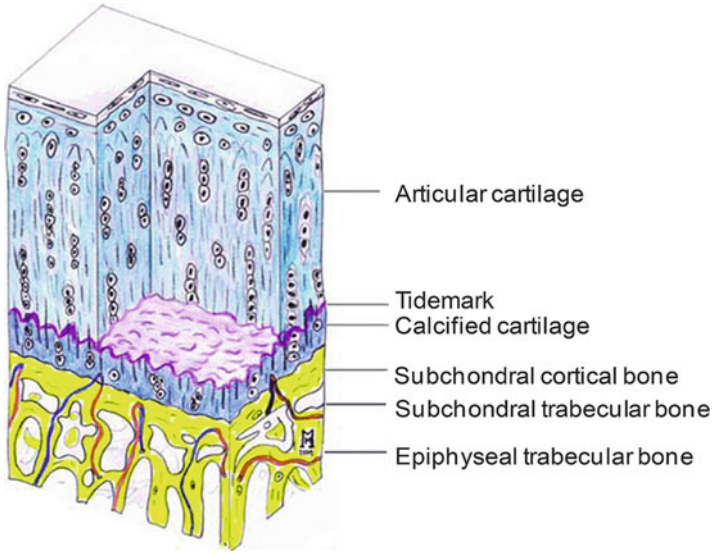

Fig. 1 Schematic drawing of the different layers of the cartilage-subchondral bone complex, including articular cartilage, subchondral cortical, subchondral trabecular, and epiphyseal trabecular bone (Modified from Imhof et al. (1999) and Madry et al. (2010), with permission from Springer)

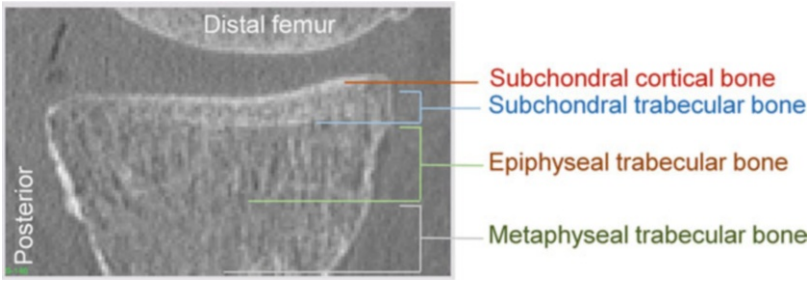

Fig. 2 Sagittal computed tomography (*CT*) image of the proximal tibia and pertinent bony components, including subchondral cortical, subchondral trabecular, epiphyseal trabecular, and metaphyseal trabecular bone. Neither cartilage nor the meniscus is observed in this image because neither tissue can absorb radiation

- Epiphyseal trabecular bone at the proximal epiphysis (Figs. 1 and 2)
- Metaphyseal trabecular bone at the proximal metaphysis (Fig. 2)

The term "subchondral bone" is used loosely to describe the subchondral bone structure, which includes subchondral cortical and subchondral trabecular bone. Subchondral bone functions as a supportive structure for the joint. Mechanically, subchondral bone acts in series with overlying cartilage to transfer energy between articulating bones. There are several subchondral bone features that have been linked to OA pathogenesis and progression. The following paragraphs define these features and discuss their role as a biomarker for OA, with a specific focus on the biomarkers linking mechanical loading and OA symptoms, particularly joint pain.

Subchondral Bone Features

Attrition

Subchondral bone attrition refers to flattening or depression of the subchondral bone surface (Bullough 1998; Neogi 2012). It is typically observed via CT or MRI (Fig. 3) and has been shown to be present in human knees with early OA and preradiographic OA (Reichenbach et al. 2008). Various studies have noted positive associations between bone attrition and OA-related pain at the knee (Dieppe et al. 2005; Torres et al. 2006; Chang et al. 2007, 2011; Javaid et al. 2012), hand (Haugen et al. 2012), and hip (Roemer et al. 2011). It is thought to be due to malalignment and/or mechanical overloading (Neogi et al. 2009a). Accordingly, attrition may be a biomarker for both overloading and OA. Interestingly, attrition presence predicted cartilage loss within the same region, suggesting that attrition may also be a biomarker for local cartilage loss (Neogi et al. 2009a).

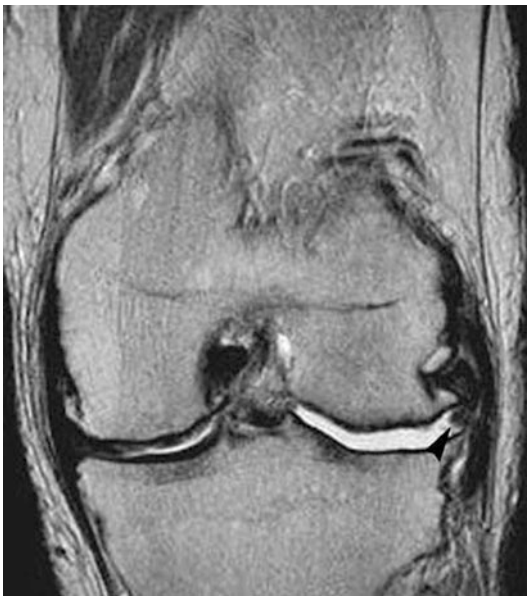

Fig. 3 Coronal magnetic resonance (*MR*) image showing bone attrition in the lateral femoro-tibial compartment. *Arrow* indicates meniscus partial maceration (From Guermazi et al. (2003), with permission from Springer)

Bone Marrow Lesions (BML)

BMLs are identified as irregular high-signal intensity areas within the subchondral region using T2-weighted MRI (Felson et al. 2001) (Fig. 4). Histologic changes associated with OA disease progression, such as necrosis, fibrosis, and trabecular abnormalities (Zanetti et al. 2000), are present in the same subchondral bone regions as BMLs, suggesting BMLs represent regions of bone trauma, micro-cracks, and high stress (Felson et al. 2001). This is supported by reports of BML presence at sites of high subchondral bone mineral density (BMD) (Lowitz et al. 2013), suggesting a possible bone modeling adaptation to altered loading or healing response after trauma. In terms of clinical characteristics, BMLs are associated with pain (Sowers 2003; Hayes et al. 2005; Torres et al. 2006; Kornaat et al. 2007) and cartilage loss (Sowers 2003). BML's are the most consistently reported image-based biomarker associated with OA-related pain (Sowers 2003; Hayes et al. 2005; Torres et al. 2006; Kornaat et al. 2007; Lo et al. 2009; Ip et al. 2011; Sowers et al. 2011; Javaid et al. 2012; Driban et al. 2013), found in 78 % of patients with painful knee OA (Felson et al. 2001), 30 % of patients with nonpainful knee OA (Felson et al. 2001), and 38 % of patients with preradiographic OA (Ip et al. 2011). Although BMLs tend to be the most consistently reported biomarker associated with OA-related pain, evidence relating BML size with pain is conflicting, with some studies showing that larger BMLs are associated with greater pain (Felson et al. 2001; Lo et al. 2009), while others suggest that BML size is not associated with pain (Driban et al. 2013). BMLs are also very dynamic in nature, changing throughout disease and pain progression (Driban et al. 2013). Overall, this evidence suggests that BMLs are plausible image-based biomarkers for OA.

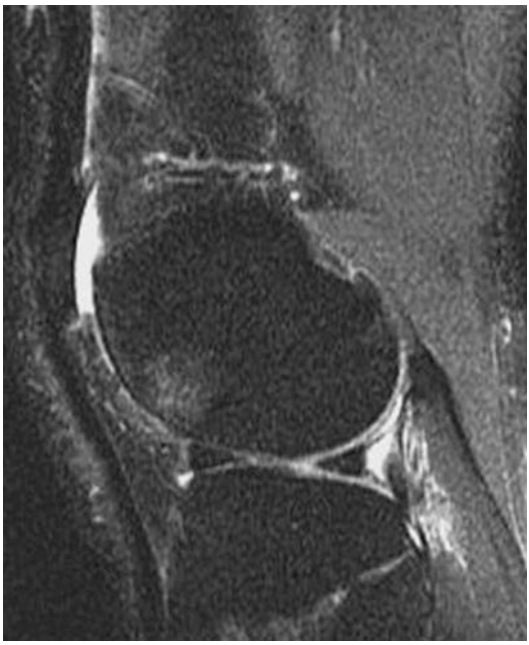

Fig. 4 Sagittal fat-suppressed T2-weighted MR image showing an anterior femoral bone marrow lesion (*BML*) (From Guermazi et al. (2003), with permission from Springer)

Bone Mineral Density (BMD)

BMD refers to the amount of bone contained within a unit area or volume. There is conflicting evidence regarding whether OA is associated with high or low BMD (Madsen et al. 1994; Karvonen et al. 1998; Wada et al. 2001; Clarke et al. 2004; Messent et al. 2005a). This discrepancy is likely due to inherent limitations of the imaging tools used to assess BMD, combined with selected analysis regions containing different types of subchondral bone (subchondral cortical, subchondral trabecular, epiphyseal trabecular bone), which differ in location and depth from the subchondral surface and may each be affected differently by OA (Burr and Schaffler 1997; Bennell et al. 2008) (discussed in more detail below). Evidence from both human and animal studies suggests that bone located nearest overlying articular cartilage plays a role in OA initiation, progression, and pain initiation (Brown et al. 1984; Burr and Schaffler 1997; Burr 1998; Lajeunesse and Reboul 2003; Burnett et al. 2015, 2016a).

To date, the majoring of imaging studies investigating the role of bone density in OA have used DXA (Madsen et al. 1994; Karvonen et al. 1998; Wada et al. 2001; Messent et al. 2005a; Lo et al. 2006). DXA is a 2D projection-based imaging tool which uses radiographic absorption (of two beams) to measure the amount of mineral within a specified two-dimensional (2D) area or region of interest (ROI) (Fig. 5); expressed as bone mineral content (BMC, g). By dividing BMC by the specified 2D area, measures of apparent density in the form of areal BMD (g/cm^2) can be obtained. Studies which compared proximal tibial areal BMD from OA and

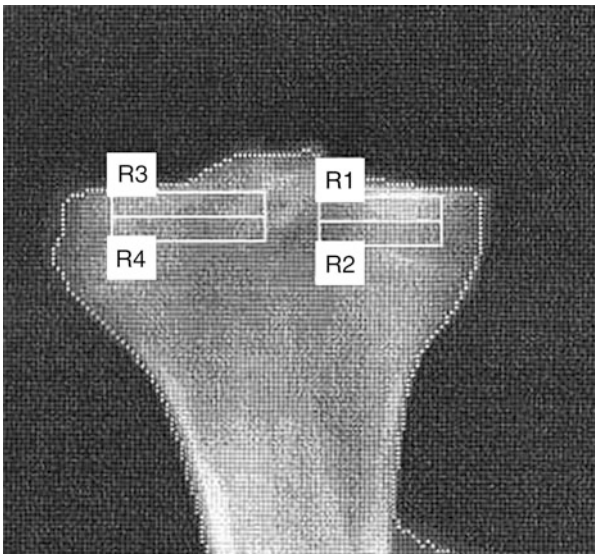

Fig. 5 DXA of a right proximal tibia (From Messent et al. (2005a) with permission from Springer)

normal knees offered conflicting results, with reports of no difference (Clarke et al. 2004; Messent et al. 2005a), 8–10 % lower (Madsen et al. 1994; Karvonen et al. 1998) and 15–26 % higher (Madsen et al. 1994; Wada et al. 2001) areal BMD in the OA groups compared to asymptomatic controls. As well, there is limited evidence regarding an association between areal BMD and joint pain. Two studies reported an association (El-Sherif et al. 2008; Akamatsu et al. 2012); one study did not (Dore et al. 2010). As noted earlier, these discrepancies may be due to inherent limitations of DXA. First, DXA represents a 3D structure as a 2D projection. Accordingly, areal BMD measure are very sensitive to subject size as larger individuals will contain more bone mineral within a specified 2D area. Second, areal BMD measures are sensitive to patient positioning (i.e., misalignment will alter BMC measures within a fixed ROI) and DXA is limited to imaging in the sagittal and coronal planes. Third, each of the DXA studies used different-sized ROIs at different locations. Conflicting results may therefore be due to the use of ROIs containing subchondral cortical, subchondral trabecular, and/or epiphyseal trabecular bone, each of which may be affected differently by OA. For example, areal BMD for OA subchondral bone near the subchondral surface (0–6.5 mm) was higher than normal, while further from the surface (16–24 mm), areal BMD did not differ (Madsen et al. 1994). OA is also associated with obesity (Felson et al. 2000) therefore findings of higher than normal areal BMD with OA may not be due to disease presence but due to obese individuals having bigger bones. The larger bone size along the projection image would have larger areal BMD – another limitation of DXA. Taken together, this evidence indicates that caution is warranted when using DXA-measured BMD as a biomarker of OA.

A promising alternative to DXA for measuring BMD is quantitative CT (QCT). This technique uses CT radiographic absorption techniques and calibration

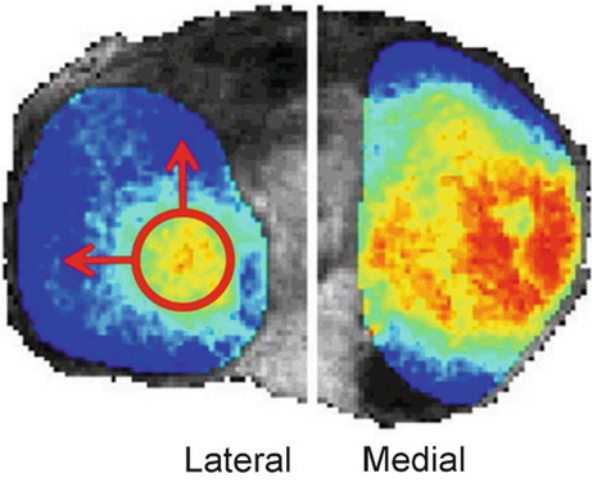

Fig. 6 Topographical colormap of proximal tibial BMD measured to a depth of 0–2.5 mm from subchondral surface. Regional analyses include average BMD of lateral and medial plateaus, as well as the average maximum BMD of a 10 mm diameter focal spot localized at the maximum value of each lateral and medial plateau

phantoms to convert grayscale CT Hounsfield units (HU) to equivalent volumetric BMD (g/cm^3) and is capable of imaging 3D cortical and trabecular density. In recent years, QCT combined with custom image processing has been increasingly used for OA research. In particular, depth-specific imaging techniques which measure BMD in relation to depth from the subchondral bone surface have been used at the proximal tibia (Johnston et al. 2009, 2010; Burnett et al. 2015) (Fig. 6), patella (Burnett et al. 2014, 2016a), proximal femur (Wright et al. 2012), acetabulum (Speirs et al. 2013), and distal tibia (Intema et al. 2011). Research to date has identified OA bone to have higher than normal BMD at sites near the subchondral bone surface (2-5-5 mm) (Johnston et al. 2010) and lower than normal BMD at distal epiphyseal sites (Bennell et al. 2008). With regards to pain, there appears to be an association between low epiphyseal trabecular BMD and high pain, found at both the tibia (Burnett et al. 2016b) and patella (Burnett et al. 2016a). It is worthwhile noting that the literature contains only a few reports of QCT assessments of OA and normal subchondral bone. This may be because, until recently, available clinical CT imaging slice thicknesses were large (1–4 mm) and therefore poorly suited for imaging thin layers of subchondral cortical and trabecular bone. Currently available clinical CT scanners now offer small isotropic voxel sizes (0.3–0.625 mm) which enable reliable data reconstructions in any plane of choice and thereby minimize imaged density dependence upon correct patient positioning. QCT also offers wide availability using whole body clinical CT scanners and very short acquisition times (seconds for hundreds of axial QCT images). Concerns regarding ionizing radiation dosage with QCT are also minimal for studying joints such as the knee due to the low presence of radiosensitive tissues. The effective dosage for a complete knee scan is comparable to 1.5x dosage from a transatlantic flight from Europe to North America (UNSCEAR 2000). CT though, like x-ray, is unable to directly image soft joint tissues such as cartilage and the tool is less ideal for studying hip OA due to the presence of radiosensitive reproductive tissues. Overall, this evidence suggests that QCT-measured BMD is a plausible image-based biomarker for OA.

One noteworthy potential limitation of using BMD as an OA biomarker pertains to its inability to distinguish the amount of mineral contained within a ROI from the amount of bone in the ROI. To clarify, BMD (which is equal to bone mass/bone volume) is equivalent to the product of bone material density (bone mass/bone volume) and bone volume fraction (bone volume/total volume). This is important because the material density of subchondral cortical, subchondral trabecular, and epiphyseal trabecular bone is lower in OA (Grynpas et al. 1991; Li and Aspden 1997a, b; Chappard et al. 2006). OA bone is hypomineralized relative to normal bone, likely due to a higher than normal bone turnover rate (Mansell and Bailey 1998). This results in less mature, hypomineralized bone and greater relative amounts of osteoid (Grynpas et al. 1991; Burr 1998). Studies at the hip showed 6–8 % lower material density of subchondral bone in late OA (Li and Aspden 1997a, b; Chappard et al. 2006). Accordingly, low BMD observed with OA (Bennell et al. 2008; Burnett et al. 2015, 2016a, b) can reflect low material density or low bone volume fraction (or both). Though, this issue may only be relevant during later stages of the disease as one study (at the proximal tibia) showed no material density differences between normal and early OA subchondral bone (Ding et al. 2001).

Bone Volume Fraction

As noted earlier, bone volume fraction pertains to the amount of bone volume contained within a specific volume. Bone volume fractions from OA and normal proximal tibia have been typically assessed using MRI (Beuf et al. 2002; Lindsey et al. 2004; Bolbos et al. 2008). The few MRI studies comparing bone volume fraction between OA and normal proximal tibiae were limited to epiphyseal trabecular bone sites distal to the subchondral surface (e.g., 5–10 mm). These studies reported lower epiphyseal trabeculae bone volume fractions in OA versus normal proximal tibiae in two studies (Beuf et al. 2002; Bolbos et al. 2008) and similar in two others (Beuf et al. 2002; Lindsey et al. 2004). Lower than normal bone volume fractions have been attributed to epiphyseal trabecular bone having thinner trabeculae (Lindsey et al. 2004; Bolbos et al. 2008) and larger spaces between adjacent trabeculae (Beuf et al. 2002; Bolbos et al. 2008). No studies have reported an association between bone volume fraction and OA-related pain. MRI is an ideal tool for assessing OA trabecular bone. It offers multiplanar 3D images, nonionizing radiation, and the ability to simultaneously image both trabecular bone and articular cartilage. MR image acquisition is, however, time consuming (multiple minutes for a single MR image), therefore bone volume results are susceptible to motion artifacts. Also, MRI is unable to account for the mineralization of subchondral bone because MRI does not directly image bone but images high-intensity fluids surrounding bone (e.g., fatty marrow, water). This is because bone shows no signal with MRI as it is primarily composed of calcium hydroxyapatite, which has only one proton which does not move. The decay time of bone is therefore very short and minimal signal arises, leading to bone appearing black with MRI (Bauer and Link 2009). By measuring fluid intensity, indirect measures of bone quantity can be obtained using

MRI. However, subchondral cortical bone and trabecular bone near the subchondral surface have minimal fluid presence; therefore MRI measures of subchondral bone quantity are primarily limited to trabecular regions apart from morphological measures (e.g., cortical thickness and/or volume). Due to an inability to quantify the mineralization of subchondral bone, combined with long scanning times, MRI is not necessarily ideal for studying subchondral cortical bone with OA.

Cysts

Cysts are essentially voids found in subchondral bone. They are observable via CT or MRI (Fig. 7). Cysts are thought to arise due to excessive loading or trauma. The 'bony contusion theory' (Ondrouch 1963; Durr et al. 2004) proposes that mechanical overloading causes trabecular microfractures, necrotic bone, and focal bone resorption, resulting in cyst development near the subchondral bone surface. Inflammatory macrophages within the lining of cysts can form into osteoclasts (Sabokbar et al. 2000), promoting further bone resorption and cyst expansion (von Rechenberg et al. 2001). Bone surrounding cysts have also been reported to be necrotic and lacking of blood vessels and normal marrow components (McErlain et al. 2012) and are thought to contribute to OA pain (Fondi and Franchi 2007). Local subchondral

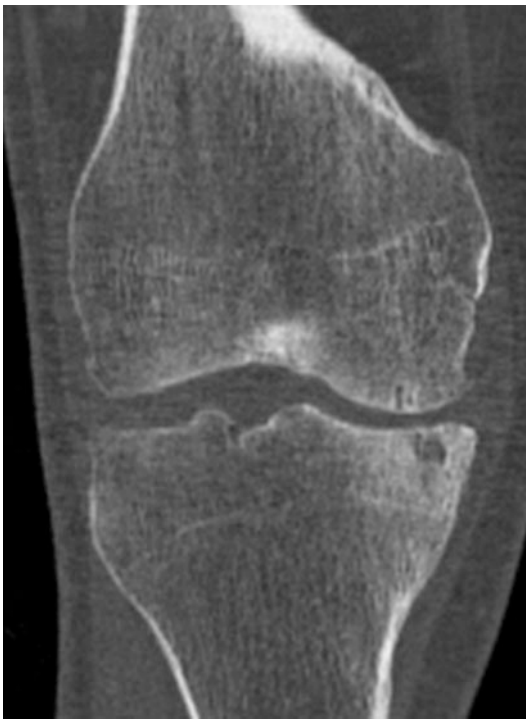

Fig. 7 Coronal CT image of proximal tibial cysts in the medial femoro-tibial compartment. Also note intensity of bone in the medial compartment of the proximal tibia, which indicates high bone mineral density (*BMD*)

bone cyst presence may increase intraosseous stress distributions, leading to pain and related immobility (McErlain et al. 2011). The evidence linking cysts and OA-related pain is contradictory. Three studies found positive associations (Hayes et al. 2005; Hayashi et al. 2012; Javaid et al. 2012); six studies found no associations (Link et al. 2003; Kornaat et al. 2006; Torres et al. 2006; Roemer et al. 2011; Sowers et al. 2011; Haugen et al. 2012). Thus, further evidence of cysts as OA biomarkers is warranted.

Osteophytes

Osteophytes are bony outgrowths appearing along joint margins (Fig. 8) and are a defining diagnostic feature of OA (Spector et al. 1993). Osteophytes are visible using radiographic tools (x-ray, DXA, CT) as well as MRI. Although a hallmark of OA, it is unclear why osteophytes develop. They may form in response to malalignment and joint laxity common with OA, whereby osteophytes are actively adapting and building a larger joint surface to accommodate shifting loads (van der Kraan and van den Berg 2007). Alternately, osteophytes may serve to stabilize and limit motion of the diseased joint (Williams and Brandt 1984). Increased metabolic activity associated with osteophyte growth and development has been shown to have a similar cellular structure and generation as the growth plate (Gelse et al. 2003). This increase in bone turnover and cellular arrangement is thought to influence nervous pathways

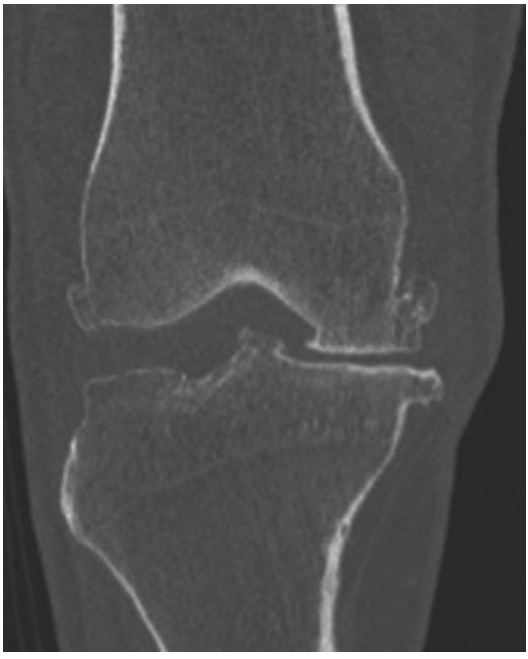

Fig. 8 Coronal CT image of osteophytes in the medial femoro-tibial compartment and lateral femur. Also note attrition in medial and lateral femorotibial compartments

and possibly pain sensation (van der Kraan and van den Berg 2007). However, there is conflicting evidence regarding the relationship between osteophyte presence and OA-related pain. Eleven studies have reported positive associations with pain (Spector et al. 1993; Boegard et al. 1998a, b; Hayes et al. 2005; Kornaat et al. 2006; Szebenyi et al. 2006; Neogi et al. 2009b; Sowers et al. 2011; Hayashi et al. 2012; Javaid et al. 2012; Kinds et al. 2013); nine studies have reported no relationships with pain (Dieppe et al. 1997, 2005; Link et al. 2003; Chang et al. 2007, 2011; Ai et al. 2010; Roemer et al. 2011; Sanghi et al. 2011; Haugen et al. 2012). Further, the relationship between osteophyte presence and pain has a tendency to change during disease progression, with early- and late-stage disease being less associated with osteophytes than mid-stage OA (Kornaat et al. 2006; Kinds et al. 2013). Further evidence of osteophytes as biomarkers of OA-related pain is needed.

Shape

Bone shape pertains to geometrical measures such as subchondral bone surface area, angulation, width, height, curvature, and congruity. These geometrical measures are generally acquired using MRI or CT. At the knee, greater proximal tibial surface area has been linked with OA progression (Cicuttini et al. 2004; Ding et al. 2008), while trochlear sulcus angle was associated with patellofemoral cartilage loss (Kalichman et al. 2007). Links to pain include the ratio of femoral-to-tibial surface area (Everhart et al. 2014), elevation of the lateral tibial plateau (relative to the medial plateau) (Haverkamp et al. 2011), as well as irregularity of the femoral condyle surface (Ochiai et al. 2010). Studies at the hip have also identified shape differences between OA and normal hips (Agricola et al. 2013). Although various studies have reported shape differences between OA and normal joints, it is unclear whether the abnormality is a structural response to OA or precursor to the disease. Accordingly, shape offers strong potential as an imaging biomarker to identify individuals at risk OA.

Texture/Micro-Architecture

Texture analyses attempt to characterize trabecular architecture or trabecular information from radiographic, CT, and MR images. An early texture method for studying OA is fractal signature analysis (FSA), which estimates differences in thickness and number of trabeculae between OA and normal proximal tibiae at sites distal to the subchondral surface (>5 mm from surface) (Messent et al. 2005a, b, c, 2006). FSA uses computational analyses of digitized 2D macroradiographs (4×-5× magnification) to define "fractal signatures" for each image (Fig. 9). Fractal signatures pertaining to OA or normal bone can then be compared to better understand trabecular bone changes in OA. For example, FSA will count the number of trabeculae with thicknesses ranging between 0.48 and

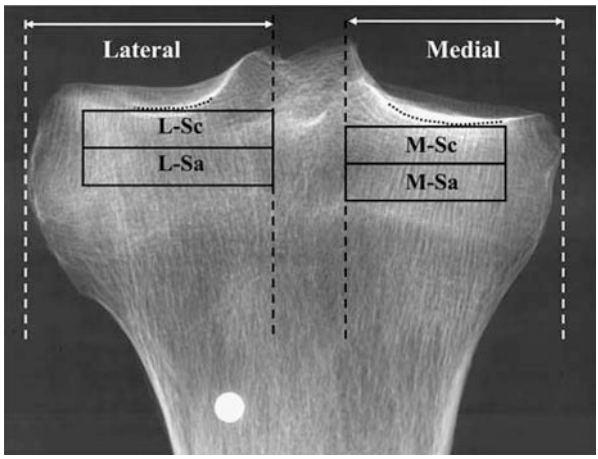

Fig. 9 Macro-radiograph of a right tibia showing placement of regions of interest for fractal signature analysis (*FSA*) (From Messent et al. (2005a) with permission from Springer)

0.60 mm for both OA and normal bone and compare those quantities to determine if OA trabecular bone is thinner or has more trabeculae. Previous studies using FSA to assess OA and normal proximal tibia showed OA trabeculae to be thinner than normal (Messent et al. 2005a, b, 2006) and fewer in number (Messent et al. 2005c) at sites distal to the subchondral surface (>5 mm). No studies were found that evaluated the association between FSA textural analyses and OA-related pain. It is important to note that due to the planar nature of FSA, the tool suffers similar limitations as DXA (e.g., cannot characterize bone properties from a complex 3D region of interest, size-related imaging artifacts, patient positioning errors). FSA is also limited to studying bone regions distal to the subchondral surface (>5 mm), since subchondral cortical and subchondral trabecular bone show minimal "signatures" in macroradiographic images (i.e., subchondral cortical and subchondral trabecular bone consistently appear primarily white).

In recent years, a statistical textural analysis (Tang 1998; Castellano et al. 2004; Szczypinski et al. 2009) has seen increased usage for studying bone (Harrison et al. 2011), with applications for normal and OA subchondral bone (Marques et al. 2013; MacKay et al. 2014, 2016). The method quantifies texture based upon the distribution of gray-level intensities within a ROI acquired using CT or MRI, providing statistical information on histogram profiles and spatial variation. The method is well suited for identifying subtle OA alterations hidden to the naked eye (MacKay et al. 2016). Statistical texture analyses have been used to identify specific knees which will develop progressive cartilage loss (Marques et al. 2013), as well as differentiate normal and OA knees (MacKay et al. 2014, 2016). Likely due to the relatively recent introduction of statistical texture analyses, no studies have identified links between statistical texture and pain. The technique, however, has potential to clarify the role of altered subchondral trabecular architecture in OA pathogenesis.

Subchondral Bone Mechanics

Importance of Biomarkers to Estimate Subchondral Bone Mechanical Properties

Approximately 30 years ago, Radin and Rose (1986) proposed that altered subchondral bone morphology and mechanical properties predisposed the joint to progressive OA by increasing stresses within the overlying cartilage. Specifically, repetitive, high-impact loading was suggested to trigger adaptation in the underlying subchondral bone, resulting in bone formation and increased subchondral bone surface stiffness (i.e., stiffness directly at the subchondral bone surface). A stiffened subchondral bone would be less able to deform under dynamic impact loading (i.e., transfer strain energy), leading to more energy being transferred through the overlying cartilage. This would result in higher internal cartilage stresses, cartilage breakdown, and eventual OA (Radin et al. 1972, 1973; Radin and Rose 1986). This theory was based upon observations of more subchondral trabecular bone microfractures in cadavers with early OA versus age-matched controls (Radin et al. 1970), as well as in animals developing OA as a consequence of repetitive loading (Radin et al. 1973). The same group also found that subchondral bone from joints with evidence of early OA transferred less strain energy during impact loading (Radin et al. 1970). This theory was supported by a FE simulation study showing increased elastic modulus (E) of subchondral cortical or trabecular bone within 1–2 mm of the subchondral surface increased cartilage stresses by ~50 % in the deepest layers (Brown et al. 1984). Accordingly, a noninvasive bone biomarker of subchondral bone surface stiffness could help explain the role of bone in OA initiation and progression.

A more recent (and related) hypothesis regarding the role of subchondral bone relates to increased bone turnover, resulting in subchondral cortical bone thickening and a decrease in cartilage thickness leading to increased cartilage stresses and eventual OA (Burr and Radin 2003). Microfractures within subchondral cortical bone resulting from impulse loading and repetitive stress are hypothesized to increase biological activity at the site of injury, resulting in increased bone turnover (Burr and Radin 2003). Increased bone turnover advances the tidemark towards the cartilage surface, resulting in thicker subchondral cortical bone and thinner cartilage. Thicker subchondral cortical bone and thinner articular cartilage, in turn, will increase cartilage stresses and result in cartilage deterioration and OA. Evidence supporting the role of increased bone turnover includes the increased presence of biochemical markers of bone formation and resorption in OA patients (Mansell and Bailey 1998; Bailey et al. 2004).

OA and Normal Subchondral Bone Mechanical Properties

Although OA subchondral bone is generally thought to be highly stiff (i.e., sclerosis = hardened), only one study has found OA subchondral bone to have a

higher than normal elastic modulus (Li and Aspden 1997). In the majority of cases, the elastic modulus of OA subchondral bone was reported lower (Zysset et al. 1994; Li and Aspden 1997) or similar (Finlay et al. 1989; Zysset et al. 1994; Day et al. 2001; Ding et al. 2001) to normal subchondral bone. A lower than normal elastic modulus for OA bone is due to hypomineralization (Li and Aspden 1997). Similar elastic moduli were explained by bone being hypomineralized but with higher than normal bone volume fraction (which increases bone's elastic modulus, thereby offsetting deficits associated with hypomineralization) (Li and Aspden 1997). Similarly, in the case of OA subchondral bone having a higher than normal elastic modulus (Li and Aspden 1997), OA bone was hypomineralized with a material density 12 % lower than normal, but the volume fraction was 60 % higher than normal, resulting in a 15 % higher elastic modulus. Although OA subchondral bone appears to have a lower than normal elastic modulus, it is unclear how altered mechanical properties are linked with OA-related pain.

A recent FE study of the OA proximal tibia investigated the individual and combined effects of OA-related morphological and mechanical alterations to subchondral and epiphyseal bone on overall surface stiffness (Amini et al. 2015). Results challenged the subchondral bone surface stiffness theory, suggesting OA subchondral bone surface stiffness was actually *lower* than normal. Findings indicated that OA-related alterations to subchondral cortical and trabecular elastic moduli or thickness had minimal effect on surface stiffness (~1 % change). Alterations to epiphyseal trabecular bone, however, had the largest effect on surface stiffness. For example, a 20 % reduction in epiphyseal trabecular elastic modulus resulted in an 11 % reduction in surface stiffness. Findings suggest that OA-related alterations to trabecular bone (e.g., lower mineralization, bone volume fraction, density, and elastic modulus) contribute to OA proximal tibiae being less stiff than normal. A QCT imaging study complemented these findings by reporting an association between low proximal tibial trabecular bone density and knee pain in OA patients scheduled for total knee arthroplasty (Burnett et al. 2016b). Given established relationships between bone density and bone's elastic modulus (Helgason et al. 2008), these results suggest a link between pain and altered subchondral bone stiffness. Accordingly, a noninvasive bone biomarker of subchondral bone surface stiffness could help explain the mechanisms underpinning pain in OA initiation and progression.

Estimating Bone Mechanical Properties Using Imaging

Imaging measures of subchondral BMD and bone volume fraction are considered surrogate measures of bone's elastic modulus (E). This is because various studies have shown direct relationships between laboratory-based measures of bone density and elastic modulus. These density-modulus relationships are typically characterized by a nonlinear power law function, i.e., $E \alpha BMD^{\gamma}$, with γ focused around a value of 2 (Anderson et al. 1992; Morgan et al. 2003). This is an important relationship

because a small change in BMD could result in a large change in bone's elastic modulus.

One important potential limitation associated with using density-modulus relationships to estimate OA subchondral bone elastic modulus is that the relationship appears to be dependent upon disease severity. A study of late OA subchondral bone from the hip (Li and Aspden 1997) showed the slope of the density-modulus curve for OA bone (subchondral trabecular and epiphyseal trabecular bone) to be approximately half that of normal bone. As discussed earlier, this discrepancy was explained by OA bone being hypomineralized with higher than normal bone volume (Li and Aspden 1997). This is an important concern because while OA bone may appear highly dense according to QCT-based measures of BMD, and therefore assumed to have a high elastic modulus, it may in fact have a lower elastic modulus than normal bone. This issue casts doubt on the utility of image-based biomarkers of subchondral bone mechanical properties for studying OA. For example, it is unclear which density-modulus should be chosen when OA status is unknown, or which density-modulus should be used in longitudinal studies of OA disease progression. However, it is important to note that this phenomenon may pertain to late OA bone only since such findings have not been noted at earlier stages of the disease (Johnston et al. 2011). Thus, for studies investigating the role of altered subchondral bone mechanics in OA initiation and early progression, a single density-modulus relationship may be valid. For studies comparing subchondral bone mechanics in normal and late OA individuals, disease specific density-modulus relationships should be employed.

It is important to note that although mechanical properties can be acquired for individual tissues using density-modulus relationships, it is not possible to predict, intuitively, how individual mechanical property variations will affect local surface stiffness. This is crucial because it is surface stiffness which reflect bone's response to articular cartilage loading and which is most relevant for studying OA progression.

Estimating Bone Mechanical Properties Using Subject-Specific Finite Element (FE) Modeling

Subject-specific FE modeling is an emerging in vivo technique in musculoskeletal research offering unique potential to clarify the role of subchondral bone in OA initiation and progression. FE modeling is a computational engineering technique used to evaluate how a structure with varying material properties and complex geometry behaves when subjected to loading. Its basic premise is to divide a complicated object into a finite number of small manageable pieces (which are known as elements), whereby the mechanical behavior of each element can be described mathematically and evaluated computationally (van Lenthe and Muller 2006). Using clinical CT or MRI, an individual's specific geometry for bone can be acquired (Fig. 10a). Tissue material properties (e.g., elastic modulus) can also be estimated using CT or MR images with previously

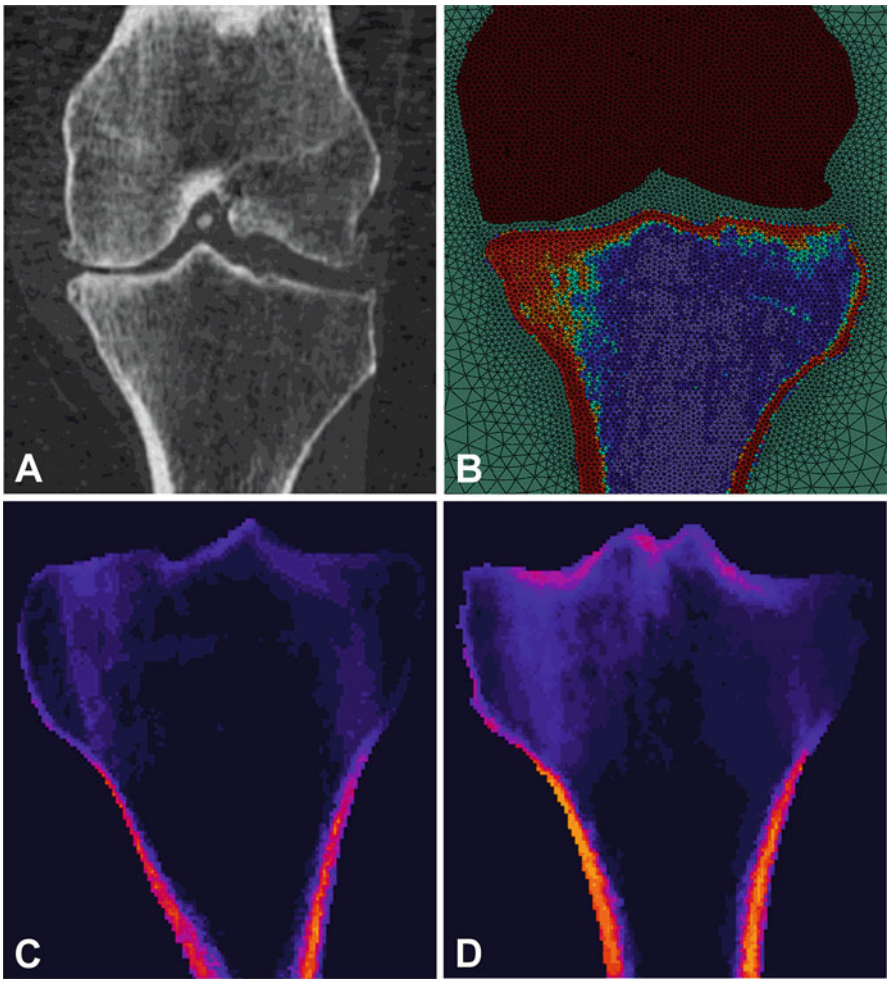

Fig. 10 (**a**) Coronal QCT image of knee. (**b**) Converted QCT image to finite element model of knee. Varying colors in the proximal tibia indicate heterogeneous distribution of elastic moduli acquired using BMD information from QCT images (*red* indicates high elastic modulus; *blue* indicates low elastic modulus). (**c**, **d**) Coronal view of mapped (von Mises) stress for a normal (**c**) and OA (**d**) proximal tibia (*orange* indicates high stress; *black* indicates low stress) (From Arjmand et al. (2016))

described density-modulus relationships or anisotropic material mapping methods (Nazemi et al. 2016) (Fig. 10b). Image-derived geometry and heterogeneous material properties can then be used to create a subject-specific FE model, which can be analyzed under varying loading scenarios (e.g., repetitive walking, impact intensive running) to simulate bone and cartilage responses to loading. Importantly, information nearly impossible to measure experimentally (e.g., internal stress and strain distributions in both cartilage and bone (Fig. 10c, d))

can be acquired using FE modeling to address possible associations with clinical symptoms, OA progression, as well as other features of OA (e.g., BMLs). The FE method can also be applied longitudinally to evaluate bone and cartilage structural behavior following OA-related morphological and mechanical alterations to these tissues.

Research to date using subject-specific FE modeling to investigate OA subchondral is at early stages. One quasi subject-specific FE study completed by McErlain et al. investigated the role of cysts in OA-related knee pain (McErlain et al. 2011). This study created virtual models of subchondral bone cysts (modeled as a hollow sphere, hence quasi subject-specific) and investigated surrounding stress. Results indicated higher stress around cysts than adjacent cyst-free regions, which could potentially explain OA-related pain and disability. A study by Fang et al. simulated BMD changes in a 3D model of the proximal tibia for both normal and OA knees (Fang et al. 2013). Results suggested that valgus (knock-kneed) malalignment could change BMD distributions in subchondral bone, potentially contributing to OA. A study by Arjmand et al. (2016) compared subchondral bone mechanical properties (internal stress and strain, medial and lateral surface stiffness) of a small sample of normal knees ($n = 7$) to OA knees ($n = 7$, early-to-moderate radiographic OA). This study found stress to be ~40 % higher in OA versus normal knees while strain and subchondral bone surface stiffness were similar between the groups. Interestingly, findings of similar strain suggest that OA subchondral bone had adapted to loading according to the mechanostat theory proposed by Frost (1987). Specifically, OA bone appears to maintain strain levels within a physiologic range, which could explain higher BMD and bone volume as adaptive responses to local stress observed with the disease.

Early subject-specific FE modeling results indicate unique potential to identify and study individuals with OA, as well as illuminate the role of altered subchondral bone morphology and mechanical properties in OA initiation, progression, and joint pain. However, it is important to highlight that subject-specific FE modeling of OA bone is in its early stages. To date, research employing subject-specific FE modeling has been primarily focused on addressing osteoporosis-related research questions (e.g., fracture strength) of bony structures not necessarily affected by OA (e.g., distal radius, femoral neck) (Keyak and Rossi 2000; MacNeil and Boyd 2008). This lack of OA-related FE research is likely due to difficulties associated with validating FE models comprised of numerous complex joint tissues, each of which have unique mechanical behavior. For example, at present it is impossible to place a device for measuring strain (i.e., a strain-gauge) in cartilage or in trabecular bone adjacent to overlying cartilage. Recently, however, new methods have arisen (Bay 2008) which have potential to validate internal strain distributions in bone and cartilage acquired using FE-modeling. These developments, combined with improvements in CT and MRI technologies and computing power, have made subject-specific FE modeling a feasible technique to offer novel computational biomarkers for addressing fundamental questions regarding the role of mechanics in OA pathogenesis and joint pain.

Conclusion

Osteoarthritis is a debilitating joint disease with no known cure, no known cause, and poor early detection. As well, the source of OA-related pain is poorly understood. Although OA is generally considered a disease of cartilage, there is increasing evidence that altered subchondral bone is involved in OA pathophysiology and pain initiation. Medical imaging offers unique, noninvasive biomarkers which can quantitatively characterize and monitor subchondral bone traits and mechanical properties. These biomarkers can help to clarify the role of subchondral bone in OA clinical symptoms, disease progress, and prevention. Of the available subchondral bone biomarkers, BMLs and attrition exhibited the most consistent evidence of an association with OA-related pain. An inverse relationship was noted between QCT-measured BMD and OA-related pain (i.e., low BMD was associated with high pain). The association between osteophytes and cysts with OA-related pain remains unclear. Subchondral shape exhibited evidence of an association with OA-related structural damage and suggests potential to identify individuals prone to OA. In terms of emerging methods, statistical texture analyses and subject-specific FE modeling indicate exciting potential to clarify the role of altered subchondral bone morphology and mechanical properties in OA onset, progression, and pain.

Potential Applications to Prognosis, Other Diseases, or Conditions

The current evidence and potential future application of subchondral bone biomarkers to improve our understanding of OA pathology and prognosis have been discussed in this chapter. In terms of applications with other diseases, BMD, bone volume fraction and shape could be applied to characterize the role of subchondral bone in relation to other forms of arthritis (e.g., rheumatoid arthritis, RA), joint injuries, and related therapies, including joint replacement surgery. For example, measures comparable to BML assessments using MRI could prove useful for characterizing osteitis present with RA. Subject-specific FE modeling also offers potential to clarify the role of bone erosion and/or formation on RA or injury-related pain. Finally, FE models of subchondral bone and related biomarkers can be used to complement development and evaluation of therapies and interventions, including joint replacements, and contribute to improved success of these therapies.

Summary Points

- This chapter focused on evidence of associations between subchondral bone features and mechanical properties as biomarkers of OA onset, progression, and pain initiation.
- BMLs and attrition exhibited the greatest evidence of an association with OA-related pain.

- An inverse relationship was noted between QCT-measured BMD distal to the subchondral surface and OA-related pain (i.e., low BMD was associated with high pain).
- The association between osteophytes and cysts with OA-related pain is unclear.
- Subchondral shape exhibited evidence of an association with OA-related structural damage and offers potential to identify individuals prone to OA.
- Statistical texture analyses and subject-specific FE modeling offer promising biomarkers and potential to clarify the role of altered subchondral bone morphology and mechanical properties in OA pathophysiology.

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Registered Micro-Computed Tomography Data as a Four-Dimensional Imaging Biomarker of Bone Formation and Resorption

25

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Abstract

There are significant clinical reasons motivating scientists to better understand how loading conditions, diseases, synthetic implants, and drug treatments affect bone formation and resorption. Changes in bone turnover have enormous impact on the quality and mechanical competence of the skeleton. Until recently, bone formation and resorption were primarily measured using biochemical markers of bone turnover or histomorphometry. However, recent advances in computed tomography allow one to follow structural changes in the cortical and trabecular bone of living animals and human patients. The aim of this chapter is to describe recently developed methods that allow the monitoring of bone modeling and remodeling processes in vivo by using registered longitudinal micro-computed tomography data, which serves as an imaging biomarker of bone formation and resorption. The chapter provides an overview of bone modeling and remodeling processes and the standard methods that have been used in the past and present to assess bone formation and resorption. Micro-computed tomography-based imaging of the bone is then discussed. A detailed description is then given of recently developed computation methods that allow monitoring of bone modeling and remodeling using registered longitudinal micro-computed tomography data as an imaging biomarker of bone formation and resorption. The chapter ends with a discussion of how these imaging-based biomarkers of formation and resorption can be used to complement and in some cases replace conventional experimental and clinical methods of monitoring bone turnover.

Keywords

Bone formation • Bone resorption • Remodeling • Modeling • Bone turnover • Imaging biomarker • Micro-computed tomography

List of Abbreviations

AFR	Activation, formation, and resorption
BALP	Bone-specific alkaline phosphatase
BFR	Bone formation rate
BMU	Basic multicellular unit
BRR	Bone resorption rate
BS	Bone surface
BSP	Bone sialoprotein
BV	Bone volume
CTX	Carboxy-terminal cross-linked telopeptide of type I collagen
DPD	Deoxypyridinoline
ES	Eroded surface
EV	Eroded volume
HR-pQCT	High-resolution peripheral quantitative computed tomography

LRP5/6	Lipoprotein receptor-related protein 5 and 6
MAR	Mineral apposition rate
microCT	Micro-computed tomography
MRR	Mineral resorption rate
MS	Mineralizing surface
MV	Mineralized volume
NTX	Amino-terminal cross-linked telopeptide of type I collagen
OC	Osteocalcin
PICP	Procollagen type I C-terminal propeptide
PINP	Procollagen type I N-terminal propeptide
PYD	Pyridinoline
TRACP5b	5b isoenzyme of tartrate-resistant acid phosphatase
TRAP	Tartrate-resistant acid phosphatase

Key Facts of Bone Modeling and Remodeling

- *Modeling can lead to large changes to bone mass and morphology.*
- *During growth, cancellous and cortical bone turnover occurs mainly through modeling processes, to shape and reshape the bone into an optimal structure depending on loading conditions.*
- *Modeling occurs when osteoblasts form the bone or osteoclasts resorb the bone, in a spatially and temporally independent manner.*
- *Modeling occurs on the periosteal, endosteal, and trabecular surface.*
- *Remodeling is a process in which bone resorption and formation are spatially and temporally coupled into a small bone packet called a basic multicellular unit.*
- *Remodeling affects all bone surfaces (trabecular, endosteal, and periosteal), and in higher vertebrates, remodeling also occurs within the cortical bone (termed intracortical or osteonal remodeling).*
- *Remodeling includes the following phases: activation of osteoclasts, resorption by osteoclasts, reversal, transition for osteoclast to osteoblast activity, and formation of osteoid by osteoblasts, mineralization, and quiescence.*
- *Remodeling is more prevalent after skeletal maturation, since the skeleton must continually repair itself over time.*

  Key facts of bone modeling and remodeling processes including when they occur, how they occur, and the structural consequences of each process to the skeleton.

Key Facts of Micro-Computed Tomography

- *Micro-computed tomography is based on the interaction of X-rays, which are electromagnetic waves, with matter (the object being imaged).*
- *X-ray computed tomography enables the nondestructive visualization of the three-dimensional (3D) internal structure of an object by producing three-dimensional images representing the X-ray attenuation distribution of the object.*

- *The attenuation at each point in the object can be reconstructed from several measurements along a series of paths.*
- *CT is very suitable to image the bone, as the X-ray attenuation coefficient of the calcium in bone is much higher than of the surrounding soft tissue.*
- *With the development of in vivo micro-computed tomography in the 2000s, it became possible to follow structural changes over time in bones of a living, anesthetized rodent.*

Key facts of micro-computed tomography including its history, the principles behind the technique, and its current experimental applications.

Definition of Words and Terms

Bone formation	The process by which osteoblasts lay down the new bone. The osteoblast first secretes a collagenous matrix called osteoid that is subsequently mineralized.
Bone modeling	The mechanism that shapes skeletal sites. Bone resorption and formation occur in an uncoupled way and on separate surfaces.
Bone remodeling	The mechanism that allows bone turnover while maintaining bone mass. It is based on the coupled and balanced activities of bone resorption and formation on the same site of the bone surface.
Bone resorption	The process by which the osteoclasts remove the bone, creating pits and tunnels in the bone. Osteoclasts resorb the bone by pumping protons to solubilize hydroxyapatite (HA) and secrete cathepsin K to degrade type I collagen.
High-resolution peripheral quantitative computed tomography	It is an in vivo clinical imaging modality based on X-rays enabling assessment of three-dimensional bone structure and density at peripheral sites (the distal tibia and distal radius).
Imaging Biomarker	A quantitative measure of a tissue property determined from in vivo image data displaying a biological or physiological process.
Micro-computed tomography	It is a nondestructive technique based on X-rays that provides three-dimensional images of the internal structure of an object at small scales and high resolution.

Osteoblasts	Cells that are derived from mesenchymal stem cells. They are responsible for forming the bone.
Osteoclasts	Cells that are derived from hematopoietic progenitors in the bone marrow. They are responsible for resorbing the bone.
Osteocytes	They are former osteoblasts that become enwalled in the bone matrix and are thought to be the mechanosensors in the bone. They sense mechanical stimuli and orchestrate the actions of osteoblasts and osteoclasts to form and resorb the bone, respectively.

Introduction

The bone is a complex, hierarchical material that forms the skeletons of most vertebrates. Although the bone has a variety of physiological functions, including structural and protective support for various internal organs, serving as a mineral reservoir for calcium and phosphorus and defensing against acidosis, its main function is mechanical. Bones form the body's load-bearing framework, the skeleton, which allows muscles to create motion via leverage against tendon anchor points. At the nanoscale, the composite material bone is made up of type I collagen fibrils and apatite mineral crystals. Mineralized collagen fiber bundles form and create a three-dimensional stiff matrix that also includes noncollagenous proteins and water. The cells of the bone include bone-resorbing (removing) osteoclasts and bone-forming osteoblasts and osteocytes. Osteoblasts are cells of mesenchymal origin, while osteoclasts are of myelomonocytic origin. In all vertebrates, with the exception of advanced teleost fish, some osteoblasts become embedded within the bone matrix, which subsequently mineralizes, and they terminally differentiate to become osteocytes (Franz-Odendaal et al. 2006). Although osteocytes are immobilized within the mineralized tissue, they are extremely metabolically active. An interconnecting network of channels called canaliculi between osteocytes allows them to communicate with one another. The osteocytic network forms a large endocrine organ that affects the bone and other tissues. Osteocytes directly control bone formation and indirectly influence bone resorption by controlling osteoclast activation. Osteocytes are thought to serve as a mechanosensor, able to orchestrate bone formation and resorption and directly form or resorb bone tissue that surrounds them by so-called osteocytic osteolysis.

There are significant clinical reasons motivating scientists to better understand how loading conditions, diseases, synthetic implants, and drug treatments affect bone formation and resorption. Changes in bone turnover have enormous impact on

the quality and mechanical competence of the skeleton. Until recently, bone formation and resorption were primarily measured using biochemical markers of bone turnover or histomorphometry. However, recent advances in imaging techniques now allow one to follow structural changes both in cortical and trabecular bones of living animals and human patients. Micro-computed tomography (microCT) and high-resolution peripheral quantitative computed tomography (HR-pQCT) are becoming increasingly popular imaging techniques to longitudinally assess the trabecular and cortical bone mass and morphology in rodents and humans, respectively. Our research group and others have introduced computational methods that enable quantitative analysis of bone formation and resorption in rodents and humans (Waarsing et al. 2004a; Schulte et al. 2011; Birkhold et al. 2014a, b). These techniques consist of overlaying two high-resolution CT imaging data sets of the same bone at different time points using an optimization algorithm based on maximization of mutual information. Bone volumes in both data sets (quiescent bone), in only the first data set (formed bone), and in only the later data set (resorbed bone) are calculated. Our group has recently introduced a computational method to monitor and quantify in vivo modeling and remodeling processes in mice (Birkhold et al. 2015), which can be extended to assess these processes in humans.

Thus, the main aim of this chapter is to describe recently developed methods that allow us to monitor bone modeling and remodeling processes in vivo by using registered longitudinal microCT data that serves as an imaging biomarker of bone formation and resorption. The chapter starts with a short introduction of bone modeling and remodeling processes. The next two sections describe two conventional methods of assessing bone formation and resorption: biochemical markers of bone turnover and bone histomorphometry. The fundamentals and current state-of the-art concerning microCT-based imaging of bone are then explained. This is followed by a detailed description of two recently developed microCT imaging-based methods. First, a 4D method of quantifying bone formation and resorption is described, followed by a 4D method of tracking consecutive surface activity events to monitor bone modeling and remodeling processes. The chapter ends with a discussion of how these imaging-based biomarkers of formation and resorption can be used to complement and in some cases replace conventional experimental and clinical methods of monitoring bone (re)modeling processes.

Bone Modeling and Remodeling

Bone structure is altered during fracture repair, growth, and aging. Fracture repair occurs through intramembranous (direct formation of bone by osteoblasts) and endochondral (cartilage is formed, calcified, and then replaced by the bone) ossification processes, which both include a final stage of remodeling that replaces the woven bone with lamellar bone. During growth, bones lengthen via endochondral ossification and increase their diameter via intramembranous ossification at the periosteal surface. Also during growth, cancellous and cortical bone turnover occurs mainly through modeling processes, to shape and reshape the bone into an optimal

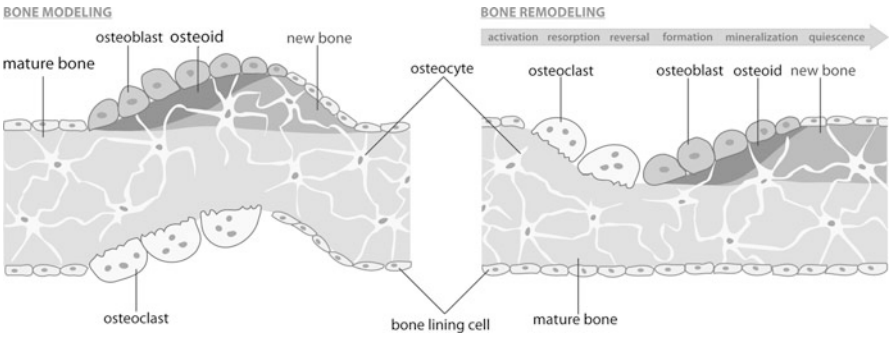

Fig. 1 Bone modeling and remodeling. Bone modeling and remodeling processes are shown. The steps of remodeling are illustrated (*arrow*)

structure depending on loading conditions (Fig. 1). During growth, periosteal apposition is thought to exceed endocortical resorption, leading to a net bone gain and cortical thickening. Remodeling processes are more prevalent after skeletal maturation, since the skeleton must continually repair itself over time. After longitudinal growth is completed, resorption and formation processes are assumed balanced. However, already in early adulthood, a negative imbalance in formation and resorption may occur; Riggs et al. (2008) reported that women experienced 6% and men experienced 15% of their total lifetime cortical bone loss before the age of 50. While the majority of cortical bone loss observed later in life is predominantly due to reductions in sex steroids and possibly loss in fat and muscle mass, the early-onset cortical bone loss observed in both sexes remains unexplained, as reductions in sex steroids are not remarkable in adults. It is thought that with advancing age resorption increases, more so on the endocortical and trabecular surface than on the periosteal, and bone formation decreases or remains constant, resulting in a net bone loss (Parfitt 1984).

Modeling can lead to large changes to bone mass and morphology. Modeling occurs when osteoblasts form the bone or osteoclasts resorb the bone, in a spatially and temporally independent manner on the periosteal, endosteal, and trabecular bone surface. Concerning terminology, ossification on endosteal and/or trabecular surfaces is called bone apposition, while it is referred to as intramembranous bone formation on periosteal surfaces because mesenchymal cells that differentiate into osteoblasts are present and appear as layers of membranes (Martin et al. 1998). Remodeling is a process in which bone resorption and formation are spatially and temporally coupled into a small bone packet called a basic multicellular unit (BMU). This process affects all bone surfaces (trabecular, endocortical, and periosteal), and in higher vertebrates, remodeling also occurs within the cortical bone (termed intracortical or osteonal remodeling), as the BMU tunnels through the bone, creating a secondary osteon since the vascular supply is essential. Remodeling occurs in three main phases termed A-R-F: activation of osteoclasts, resorption by osteoclasts, and formation by osteoblasts. However, for clarity the process can be further subdivided,

to also include a reversal phase (transition for osteoclast to osteoblast activity) between resorption and formation and mineralization and quiescent phases (Fig. 1). Thus, the formation phase only refers to the formation of osteoid by osteoblasts followed by phase that involves mineral deposited within and between the collagen fibers. Osteoblasts form human bone at a rate of approximately 1 μm/day, while osteoclasts remove the bone much faster (~40 μm/day) (Jaworski and Lok 1972). The whole remodeling process lasts a median of 120 days in healthy cortical human bone, with activation accounting for 3 days, resorption and reversal 30–40 days (Eriksen et al. 1984b), and formation and mineralization 100 days (Eriksen et al. 1984a). Healthy human cancellous bone remodeling lasts for approximately 200 days (Eriksen 1986). Remodeling usually results in minimal changes occurring in overall bone mass and morphology. The activation of remodeling is categorized as targeted or nontargeted. Targeted remodeling is thought to occur when microdamage initiates osteocyte apoptosis and osteoclasts are subsequently recruited to these sites. Thus the function of remodeling is primarily the replacement of old bone with new bone to presumably maintain its load-bearing capacity. Hormones, drug treatments, and possibly the need to participate in mineral homeostasis can initiate nontargeted (stochastic) remodeling. Remodeling may also be influenced by the mechanical loading environment to alter the morphology of the skeleton, but experimental evidence supporting this idea is missing.

Methods of Assessing Bone Remodeling

Biochemical Markers of Bone Turnover

Biochemical markers that reflect enzymatic activity of bone cells (osteoblasts or osteoclasts) or bone tissue breakdown products are widely used to clinically assess bone modeling and remodeling processes. Evaluation of these markers using blood serum and urinary assays is common practice in monitoring the clinical treatment of various metabolic bone diseases, such as osteoporosis. Current bone formation markers are either derived from osteoblasts or procollagen, which are precursor molecules of collagen type I that is secreted by osteoblasts during bone formation. There exist four common markers for bone formation. Bone-specific alkaline phosphatase (BALP), measured in serum, is secreted by osteoblasts. Osteocalcin (OC), measured in serum and urine, is a noncollagenous hydroxyapatite-binding protein also produced by osteoblasts during bone formation. The other two common formation markers can be found at each end of the procollagen molecule: procollagen type I N-terminal propeptide (PINP) and procollagen type I C-terminal propeptide (PICP). Both PINP and PICP are released into the blood by osteoblasts during bone formation.

Although the majority of bone resorption markers are degradation products of type I collagen, there are also a few markers that are not related to collagen. Two noncollagenous markers are the osteoclast enzyme serum 5b isoenzyme of tartrate-resistant acid phosphatase (TRACP5b) and cathepsin K. Also, the noncollagenous

glycoprotein of bone matrix and bone sialoprotein (BSP) is a marker that is produced by osteoblasts and osteoclasts and is measured in serum. Common collagen-associated markers include the pyridinium cross-links, pyridinoline (PYD), and deoxypyridinoline (DPD) that are released during bone resorption and excreted into the urine. The carboxy-terminal cross-linked telopeptide of type I collagen (CTX) and amino-terminal cross-linked telopeptide of type I collagen (NTX) are released into the blood during resorption and are excreted in urine. There are several other markers that are not mentioned here, since they are less commonly used.

Limitations and Recommendations

Despite their widespread use, both markers of bone formation and resorption have a variety of limitations. Many markers are not bone specific but are also found in other tissues. They also reflect the whole skeleton, rather than the metabolic activity at a particular site. In general the largest drawback to using biochemical bone turnover markers is related to their variability. This variability can be attributed to a variety of sources categorized as (1) pre-analytical, (2) analytical, and (3) post-analytical. Contributors to pre-analytical variability include age, menopausal status, gender, fracture status, pregnancy and lactation, drugs, comorbidity, mobility/exercise, geography, ethnicity, technical causes, intrinsic biological variation, circadian rhythm, fasting status, exercise, menstrual, seasonal variation, and diet. Analytical variability comes from intra- and interlaboratory variations. Finally, post-analytical variability is based on the interpretation of results in light of the first two types of variability. For a detailed discussion on the importance and nature of variability in bone turnover marker assessment, please refer to some excellent reviews on the topic (Vasikaran et al. 2011; Hlaing and Compston 2014). Several governmental agencies and bone-related societies have issued various standards and recommendations to minimize variability. In an attempt to establish international reference standards for bone turnover markers, the International Osteoporosis Foundation and International Federation of Clinical Chemistry advocated for the use of the bone formation marker, serum PINP, and bone resorption marker, serum CTX (Vasikaran et al. 2011).

Histomorphometry to Assess Bone Modeling and Remodeling

Historical Perspective

The ability to perform bone histomorphometry only became possible with the advent of embedding, staining, and fluorochrome labeling methods that were greatly advanced during the latter part of the previous century. Methyl methacrylates were first introduced in the 1940s as embedding media for mineralized bone. The use of fluorochrome labeling in human bone began in the 1950s (Milch et al. 1958), when it was discovered that the antibiotic tetracycline incorporated into bone surfaces that were actively mineralizing. Other fluorescent dyes that are not safe in humans, such as alizarin, calcein green, calcein blue, and xylenol orange, are commonly used to label the bone of animals. Sequential fluorochrome dyes can be administered, at which time they bind to newly forming hydroxyapatite, and thereby provide labels,

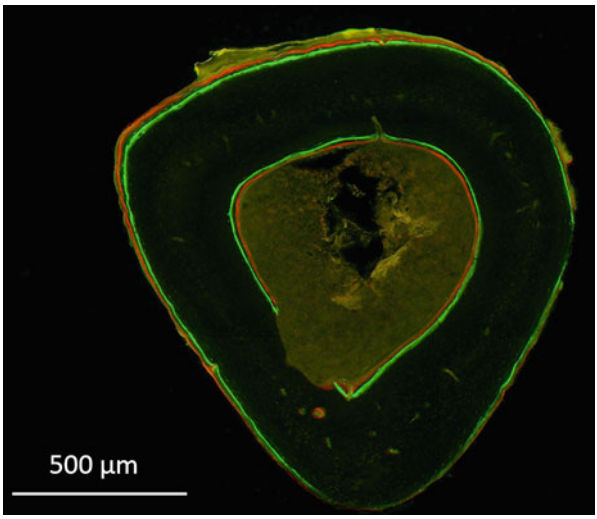

Fig. 2 Fluorochrome-labeled image of the bone. Fluorochrome-labeled histological section of a mouse tibia at the mid-diaphysis, sequentially labeled with calcein and alizarin *red*

which can be used to quantify dynamic active bone formation over a known time period (Fig. 2). For example, mineral apposition rate (MAR) is calculated as the mean distance between the two fluorochrome labels divided by the time between their administration (Frost 1969). A variety of static and dynamic measurements can be made using histomorphometry to assess bone (re)modeling processes. In humans this is most commonly performed on bone biopsies from the iliac crest. It became easier to compare results of bone histomorphometry after efforts were made in the 1980s that continue today, to standardize nomenclature and methods (Parfitt et al. 1987; Dempster et al. 2013). A review of bone histomorphometry can be found in the book, *Bone Histomorphometry: Techniques and Interpretation* (1983), as well as a variety of excellent papers on the topic (Hattner et al. 1965; Frost 1969; Parfitt 1994; Erben and Glösmann 2012).

Limitations and Assumptions

Although histomorphometry has greatly advanced our understanding of bone modeling and remodeling processes, it is far from a perfect method. There are a variety of limitations associated with its two-dimensional (2D) nature and many assumptions. For example, label escape can occur when bone remodeling is initiated after the first label is given or remodeling is stopped before the second label was administered, resulting in the presence of only one label. The resorption and, remodeling periods, as well as the activation frequency are all histological measure of bone turnover (Hernandez et al. 1999; Dempster et al. 2013), which are inferred indirectly from two-dimensional formation velocity perpendicular to the cutting direction. These measures are estimated histologically (MAR) by assuming a steady state in which fractions of space are equal to fractions of time (Eriksen et al. 1984a, b; Parfitt et al. 1987). This central assumption that remodeling is in a steady state (new BMUs form at a constant rate and do not change over with time) prohibits the accurate

assessment of transient alterations in remodeling. Specifically, histomorphometry allows the assessment of formation velocities in a system, which is in a steady state, meaning that changes occur at the same rate. In response to mechanical signals or to drug administration, differentiation between a fast response and a time delay in the response is not possible, due to its averaging character. Temporal subdivisions of bone turnover and the duration times of formation, resorption, and quiescence period are not determined with conventional histomorphometry methods but are to date inferred from fluorochrome label-based formation velocity measures.

Several attempts have been made to differentiate between bone modeling and remodeling using histomorphometric methods (Hattner et al. 1965; Frost 1969; Erben 1996; Dempster et al. 2013). These studies classify fluorochrome-labeled formation surfaces in 2D histological slides as either modeling or remodeling driven, based on the shape of the underlying cement or reversal lines. Scalloped reversal cement lines are assumed to indicate a prior resorption of the bone; a smooth resting cement line (arrest lines) is interpreted as modeling-based formation sites. However, the assumption that a smooth cement line reflects modeling has so far not been confirmed experimentally. Additionally, this method disregards half of modeling, since only activation of formation is quantified, but not resorption.

While fluorochrome labeling allows dynamic measurement of bone apposition, measures of resorption are limited to identifying a scalloped or eroded surface, which may or may not have active resorption occurring. Resorption has also been detected using stereological methods, but these methods have remained relatively unpopular as they are quite labor intensive. More commonly, tartrate-resistant acid phosphatase (TRAP) staining of osteoclasts is used to identify bone resorption. However, TRAP staining is a static measure that does not allow investigation of temporal changes or the spatial, 3D distribution, and volume of resorption. Serial block face imaging has more recently been reported, allowing quantification of number and size of resorption cavities (Slyfield et al. 2012). However it is limited to measuring small sample volumes, resorption cavities are identified with the presence of an eroded surface, it allows examination of only one time point within a particular specimen, and it is not commercially available.

Although histomorphometry is still considered the gold standard in evaluating bone (re)modeling, microCT has largely replaced histomorphometry to assess bone microarchitecture. While in histomorphometry, structural parameters are derived from stereologic analysis of a few two-dimensional sections, which assumes an underlying platelike structure, microCT is able to measure three-dimensional volumes. A more detailed description of microCT evaluation of bone structure can be found in the next section.

Micro-Computed Tomography of the Bone

MicroCT has become an essential tool for investigating static 3D bone microstructure and quality (Bouxsein et al. 2010) in preclinical small animal models and in human bone biopsies and cadaveric samples. It is a valuable method to assess

metabolic bone diseases and test the efficacy of antiresorptive and anabolic therapeutics. In addition to providing static measures, since it is a noninvasive technique, microCT can be used to monitor structural changes of the bone in longitudinal studies of living mice and rats. Longitudinal in vivo microCT enables following the complex bone restructuring processes in a single animal using a 4D approach with spatiotemporal resolution. This approach also provides more internally consistent data and reduces the number of animals needed in preclinical studies. This in vivo imaging method is particularly useful to detect the onset of a disease, monitor the progression of this disease, or the response to therapy over time. The obtained quantitative measures change in response to maturation and aging or disease because of pharmacological treatment or environmental conditions.

Fundamentals and Historical Perspective

MicroCT is based on the interaction of X-rays, which are electromagnetic waves, with matter (key facts). An X-ray beam traversing an object can be attenuated; hence its amplitude changes. In the range of hard X-rays, which are mainly used in X-ray computed tomography, three main processes contribute to this attenuation: the photoelectric effect and inelastic (Compton) and elastic (Rayleigh) scattering. The most common and technically direct mode of X-ray scanning is attenuation-based imaging. The basic mechanism behind this imaging approach is the generation of a shadow graph which is quantitated by detecting the reduction in the local X-ray intensity. The total attenuation of the beam corresponds to the total contribution of these processes, given by the linear attenuation coefficient μ, which is established by the Beer-Lambert law stating that the intensity I of a monochromatic beam with a given energy E traversing a homogenous object diminishes exponentially, as a function of the path length x and the material's linear attenuation coefficient μ:

$$I = I_o \times e^{-\mu x},$$

where I is the X-ray intensity detected by a detector pixel after passing through the specimen with the thickness x and I_o is the incident X-ray intensity of the same beam. The microCT technology is based on X-ray computed tomography, which enables the nondestructive visualization of the 3D internal structure of an object, producing 3D images representing the X-ray attenuation distribution of the object. The attenuation at each point in the object can be reconstructed from several measurements along a series of paths.

Tomography became feasible with the development of computer technologies in the 1960s. However, the idea on which tomography is based was developed in 1917, by the mathematician Radon. He proved that the distribution of a material property in an object layer can be determined from the integral values along any number of lines passing through the same layer (Radon 1971). The first experimental medical applications of tomography were carried out by the physicist Allan M. Cormack (1963), and in the 1970s, the English engineer Godfrey Hounsfield developed the first CT scanner

(Hounsfield 1973). In the 1980s, the application of the tomography was then extended to research, allowing higher energies, resolution, and longer exposure times, since patient safety is of lower priority in these applications (Elliott and Dover 1982). These systems provide an increased resolution, at the cost of a much smaller field of view and corresponding sample size. The X-ray attenuation-based CT technology allows acquisition of images of the internal structure of bones, as CT provides a great contrast of the bone to soft tissue. Therefore, new biomedical developments of this technique were often first implemented in the bone research field. The application of microCT to investigate the bone at a high resolution was introduced by Feldkamp et al. (1989) in 1989, who build a microCT scanner for the evaluation of the 3D microstructure of trabecular bone. In 1994, the first commercially available bone microCT scanner was introduced (Rueegsegger et al. 1996). Besides the higher resolution, these microCT systems provide several advantages over clinical CT systems in a research setting, as imaging protocols can be adapted more freely and the raw data are readily available for post-processing. Since this time, microCT has changed from an experimental technique available to a few research groups to a standard tool in bone research. Today, the technology is the gold standard for the evaluation of static bone morphology and microarchitecture in mice and other small animal models (Bouxsein et al. 2010). MicroCT scanners are available from numerous manufacturers providing several different scanner types for various applications ranging from in vivo measurements to the analysis of bone tissue at the micrometer scale.

Limitations

CT is very suitable to image the bone, as the X-ray attenuation coefficient of the calcium in the bone is much higher than that of the surrounding soft tissue. Thus, when discussing CT imaging of the bone, only the mineralized bone is meant. In desktop microCT systems, an X-ray tube emits a spectrum of photon energies. This polychromatic electromagnetic beam consists of energies typically in the range of 20–100 kV. Passing through the sample, the X-ray photons are attenuated by absorption or scattering effects, depending on the energy. Low-energy X-rays ($<$50 keV) interact mainly by the photoelectric effect depending on the atomic number of the material. Differentiation into the bone and marrow is best at low energies; however, because of the high total attenuation of the X-rays, only small objects can be measured at low energies, as otherwise noise hinders a quantitative analysis. Accurate data interpretation requires knowledge concerning all possible sources of artifacts. Signal noise is a key issue in microCT imaging, as the small spot size of the X-ray source limits source power and X-ray flux. Further negative impact on the density estimation can be attributed to the cone-shaped beam geometry used in microCT and beam divergence (Chappard et al. 2006). Other imaging artifacts are characteristic for CT, such as ring artifacts, caused by the interplay of incremental rotation of the object-camera geometry with nonhomogeneity of the X-ray detection and beam hardening. For a monochromatic beam, the X-ray attenuation coefficient has been shown to be proportional to the bone tissue degree of mineralization

(Nuzzo et al. 2002b). Beam hardening results from the polychromatic X-ray emission of the microCT source, as the X-ray beam energy spectrum shifts progressively higher when passing through solid; low-energy photons interact preferentially with the solid. This leads to an underestimation of the attenuation coefficient of the material toward the center of the object. To minimize this effect, compensation software and hardware filter should be applied (Meganck et al. 2009). The use of polychromatic microCT as a method to measure mineral density has become more accepted with the use of density calibration, the beam hardening correction, and the implementation of advanced image acquisition and reconstruction protocols (Nuzzo et al. 2002a). After acquisition, the data sets are converted into images; this image reconstruction solves an inverse problem by mapping the cone beam projection data to a 3D matrix corresponding to a discretized, tomographic model of the sample. Two major types of reconstruction algorithms exist, filtering back projection-based algorithms and iterative algorithms (Herman 2009). MicroCT scanners must be calibrated regularly against a hydroxyapatite (HA) mineral phantom and for determining in-plane spatial resolution.

Longitudinal In Vivo MicroCT of Rodents

With the development of in vivo microCTs in the 2000s, it became possible to follow structural changes over time in bones of a living, anesthetized rodent (David et al. 2003; Waarsing et al. 2004a). In vivo scanners have a different scanner geometry compared to ex vivo machines; the object remains stationary during imaging, while the X-ray source and detector are mounted on a gantry that rotates around the sample. This scanning setup is the same as for a clinical CT scanner; however the resolution goes down to below 10 μm. In contrast to ex vivo applications, in vivo imaging requires some special preparations. Imaging is generally limited to rodents, due to size of the borehole in current preclinical scanners. The animal has to lie comfortably during a scan, e.g., in a mouse bed with minimal X-ray absorption (Fig. 3 left). Temperature must be controlled to maintain the animal's body temperature during a scan. Anesthesia needs to be properly administered to have the animal remain anesthetized for the entire duration of the scan. Spatial and temporal resolution of an imaging modality must match the size of the expected measurement. MicroCT has several advantages over histomorphometry; however, the in-plane resolution of microscopic techniques is in general greater. Mice bones are approximately 20 times smaller than those of humans (Duyar and Pelin 2003; Somerville et al. 2004). For example, the cortical thickness at the tibia mid-diaphysis reaches a peak of 230 μm at 6 months and declines afterward to 185 μm at 2 years of age (Halloran et al. 2002), whereas the cortical thickness at the metaphysis is on average only 190 μm. Trabecular thickness (Tb.Th) is even smaller in mice; it increases from 60 μm in 12-week-old mice up to 80 μm (Buie et al. 2008). Therefore a small voxel size is preferred, and current resolution of approximately 10 μm is sufficient.

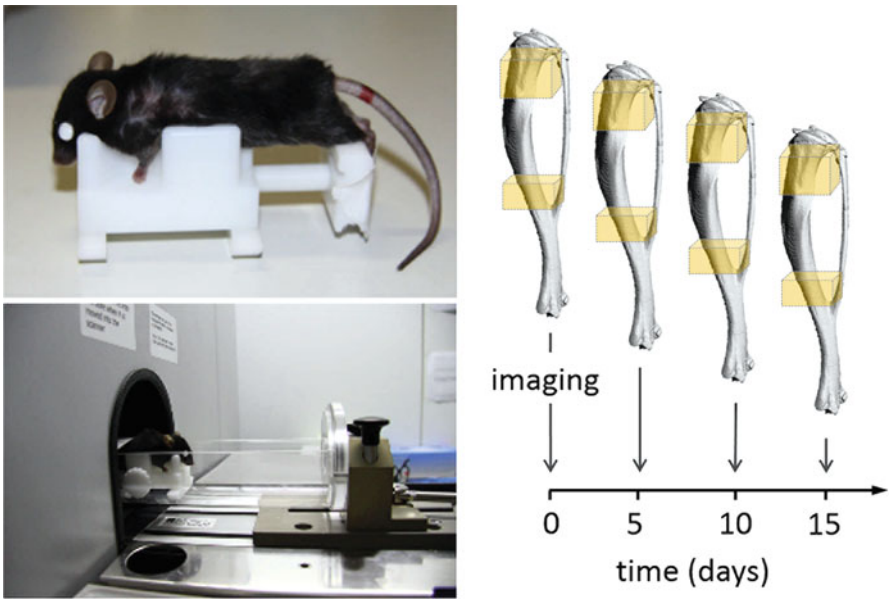

Fig. 3 Micro-computed tomography imaging of mouse bone. *Left*: In vivo imaging of a mouse. *Left top*: During imaging, the animal has to be fixed, for example, in a mouse bed. *Left bottom*: The mouse remains stationary during imaging, while the X-ray source and detector rotate. *Right*: Possible scan regions and imaging protocol to assess bone remodeling in mice

Radiation Effects

Ionizing radiation delivered during an in vivo microCT scan, especially in longitudinal studies where animals are scanned multiple times, may cause unwanted side effects to the tissues, to physiological processes being investigated, or to the animal in general. X-ray exposure results in direct disruption of chemical bonds and generates super radicals (Boone et al. 2004), which may affect cell reproduction and control (Ford et al. 2003). Several studies showed that the magnitude of a single exposure, the frequency of the exposures, and the photon energy of the exposure have an impact on the severity of the biological reaction to the radiation (Boone et al. 2004; Carlson et al. 2007). Furthermore, effects of radiation depend on several factors such as animal age or genetic strain (Klinck et al. 2008). The radiation exposure reported by Waarsing et al. (2004a) investigating dynamic structural changes in a rat hind limb bone (0.4 Gy for a single 20 min microCT scan at 10 mm voxel size) is not expected to have a significant effect on bone cells; however multiple exposures may result in greater effects. Klinck et al. (Klinck et al. 2008) performed weekly in vivo microCT scans of the proximal tibia of rats and mice starting at 12 weeks of age and compared the irradiated with the contralateral nonirradiated limbs. They found trabecular bone volume to be 8–20% lower due

to radiation (0.5 Gy per scan). However, they found no effects of radiation on the animals' general health. We investigated the effect of radiation on trabecular bone in young (10-week-old) and adult (26-week-old) mice tibiae, by comparing morphometric parameters of animals being radiated four times with a time lapse of 5 days to animals being radiated only once (Willie et al. 2013). Our data showed that radiation has affected the trabecular volume and trabecular separation in the young animals. No other microCT or histomorphometric parameters showed differences between the repeatedly radiated group and the single-radiated group in the young mice, and no differences were observed in any measured parameters for the adult mice. In contrast, Buie et al. (2008) reported no differences in trabecular BV/TV from the proximal tibia of 6-week-old female C57Bl/6 J mice that underwent 12 scans compared to 6 scans on a weekly basis. Therefore, studies performing repeated in vivo microCT analyses should consider these effects, especially when examining skeletally immature animals.

4D MicroCT Data as an Imaging Biomarker of Bone Turnover

An automated evaluation of longitudinal in vivo microCT is achieved by combining 3D image registration, automated volumetric segmentation, and morphological evaluation to visualize and volumetrically quantify spatial bone (re)modeling events on bone surfaces of living animals (Schulte et al. 2011, Birkhold et al. 2014a, b). This method identifies quiescent bone volumes, resorbed bone volumes, and formed bone volumes. Moreover, this computational method enables the extraction of the temporal character of (re)modeling by analyzing the underlying spatiotemporal processes. By tracking bone (re)modeling events on the bone surface over time to identify bone (re)modeling sequences, bone turnover can be analyzed in a temporal manner (Birkhold et al. 2015). This allows one to study the temporal character of resorption processes, the kinetics and balances of resorption and formation processes, and the extraction of the periods of the underlying (re)modeling sequences. With these recent methods, detailed information on biological processes can be provided, in addition to or instead of standard histomorphometry, which is expected to lead to a more complete analysis of bone biology. By being able to monitor the target bone and study 3D (re)modeling longitudinally, the imaging method provides new experimental readouts. 4D microCT provides a possible platform for future drug screening in translational or clinical studies as continuous monitoring of bone surfaces can be performed in vivo, which will improve the understanding of bone (re)modeling dynamics in human disease and may ultimately yield new therapies. A detailed description of the methods is provided in the following subsections ("Image Acquisition and Temporal Resolution," "Image Registration and Fusion," "Image Segmentation," "Three Dimensional Analysis of Bone Morphology," "4D Analysis of Spatio-Temporal Bone Remodeling Processes," "Surface Event Identification, Visualization and Quantification," and "Bone Surface Monitoring").

Image Acquisition and Temporal Resolution

Visual and quantitative information of bone (re)modeling can be extracted from image data acquired at different time points (Fig. 3, right). When choosing the time points, one must consider the required temporal resolution, the interval of time between imaging sessions. To extract dynamic bone formation and resorption indices of longitudinal microCT images, the temporal resolution of the method must be adjusted to the temporal character of the physiological processes. This includes the velocities of the processes (formation and resorption) and the individual durations of the processes. An indicator of the time frame of structural processes can be estimated from what we know concerning the mean active life span of the bone cells involved. The mean active life span of a murine osteoblast is thought to be between 10 and 14 days (Weinstein et al. 1998) and the mean active life span of a murine osteoclast is around 3 days (Weinstein et al. 2002). Weinstein et al. (1998) reported a remodeling period of about 2 weeks in mice. The activation frequency is reported to be approximately 10*/year* in mice (Gonzalez et al. 2002); however this was assessed with histological techniques, which cannot assess resorption and reversal period directly. Human cells have a life span of 90 days (osteoblasts) and 21 days (osteoclasts) (Parfitt 1998). To resolve these processes, an interval of 4 weeks was used for rat bone (Waarsing et al. 2004a). For mice, which have a faster (Dempster et al. 2013) metabolism, a time lapse of 5 days to 1 week is sufficient to reveal formation and resorption processes (Schulte et al. 2011; Birkhold et al. 2014a, b).

Image Registration and Fusion

High resolution and in vivo application make a reproducible positioning and orientation of the bone during image acquisition unachievable. Consequently, methods to fuse these data sets into one 4D data set with consistent spatial information are necessary. This spatial alignment of image data sets is done using registration techniques (Oliveira and Tavares 2014). A registration is an optimization problem, which requires the maximization of a measure of similarity or minimization of a measure of difference. The aim of this procedure is to find the geometric transformation T between the data sets obtained at different imaging procedures. It involves several components: a transformation model, a similarity metric, an optimization method, and a validation protocol (Deserno 2011).

The transformation model defines the geometric transformation between the images; several types of transforms including rigid, affine, and deformable transforms may be used. For the 4D analysis of bone (re)modeling, rigid transformations have been implemented (Waarsing et al. 2004a; Schulte et al. 2011; Birkhold et al. 2014a, b). As target coordinate system, usually the local coordinate system of the first image is chosen, although the results of the registration are independent of the coordinate systems. A similarity metric measures the degree of alignment between

the images. Feature-based or intensity-based similarity measures are commonly used in medical image registration. As the aim of the 4D analysis of the bone is to extract temporal changes of the bone, which is restructuring over time, features such as landmarks, edges, or surfaces are not an ideal measure of alignment. Therefore, the image intensities representing the local mineralization dynamics are used to determine the matching of the images. This approach has the advantage that the mineralizing kinetics of the bone are included in the registration approach, as the newly formed bone has a lower density and the existing bone gets demineralized before it resorbs (Lukas et al. 2013). In medical image processing, a commonly used intensity-based similarity measure is the normalized mutual information (Wells et al. 1996; Pluim et al. 2003), which has also been introduced for time-lapsed bone imaging (Waarsing et al. 2004a) and has been validated for alignment of the vertebra (Schulte et al. 2011) and metaphyseal (Birkhold et al. 2014a) and diaphyseal (Birkhold et al. 2014b) bone of the lower limb. Furthermore, an optimization method that maximizes the similarity measure is applied. To reduce the risk of finding local minima instead of the global minimum, hierarchical strategies have been proposed, starting at a coarse resampling of the data sets and proceeding to finer resolutions (Thévenaz et al. 1998). The process requires interpolations of the original intensities to produce a reoriented image. Commonly applied interpolation methods are nearest neighbor interpolation, linear interpolation, and B-spline or Lanczos interpolation (Meijering 2000). The applied interpolation affects the intensity information, especially as it is required twice, or the search for the optimal transformation matrix and for the actual transformation of the image. Therefore, a validation protocol that measures the performance of the registration techniques is needed (Deserno 2011). Following the registration and transformation into the reference coordinate system, the data sets are fused to obtain one 4D data set including spatial and temporal information (Fig. 4).

Fig. 4 Image registration and fusion. Consecutive acquired images of the bone are aligned and fused in a registration procedure. The resulting four-dimensional data sets include spatial and temporal information

Image Segmentation

To enable a quantitative analysis, the acquired sets have to be segmented. Segmentation, the algorithmic delineation of objects, is necessary to extract structures of interest from the raw data and to generate 3D models for visualization and further analysis. Quantitative parameters like volumes can only be extracted from segmented objects. Segmentation of anatomical objects is one of the key steps in medical image processing. Automating this process is a challenging task, as segmentation quality depends on contrasts, imaging-related problems like noise or artifacts, and the anatomical variability of tissues. Another challenge is biological variation between structures, especially of trabecular bone, as its microstructure differs strongly between different subjects and changes tremendously with aging. However, an automated segmentation has to produce similar results for different groups. Consequently, segmentation algorithms must be robust against geometric changes. Bone segmentation algorithms consist of several steps: filtering, thresholding, and segmentation into trabecular and cortical compartments and further subregions, such as segmentation into endocortical and periosteal compartments or bone surface extraction to track surface changes over time.

The application of image preprocessing steps, such as filtering to reduce statistical noise, improves the quality of the image data and the content of information included by reducing the impact of noise. This facilitates the later image processing steps, especially the visualization of structures. Removal of image noise requires a low-pass filtering; however this blurs the image. In contrast, edge enhancement has achieved a high-pass filter, which may result in increased noise. In general, a Gaussian filter balances these competing goals.

Thresholding

Quantitative measures are in general calculated from binary images; therefore thresholding transfers gray value images into bone and non-bone (background such as soft tissue and fluids) voxels. A threshold is an intensity value that represents the gray value, and a voxel in the image should exceed to be considered a voxel of the relevant tissue, here the bone.

Global thresholding applies the same threshold value to every voxel in the image. This global threshold value is often determined by visually selecting an appropriate threshold; however, automatic threshold selection methods exist as well. Enabling a more robust selection of the threshold value, these methods are mostly based on analysis of a histogram of image intensities (Buie et al. 2007; Burghardt et al. 2007). Depending on the analyzed bones, it further may make sense to use specimen-specific thresholds or to apply different thresholds to different regions of the bone, e.g., trabecular and cortical. Local adaptive threshold using the gray values of neighborhood voxels (Dufresne 1998; Burghardt et al. 2007) or the local gradient to identify bone marrow edges (Waarsing et al. 2004b) may be used as well. However, applying a fixed global threshold represents the most widely used technique in segmentation into the bone and background (Bouxsein et al. 2010). With the

high ratio between the bone and marrow X-ray attenuation, a global threshold results in most cases in a good delineation of bone form background. In all cases, the threshold value has to be selected carefully, as it has a significant impact on the results.

Automated Segmentation into Different Regions for Separate Analysis

Microstructure, as well as physiological activity, varies between different regions of the bone. Cortical bone is less active than trabecular and endocortical, and periosteal regions may show different remodeling activities. Therefore, separate analysis of these compartments is necessary; consequently, they have to be separated in the images, which can be achieved via segmentation (Fig. 5).

To date, the segmentation gold standard is a semiautomated slice-by-slice hand contouring approach (Bouxsein et al. 2010). This segmentation is a very important, however also very time-consuming, task. Moreover, the user interaction introduces a subjective element to the image analysis. Thus, to achieve high accuracy and precision, a high degree of automation is desirable. However, this is challenging because the bone can have such a varied appearance. Semiautomated algorithms based on region growing, energy-minimizing spline curves, and deformable model methodology (Gelaude et al. 2006) or using distance maps (Dufresne 1998) have been introduced as well. Another promising approach for fully automated segmentation is to use the mathematical morphology (Buie et al. 2007; Kohler et al. 2007), as this method has been shown to be robust against microstructural variations in trabecular morphometry (Birkhold et al. 2014a).

Three-Dimensional Analysis of Bone Morphology

This section provides an overview of the most common morphometric indices that can be derived from the 3D images. The 3D analysis of static parameters does not

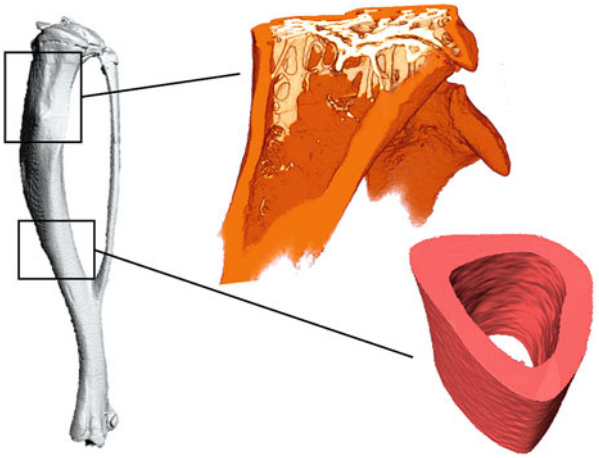

Fig. 5 Segmented microCT images of a mouse bone. Micro-computed tomography images of the tibia of a mouse. *Left*: Full bone. *Right*: Segmented regions. *Right top*: Proximal region segmented into trabecular and cortical compartment. *Right bottom*: Segmented mid-diaphyseal region

require registration; although it has been shown that alignment of consecutive images improves the reproducibility of bone morphometric indices (Nishiyama et al. 2010). A comprehensive review of the morphometric parameters that can be extracted from bone microCT images has been published by Bouxsein et al. (2010).

The morphometric analysis of the bone includes analysis of trabecular and cortical bone compartments for architectural parameters derived from histomorphometry. However, the microCT analysis is based on 3D data sets. The commonly assessed 3D parameters, which have been adapted from previous 2D histomorphometric parameters (Parfitt et al. 1983), for the trabecular bone region include relative trabecular bone volume (*BV/TV*), trabecular thickness (*Tb.Th*), and trabecular separation and number (*Tb.Sp, Tb.N*). The commonly assessed parameters for the cortical bone region include total cross-sectional area (*Tt.Ar*), marrow area (*Ma.Ar*), and cortical bone area (*Ct.Ar*); maximum (*Imax*), minimum (*Imin*), and polar (*J*) area; moments of inertia; cortical bone area fraction (*Ct.Ar/Tt.Ar*); and cortical thickness (*Ct.Th*). If image resolution is sufficient, cortical porosity can also be reported.

4D Analysis of Spatiotemporal Bone Remodeling Processes

The best approach to study morphological changes of anatomic features and physiological processes is to visualize and quantify their dynamics in four dimensions (4D), which means, in 3D space and over time. Recently, 4D in vivo microCT experiments have been performed to study bone adaptation in animal models using tomographic sequences of a few minutes exposure time per image interrupted by longer nonexposure time spans (Waarsing et al. 2004a; Boyd et al. 2006; Willie et al. 2013). This method allows one to follow the relatively slow dynamics and morphological changes during bone (re)modeling with 10 μm resolution over total time intervals of several days in the living animal. Combining the information of these time-lapsed images enables one to quantify spatiotemporal morphological processes (Schulte et al. 2011; Birkhold et al. 2014a, b) and to track pattern-forming mechanisms over time (Birkhold et al. 2015). This approach enables us to gain insights into 4D mechanisms at the level of individual sites of formation and resorption. It allows us to understand of how the bone acquires its architecture and to understand the effect of agents such as gene products or drugs on bone structure.

Surface Event Identification, Visualization, and Quantification

In spatially fused and segmented images, temporal-structural changes on the bone surfaces can be identified, localized, visualized, and further quantified. Comparing data sets of the same region of one bone acquired at different time points enables one to detect structural changes that occur over time. To identify whether a bone surface is forming, resorbing, or quiescent, the detected changes can be classified by identifying if a certain voxel appeared or disappeared over time. If a voxel changes

from being classified as background into being classified as the bone, it is considered as the newly formed bone. In contrast, if a voxel changes from being classified as the bone into being classified as background, it is considered as the resorbed bone. Voxels that have been classified as the bone and remain bone are defined as the quiescent bone. From the obtained matrices, 2D and 3D models visualizing the distribution of the formation and resorption patches on the bone surfaces are created (Fig. 6). An early approach was to create cross-sectional images comparable to histological images (Waarsing et al. 2004a). However, nowadays 3D surface and volume rendering (Schulte et al. 2011; Birkhold et al. 2014a, b) or 4D video data including temporal changes over time have been used to visualize bone (re)modeling (Birkhold et al. 2014a, b). For quantification, 3D dynamic in vivo morphometry further describing the shape of individual's newly formed bone packets and resorption cavities has been introduced. From the voxel-based model, therefore volumes, surface areas, and distribution of thicknesses and depths of the three processes, formation, resorption, and quiescence, can be calculated.

Several parameters quantifying the formation dynamics can be extracted (Fig. 7), such as (Schulte et al. 2011; Birkhold et al. 2014a, b):

- Volume of the newly formed bone normalized to the total bone volume (mineralized volume: *MV/BV*)
- Surface area, where this formation took place, normalized to the total bone surface area (mineralizing surface*: MS/BS*)
- Distribution of thickness of the patches of the newly formed bone (mineralizing thickness: *MTh*)

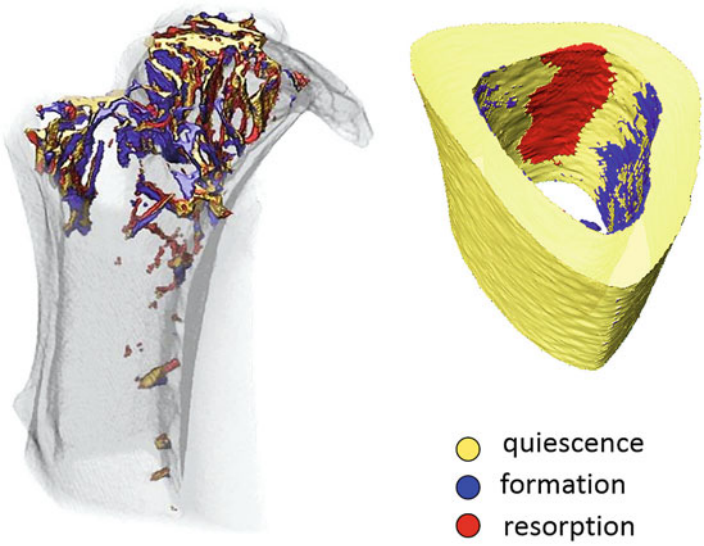

Fig. 6 Visualization of bone remodeling . Visualization of bone formation (*blue*) and resorption (*red*) patches at the trabecular bone of the metaphyseal mouse tibia

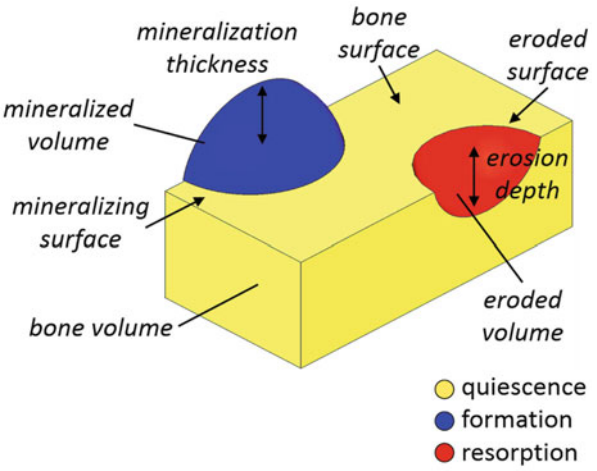

Fig. 7 Quantification of bone remodeling . Dynamic in vivo morphometry measures enabling a quantification of bone formation sites and resorption cavities in a three-dimensional manner

- Rate of bone volume formed over a certain period of time (3D bone formation rate: *3D BFR*)
- Rate of bone formation perpendicular to bone surface (3D mineral apposition rate: *3D MAR*)

In the same manner, parameters quantifying the resorption dynamics can be extracted, such as:

- Volume of the resorbed bone normalized to the total bone volume (eroded volume: *EV/BV*)
- Surface area, where this resorption took place normalized to the total bone surface area (eroded surface: *ES/BS*)
- Distribution of depths of the resorption cavities (erosion depth: *ED*)
- Rate of bone volume resorbed over a certain period of time (3D bone resorption rate: *3D BRR*)
- Rate of bone resorption perpendicular to bone surface (3D mineral resorption rate: *3D MRR*)

Bone Surface Monitoring

We recently introduced an approach to include the temporal character of local (re) modeling sequences in the investigation of bone restructuring based on tracking the position of the bone surfaces over time (Birkhold et al. 2015) (Fig. 8). Based on the identification of spatially correlated but temporally sequenced surface events, the duration of formation and resorption processes can be identified; furthermore the temporal succession of formation and resorption events or the duration of the reversal phase in between resorption and consecutive formation can be determined.

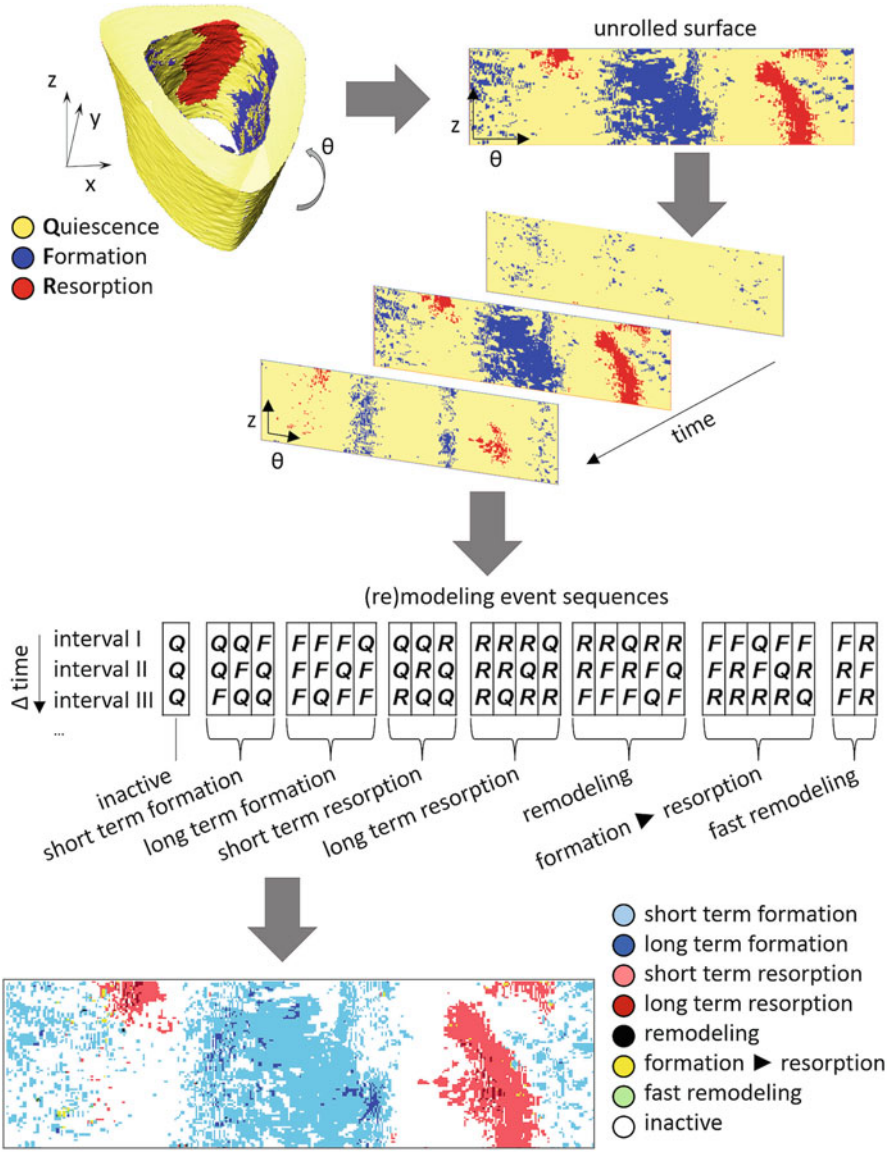

Fig. 8 Bone surface monitoring. Image processing chain to monitor bone surface movements over time. A remodeling map of the bone surface is created by combined evaluation of several consecutive imaging intervals

Consecutive surface events are combined in a temporal fusion process in one matrix to track how (re)modeling occurs site specifically over time. Eight biologically relevant (re)modeling sequences are identified: *(1) inactive*; (2) *short-term formation*; (3) *long-term formation*; (4) *short-term resorption*; (5) *long-term*

resorption; (6) mixed sequences in which resorption precedes formation, which is defined as *remodeling* (Frost 1969); (7) mixed sequences in which first formation and then resorption occurred, *formation ▸ resorption*; and (8) *fast remodeling*, representing 1.5 remodeling cycles. Graphs representing the unrolled and flattened bone surfaces visualize the spatial distribution of (re)modeling sequences on the bone surfaces, visualizing the probability to find a specific remodeling sequence on the observed bone surface. Number and size of individual processes can be determined using these remodeling maps.

Another aspect of bone restructuring is the contribution of modeling and remodeling processes to the total surface changes. Using 4D images a differentiation into modeling (independent formation or resorption) and remodeling (resorption followed by formation) is feasible. However, for a differentiation in line with the histological definition of modeling/remodeling long time frames of monitoring would be necessary, as histology-based modeling is defined as formation on a surface on which never in the whole life of the individual has a previous resorption event occurred.

Potential Applications to Prognosis, Other Diseases, or Conditions

Preclinical Applications of the Method

Four-dimensional microCT as an imaging biomarker of bone turnover has the strengths of 3D/4D visualization of spatiotemporal processes and in-depth quantification of the underlying (re)modeling processes. In addition to achieving 3D measures similar to traditional 2D histology, this computational approach enables several new ways to examine bone (re)modeling. These novel insights include the temporal character of resorption processes, the kinetics and balances of resorption and formation processes, and the extraction of the underlying (re)modeling sequences. This approach was first introduced in 2004 to visualize structural changes occurring in between two time-lapsed scans in trabecular rat bones (Waarsing et al. 2004a). Recently, more detailed 3D dynamic in vivo morphometry of formation and resorption processes has been performed to investigate bone adaptation processes, including studies examining trabecular bone in mice, including at the vertebrae (Schulte et al. 2011) and tibiae (Birkhold et al. 2014a). Cortical bone adaptation (Birkhold et al. 2014b) and bone osseointegration around implants (Kettenberger et al. 2014) has been analyzed. The method further allows detailed analysis of mechanoregulatory processes (Schulte et al. 2013; Razi et al. 2015). More detailed analysis of the duration of modeling-based formation and resorption processes during adaptation (Birkhold et al. 2015) and mineralization (Lukas et al. 2013) has also been performed.

The 4D analysis technique offers many advantages over standard microCT and histomorphometry analysis, as it also enables accurate detection, tracking and quantification of the magnitude, and time course of narrowly distributed structural

changes, which cannot be accurately quantified using histological or standard microCT-based methods. Besides increased efficacy, the automated method has other critical benefits, such as improved precision. The replacement of subjective manual evaluations enables a better comparison of findings across different studies and a higher throughput of data. Moreover, detailed information on the dynamics of biological processes can be provided, either in addition to or instead of standard histomorphometry, which is expected to lead to a more complete analysis of bone biology. The computational methods can be used for the detection and improved localization of bone (re)modeling events and sequences, e.g., in bone disease models, preclinical application to test new therapies or in other bone restructuring scenarios. Longer time intervals will possibly allow in future applications to investigate full remodeling cycles and to differentiate between modeling and remodeling. The method, thus, is not intended to completely replace histomorphometry but concentrates on an important, single, and precisely specified aspect in bone biology: the spatiotemporal dynamics of the process that is occurring.

Clinical Applications of the Method

Methodological innovations in clinical practice often rely on the development and existence or evolving technologies from basic research. The declining radiation dose of high-resolution CT imaging allows one to apply the novel computational approaches used in mice to human bone. To translate and apply this technique to the clinic requires the ability to resolve the spatiotemporal processes with tomography systems suitable for imaging of humans, with respect to image size and resolution as well as radiation dose. The rate of bone turnover of the whole human skeleton is only 10% per year (Manolagas 2000). In contrast to 0.7% per day in mice (Weinstein et al. 1998), even the rapidly (re)modeling trabecular bone has only a turnover rate of 0.1% per day in humans. Slower turnover along with a longer life span of osteoblasts (3 months) and osteoclasts (2 weeks) results in a tremendously longer remodeling period in human bone (Parfitt 1994). This is beneficial, in that it allows for a longer time interval between the imaging sessions for human bone and would therefore reduce the necessary doses of ionizing radiation. The computational 4D methods enable a fast translation to human bone without significant adjustments. Recent advances in HR-pQCT technology allow the application in clinical research, as novel scanners are designed to measure the static bone density and to quantify the static 3D human bone microarchitecture at the distal tibia and radius for the clinical in vivo assessment of osteoporosis. Human bone in a research setting has recently been published (Christen et al. 2014). Unfortunately, HR-pQCT is currently primarily used as a research method and is not widely available. Although it has many advantages over dual energy X-ray absorptiometry, it has higher doses of radiation. Combining the current application of standard structural measures with the novel dynamic measures could result in a powerful tool for fracture risk assessment in humans in the future.

Summary Points

- There is a clinical need to better understand how loading conditions, diseases, synthetic implants, and drug treatments affect bone formation and resorption.
- Changes in bone formation and resorption, termed bone turnover, have enormous impact on the quality and mechanical competence of the skeleton.
- Current methods of quantifying bone formation and resorption, biochemical markers of bone turnover, and histomorphometry have a range of limitations.
- Computed tomography can be used to monitor structural changes in the cortical and trabecular bone of living animals and human patients.
- This chapter aims to describe recently developed methods that use 4D microCT data as an imaging biomarker of bone formation and resorption to monitor bone (re)modeling.
- Detailed spatiotemporal analysis of cortical and trabecular bone dynamics allows to better understand changes that occur with age, disease, sexes, and the effects of treatment.

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Spine Bone Texture and the Trabecular Bone Score (TBS)

26

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Abstract

Trabecular bone score (TBS) is a gray-level texture measure that can be extracted from two-dimensional lumbar spine dual-energy X-ray absorptiometry (DXA) images and provides skeletal information beyond standard bone mineral density (BMD) measurement. This chapter reviews scientific and clinical evidence on the development, validation, and clinical application of lumbar spine TBS. TBS should not be used alone to determine treatment recommendations, but can be used in association with the World Health Organization fracture risk assessment tool (FRAX) to adjust FRAX probability of fracture in postmenopausal women and older men and additionally guide decisions regarding treatment initiation.

Keywords

Osteoporosis • Fracture • Trabecular bone score • Bone texture • Dual-Energy X-ray Absorptiometry

Key Facts About Tbs (For The Lay Person)

- Lumbar spine trabecular bone score (TBS) is a bone texture measurement obtained from bone density scans of the lumbar spine, a routine medical procedure for osteoporosis assessment.
- TBS provides information about bone structure in the spine that reflects bone strength and risk for fracture.
- TBS alone should not be used to make treatment decisions, but can provide an improved estimate of fracture risk when used in conjunction with the World Health Organization Fracture Risk (FRAX) tool.

Definitions of Words and Terms

Biomarker:	A distinctive biological or biologically derived indicator of a process, event or condition (as aging or disease).
Dual-energy X-ray absorptiometry (DXA, DEXA):	A means of measuring areal bone mineral density (BMD) utilizing two X-ray beams with different energy levels. The two energies exploit differential absorption by soft tissue and bone.
Osteoporosis:	World Health Organization: "A disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk." NIH Consensus Development Panel: "A skeletal disorder

	characterized by compromised bone strength predisposing to an increased risk of fracture."
Predictive biomarker:	Unlike prognostic biomarkers, predictive biomarkers are linked to treatment, as they provide a forecast of the potential for a patient to respond, in some identified manner (which may be favorable or unfavorable), to one or more specific treatments.
Prognostic biomarker:	Provides information on the likely course of disease in an untreated individual. A prognostic biomarker informs about the aggressiveness of the disease and/or the expectation of how a particular patient would fare in the absence of therapeutic intervention.
Response biomarker:	Dynamic assessments that show a biological response have occurred in a patient after having received a therapeutic intervention.
Texture:	A loosely defined term in image processing referring to local variation in brightness from one pixel to the next or within a small region. If brightness is interpreted as surface elevation in a representation of the image, then texture can be considered to reflect surface "roughness," a related term again without an accepted or universal quantitative meaning.
T-score:	Number of standard deviations that BMD is above (positive) or below (negative) the average mean value for a young healthy reference population. The World Health Organization operational definition of osteoporosis is a T-Score ≤ -2.5 based upon a standardized reference site (the femoral neck) and a standard reference range for both men and women (the NHANES III data for women aged 20–29 years).
Z-score:	Number of standard deviations that BMD is above (positive) or below (negative) the average mean value for an age, gender and (where available) ethnicity-matched reference population.

Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength and degradation of bone microarchitecture predisposing to increased fracture risk, particularly of the hip, proximal humerus, vertebra, and forearm (often termed the

"major osteoporotic fracture" sites), though other skeletal sites contribute to the global fracture burden in osteoporosis (Stone et al. 2003). In the absence of a fragility fracture, osteoporosis is typically diagnosed from bone mineral density (BMD) measured with dual-energy X-ray absorptiometry (DXA). The World Health Organization operational definition of osteoporosis is a BMD value that lies 2.5 standard deviations (SD) or more below the average young normal mean BMD value (i.e., a T-Score ≤ -2.5) based upon a standardized reference site (the femoral neck) and reference population (National Health and Nutrition Examination Survey [NHANES] III data for White women aged 20–29 years) (Kanis et al. 1994, 2008; Looker et al. 1998).

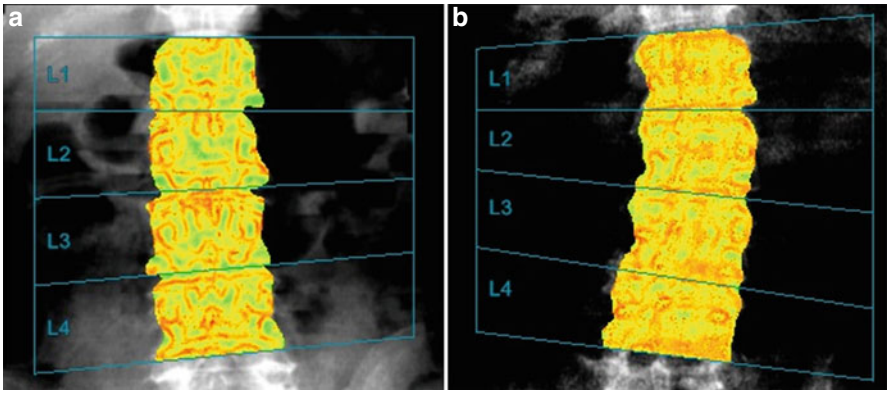
DXA BMD measurement provides a relative estimate of fracture risk, increasing 1.4 to 2.6 fold for every SD reduction in BMD (Johnell et al. 2005; Marshall et al. 1996). Despite the deceptive simplicity of a BMD-based approach to osteoporosis management, most fractures occur in individuals who have a BMD T-score better than -2.5, the cutoff defining osteoporosis (Siris et al. 2004; Schuit et al. 2004; Stone et al. 2003; Cranney et al. 2007). It is therefore apparent that BMD measurement alone does provide not an ideal approach for identifying individuals who will sustain fragility fractures.

Although BMD as measured by DXA is strongly associated with fracture risk, many other skeletal parameters that can be derived from DXA and non-DXA methodologies also influence bone strength and fracture risk, e.g., the macrogeometry of bone, cortical porosity, microarchitecture of trabecular bone, bone microdamage, and bone mineralization. Until recently, there was no clinically available methodology to assess bone microstructure.

Lumbar spine trabecular bone score (TBS), a recently described method for bone texture evaluation, was developed to provide an assessment of skeletal microarchitecture independent of BMD (Fig. 1). TBS evolved into a commercial product (TBS iNsight®, Med-Imaps, France) that was cleared for clinical use in the United States in 2012 with the following labeling: "TBS is derived from the texture of the DEXA image and has been shown to be related to bone microarchitecture and fracture risk. This data provides information independent of BMD value... The TBS score can assist the health care professional in assessment of fracture risk..." TBS can be considered a prognostic and response biomarker as it provides a measurable indication of skeletal structure and strength and also exhibits responses to pharmacologic interventions consistent with the expected change in skeletal strength and fracture risk (Silva et al. 2015). This chapter reviews scientific and clinical evidence on the development, validation, and clinical application of lumbar spine TBS.

Conceptual Aspects of Trabecular Bone Score (TBS)

The concept of TBS arose out of the desire to extract structural information on 3D bone morphology from the 2D projection-based images provided by DXA (Pothuaud et al. 2008). Based upon X-ray absorption, gray-level variations in the



L1-4 BMD = 0.891 g/cm^2
TBS = 1.431

L1-4 BMD = 0.893 g/cm^2
TBS = 1.161

Fig. 1 Sample lumbar spine TBS printouts that are (**a**) low and (**b**) normal. The TBS calculation is performed over the same region of interest as the areal bone mineral density measurement. A low TBS value indicates few gray-level variations of large amplitude and is interpreted as low quality bone texture. These two postmenopausal women have virtually identical L1-L4 BMD and T-score (−2.4). However, their TBS values differ

2D projection-based DXA image are presumed to correlate with absorption properties in the 3D bone volume according to a predictable mathematical relationship. At an intuitive level, higher TBS is intended to reflect more homogeneously textured bone characterized by high frequency/low amplitude fluctuations in gray level. By contrast, less well-textured bone is characterized by lower frequency/higher amplitude fluctuations in gray level. The mathematical details were published in 2008 and exploited the concept of the experimental variogram, which expresses variance in gray levels in the DXA image as a function of distance (Pothuaud et al. 2008). Experimental variograms are also used in nonmedical image processing to characterize spatial variation. For example, in mining a variogram can provide a measure of how much two samples taken from an area vary in gold percentage depending on the distance between the samples. In the skeleton variations are predicted to be greater at larger distances, and it is the slope in this relationship that critically defines the TBS measurement. Specifically, TBS is defined as the slope at the origin in the log-log representation of the variogram expressed as variation in gray levels (Y-axis) versus pixel distance per mm (X-axis) as evaluated from least squares regression (Pothuaud et al. 2008). The slope at the origin reflects the "roughness" in gray levels: the lower the slope, the greater the regularity (homogeneity) in gray levels. A 3D image of a tight network of trabeculae produces a 2D projection image with many gray-level variations of small amplitude and, therefore, greater "roughness" with a steep variogram slope and a high TBS value (architecture associated with good mechanical strength). A low TBS value, in contrast, indicates poor-quality architecture with fewer gray-level variations of larger amplitude and, therefore, a reduced slope at the

origin of the variogram. Importantly, TBS is calculated retrospectively from the DXA image, allowing for the estimation of skeletal texture from a previously acquired scan.

Technical Aspects of Trabecular Bone Score (TBS)

Although TBS is dependent on bone texture, DXA image texture can be affected by factors unrelated to bone. This includes the DXA scan acquisition mode, soft tissue composition overlying bone and differences between densitometer manufacturers; to be clinically useful, the TBS algorithm must accommodate these factors. An in vivo reproducibility assessment in 210 men and women undergoing paired DXA precision assessments on lower and higher resolution DXA scanners found moderately high correlations (72–81% explained variance) but with significant bias offsets that may have been related to image resolution (Krueger et al. 2015). As such, new installations of TBS software require use of a specially constructed phantom during the initial setup and calibration.

The importance of soft tissue composition was highlighted in early investigations of TBS in men versus women. The original TBS algorithm had been optimized for women and paradoxically gave lower TBS measurements in men than in women despite the fact that men have higher BMD and lower fracture risk and would be expected to have a less degraded trabecular structure (Leslie et al. 2014c). The Manitoba BMD Database was used to compare TBS measurements in men and women before and after sequential matching for age, body mass index (BMI), abdominal soft tissue thickness, and abdominal percent fat. Men had lower TBS measurements after age and BMI matching, but not after abdominal tissue thickness and percent fat matching. The presumed mechanism is that image texture tends to degrade (decrease) with increasing adiposity. As adiposity in men tends to be more abdominal/truncal than in women, a simple TBS adjustment based upon BMI would underestimate the effect of abdominal adiposity on the TBS measurement in men.

The TBS algorithm was subsequently modified in version 2.x to address these technical issues and became applicable to both women and men (Leslie et al. 2014d). The clinical performance of this updated algorithm was assessed in the Manitoba BMD Cohort using 47,736 women and 4,348 men. The initial TBS software (version 1.x) showed significantly lower spine TBS in men versus women (1.080 ± 0.145 vs. 1.244 ± 0.127, $P < 0.001$), but this was reversed in version 2.x (men 1.360 ± 0.132 vs. women 1.318 ± 0.123, $P < 0.001$) consistent with the lower fracture risk and higher BMD seen in men. There was a strong negative association between TBS and BMI in version 1.x for men ($r = -0.40$) and women ($r = -0.18$), despite a positive correlation between BMI and BMD at the lumbar spine (men $r = 0.14$, women $r = 0.33$). Under TBS version 2.x for GE/Lunar scanners, the BMD dependency was eliminated (men $r = 0.01$, women $r = -0.01$). Fracture prediction under the version 2.x algorithm was also slightly improved with an increase

in AUC for major osteoporotic fracture prediction of +0.021 for men (AUC version 2.x 0.574, 95% CI 0.535–0.614 vs. version 1.x 0.553, 95% CI 0.515–0.591) with an increase of +0.012 for women (AUC version 2.x 0.640, 95% CI 0.630–0.650 vs. version 1.x 0.628, 95% CI 0.618–0.638). An even larger improvement was seen for hip fracture prediction in men of +0.046 (AUC version 2.x 0.669, 95% CI 0.585–0.753 vs. version 1.x 0.623, 95% CI 0.544–0.703) and women +0.020 (AUC version 2.x 0.699, 95% CI 0.680–0.718 vs. version 1.x 0.679, 95% CI 0.660–0.667). Therefore, the updated TBS algorithm for GE/Lunar is less affected by soft tissue, gives results for men that are consistent with their lower fracture risk, and improves fracture prediction in both men and women compared with the original version. As a result of this BMI dependence, the manufacturers of TBS software recommend that it not be used in individuals with BMI outside of the 15–37 kg/m^2 range.

Interestingly, although BMD measured in the lumbar spine is notoriously sensitive to age-related degenerative spondylosis and other age-related artifact (typically producing overestimation), TBS may be relatively unaffected by these changes. In the largest analysis to date, Kolta et al. (2014) correlated DXA measurements with lumbar spine X-rays where lumbar osteoarthritis was graded according to the Kellgren and Lawrence classification (grades 0–4). Although BMD increased with higher grades of osteoarthritis, spine TBS was no different between those with and without lumbar osteoarthritis (grade 2 or higher) and was not correlated with Kellgren-Lawrence grade. Over 6 years, lumbar spine BMD was unchanged (although there was a significant decrease in hip BMD), while lumbar spine TBS showed a significant decrease over time that was independent of Kellgren-Lawrence grade.

Experimental Data on Trabecular Bone Score (TBS)

In Vitro Validation Studies

TBS was developed in an attempt to find an effective, efficient solution to the routine clinical evaluation of trabecular bone microarchitecture (Pothuaud et al. 2008). Correlations have been reported between gray-level variation in DXA images as reflected by TBS with trabecular bone microarchitecture, but theoretical and technical concerns have been raised (Bousson et al. 2012) including the limited spatial resolution with DXA, which is considerably worse than the microarchitectural parameters typically defined from high-resolution imaging (voxel size 93 μm and below): bone volume/total volume (BV/TV), trabecular thickness, trabecular spacing, trabecular number, and connectivity.

The initial description of TBS used human cadaveric lumbar spine, femoral neck, and distal radius bone samples examined using micro-CT in comparison to TBS using 2D projection images derived from the reconstructed 3D data (Pothuaud et al. 2008). Significant correlations were found, particularly for bone volume fraction, trabecular spacing, and trabecular number. A subsequent analysis of

30 human cadaveric vertebrae examined with micro-CT was compared with ex vivo DXA scans of the same samples (Hans et al. 2011a). Once again, significant correlations were found between TBS and the bone microarchitecture parameters (highest for TBS vs. connectivity density, $r = 0.821$). Multivariable regression suggested that TBS was able to differentiate samples of differing microarchitecture despite similar BMD measurements. Effects of image resolution and noise in these samples were studied using simulated degradation in resolution from 93 μm to 1488 μm. Correlations between TBS and microarchitecture parameters (connectivity density, trabecular number, trabecular spacing) were only minimally affected by image resolution up to a pixel size of 1023 μm.

Clearly, this raises the question of how TBS is able to assess such small microarchitectural parameters given the inherent limitations in DXA resolution (Bousson et al. 2012). A complete explanation has not yet emerged. One possibility is that TBS is simply capturing other macroscopic skeletal parameters that in turn are correlated with microstructure, thereby serving as an indirect proxy measure for degraded skeletal microarchitecture. For example, areal BMD itself was found to correlate with microstructure in these studies, but clearly is not a direct measure of microarchitecture. Importantly, studies of TBS have not reported the partial correlation between TBS and microarchitecture after adjusting for important and confounding factors (such as age, sex, BMI, and BMD). Alternatively, TBS may directly assess degradation in skeletal macroarchitecture (e.g., trabecular network porosity) which, in turn, shows a coupled degradation with skeletal microarchitecture (below the resolution limits of DXA). A simple analogy is an iceberg whereby the visible portion of the iceberg corresponds to the architectural aspects of bone visible to DXA (i.e., image texture), whereas finer microarchitectural details, like the majority of an iceberg, are hidden below the surface. As an iceberg melts (degrades), the visible and submerged components are both affected. As noted by others (Silva et al. 2015), direct evidence that spine TBS measures vertebral trabecular microarchitecture would best be established in an experimental framework whereby TBS is derived from cadaveric lumbar spines scanned in situ and then correlated with ex vivo vertebral microarchitecture adjusted for important covariates (age, sex, soft tissue thickness, BMD). Until such time, spine TBS is best considered as an index of bone texture that may serve as an indirect proxy of skeletal microarchitecture.

Regardless of what TBS actually measures, its clinical utility depends on the ability to assess bone strength/fracture risk independent of BMD and other covariates. In this regard, an ex vivo study of vertebral compressive strength testing was performed using whole L3 vertebral bodies with TBS measured from in situ DXA scans of the lumbar spine (Roux et al. 2013). Although the number of samples was extremely small (16 donors), there was a moderate correlation between TBS and trabecular bone volume ($r = 0.58$) and structure model index ($r = -0.62$), and TBS correlated with vertebral body stiffness ($r = 0.64$). Importantly, TBS explained less than 50% of the variance in these parameters, suggesting that it is a relatively weak

direct measurement. Correlations would likely be even worse if adjusted for other covariates.

Using 743 micro-CT trabecular bone reconstructions (bone samples from femur, radius, vertebrae, iliac crest), Maquer et al. (Maquer et al. 2015) looked at morphologic parameters predicting stiffness from microfinite element analysis. Bone volume fraction was the major determinant of bone elastic properties (80% of explained variance) followed by fabric anisotropy (10% additional explained variance) without any significant additional contribution from TBS or other morphologic variables (though the residual unexplained variance was probably too small). A subsequent analysis from the same group examined compressive failure of 62 human lumbar vertebrae scanned with high-resolution peripheral quantitative computed tomography (HR-pQCT) (Maquer et al. 2016). Simulated DXA images were used to estimate the initial slope of the variogram. Although simulated areal BMD predicted failure load and failure stress, the initial slope of the variogram was a poor surrogate for vertebral strength. Whether similar results would be seen with the commercial version of the TBS algorithm is uncertain.

Recently, a retrospective analysis of transiliac bone biopsies was performed using material from 80 premenopausal women and 43 men with idiopathic osteoporosis (Muschitz et al. 2015). Micro-CT of the specimens was used to study microarchitectural indices and their relationship with gender, bone turnover markers, and DXA-derived parameters in multiple regression models. TBS was independently associated with structural model index (SMI), reflecting the rod- versus plate-like nature of the structure, trabecular number, trabecular spacing, and BV/TV. The incremental contribution of TBS to the other covariates was not reported. The authors concluded that TBS was a practical, noninvasive, surrogate technique for assessment of trabecular bone microarchitecture.

In Vivo Validation Studies

Attempts have been made to study skeletal microarchitecture in humans using HR-pQCT and relate this to TBS. Unfortunately, correlations of HR-pQCT and TBS are inherently limited by assessment at different skeletal sites in that HR-pQCT measurements are only feasible for the distal radius and tibia, whereas TBS is derived from the lumbar spine. In a study of 22 menopausal women with primary hyperparathyroidism, Silva et al. (Silva et al. 2013a) found that TBS correlated with estimated whole bone stiffness and most HR-pQCT indices of trabecular microarchitecture. Correlations were moderate with TBS alone explaining 20% to 52% of variance in HR-pQCT measurements at the radius and tibia. A subsequent study from the same group examined 115 pre- and postmenopausal, White and Chinese American women (Silva et al. 2013b). Once again, TBS showed a significant but modest correlation with most HR-pQCT indices ($r = 0.20–0.52$). However, after adjustment for age, ethnicity,

and BMI, there was no significant residual association between TBS and any of the HR-pQCT indices.

In a study of 72 premenopausal women, Popp et al. (Popp et al. 2014) found that TBS showed a moderate correlation with trabecular microstructural parameters ($r = -0.43$ to -0.57, $r = 0.42$ to 0.46 for connectivity). Additional adjustments for BMD and other covariates were not performed. Among these healthy young women, TBS correlated with the BMD at the lumbar spine and femoral neck ($r = 0.66$ and 0.53).

In the largest such study to date, Amstrup et al. (Amstrup et al. 2015) studied 125 postmenopausal women using DXA, QCT, and HR-pQCT. TBS correlated weakly with microarchitectural indices derived from HR-pQCT at the radius ($r = -0.24$ to 0.31) and tibia ($r = -0.16$ to 0.13) and were not adjusted for additional covariates.

These data were recently reviewed by Bousson et al. (Bousson et al. 2015). It was concluded that the evidence to date suggests that TBS explains relatively little of the variance in trabecular microarchitectural parameters as assessed in vivo, leaving open the question of what skeletal properties TBS primarily measures that account for its ability to predict fracture risk.

Clinical Correlates

In a large cross-sectional analysis of the Manitoba BMD Database, lumbar spine TBS was studied in 29,407 women age 50 years and older at the time of baseline hip and spine DXA scanning. BMD explained only a small amount of variation in TBS (7–11%). Lower lumbar spine TBS was associated with older age, recent glucocorticoid use, prior major fracture, rheumatoid arthritis, chronic obstructive pulmonary disease, high alcohol intake and higher BMI, while recent osteoporosis therapy was associated with a lower likelihood for reduced TBS. These findings were unaffected by adjustment for BMD measured at the lumbar spine or femoral neck.

Subsequent studies have confirmed the low correlation between lumbar spine TBS and BMD and the age-related reduction in TBS (El et al. 2014; Bazzocchi et al. 2015; Dufour et al. 2013; Simonelli et al. 2014; Iki et al. 2015b; Sritara et al. 2015; Aloia et al. 2015) in women and/or men from Lebanon, Italy, France, the United States (White, Chinese, African American), and Japan. To date, there has been relatively little description of ethnic variation in TBS. No difference between American Chinese and White women was identified (Silva et al. 2013b), whereas TBS was found to be higher in African American women compared with literature values for White women (Aloia et al. 2015). Although several groups have attempted to establish local reference data for TBS (Bazzocchi et al. 2015; Dufour et al. 2013; Simonelli et al. 2014; Iki et al. 2015b; Sritara et al. 2015), it is currently uncertain whether the population-specific differences need to be considered when using TBS clinically. As discussed later, current clinical applications of TBS are based upon an assumption of population independence.

Clinical Data on Trabecular Bone Score (TBS)

Cross Sectional Studies

Several cross-sectional studies (summarized in Table 1) have examined the association between TBS and prior fracture. The initial assessment of TBS as a complement to BMD was reported by Pothuaud et al. (Pothuaud et al. 2009) in 45 women with osteoporotic fractures (5 hip, 20 vertebral, 20 other) and 155 women without fracture matched for age and spine BMD. TBS was significantly lower in fracture versus nonfracture women. A larger confirmatory case–control study was then conducted in 81 women with vertebral fractures and 162 age-matched controls without fractures (postmenopausal Caucasian women, age 50–80 years, BMD T-scores between −1.0 and −2.5). The odds ratio (OR) for fracture based upon each standard deviation (SD) reduction in TBS was 2.53 (95% confidence interval [CI] 1.82–3.53) and was greater than for BMD (OR 1.54, 95% CI 1.17–2.03), but this may relate to the fact that the population was constrained to a narrow range of BMD T-scores (between −1.0 and −2.5).

A multicenter retrospective case–control study was reported by Rabier et al. (2010). Forty-two women with osteoporotic vertebral fractures (ages 50–80 years) were age-matched with 126 women without fracture. In this cohort, the OR per SD reduction in TBS was 3.20 (95% CI 2.01–5.08) with AUC 0.746 compared with OR per SD decrease in BMD of 1.95 (95% CI 1.34–2.84) with AUC 0.662 ($P = 0.011$ for AUC comparison). Similar results were seen when women with osteopenic or osteoporotic BMD were studied separately (OR per SD decrease 3.36 and 2.82, respectively). A subsequent report in 191 women age 50 years and older included 83 women with nontraumatic femoral neck fractures and also showed significant discrimination for TBS (OR per SD decrease 2.05, 95% CI 1.45–2.89 unadjusted, 1.71, 95% CI 1.15–2.79 age adjusted). The unadjusted AUC for TBS was 0.668 (95% CI 0.597–0.734).

Leib et al. (2014b) studied a clinical referral database of 2165 women age 40 years and older without illness or medication known to affect bone metabolism to identify a subset with one or more low energy fractures ascertained from review of the medical records ($N = 289$). The most common fracture sites were lower leg (31%), upper arm (30%), and forearm (21%). TBS discriminated prior fracture (unadjusted OR per SD decrease 1.38, 95% CI 1.22–1.56; age and BMD adjusted 1.28, 95% CI 1.13–1.46). The unadjusted AUC was 0.598 (95% CI 0.563–0.633). The lower level of discrimination was likely related to the more diverse fractures evaluated.

In the context of a Fracture Liaison Service, Nassar et al. (Nassar et al. 2014) examined 362 individuals hospitalized after nonvertebral fracture (mean age 74.3 years, 77.3% women, 51.4% hip fracture). Vertebral fracture assessment (VFA) was performed to identify vertebral fractures using the Genant semiquantitative method. Vertebral fracture prevalence was 36.7% ($N = 133$), with 52.2% ($N = 189$) of the patients osteoporotic according to BMD. Although all patients had nonvertebral fractures by definition, TBS provided significant discrimination for

Table 1 Cross-sectional studies of lumbar spine TBS for fracture discrimination (Reprinted with permission Binkley and Leslie (2016))

Reference (source)	Population	Prevalent Fractures	TBS Results
(Pothuaud et al. 2009) (university hospitals in Lausanne, Switzerland and Bordeaux, France)	200 postmenopausal women (case–control)	45 (5 hip, 20 vertebral, 20 other)	OR per SD 1.95 (1.31–2.89) AUC 0.685 (0.599–0.762)
(Winzenrieth et al. 2010) (hospital in Avignon, France)	243 postmenopausal women (age-matched case–control) with BMD T-scores between −1 and −2.5	81 vertebral	OR per SD 2.52 (1.82–3.53). AUC 0.721 (0.660–0.777)
(Rabier et al. 2010) (hospitals Libourne, Médoc-Lesparre, France)	168 postmenopausal women (age-matched case–control)	42 osteoporosis related vertebral	Overall: OR per SD 3.20 (2.01–5.08), AUC 0.746 Osteoporotic: OR per SD 3.36 (1.90–5.92), AUC uncertain Osteopenic: OR per SD 2.82 (1.27–6.26, AUC 0.716 (0.572–0.833
(Del Rio et al. 2013) (medical clinic, Barcelona, Spain)	191 women age 50+ year	83 mild or moderate trauma femoral neck	OR per SD 2.05 (1.45–2.89) [unadjusted], 1.71 (1.15–2.79) [age] AUC 0.668 (0.597–0.734)
(Leib et al. 2014b) (university hospital, Burlington, United States)	2,165 women age 40+ year	289 low-energy	OR per SD 1.38 (1.22–1.56) [unadjusted], 1.28 (1.13–1.46) [age] AUC 0.598 (0.563–0.633)
(Krueger et al. 2014) (university hospital, Madison, United States)	429 women, mean age 71.3 year	158 low-energy nonvertebral or vertebral on VFA	OR any fracture 2.46 (1.9–3.1), vertebral 2.49 (1.9–3.3) [age and BMI]; AUC any fracture 0.74, vertebral 0.73 OR any fracture 2.36 (1.8–3.0), vertebral 2.44 (1.8–3.3) [age, BMI, BMD]
(Nassar et al. 2014) (Fracture Liaison Service in Paris, France)	362 age 50+ year patients hospitalized after nonvertebral fracture, 51.4% hip fracture, 77.3% women, mean age 74.3 year	133 vertebral on VFA	Overall: AUC 0.677 (CI unknown) for vertebral fracture. Nonosteoporotic BMD: AUC TBS 0.671 (CI unknown) for vertebral fracture.

(*continued*)

Table 1 (continued)

Reference (source)	Population	Prevalent Fractures	TBS Results
(Ayoub et al. 2014) (public advertisements, Lebanon)	1000 postmenopausal women age 45–89 year, mean 61.1 year	164 MOF	OR significant ($P < 0.001$) [unadjusted]. OR significant ($P = 0.01$) [BMI, age, lumbar spine BMD]
(Leib et al. 2014a) (university hospital, Burlington, United States)	180 men age 40+ year (age and BMD-matched case–control)	45 low-energy any site	Overall: OR per SD 1.59 (1.09–2.20), AUC 0.614 (0.539–0.685) Vertebral fracture: OR per SD 2.07 (1.14–3.74), AUC 0.695 (0.615–0.767)
(Touvier et al. 2015) (university hospital, Orleans, France)	255 postmenopausal women, mean age 65 year (range 40–92)	79 fragility fractures	OR 2.25 (1.58–3.29) [age] OR 1.83 (1.24- 2.77) [age, hip BMD, height, weight, calcaneus Hurst parameter]

OR odds ratio (95% confidence interval) per SD decrease [covariate adjustments], *SD* standard deviation, *AUC* are under the curve (95% confidence interval), *VFA* vertebral fracture assessment, *BMI* body mass index, *MOF* major osteoporotic fractures (hip, clinical spine, forearm, humerus)

vertebral fracture risk (AUC 0.677 in the overall cohort; 0.671 in nonosteoporotic individuals).

Ayoub et al. (2014) invited 1000 Lebanese postmenopausal women aged 45–89 years to participate in BMD measuring through advertisements, among whom 164 had a history of major osteoporotic fracture (hip, clinical spine, forearm, or humerus). The adjusted OR per SD lower TBS was statistically significant ($P < 0.001$) and remained significant after adjustment for age, BMI, and lumbar spine BMD ($P = 0.01$). Unfortunately, ORs and AUCs were not reported in this study.

In the only cross-sectional study that specifically evaluated men, Leib et al. (2014a) studied 45 men with low energy fracture and 135 men matched for age and BMD (age 40 years and older). For all fractures combined, the OR per SD decrease in TBS was 1.59 (95% CI 1.09–2.20) with AUC 0.614 (95% CI 0.539–0.685). When analysis was limited to men with vertebral fractures, there was even higher discrimination (OR per SD decrease 2.07, 95% CI 1.14–3.74; AUC 0.697, 95% CI 0.615–0.767).

Finally, Touvier et al. (2015) examined fracture discrimination using two texture methods, lumbar spine TBS and calcaneus Hurst parameter. Among 255 postmenopausal women, mean age 65 years (range 40–92), there were 79 with previous fragility fractures. The age-adjusted OR per SD decrease in

TBS was 2.25 (95% CI 1.58–3.29). This was slightly attenuated after adjustment for age, hip BMD, height, weight, and calcaneus texture (OR per SD decrease 1.83, 95% CI 1.24–2.77).

In summary, there is a consistent evidence base for the ability of lumbar spine TBS to discriminate previous fractures. Analyses suggest that this discrimination is independent of other commonly used risk factors including BMD. Although the evidence base is largest for women, one study supports a similar effect of TBS in men.

Longitudinal Studies

Longitudinal studies are summarized in Table 2. The largest assessments of lumbar spine TBS for prediction of incident osteoporotic fractures have been performed in the Manitoba BMD Database from Canada. The first such analysis was reported in 2011 based upon 29,407 women aged 50 years or older at the time of baseline DXA scanning performed through a regionally based clinical program (Hans et al. 2011b). Fractures were ascertained from linkage to population-based hospitalization and physician billing records. During mean follow-up of 4.7 years, 1,668 women developed one or more major osteoporotic fractures (including 293 hip fractures). Lumbar spine TBS was significantly lower in fracture versus nonfracture women ($P < 0.001$), and there was a gradient of risk across lumbar spine TBS tertiles overall and within each WHO BMD category. Adjusted for multiple clinical risk factors (age, comorbidity score, rheumatoid arthritis, chronic obstructive pulmonary disease, diabetes, substance abuse, BMI, prior osteoporotic fracture, recent corticosteroid use, and recent osteoporosis treatment) and femoral neck BMD, lumbar spine TBS was independently associated with any major osteoporotic fracture (hazard ratio [HR] 1.20 [95% CI 1.14–1.26]), clinical vertebral fracture (HR 1.45, 95% CI 1.32–1.58), and hip fracture (HR 1.28, 95% CI 1.13–1.46). There was a small but statistically significant improvement in AUC when lumbar spine TBS was combined with any BMD measurement (femoral neck, total hip, or lumbar spine).

The prospective OFELY cohort from France (Boutroy et al. 2013) assessed lumbar spine TBS in 560 postmenopausal women (age range 31–89 years). During mean 7.8 years of follow-up, 94 women sustained one or more fragility fractures affecting diverse sites (most common wrist and vertebral). Mean age for women with fractures was 70.4 years versus 65.3 years for women without fractures. Lumbar spine TBS was 3.6% lower in women with fractures than without ($P < 0.001$). The unadjusted OR for fracture per SD reduction in TBS was 1.57 (95% CI 1.25–1.98), which was similar to lumbar spine BMD (1.42, 95% CI 1.17–1.72) but lower than total hip BMD (2.12, 95% CI 1.62–2.77). The corresponding AUC measurements were 0.63 (95% CI 0.57–0.68), 0.62 (95% CI 0.55–0.68), and 0.68 (95% CI 0.62–0.74), respectively. Lumbar spine TBS predicted fracture independently of

Table 2 Longitudinal studies of lumbar spine TBS for fracture prediction (Reprinted with permission Binkley and Leslie (2016))

Reference (cohort)	Population	Incident Fractures	TBS Results
(Hans et al. 2011b) (Manitoba, Canada)	29,407 women age 50+ year, mean age 65.4 year	1668 one or more MOF (including 439 vertebral and 293 hip)	Any MOF: AUC 0.63 (0.61–0.64), HR 1.35 (1.20–1.42) [unadjusted], HR 1.20 (1.14–1.26) [multiple clinical risk factors and femoral neck BMD] Vertebral: AUC 0.66 (0.64–0.69), HR 1.45 (1.32–1.58) [unadjusted], HR 1.22 (1.10–1.34) [multiple clinical risk factors and femoral neck BMD] Hip: AUC 0.68 (0.65–0.71). HR 1.46 (1.30–1.63) [unadjusted], HR 1.28 (1.13–1.46) [multiple clinical risk factors and femoral neck BMD]
(Boutroy et al. 2013) (OFELY, France)	560 postmenopausal women age range 31–89 year	94 with 112 fragility fractures (35 wrist, 32 vertebral, 16 tibia, 8 hip, 7 ribs, 6 humerus, 3 metatarsal, 2 scaphoids, 1 scapula, 1 elbow, and 1 patella)	OR AUC 0.63 (0.57–0.68), 1.57 (1.25–1.98) [unadjusted] OR not significant with total hip BMD; not significant with age, prevalent fracture, lumbar spine BMD
(Briot et al. 2013) (OPUS, European centers Kiel, Paris and Sheffield)	1007 women age 55+ year, mean age 65.9 year	82 clinical osteoporotic (80 with TBS), 46 radiographic vertebral	Clinical osteoporotic: AUC 0.62 (0.56–0.69), OR 1.62 (1.30–2.01), [unadjusted] Radiographic vertebral: AUC 0.63 (0.54–0.72), OR 1.54 (1.17–2.03) [unadjusted]
(Iki et al. 2014) (JPOS, Japan)	665 women aged 50+ year, mean age 64.1 year	92 with incident vertebral fracture by VFA	OR AUC 0.682 (0.662–0.773), 1.98 (1.56–2.51) [unadjusted], OR 1.52 (1.16–2.00) [age, prevalent vertebral deformity, spine BMD adjusted]

(*continued*)

Table 2 (continued)

Reference (cohort)	Population	Incident Fractures	TBS Results
(Leslie et al. 2014a) (Manitoba, Canada)	3620 women age 50+ year, mean age 67.6 year	183 one or more MOF (including 91 clinical vertebral and 46 hip)	Any MOF: AUC 0.59 (0.55–0.63), HR 1.22 (1.05–1.41) [clinical FRAX score and osteoporosis treatment], HR 1.12 (0.96–1.30) [above plus hip BMD] Hip: AUC 0.67 (0.59–0.75), HR 1.60 (1.21–2.11) [clinical FRAX score and osteoporosis treatment], HR 1.36 (1.01–1.83) [above plus hip BMD] Vertebral: AUC 0.57 (0.51–0.63), HR 1.12 (0.91–1.38) [clinical FRAX score and osteoporosis treatment], HR 1.09 (0.88–1.34) [above + hip BMD]
(Popp et al. 2015) (SEMOF, Switzerland)	556 women mean age 76.1 year	52 clinical fragility (20 forearm, 6 hip, 10 clinical vertebral, 9 humerus, 2 pelvis, 3 ankle, 1 clavicle, 1 elbow)	AUC 0.69 (0.62–0.77), HR 2.01 (1.54–2.63) [age and BMI], 1.66 (1.25–2.22) [above + femoral neck BMD]
(Iki et al. 2015a) (JPOS, Japan)	2012 community-dwelling men aged 65+ year, mean age 73.0 year	22 MOF (by interviews or mail and telephone surveys)	AUC 0.669 (0.548–0.790), OR 1.89 (1.28–2.81) [unadjusted]
(Leslie et al. 2014b) (Manitoba, Canada)	33,352 women aged 40–100 y. mean age 63 year	1872 one or more MOF, 1754 deaths	MOF: HR 1.36 (1.30–1.42) [time since baseline and age], HR 1.18 (1.12–1.23) [clinical risk factors and femoral neck BMD] Death: HR 1.32 (1.26–1.39) [time since baseline and age], HR 1.20 (1.14–1.26) [clinical risk factors and femoral neck BMD]

(*continued*)

Table 2 (continued)

Reference (cohort)	Population	Incident Fractures	TBS Results
(McCloskey et al. 2015) (Manitoba, Canada)	33,352 women aged 40–100 years,	1754 deaths, 1639 one or more MOF excluding hip, 306 women one or more hip	Death HR 1.20 (1.14–1.26), MOF excluding hip HR 1.18 (1.12–1.24), hip 1.23 (1.09–1.38) [all FRAX risk variables]
(Schousboe et al. 2016) (MrOS, United States)	5979 men aged 65+ year	448 one or more MOF including 181 hip	MOF 1.27 (1.17–1.39) and hip 1.20 (1.05–1.39) [FRAX with BMD and prevalent vertebral fracture]
(McCloskey et al. 2016) (14 international cohorts)	17,809 (41% men and 59% women), mean age 72 year	1109 one or more MOF including 298 hip	MOF HR 1.44 (1.35–1.53) [age and time since baseline], HR 1.32 (1.24–1.41) [FRAX MOF probability] Hip HR 1.44 (1.28–1.62) [age and time since baseline], HR 1.28 (1.13–1.45) [FRAX hip probability]

HR hazard ratio (95% confidence interval) per SD decrease [covariate adjustments], *SD* standard deviation, *AUC* are under the curve (95% confidence interval), *BMI* body mass index, *MOF* major osteoporotic fractures (hip, clinical spine, forearm, humerus)

lumbar spine BMD, but was not independently predictive of fracture in the presence of total hip BMD. It also did not significantly improve fracture prediction when combined with age, prevalent fracture, and lumbar spine BMD.

Lumbar spine TBS was retrospectively calculated from DXA scans obtained as part of the OPUS study performed in ambulatory European women age 55 years and older (mean age 65.9 years) recruited from three centers (Kiel, Paris, and Sheffield) (Briot et al. 2013). Among the 100,007 women available for analysis, incident radiographic vertebral fractures were identified in 46 women and incident clinical osteoporotic fractures were identified in 82 women (80 with TBS available) during mean follow-up of 6.0 years. Lumbar spine TBS was significantly lower in fracture versus nonfracture women. Each standard deviation reduction in TBS was associated with an OR 1.62 (95% CI 1.30–2.01) for incident clinical osteoporotic fracture which was similar to femoral neck BMD (OR 1.60, 95% CI 1.26–2.06) and total hip BMD (OR 1.65, 95% CI 1.30–2.11) and better than lumbar spine BMD (OR 1.47, 95% CI 1.16–1.89). The combination of TBS and hip BMD resulted in slightly higher ORs than either measurement alone (TBS with femoral neck BMD OR 1.69, 95% CI 1.40–2.04; TBS with total hip BMD OR 1.72, 95% CI 1.43–2.07).

Complementary results were seen in AUC measurements. For radiographic vertebral fractures, each SD reduction in lumbar spine TBS was associated with OR 1.54 (95% CI 1.17–2.03), which was similar to femoral neck BMD (OR 1.54, 95% CI 1.12–2.14) but lower than lumbar spine BMD (OR 1.75, 95% CI 1.25–2.48) or total hip BMD (OR 1.73, 95% CI 1.26–2.38). Inexplicably, there was worse performance when TBS was combined with lumbar spine BMD (OR 1.54, 95% CI 1.18–1.99), total hip BMD (OR 1.56, 95% CI 1.25–1.93), and femoral neck BMD (OR 1.50, 95% CI 1.17–1.89). No adjustments for age or other covariates were performed. Analyses were also conducted in 672 women with BMD T-scores that were not in the osteoporotic range. Odds ratios tended to be slightly lower for TBS: clinical osteoporotic fracture OR 1.43 (95% CI 1.08–1.88) with AUC 0.59 (95% CI 0.50–0.68), radiographic vertebral fracture OR 1.20 (95% CI 0.87–1.84) with AUC 0.55 (95% CI 0.44–0.67).

The Japanese Population-Based Osteoporosis Cohort Study (JPOS) retrospectively derived lumbar spine TBS measurements for 665 women age 50 years of older at baseline (mean age 64.1 years) (Iki et al. 2015a). Vertebral fractures were identified from VFA performed up to 10 years, with 92 women developing 140 incident vertebral fractures. In women with versus without incident vertebral fractures, the unadjusted mean TBS was 5.7% lower (1.132 vs. 1.200, $P < 0.0001$). This difference was attenuated in sequential adjustments for age (3.4% difference, $P < 0.0001$), height and weight (3.1% difference, $P = 0.0001$), prevalent vertebral deformity (difference 2.9%, $P = 0.0003$), spine BMD (1.4% difference, $P = 0.0438$), and osteoporosis treatment at baseline (1.5% difference, $P = 0.0386$). Unadjusted OR per SD reduction in TBS was 1.98 (95% CI 1.56–2.51) with AUC 0.682 (95% CI 0.621–0.743). After adjustment for age, prevalent vertebral deformity, and spine BMD, TBS remained significantly associated with incident vertebral fracture (OR 1.52, 95% CI 1.16–2.00). Models that compared TBS and lumbar spine BMD alone did not show a significant improvement in AUC when these were used in combination ($P = 0.1710$).

The first assessment of incident fracture prediction in men was performed in the Manitoba BMD database (Leslie et al. 2014a). Fracture data on 3,620 men age 50 years and older at the time of baseline DXA (mean age 67.6 years) were obtained from Health Services records during mean follow-up 4.5 years. One or more major osteoporotic fractures were identified in 183 (5.1%) of the men, of which 46 involved hip and 91 were clinical vertebral fractures. As had been seen in women, the correlation between spine BMD and spine TBS was relatively low ($R = 0.31$) and much less than the correlation between spine and hip BMD ($R = 0.63$). In men with versus without incident fractures, spine TBS was significantly lower for major osteoporotic fracture, hip fracture, and clinical vertebral fracture with unadjusted AUC measurements of 0.59 (95% CI 0.55–0.63), 0.67 (95% CI 0.59–0.75) and 0.57 (95% CI 0.51–0.63), respectively. When adjusted for the clinical FRAX score (without BMD) and osteoporosis treatment, spine TBS was associated with a significant increase in risk for major osteoporotic fracture (HR per SD reduction 1.22, 95% CI 1.05–1.41), but this was no longer significant after adjustment for femoral neck BMD or spine BMD. In contrast, the association between spine TBS

and hip fracture was significant when adjusted for risk factors without BMD (HR 1.60, 95% CI 1.21–2.11), after additional adjustment for femoral neck BMD (HR 1.36, 95% CI 1.01–1.83) or spine BMD (HR 1.44, 95% CI 1.07–1.94). Prediction of clinical vertebral fractures was not significant when spine TBS was adjusted for clinical risk factors (HR 1.12, 95% CI 0.91–1.38).

The SEMOF Cohort from Switzerland (Popp et al. 2015) examined fracture outcomes in 556 women, mean age 76.1 years, identified through the University Hospital of Berne, Switzerland. During a mean follow-up of 2.7 years, 52 women sustained clinical fragility fractures at mixed sites (20 forearm, 6 hip, 10 clinical vertebral, 9 humerus, 2 pelvis, 3 ankle, 1 clavicle, 1 elbow). Women with incident fracture had significantly lower spine TBS ($P < 0.001$). AUC for fracture discrimination was 0.69 (95% CI 0.62–0.77), which was significantly higher than for lumbar spine BMD (AUC 0.62, 95% CI 0.55–0.70, $P = 0.03$) and not significantly different from hip BMD measurements. The HR per SD reduction in TBS (adjusted for age and BMI) was 2.01 (95% CI 1.54–2.63) and remains statistically significant when additionally adjusted for femoral neck BMD (HR 1.66, 95% CI 1.25–2.22).

Iki et al. (2015a) also reported on 2,012 community-dwelling men age 65 years and older (mean age 73.0 years) from the JPOS Cohort. In follow-up interviews, mail, and telephone surveys, 22 men sustained major osteoporotic fractures. TBS provided fracture discrimination with AUC 0.669 (95% CI 0.548–0.790) with unadjusted OR 1.89 (95% CI 1.28–2.81).

To broadly summarize these clinical data, there is consistent evidence that TBS is associated with fracture risk in multiple studies although the effect sizes vary considerably. In general, the strength of the reported effects is larger in studies that are cross-sectional and/or smaller in size and weaker in those that are longitudinal and/or based upon larger populations. This variability may, in part, reflect an element of reporting bias, but does not negate the potential for TBS to provide insights into fracture risk independently of BMD.

Potential Clinical Applications

Improved Fracture Risk Prediction

The suboptimal performance of BMD alone for fracture prediction led to the development of the Fracture Risk Assessment (FRAX) tool by the WHO Collaborating Centre for Metabolic Bone Diseases to estimate an individual's 10-year probability of major osteoporotic fracture (composite of clinical spine, hip, forearm, proximal humerus) and hip fracture (Kanis et al. 2009). The input variables were selected following a series of meta-analyses using data from nine prospective international population-based cohorts (Kanis and on behalf of the World Health Organization Scientific Group 2007). In addition to age, sex, and body mass index (BMI), additional clinical risk factors (CRFs) for fractures include prior fragility fracture, a parental history of hip fracture, prolonged use of glucocorticoids, rheumatoid arthritis, current cigarette smoking, alcohol intake of 3 or more units/day, and

secondary osteoporosis. Femoral neck BMD is an optional input that can refine the risk estimate, but historically was the only skeletal parameter considered by FRAX. For fracture, competing death is particularly important to consider in order to obtain unbiased estimates of fracture risk since, following death, fracture is no longer possible. Therefore, FRAX adjusts for competing mortality as failure to do so overestimates major fracture probability. In recognition of the large international variability in fracture and mortality rates (Kanis et al. 2012), population-specific FRAX tools are customized to the fracture and mortality epidemiology in a specific region (Kanis et al. 2009).

In order to determine whether TBS provides information on fracture risk beyond that provided by the FRAX algorithm, 33,352 women age 40–100 years (mean age 63 years) from the Manitoba BMD Program in Canada were analyzed. FRAX 10-year fracture probability at the time of baseline DXA was calculated with and without femoral neck BMD. During mean follow-up of 4.7 years, 1,754 women died and 1,872 women sustained one or more major osteoporotic fractures. Lower TBS was a significant risk factor for both fracture and death. The HR for major osteoporotic fracture per SD reduction in TBS was 1.36 (95% CI 1.30–1.42) adjusted for time since baseline and age; when additionally adjusted for the clinical risk factors in FRAX and femoral neck BMD, the HR was slightly attenuated (1.18, 95% CI 1.12–1.23). Each SD reduction in TBS was also associated with a 32% increase in death (HR 1.32, 95% CI 1.26–1.39) adjusted for time since baseline and age, and this remains significant when further adjusted for FRAX clinical risk factors and femoral neck BMD (HR 1.20, 95% CI 1.14–1.26). Similar results were seen when lumbar spine BMD was used instead of femoral neck BMD in the models. The clinical risk factors included in the analysis included BMI, previous fracture, chronic obstructive pulmonary disease (a smoking proxy), glucocorticoid use greater than 90 days in the prior year, rheumatoid arthritis, secondary osteoporosis, and high alcohol use. Models were constructed to estimate major osteoporotic fracture probability that accounted for competing mortality. These showed that low TBS (10th percentile) increased risk for major osteoporotic fracture 1.5–1.6 fold relative to a high TBS measurement (90th percentile), and this was consistent across a broad range of ages and femoral neck T-scores. This study paved the way for developing an adjustment to the FRAX probability measurement based upon lumbar spine TBS as described by McCloskey et al. (2015). Using the same Manitoba BMD cohort, fractures were categorized as major osteoporotic fracture excluding hip (N = 1,639) and one or more hip fracture (N = 306). Importantly, this study examined interactions with other risk factors and incorporated competing mortality into the procedure. Even when adjusted for FRAX clinical risk factors and femoral neck BMD, spine TBS was a risk factor for nonhip major osteoporotic fracture (HR 1.16, 95% CI 1.12–1.24) and hip fracture (HR 1.23, 95% CI 1.09–1.38). A significant interaction was found between TBS and age, with TBS exerting a stronger effect on major osteoporotic fracture risk and hip fracture risk in younger women and a progressively weaker effect in older women. The reason for the waning effect of TBS with advancing age is uncertain, but may reflect the multifactorial pathophysiology of age-related fractures which is influenced by both skeletal strength and falls risk.

Among the elderly, falls are common and may play a larger role in determining overall fracture risk (especially for hip fractures) than compromised skeletal strength, whereas in younger individuals falls are less frequent and measures of skeletal strength may be of greater importance. This interaction was incorporated into the final models developed for predicting TBS-adjusted major osteoporotic fracture probability and hip fracture probability.

In 2015, a method for adjusting FRAX probability measurements based upon lumbar spine TBS which also includes the effect of competing mortality was developed (McCloskey et al. 2015):

Hip fracture: 10-year probability calculated with TBS is $100/(1 + e^{-W})$

where $W = 15.420–12.627 \times TBS - 0.194 \times age + 0.157 \times TBS \times age + 0.920 \times L$, $L = -\ln(100/p - 1)$, p is the 10-year probability calculated without TBS.

Major Osteoporotic Fracture: 10-year probability calculated with TBS is $100/(1 + e^{-W})$

where $W = 5.340–4.213 \times TBS - 0.0521 \times age + 0.0393 \times TBS \times age + 0.897 \times L$, $L = -\ln(100/p - 1)$, p is the 10-year probability calculated without TBS.

This adjustment contains a "TBS x age" interaction term which reflects the declining strength of the TBS adjustment on FRAX with increasing age.

To further validate, the TBS adjustment developed using the Manitoba BMD Cohort, 14 prospective international cohorts were assembled (McCloskey et al. 2016). In total, 17,809 participants were included (59% women, 41% men) spanning the age range from 40 to 90 years (mean 72 years). During follow-up, 1,109 participants experienced a major osteoporotic fracture, while 298 participants experienced an incident hip fracture. TBS adjusted for time since baseline and age was significantly associated with both major osteoporotic fracture (HR per SD 1.44, 95% CI 1.35–1.53 men and women combined; 1.50, 95% CI 1.36–1.66 men only; 1.40, 95% CI 1.30–1.52 women only) with slight attenuation when adjusted for FRAX probability (HR per SD 1.32, 95% CI 1.24–1.41 men and women combined; 1.35, 95% CI 1.21–1.49 men only; 1.31, 95% CI 1.21–1.42 women only). The gradient of risk using TBS-adjusted 10-year major osteoporotic fracture probability (1.76, 95% CI 1.65–1.87 men and women combined) was greater than FRAX probability alone (1.70, 95% CI 1.60–1.81). For prediction of hip fracture, each SD reduction in spine TBS was again associated with a significant increase in risk adjusted for time since baseline and age (1.44, 95% CI 1.28–1.62 men and women combined; 1.47, 95% CI 1.23–1.75 men only; 1.42, 95% CI 1.21–1.67 women only), which was still significant after adjustment for FRAX probability (1.28, 95% CI 1.13–1.45 men and women combined; 1.27, 95% CI 1.06–1.53 men only; 1.29, 95% CI 1.09–1.52 women only). No significant difference between genders was identified ($P > 0.10$). For both hip fracture and major osteoporotic fracture, incorporation of the TBS-adjustment factor resulted in an improvement in the gradient of risk. The gradient of risk for hip fracture prediction using the TBS-adjusted 10-year probability was 1.76 (95% CI 1.65–1.87) for men and women combined

versus 1.70 (95% CI 1.60–1.81) for FRAX probability alone. These findings were felt to support the use of TBS as a potential adjustment for FRAX probability, but did not directly assess the impact of the adjustment in the context of clinical practice guidelines.

This evidence base supports a clinical role for TBS in fracture risk estimation and, as such, this is now integrated into the online FRAX calculator as seen in Figs. 2 and 3. As noted earlier, the magnitude of the TBS adjustment is greater in younger individuals than older individuals due to the age–TBS interaction, and this is clearly depicted in Fig. 4.

Clinical Practice Guidelines

The International Society for Clinical Densitometry (ISCD) is a nonprofit professional organization dedicated to the advancement of musculoskeletal health. The ISCD recently held a Position Development Conference around the theme "fracture prediction beyond BMD," and one of the technologies evaluated was TBS. Through a systematic evidence-based review and position development process

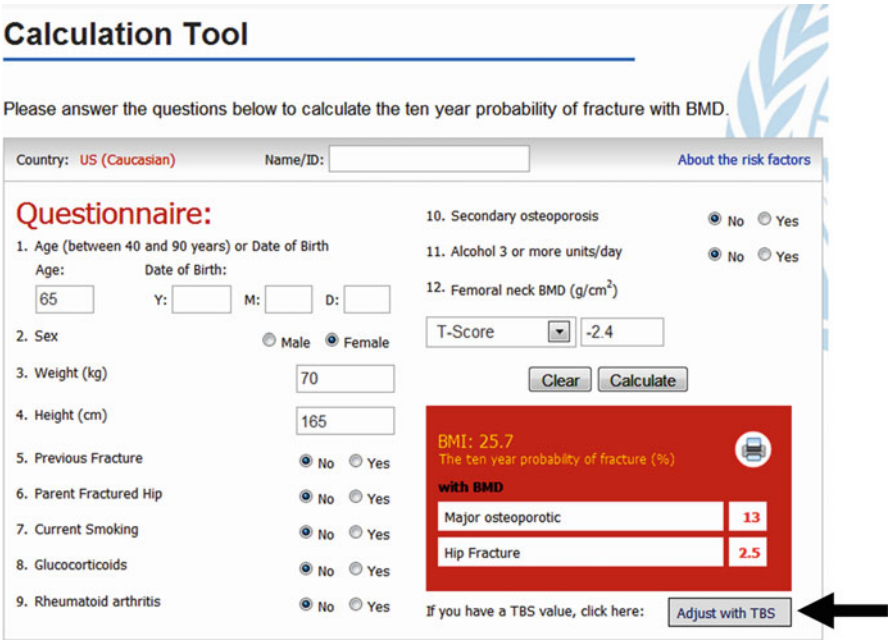


Fig. 2 Sample screenshot for FRAX® (US Caucasian tool). 10-year probability for major osteoporotic fracture is 13 % and for hip fracture is 2.5 % in a woman age 65 years, weight 70 kg, height 165 cm, femoral neck T-score −2.4 and no additional clinical risk factors. The arrow indicates button for entering TBS if available

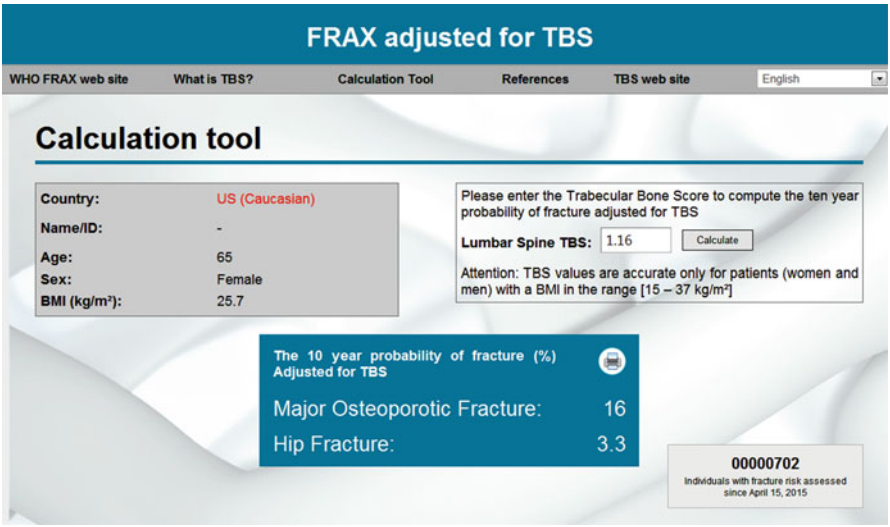


Fig. 3 Sample screenshot for adjusting FRAX® probability based upon lumbar spine TBS. Assuming a reduced TBS measurement of 1.160 (10th percentile), the 10-year probability for major osteoporotic fracture increases from 13 % to 16 %, and for hip fracture from 2.5 % to 3.3 %

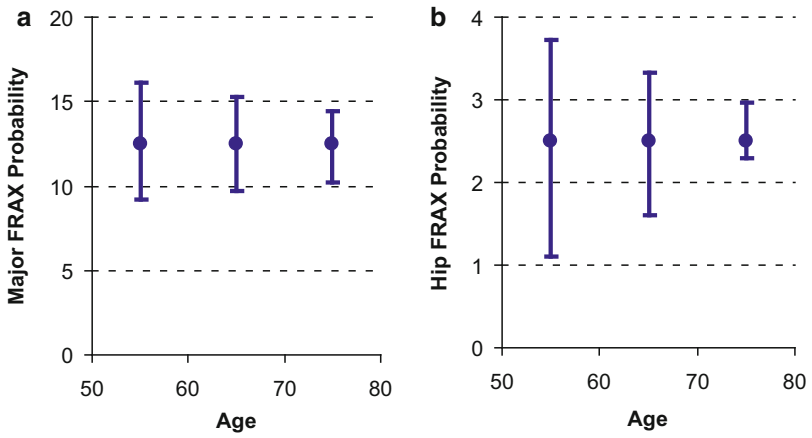


Fig. 4 Effect of age interactions on the FRAX TBS adjustment. The circle indicates FRAX probability in the absence of TBS, whereas the ends of the error bars indicate how FRAX probability is affected by low TBS 1.160 (10th %ile, upper error bar) and high TBS 1.470 (90th %ile, lower error bar)

based upon the RAND/UCLA Appropriateness Method (RAM), a number of positive statements regarding the clinical use of TBS were developed and approved (Silva et al. 2015). Some of these positions appear in the summary points. A working group from the European Society for Clinical and Economic Aspects of

Osteoporosis and Osteoarthritis (ESCEO) performed a scientific literature review and reached similar conclusions that TBS had predictive value for fracture independent of fracture probabilities obtained using the FRAX algorithm (Harvey et al. 2015). Additionally, a potential role in fracture risk in certain forms of secondary osteoporosis (diabetes, hyperparathyroidism, and glucocorticoid-induced osteoporosis) was highlighted.

A recent reanalysis of the Manitoba BMD Database was performed to assess net reclassification improvement (NRI) with the TBS-adjusted FRAX probability in 34,316 women age 40–100 years (mean age 64 years) (Leslie et al. 2015). During mean follow-up 8.7 years, 3,503 women sustained an incident major osteoporotic fracture including 945 with an incident hip fracture. Reclassification was assessed looking at specific FRAX-based intervention criteria (major osteoporotic fracture 20%, hip fracture 3%, age-specific major osteoporotic fracture) and three national clinical practice guidelines (Osteoporosis Canada, US National Osteoporosis Foundation [NOF], and UK National Osteoporosis Guidelines Group [NOGG]). Overall proportions of women reclassified under the TBS-adjusted FRAX probability were small (<5%). However, for women close to an intervention cutoff, reclassification rates were much higher: 17.5% (major osteoporotic fracture 20% ± 5% [absolute]), 17.9% (hip fracture 3% ± 1% [absolute]), and 25.3% (age-specific ± 20% [relative]). Outside of these ranges, reclassification rates were <1%. Based upon a treatment threshold of major osteoporotic fracture 20%, the NRI was +1.4% for fracture cases ($P < 0.001$) and −0.4% for nonfracture cases ($P < 0.001$) with overall NRI +0.011($P < 0.01$). For a treatment threshold of hip fracture 3%, the NRI for fracture cases was +3.0% ($P < 0.001$), for nonfracture cases was −1.1% ($P < 0.001$) with overall NRI +0.018 ($P < 0.001$). For the age-specific treatment criterion, the NRI for fracture cases was +1.7% ($P < 0.001$) and for nonfracture cases was −1.0% ($P < 0.001$) with overall NRI +0.007 (not significant). When NRI was computed for specific clinical practice guidelines, overall NRI was significantly improved for guidelines from Osteoporosis Canada (+0.008, $P < 0.05$), for the US NOF (0.005, $P < 0.05$) and for UK NOGG (+0.011, $P < 0.01$). Overall, these data were consistent with a small but significant improvement in fracture risk assessment using the TBS-adjusted FRAX probability. NRI was consistently greater in women less than age 65 compared with women over age 65, consistent with the age–TBS interaction incorporated into the adjustment. The improvement in risk classification was not specific to a particular clinical practice guideline, but almost all of the benefit in terms of risk recategorization was seen in individuals close to the intervention threshold.

In summary, although TBS should not be used alone to determine treatment recommendations, it can be used in association with FRAX and BMD to adjust FRAX probability of fracture in postmenopausal women and older men and guide treatment initiation. For those individuals close to the intervention threshold, inclusion of TBS will have a greater impact upon treatment initiation decisions.

TBS in Special Populations

Diabetes Mellitus

TBS may have particular value in patients with Type 2 diabetes where fracture risk is paradoxically increased despite higher BMD, leading to underestimation of fracture risk from the FRAX algorithm (Giangregorio et al. 2012; Schwartz et al. 2011). The Manitoba BMD Database was used to study 29,407 women age 50 years and older among whom 2,356 had previously diagnosed diabetes (predominantly Type 2) (Leslie et al. 2013). Although diabetes was associated with higher BMD, lumbar spine TBS was significantly lower in unadjusted and adjusted models. Importantly, lumbar spine TBS was able to predict incident major osteoporotic fractures in those with diabetes (adjusted HR 1.27, 95% CI 1.10–1.46) as well as in those without diabetes (HR 1.31, 95% CI 1.24–1.38). The effect of diabetes attenuated when lumbar spine TBS was added to the prediction model, but was paradoxically increased from adding a BMD measurement. It was concluded that lumbar spine TBS captures a larger proportion of the diabetes associated fracture risk than does BMD.

Other studies have confirmed lower spine TBS in type 2 diabetes (Dhaliwal et al. 2014; Kim et al. 2015). One study did not find lower TBS in 99 postmenopausal women with type 2 diabetes versus 107 nondiabetic controls, though TBS discriminated prevalent radiographic vertebral fractures in the women with type 2 diabetes (Zhukouskaya et al. 2016). The prevalence of vertebral fracture in the women with type 2 diabetes was 34.3% compared with 18.7% in controls ($P = 0.01$). Mean BMD was higher in women with type 2 diabetes than controls for the lumbar spine (mean T-score -0.8 ± 1.44 vs. -1.39 ± 1.28, $P = 0.002$) and for the femoral neck (mean T-score -1.06 ± 1.08 vs. -1.45 ± 0.91, $P = 0.006$). TBS was lower in diabetic women with vertebral fracture than those without vertebral fractures (1.072 ± 0.15 vs. 1.159 ± 0.15, $P = 0.006$) with AUC 0.69 ($P < 0.0001$). The definition of vertebral fracture in this study was based upon reduction of more than 20% in anterior, middle, or posterior vertebral height, which included many grade 1 deformities that would not be considered true fractures under some other definitions. The authors found that a combination of TBS of ≤ 1.130 and femoral neck BMD < -1.0 had the lowest sensitivity (63.6%) but highest specificity (73.8%) compared with other combinations. One small cross-sectional study of 57 women with type 2 diabetes found that TBS was higher in subjects with good glycemic control (defined as HgbA1C $\leq 7.5\%$) compared to those with poor glycemic control (mean 1.254 ± 0.148 vs. 1.166 ± 0.094, $P = 0.01$) (Dhaliwal et al. 2014). A much larger study of 1,229 older men (325 with diabetes) and 1,529 postmenopausal women (370 with diabetes) assessed measures of fasting glucose, pancreatic beta cell function, and insulin resistance in relation to TBS (Kim et al. 2015). In a model adjusted for age, BMI, and other covariates, men with diabetes were found to have lower TBS than nondiabetic men (mean 1.294 vs. 1.316, $P < 0.001$), but after similar adjustments differences were not significant for women (1.345 vs. 1.350, $P = 0.241$). In unadjusted analyses, TBS was lower in subjects with versus without

diabetes (men $P < 0.001$, women $P < 0.001$). However, for women younger than age 65 years, TBS was significantly lower in women with diabetes even after covariate adjustments (mean 1.361 vs. 1.392, $P < 0.001$). TBS was negatively correlated with HgbA1C, fasting blood glucose, fasting insulin, and the homeostasis model assessment for insulin resistance (HOMA-IR), although the magnitude of the correlations was attenuated by age and BMI adjustment (men adjusted R −0.144 to −0.073, women −0.150 to −0.061, all $P < 0.05$). It was suggested that the inverse association between lumbar spine TBS and insulin resistance may offer an indication of skeletal deterioration in diabetic patients.

To date only a single study has examined TBS in type 1 diabetes (Neumann et al. 2016). In a cross-sectional comparison of 119 type 1 diabetic patients (59 men, 60 premenopausal women, mean age 43.4 years) and 68 healthy controls (matched for gender, age, and BMI), TBS did not differ between the two groups ($P = 0.075$). Prevalent fractures were reported in 24 of the type 1 diabetic patients, and TBS was significantly lower in these individuals than in the diabetic patients without fractures (mean 1.309 ± 0.125 vs. 1.370 ± 0.127, $P = 0.04$). The most common sites of fracture were forearm/hand (in 15) and lower leg/foot (in 13). Diabetic patients with fractures had significantly higher HgbA1c and lower total hip BMD and lumbar spine TBS. The AUC for fracture discrimination among type 1 diabetic subjects was 0.63 (95% CI 0.51–0.74) for TBS, which was similar to total hip BMD (0.64, 95% CI 0.51–0.78). The odds ratio (adjusted for age and BMI) for lumbar spine TBS in the lowest tertile (versus highest tertile) in the comparison between type 1 diabetic patients and controls was significantly greater for TBS (OR 2.59, 95% CI 1.08–6.21), whereas no significant effect for any BMD measurement was seen between the two groups. In a multivariable model, both TBS ($P = 0.049$) and HgbA1c ($P = 0.036$) were independently associated with prevalent fractures in the type 1 diabetic subjects. No studies assessing prediction of incident fractures have been reported for type 1 diabetes.

Glucocorticoid Exposure

A number of studies have assessed the potential usefulness of TBS for assessing fracture risk and skeletal changes in individuals with exogenous or endogenous excess glucocorticoid exposure. In a cross-sectional and longitudinal assessment of 102 patients with adrenal incidentalomas (34 with subclinical hypercortisolism) and 70 matched controls, TBS was found to be lower in those with subclinical hypercortisolism (Z-score -3.184 ± 1.211) than adrenal incidentalomas without subclinical hypercortisolism (-1.704 ± 1.541, $P < 0.001$) and controls (-1.189 ± 0.991, $P < 0.0001$). Prevalent vertebral fractures assessed from conventional spinal radiography were associated with lower TBS (OR 1.38, 95% CI 1.04–1.85) adjusted for age, lumbar spine BMD, BMI, and gender. The AUC for vertebral fracture discrimination approached but did not achieve statistical significance (0.657, $P = 0.07$).

Forty patients were followed longitudinally and the occurrence of new vertebral fractures after 24 months was significantly associated with lower TBS (OR 11.2, 95% CI 1.71–71.41) adjusted for age, BMI, and lumbar spine BMD.

Exogenous glucocorticoid therapy results in lower TBS measurements in older women compared to those who are glucocorticoid naïve (mean 1.011 ± 0.152 vs. 1.132 ± 0.136, $P < 0.001$) (Paggiosi et al. 2015). Among glucocorticoid-treated women, TBS was able to discriminate those with and without recent prior fracture (within 6 months) with AUC 0.71 (95% CI 0.670–0.768), which was better than lumbar spine BMD (0.572, 95% CI 0.518–0.625).

In a large clinical dataset, Leib et al. (Leib and Winzenrieth 2016) identified 416 glucocorticoid-treated men and women aged 40 years and older (dose $\geq$5 mg per day for $\geq$ 3 months) who were matched with 1,104 control subjects (matching variables age, sex and BMI). Prior osteoporotic fractures were documented from the medical report in 16.3% of glucocorticoid-treated and 13.1% of control subjects. Mean TBS was significantly lower in glucocorticoid-treated patients than controls (1.267 vs. 1.298, $P < 0.001$). Among glucocorticoid-treated patients, those with fracture ($N = 68$) compared to those without fracture ($N = 348$) had significantly lower mean TBS (1.22 ± 0.131 vs. 1.276 ± 0.134, $P < 0.05$) with significant discrimination as assessed from AUC (0.620, 95% CI 0.571–0.667) and logistic regression (OR 1.44, 95% CI 1.09–1.89). The association between TBS and prior fracture remained significant after multiple covariate adjustments. Interestingly, BMD at the spine, total femur, and femoral neck was not useful for fracture discrimination in this study population.

A single study examined TBS in recent kidney transplant recipients ($N = 327$, mean age 45 years, 39% men) (Naylor et al. 2016). Kidney transplant recipients had significantly lower lumbar spine TBS than matched controls (1.365 ± 0.129 vs. 1.406 ± 0.125, $P < 0.001$), and this persisted after multivariable adjustment. TBS was more than twice as likely to be in the lowest versus the highest tertile for kidney transplant recipients (adjusted OR 2.1, 95% CI 1.47–3.07). TBS was associated nontraumatic incident fractures ($n = 31$) with AUC 0.64 (95% CI 0.53–0.74, $P = 0.012$), and this was independent of the FRAX score (using femoral neck BMD) and spine BMD (adjusted HR 1.55, 95% CI 1.06–2.27). It is unclear if the lower TBS values reflect prior renal disease and/or glucocorticoid use. Nonetheless, TBS may prove to be a useful tool in the quest to better predict fracture risk in this unique patient population, but confirmatory studies are needed.

Other Conditions

TBS has been assessed in a number of other conditions, but these reports have not evaluated incident fracture prediction. Among 65 women with systemic sclerosis, TBS was found to be significantly lower than in age-matched controls ($P < 0.0001$) and similar to age-matched women with rheumatoid arthritis ($P = 0.128$). In women

with systemic sclerosis receiving higher doses of glucocorticoids (≥5 mg per day), TBS was significantly lower than in those receiving lower doses ($P = 0.001$). No association between glucocorticoid use and TBS was found in women with rheumatoid arthritis. TBS was lower in systemic sclerosis patients with prevalent fractures than in those without a history of fracture (1.145 ± 0.166 vs. 1.233 ± 0.129, $P = 0.019$), and a similar pattern was seen in the rheumatoid arthritis population (1.214 ± 0.131 vs. 1.265 ± 0.135, $P = 0.045$). BMD also provided fracture discrimination in both populations ($P \leq 0.001$). The independent association between TBS and BMD was not assessed, and no covariate adjustments were conducted for fracture discrimination. In rheumatoid arthritis, TBS was able to discriminate the presence of prevalent vertebral fractures assessed from VFA (Breban et al. 2012). In primary hyperparathyroidism, TBS has been found to be significantly lower and to be associated with prevalent vertebral fracture (Eller-Vainicher et al. 2013) (Romagnoli et al. 2013). TBS also appears to be adversely affected by thalassemia major (Baldini et al. 2014), anorexia (Donaldson et al. 2015), but within inconsistent findings in osteogenesis imperfecta (Kocijan et al. 2015).

In summary, TBS appears likely to provide additional information in the clinical assessment of fracture risk in patients with diabetes, in those with endogenous or exogenous glucocorticoid exposure and potentially in other conditions. Further studies evaluating these possibilities are needed.

Monitoring and Treatment Responsiveness

As noted earlier, many studies demonstrate cross-sectionally and longitudinally that lumbar spine TBS measurements show an age-related decline in the absence of osteoporosis pharmacologic therapy. Several studies have examined the response of TBS to antiosteoporosis therapies. In general, these confirm a small but significant increase in TBS though the magnitude of the effect is often considerably less than observed with BMD change.

The Manitoba BMD Database was assessed to identify 534 women age 50 years and older who initiated antiresorptive drug therapy (86% bisphosphonate) and subsequently had a high level of adherence (medication possession ratio at least 80% during the follow-up interval, mean 3.7 years) (Krieg et al. 2013). The treated women showed an annual spine BMD increase of 1.86% ($P < 0.002$) while TBS increased only 0.20% per year ($P < 0.001$). For comparison, in 1,150 untreated women, there was a similar annualized decrease in these measurements (BMD 0.36% per year, TBS 0.31% per year).

In a 3-year randomized study of zoledronate ($N = 54$) versus placebo ($N = 53$), the overall increase in lumbar spine BMD with active treatment (9.58%) was considerably greater than seen with TBS (1.41%). No significant correlation was seen between change in BMD and change in TBS, though this finding is difficult to interpreted given the associated measurement error in both measurements and low level of correlation in the baseline measurements ($r = 0.20$). Another randomized trial of zoledronate was conducted in premenopausal women with breast cancer

(Kalder et al. 2015). In women receiving zoledronate (N = 34), lumbar spine BMD at 24 months increased by 3.14% versus mean increase in TBS of 0.75%. Among the 36 women receiving placebo, lumbar spine BMD decreased at 24 months by 6.43% versus 2.16% for TBS. Denosumab was studied in 60 patients with postmenopausal osteoporosis, 30 receiving glucocorticoid therapy and 30 not receiving glucocorticoid therapy (Petranova et al. 2014). Twelve months of denosumab treatment increased BMD by 6.1% at the spine, 2.1% at the hip, but only 0.3% in TBS (Petranova et al. 2014).

TBS may show a larger increase in response to anabolic therapy. In a nonrandomized comparison of 2 years of treatment with teriparatide (N = 65) and intravenous bisphosphonate therapy with ibandronate (N = 122) over 2 years, TBS increased by 2.9% with the former and 0.3% with the latter ($P < 0.0001$), though this was less than the increase in lumbar spine BMD (7.6% vs. 4.3%, $P < 0.0001$).

In a small study of 36 women receiving tamoxifen (N = 17) or the aromatase inhibitor exemestane (N = 19), there was a detectable increase in TBS with the former but a significant decrease with the latter (Roux et al. 2013). In a retrospective analysis of estrogen deprivation occurring from spontaneous menopause (N = 29) versus aromatase inhibitor therapy (N = 34) found that over a mean follow-up of 2–3 years that aromatase inhibitor therapy produced a greater decrease in BMD than TBS (5.9% vs. 2.1%, $P = 0.002$). In 25 men with idiopathic osteoporosis treated with ibandronate, there was a significant increase in lumbar spine BMD (+3.4%, $P = 0.002$), total hip BMD (4.1%, $P < 0.0001$) and TBS (4.4%, $P = 0.0130$)

Human growth hormone replacement led to a significant increase in TBS after 24 months of therapy (4% change, $P = 0.02$) though this was less than the BMD increase seen (15% in males, 10% in females) (Kuzma et al. 2014). In a randomized placebo-controlled trial of vitamin D supplementation 2800 IU per day in primary hyperparathyroidism, no significant change in TBS was observed prior to surgery, but there was a significant increase following parathyroidectomy (1.9%, 95% CI 0.3–3.4%) (Rolighed et al. 2014).

A nonrandomized analysis of 390 patients age 40 years and older (including 72 men) examined the longitudinal effect of various osteoporosis treatments on TBS and lumbar spine BMD (Di et al. 2015). After 24 months, significant improvements were seen in BMD and TBS for alendronate (4.1% vs. 1.4%, respectively), denosumab (8.8% vs. 2.8%), and teriparatide (8.8% vs. 3.6%). Significant BMD increases were seen for risedronate without a significant change in TBS.

Although many of these studies are limited by small sample sizes, the general pattern is one showing that treatment-related changes in TBS are statistically significant in groups of subjects but that the magnitude of the increase is considerably smaller than seen with BMD. The overall increase in TBS would likely be very difficult to detect in an individual patient based upon measurement precision that is similar to or slightly worse than BMD (Bandirali et al. 2015). To date, no studies have demonstrated than BMD change on therapy correlates with antifracture effect and therefore the significance of an increase (versus stability

or decrease) in TBS remains uncertain. For this reason, the ISCD has recommended against use of TBS for monitoring bisphosphonate therapy (Shepherd et al. 2015). It is possible that more potent antiosteoporosis agents will induce a more robust change in TBS and provide a surrogate for antifracture effect, but this awaits supporting evidence. In conclusion, at present we do not have evidence that TBS can be used as a predictive biomarker to monitor current pharmacologic osteoporosis therapy.

Summary Points

- Trabecular bone score (TBS) is a gray-level texture measure that can be extracted from the two-dimensional lumbar spine dual-energy X-ray absorptiometry (DXA) images and provides skeletal information beyond the standard bone mineral density (BMD) measurement.
- TBS is associated with vertebral, hip, and major osteoporotic fracture risk in postmenopausal women.
- TBS is associated with hip fracture risk and with major osteoporotic fracture risk in men 50 years and older.
- TBS can be used in association with FRAX and BMD to adjust FRAX probability of fracture in postmenopausal women and older men.
- TBS can be considered a prognostic and response biomarker of skeletal structure and strength.
- TBS shows treatment-related increases, but these are considerably smaller than seen with BMD and, at present, there is insufficient evidence that TBS can be used as a predictive biomarker to monitor pharmacologic treatment.

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Determining Skeletal Geometry

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Abstract

Areal bone mineral density (aBMD), assessed by dual-energy x-ray absorptiometry (DXA), at the lumbar spine, total hip, or femoral neck is the single most important criterion for the diagnosis of osteoporosis, i.e., bone fragility, in postmenopausal women and in men aged 50 years old. However, technological and scientific advances during the last two decades have led to the realization that bone fragility and the associated risk of fracture are not solely determined by the aBMD. In response, the International Society for Clinical Densitometry has recently published a position paper on the relevance of the use of geometric bone measurements to estimate the risk of fracture using DXA and quantitative computed tomography (QCT).

The geometry of the skeleton is determined by the bone shape and size and can be quantified using measures that include (a) cross-sectional measures – areas, moments of inertia, radii, circumferences, and cortical thickness – and (b) other specific measures of proximal femur including hip axis length (HAL), neck-shaft angle (NSA), and femoral neck width (FNW). In general larger cross-sectional measures are associated with a smaller risk of bone fracture, while the larger specific measures of the proximal femur are associated with a greater risk of bone fracture. The current evidence concerning the geometry of the skeleton and risk of fracture is not equally conclusive for all measures. At this time, only HAL, derived from DXA, is recommended for evaluation of hip fracture risk in postmenopausal women. Cross-sectional measures or other specific measures of hip geometry parameters should not be used to assess fracture risk.

The risk of bone fracture is conditioned by the biomechanical characteristics of the bone structure in relation to forces resulting from axial mechanical loads, bending, and torsion. Bone fracture can be described as a structural failure due to a force higher than bone's mechanical resistance. However loads applied during everyday movements, i.e., physical activity within physiological limits, are key determinant for the optimization of bone geometry even without improvements in aBMD. This is particularly the case during the years of growth. Other determinants of bone geometry include interethnic differences, age, and gender. Because these latter determinants are nonmodifiable, they have little potential for intervention at the level of skeletal phenotype.

Keywords

Bone geometry, bone strength, and dual-energy x-ray absorptiometry (DXA) • Finite element analysis (FEA) • Fracture risk • Hip axis length (HAL) • Mechanical loading • Neck-shaft angle (NSA) • Quantitative computed tomography (QCT)

List of Abbreviations

aBMD	Areal bone mineral density
AHA	Advanced hip assessment
CSA	Cross-sectional area
CSMI	Cross-sectional moment of inertia
DXA	Dual-energy x-ray absorptiometry

FEA	Finite element analysis
FNAL	Femoral neck axis length
FNL	Femoral neck length
FNW	Femoral neck width
HAL	Hip axis length
HSA	Hip structural analysis
ISCD	International Society for Clinical Densitometry
NSA	Neck-shaft angle
PMI	Polar moment of inertia
pQCT	Peripheral quantitative computed tomography
QCT	Quantitative computed tomography
vBMD	Volumetric bone mineral density
Z	Section modulus

Key Facts of Sedentary Behaviors and Physical Activity

- Sedentary is a behavior associated with activities in a sitting or reclining posture that involve a low-energy expenditure ranging from 1.0 to 1.5 MET (basal metabolic rate) such as watching television or other forms of recreation or work with screens.
- Sedentary behavior is associated with adverse effects on health outcomes such as obesity, type II diabetes, cardiovascular diseases, pulmonary disease, cancer, and hip fracture.
- Sedentary behavior has adverse consequences even among those meeting the moderate-to-vigorous physical activity guidelines.
- Age-related declines in bone strength are much greater than reductions in aBMD; reductions in fracture risk with pharmaceuticals treatment (35–50% reduction) are also much greater than changes in aBMD (1–3% per year).
- Skeletal geometry alterations associated with mechanical loading from physical activity are principally cross-sectional because the skeleton responds to physical activity by increased apposition of the periosteal surface and/or by diminished resorption at the endocortical surface. These changes lead to greater periosteal circumference, bone area, cortical thickness, moments of inertia, section modulus, and buckling ratio.
- Physical activity may have considerable effects on bone geometry and consequently in bone strength in adults and older adults despite modest effects on aBMD (prevention of the loss of aBMD about 1.0–1.6% per year).

Definitions of Words and Terms

BMD	Bone mineral density or bone density is the amount of bone mineral in bone tissue expressed in grams per square centimeter of bone surface

	(g/cm^2, aBMD) when evaluated by DXA and in grams per cubic centimeter of bone volume (g/cm^3, vBMD) when evaluated by QCT.
Bone strength	Is the general integrity of bone that comprises the bone material properties (organic and inorganic composition), cellular activity, and bone structural properties (micro- and macro-architecture).
Cross-sectional bone geometry	Is a view of a three-dimensional bone in two dimensions allowing to see inside the internal macro-architecture (areas, moments of inertia, radii, circumferences, and cortical thicknesses); cross-sectional (tomographic) images of specific areas of bone are produced by QCT scans.
DXA	Dual-energy x-ray absorptiometry is the clinical tool used to assess skeletal health, namely, diagnosis of osteoporosis for which it was originally developed, although also assess body composition and can incorporate analytical tools that perform measurements on DXA images regarding macro-architecture of the proximal femur (bone geometry) and the micro-architecture of the lumbar spine (trabecular connectivity expressed by the trabecular bone score (TBS)).
FEA	Finite element analysis is a numerical technique that subdivides a large problem, usually related with structural or performance issues into smaller, simpler parts called finite elements.
Geometry of the skeleton	Characteristics of whole bone macro-architecture that provide dimensional information of whole bone.
In vitro studies	Studies that are conducted using components of an organism that have been isolated from their usual biological surroundings; in the case of bone strength measurement, in vitro studies are conducted with bone specimens.
In vivo studies	Studies that are conducted in animals including humans and whole plants.
Moment arm	Perpendicular distance from the line of action of a force and the rotation center.
Osteoporosis	Is a silent skeletal disease (there are no symptoms) characterized by low bone mass and structural deterioration of bone tissue leading to decreased bone strength and an increased risk of low-energy fractures.

QCT	Quantitative computed tomography is a technique that measures BMD using a standard x-ray computed tomography (CT) scanner with a calibration standard to convert Hounsfield units (HU) of the CT image to BMD values and is also the technique used to assess bone architecture.

Introduction

The geometry of the skeleton is defined by the characteristics of whole bone macro-architecture and provides dimensional information about whole bone. Skeletal geometry can be determined using different approaches depending on research fields and the type of research question being explored. In the health sciences, the study of skeletal geometry has been focused on the relation of size and shape to osteoporotic or fragility fractures, i.e., fractures that occur in the absence of trauma or as result of a low trauma usually following a fall from standing height or less; this is because skeletal geometry is an important contributing factor to bone strength, i.e., the resistance to loading forces (Bouxsein 2005; Friedman 2006).

Bone strength describes the general integrity of bone and comprises the bone material properties (organic and inorganic composition), cellular activity, and bone structural properties (micro- and macro-architecture). However, the clinical criteria to evaluate the risk of fragility fractures are generally limited to inorganic composition, expressed as areal bone mineral density (aBMD, g/cm^2) and measured using dual-energy x-ray absorptiometry (DXA). On average, a one standard deviation (SD) decrease in the aBMD (about 10% below the peak bone mass reached at around 20–30 years) is associated with a twofold increase in fracture risk (Cummings et al. 2002).

Although aBMD appears to be the single most important factor affecting fracture risk, it does not explain all of bone strength (Bousson et al. 2006; Cheng et al. 1997; Moro et al. 1995). Many people diagnosed with osteoporosis (aBMD $\leq$ 25% of peak bone mass $=$ T-score ≤ -2.5 SD) do not sustain fractures, while other people without osteoporosis sustain fragility fractures. For example, increasing the outer diameter of a long bone can improve its resistance to bending and torsional loading without necessarily increasing aBMD (Bouxsein and Karasik 2006). This example points to the need of describing more features of bone strength within clinical settings and using the technology available in these settings. However until now there have been no geometric measures that could be used clinically, despite important progress in advanced imaging procedures, in understanding the important role of skeletal geometry to bone strength.

This chapter reviews the general concepts of bone geometry (Fig. 1) as well as the key findings from studies concerning its main determinants – age, sex, ethnicity, and mechanical loading. Moreover, the role of skeletal geometry on bone strength and

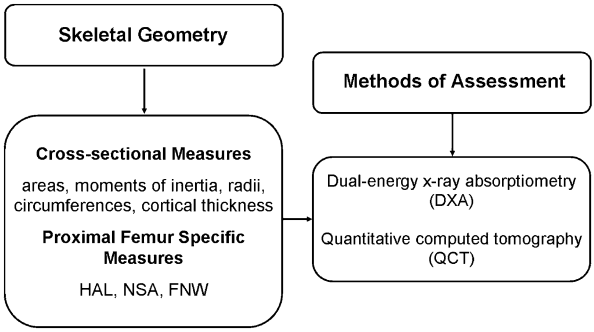

Fig. 1 Bone geometry and methods of assessment. This figure shows the main measures that characterize the whole bone geometry as well as the methods of assessment. *HAL* hip axis length, *NSA* neck-shaft angle, *FNW* femoral neck width

fracture risk as well as the methods used to assess the skeletal geometry are addressed. The attention was concentrated on technologies commonly available in clinical settings, specifically DXA and quantitative computed tomography (QTC). However finite element analysis (FEA) which can be used to evaluate biomechanical effects due to bone material and macrostructural (geometry) properties is also addressed.

Skeletal Geometry and Mechanical Loads

One of the main functions of the skeleton is to support the body and, together with the muscles, to provide movement. This implies that bones are subject to mechanical loads from body weight and muscle forces. Therefore it is not surprising that some features of skeletal geometry are determined by loads since bone (re)modeling depends, among other factors, on the mechanical environment. In fact, bone structural properties change in order to improve the bone strength for the applied loads. In turn, the intensity of load detected by a bone can depend on its geometry. For example, the force acting on the femoral head during gait (due to the body weight) depends on the femoral neck length (FNL) because the moment arm (perpendicular distance from the line of action of a force and the rotation center) changes with the distance.

In general, a long bone is subject to axial loading, bending, and torsion (Fig. 2). Assuming the shaft of a long bone is a circular crown (Fig. 3) and considering bone strength as the magnitude of fracture force, the maximum admissible force can be estimated. This force is proportional to the cross-sectional area (CSA) during only axial loading, and it is proportional to the ratio between the cross-sectional moment of inertia (CSMI) and the product of the diameter (D) by the bone length (L) (CSMI/LD) for bending. For torsion, it is proportional to the ratio between the polar moment of inertia (PMI, a measure of how bone mass is distributed around the shaft axis and it corresponds to two times CSMI) and the product of the diameter by the bone length (PMI/LD).

The relations among geometry, loads, and strength can be used to define skeletal geometry by cross-sectional measures – areas, moments of inertia, radii,

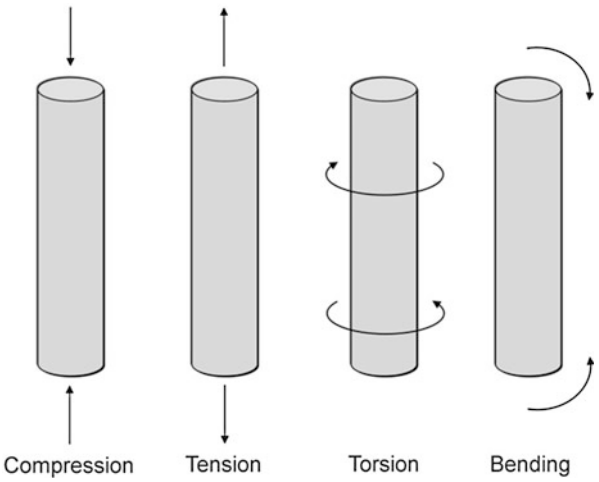

Fig. 2 Mechanical loading of long bones. This figure shows the types of mechanical loading of long bones: axial loading (compression, tension), torsion, and bending

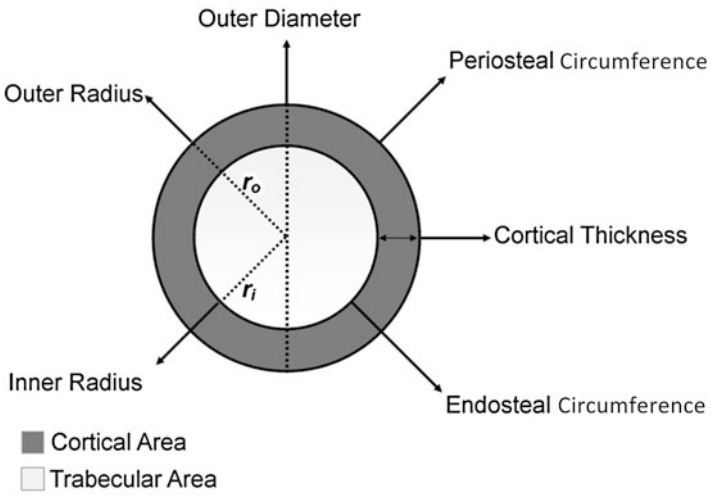

Fig. 3 Schematic illustration of the cross section of the shaft of a long bone. This figure shows the main parameters that characterize the bone cross-sectional geometry. The entire area comprises the bone cross-sectional area (*CSA*)

circumferences, and cortical thickness – and by other specific measures of proximal femur including hip axis length (HAL), neck-shaft angle (NSA), and femoral neck width (FNW) (Fig. 4), which are frequently used as parameters to assess the bone strength. Brief definitions of these measures are provided in the following text.

CSAs (total, cortical, and trabecular) are the size of the surface occupied by the integral bone or its compartments; the total CSA of the bone, also reported as total

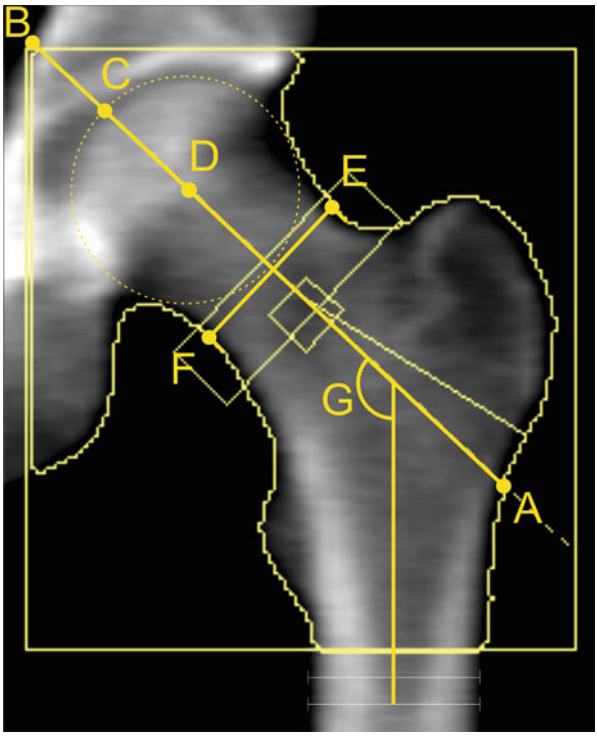

Fig. 4 Typical image of the proximal femur taken from DXA illustrating specific geometric measures. (**a**, **b**) Hip axis length (*HAL*), (**a**–**c**) femoral neck axis length (*FNAL*), (**a**–**d**) femoral neck length (*FNL*), (**e**, **f**) femoral neck width or narrow neck width, (**g**) femoral neck-shaft angle (*NSA*)

area, reflects bone's resistance to axial compression, as mentioned earlier. The periosteal and endosteal circumferences are the measures of the outer and inner circular contour, respectively, of the cortical compartment of the bone (Zemel et al. 2008), while the cortical thickness is the distance between the periosteal and endosteal circumferences. The cortical thickness is the result of both bone apposition at the periosteal side and bone reabsorption at the endosteal side. The outer diameter or subperiosteal width is the straight line segment that passes through the center of the bone with endpoints on the periosteal circumference (Burnham et al. 2007). The CSMI is a measure of how bone mass is distributed in relation to an axis containing the diameter of the cross section and is indicative of bone bending strength. The CSMI can be calculated as:

$$\text{CSMI} = I = \frac{\pi}{4}\left(R_o^4 - R_i^4\right)$$

where R_o is the outer radius and R_i is the inner radius (Zemel et al. 2008). For example, a bone diaphysis with its bone mass located closer to the axis will have lower bending strength compared to another one with the same amount of bone mass but distributed further away from the axis. Moreover, small increases in the outer

radius have a greater effect on the CSMI than relatively larger increases in the inner radius. This is because radial values are raised to the fourth power.

Relationships among cross-sectional measures of bone allow the estimation of biomechanical parameters of bone strength, i.e., section modulus and bucking ratio. The section modulus, expressed by Z or sometimes abbreviated as SM, results from the division of the CSMI by the outer radius (Choi 2016) and is also indicative of bone bending strength. The buckling ratio is the fraction of the outer radius to the cortical thickness and is suggestive of local structural instability. For example, during aging, the bone periosteal apposition increases at the outer radius which partially offsets the endocortical resorption. This phenomenon may improve the bone bending strength, again primarily determined by the distribution of bone in relation to its axis. However the strength of bone is not fully explained by resistance to bending, axial loading, and torsion. The risk of structural failure due to local buckling increases with a reduction of the cortical thickness. Thus, a large bone with a thin cortical thickness may result in a structural instability increasing the risk for local buckling.

In addition to measures of bone cross section that are common to long bones, other measures that are specific to bone regions at high risk for fracture are important to fully understanding the contribution of skeletal geometry to bone strength. One such set of measures is used to assess in the proximal femur and will be described in the following text.

The HAL is the distance along the femoral neck axis from the inner pelvic border to the outer edge of the greater trochanter. The femoral neck axis length (FNAL) is similar to HAL but does not include the acetabular structure. The FNL is the distance from the center of femoral head to the angle between derived axes of neck and shaft (NSA). Finally, the narrow width of femoral neck (FNW) is the narrowest width of the neck.

The relation between loads and the geometric measures defined above can be addressed by suitable computational and mathematical methods such as the FEA and bone adaptation models. In Machado et al. (2014), the influence of the FNL, NSA, and FNW on bone mass distribution of the proximal femur was studied. Authors reported the effect of these measures on the cortical thickness, as shown in Fig. 5, for the NSA variations.

Evaluation of Skeletal Geometry

DXA and QCT are the predominant approaches for evaluating bone geometry. Although DXA is usually thought of as an imaging technique for aBMD, geometric measurements of the proximal femur can be obtained through manual or automatic approaches with specific software such as hip structural analysis (HSA, Hologic Inc., Bedford, MA) and advanced hip assessment (AHA, GE Healthcare, Madison, WI). These approaches are gaining acceptance and providing new insights to skeletal geometry. Details of the manual approach are provided in the following text.

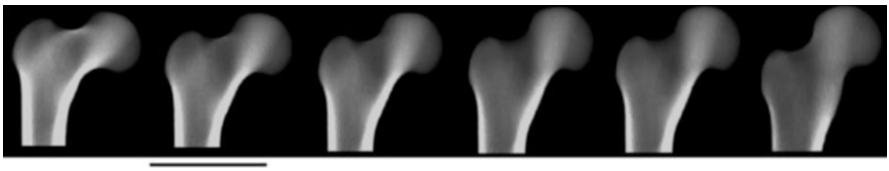

Fig. 5 Simulated proximal femur DXA images by computational analysis of finite element models according variations in the neck-shaft angle. Higher neck-shaft angles correlate with less bone mass (*in white color*) at both the femoral neck and shaft regions, a result that may be explained by a decreased stress that more vertical femoral necks are subject during gait

Determination of femur geometry manually using DXA images requires the use of a conventional ruler and goniometer or the use of software that provides these tools. The manual measures include linear distances and angles obtained directly on hip images, namely, the HAL, the NSA, and the FNW. These measures can be obtained more easily with the use of HSA or AHA software sold/provided by DXA manufacturers. In addition, the software estimates a broader set of measurements at the proximal femur including cross-sectional geometry at the neck, intertrochanter, and shaft regions, i.e., CSA, outer diameter, CSMI, section modulus, and buckling ratio. A full description of these methods and measurements can be found elsewhere (Beck and Broy 2015). One of the main limitations of these methods is the 2D nature of DXA to measure a complex 3D geometry, a limitation surpassed with the use of QCT technologies.

QCT is a 3D imaging technology which enables the quantification of bone density (mg/cm^3) and geometry at the integral or at the trabecular and cortical compartments, separately. The designation of central QCT defines QCT performed at the spine and proximal femur. The distal forearm can be assessed with QCT but also with small dedicated peripheral QCT scanners (pQCT). The appendicular skeleton sites, such as the forearm and tibia, can also be imaged with high-resolution peripheral CT (hr-pQCT). This device can evaluate the macrostructure (geometry) and the micro-bone structure including the trabecular connectivity and the cortical porosity.

The most common QCT geometric parameters are CSA (total, trabecular, and cortical), CSMI, and endosteal and periosteal circumferences. These parameters are used to estimate measures of biomechanical parameters of bone strength such as section modulus and buckling ratio. Reference data for bone cross-sectional geometry in pediatric ages (Ashby et al. 2009; Rauch and Schönau 2008) and young adults (Rauch and Schönau 2008) were provided for radius (Ashby et al. 2009; Rauch and Schönau 2008) and the tibia (Ashby et al. 2009; Moyer-Mileur et al. 2008).

Bone strength can only be experimentally evaluated by a mechanical test that determines in ex vivo the ultimate force necessary to fracture a bone. In vivo, the estimation of failure loads is only possible using the FEA. QCT is also the basis for FEA since finite element models are created from digital images obtained with QCT.

FEA is an engineering method that uses volumetric bone mineral density (vBMD) and bone geometry to estimate bone mechanical strength, i.e., the stress (concentration of mechanical force) and the strain (amount of physical deformation) under

various loading conditions such as axial compression, anterior bending, and torsion in the vertebrae and for stance (load experienced in walking and other daily activities) and fall in the proximal femur.

FEA of the hip and spine has been also used to provide insights into the biomechanical effects of a variety of factors such as age, sex, bone loss, pharmaceuticals, and mechanical loading. Additional information about steps required to perform QCT-based FEA can be found in the literature (Carpenter 2013).

Skeletal Geometry, Fracture Risk, and Bone Density

Cross-sectional geometry derived from DXA seems to predict fractures in older people. However, it is unclear whether these measures are independent of aBMD (Ahlborg et al. 2005; Kaptoge et al. 2008; Rivadeneira et al. 2007; Szulc et al. 2006). Moreover, most of the studies were conducted with postmenopausal Caucasian women, and there are only few data which explored the associations of cross-sectional geometry derived from DXA with fractures in men and other racial/ethnic groups whether they be male or female. Besides geometric measures derived by DXA, geometric parameters assessed by QCT and pQCT also predict or discriminate fractures in vivo and in vitro. A brief narrative of the literature is provided in the following text.

Healthy children with forearm fracture appear to have smaller measures of cross-sectional geometry (total and cortical area) at the radius compared with controls (Kalkwarf et al. 2011; Skaggs et al. 2001) (Table 1). In older adults, a smaller CSA of femoral neck was independently related to increased hip fracture risk in men (Black et al. 2008). In elderly women the cortical thickness was significantly thinner in the neck, trochanter, intertrochanter, and proximal shaft of the femur in a hip fracture group compared with controls (Bousson et al. 2011). Also in older adults, postmenopausal women with vertebral fractures had larger cross-sectional and endocortical areas but a significantly smaller cortical thickness (Melton et al. 2007). This finding suggests the importance of assessing multiple geometries when assessing risk.

Studies in vitro (conducted with bone specimens) verify that bone cross-sectional geometry and derived parameters (total area, cortical area, and section modulus) at the tibial midshaft were strongly correlated with bone mechanical properties including 62–77% of the variance in the failure load, i.e., the force required to break the bone (Liu et al. 2007). At the distal radius, cortical area and thickness and the minimum moment of inertia (cross section with the smallest diameter) have also been associated with the failure load (Augat et al. 1996). At the spine, geometric dimensions, specifically the area of the vertebral end plates (Biggemann et al. 1988; Brinckmann et al. 1989) and the CSA (Singer et al. 1995), improved the associations between trabecular bone density and compressive strength of vertebral bodies (Brinckmann et al. 1989). In the femur, the femoral neck CSA and the FNAL in combination with vBMD explained 87–93% of femoral strength assessed in vitro, while BMD alone explained 48–77% (Lang et al. 1997).

Table 1 Quantitative computed tomography (*QCT*) and peripheral QCT of in vivo studies showing associations between bone geometry and bone fractures. This table summarizes findings from studies that used QCT and pQCT methodology to demonstrate that bone geometry may predict or discriminate bone strength or fractures in vivo. Abbreviations: *CSA* cross-sectional area, *HR* hazard ratio (relative risk of fracture based on comparison of fracture rates), *VOI* volumes of interest

Authors (year)	Study design	Population description	Number of subjects	Bone region	Bone geometry outcomes
Kalkwarf et al. (2011)	Cross-sectional study	Sex, 209 boys and 215 girls Age, 5–16 years Race, no restriction regarding race/ethnicity	424	Radius	At 20 % (diaphysis) from the distal end of the radius fracture, cases had lower cortical area (−2.8 %) than controls. There were no differences in periosteal or endosteal circumference and cortical thickness
Skaggs et al. (2001)	Cross-sectional study	Sex, 100 girls Age, 4–15 years Race, white	100	Radius	Girls with fractures had, on average, 8 % smaller CSA at the distal radius (1.82 ± 0.50 cm^2 vs. 1.97 ± 0.42 cm^2; $p < 0.0001$) than controls
Black et al. (2008)	Prospective study Follow-up, an average of 5.5 years	Sex, 3347 men Age, ≥65 years Race, ethnically diverse	3347	Femur	Smaller neck CSA (HR, 1.6; 95 % CI, 1.2–2.1) independently related to increased hip fracture risk
Bousson et al. (2011)	Prospective study	Sex, 107 women Age, ≥ 60 years Race, white	107	Femur	Cortical thickness was significantly thinner in the neck, trochanter, intertrochanter, and proximal shaft VOIs in the hip fracture group with percentage differences between mean values of 4.31 %, 4.44 %, 5.73 %, and 13.22 %, respectively
Melton et al. (2007)	Cross-sectional study	Sex, 80 women Age, 39–97 years Race, original sample ($n = 248$), 98 % White	80	Lumbar spine	Women with vertebral fractures had smaller cortical thickness (1.6 ± 0.2 vs. 1.8 ± 0.2 mm)

We now turn attention from the general discussion of bone geometry to a site of great clinical significance, i.e., the hip. Hip fractures are of clinical concern due to their high rate of morbidity and mortality. Although they constitute only 14% of all incident of osteoporotic fracture, they account for ~72% of the fracture cost treatment (Burge et al. 2007). This high personal and economic cost provides a compelling reason to better understand the biomechanics of hip fractures.

A longer HAL has been associated with hip fractures (Faulkner et al. 2006; Gnudi et al. 2012; Li et al. 2013; Szulc et al. 2006), but some divergence in the literature exists (Bousson et al. 2011; Pande et al. 2000). Disagreements can be attributed to variations in study design, measurement techniques, sample size, and statistical design, particularly covariate selection including adjusting (or not as the case may be) for aBMD. The adjustment for aBMD is particularly important because a longer HAL is usually associated with larger bones and a higher aBMD, and the risk from a longer HAL can be compensated by an increased aBMD (Broy et al. 2015).

The positive association between HAL and hip fractures has mainly been reported in studies of postmenopausal Caucasian women; there is insufficient evidence that HAL predicts fracture in men (Pande et al. 2000). However, a recent report from the Manitoba database (Canada) found that in men and women aged 40 years and older, the mean HAL was significantly greater in those with an incident of hip fractures compared to those without an incident of hip fractures (men, 123.0 $\pm$ 7.6 vs. 121.3 $\pm$ 7.4 mm; women, 106.9 $\pm$ 6.2 vs. 104.6 $\pm$ 6.2 mm) (Leslie et al. 2016). When adjusted for age and femoral neck aBMD, each millimeter increase in HAL was associated with an increased hip fracture risk of 3.6% in men and 4.6% in women.

The mechanism for the association between HAL and hip fractures is not known. One possible explanation is that a long HAL contributes to a long moment arm of force on the proximal femur during sideways fall (Wang et al. 2009). Although at the present time, HAL cannot be used to quantify the risk of hip fracture; it shows a great promise for improving fracture risk assessment in the clinical setting and in postmenopausal women without requiring imaging technology other than DXA.

The evidence for NSA and the risk of hip fracture is mixed. Some studies have reported positive associations (Alonso et al. 2000; Gnudi et al. 2004; Kaptoge et al. 2008) and another no associations (Bergot et al. 2002). In general a greater NSA appears to be associated with hip fracture in older men and women. The mechanism of how NSA might contribute to hip fracture seems to be similar to the HAL: a long moment arm associated with a greater NSA may induce a bigger impact on the proximal femur during a sideways fall (Wang et al. 2009). Nevertheless it is unclear if this risk is independent of aBMD since it is possible that NSA might be a moderator of aBMD. Recently, the 2015 International Society for Clinical Densitometry (ISCD) Position Development Conference reported that HAL derived from DXA is associated with hip fracture in postmenopausal women independently of aBMD, but the CSA, periosteal circumference, section modulus, CSMI, and NSA should not be used to assess fracture risk (Broy et al. 2015).

Computational bone remodeling models such as the FEA evaluate the stress and strain of bone and have been used to determine bone mass distribution effects due to

load changes, disease conditions (Fernandes et al. 1999; Jacobs et al. 1997; Weinans et al. 1992), and specific bone geometry (Machado et al. 2014). Although phenomenological models for bone remodeling do not include all biological mechanisms involved in this complex process, they do reproduce the bone distribution at a macroscale and, therefore, represent powerful tools to assess how several important conditions and features influence bone (Folgado et al. 2009; Machado et al. 2013; Quental et al. 2014).

Since bone mass distribution at the proximal femur is associated with hip fracture risk (Yang et al. 2012), a reasonable approach to link hip geometry and hip strength is to analyze how variations of proximal femur geometric characteristics create specific spatial bone mass distribution patterns. For example, FEA suggests hip geometry is a moderator of the mechanical loading influence on bone mass distribution at the proximal femur (Machado et al. 2014).

Determinants of Skeletal Geometry

Age and Sex

Among the demographic factors that influence skeletal geometry, age and sex are likely the most important. Bone geometry changes throughout life by modeling and remodeling (Seeman 2009). These changes are more pronounced during the years of growth and include changes in linear (length), cross-sectional (areas, circumferences, thickness, and moments of inertia) and angular (NSA) dimensions. However, changes also occur during adulthood especially later in life. The most commonly reported changes in bone geometry with aging are cross-sectional and include the increased periosteal circumference of long bones. These changes have been suggested as a compensatory adjustment to unfavorable modifications in bone material properties and micro-architecture including less trabecular connectivity and more cortical porosity.

Changes in bone geometry appear to be sex specific with the male skeleton showing greater bone robustness (bone width relative to bone length) than the female skeleton. This robustness can be observed during adolescence at bone sites where muscle tendons are inserted reflecting the combination effects of sex steroids and mechanic loading imposed by muscle forces (Lang 2011). However, sexual dimorphism in cross-sectional bone geometry is established at younger ages (Forwood et al. 2004); e.g., during early growth, the total and cortical CSA of long bones increases with body height in males and females; nonetheless between 5 and 19 years old, the increments are greater in boys than girls at each body height interval (Moyer-Mileur et al. 2008; Rauch and Schönau 2008). Long bones widen by periosteal apposition (Duan et al. 2003; Riggs et al. 2004); this geometric change is observed in males and females, but periosteal apposition appears to be greater in males than in females and contributes to a greater cross-sectional geometry (periosteal circumference, cortical thickness, CSMI, and section modulus). However, if the

apposition is lower than endocortical reabsorption which is often the case in older adults, cortical thinning occurs which may lead to structural failure via local buckling.

Few studies have investigated the change of HAL with age. Limited findings show that HAL increases with growth (Flicker et al. 1996; Goulding et al. 1996; Wang et al. 2015), with no consensus about the age at the peak of HAL (maximum length achieved) or about HAL changes in adulthood. A study spanning three generations of women (White young girls, adult mothers, and old grandmothers) suggested that HAL increases from premenarche to the end of adolescence, reaches a plateau during adulthood (similar HAL between 18-year-old females and their premenopausal mothers, 102.8 ± 5.7 mm vs.103.9 ± 5.8 mm, respectively), and probably continues to increase during late adulthood since the HAL of grandmothers was longer (104.3 ± 5.7 mm) than that of girls and their mothers (after correction for body height) (Wang et al. 2015) (Table 2). On the other hand, Zhang et al. (2011) reported that in Chinese subjects, HAL reaches a peak at 35–39 years in men and in women (112.0 ± 6.2 mm vs. 98.6 ± 5.4 mm, respectively) and over 85 years of age HAL is 4% and 2% shorter in women and in men, respectively, compared to peak values (not height and weight adjusted). After adjustment for body height and weight, HAL increased with age after 50 years in women and 55 years in men (Zhang et al. 2011). Longitudinal studies with a large range of age and greater follow-up are necessary to obtain a clearer perspective of changes in HAL with age.

HAL varies by sex with men having on average a longer HAL than women (Gao et al. 2008; Leslie et al. 2016; Nissen et al. 2005). Indeed twin studies have suggested that variation in HAL is strongly determined by genetics (~80%) and in lesser percentage (~20%) by environmental factors. In particular body height which is itself largely under genetic influence appears to explain about one-fourth of the HAL variation (Flicker et al. 1996). Because of this strong relation of HAL with body height, taller individuals tended to have longer HAL than shorter individuals. Thus, it is not surprising that HAL is generally longer in men than women, since men are generally taller than women. Therefore studies analyzing this measure should be adjusted for body height. In individuals of Chinese descent, even after adjustment for body height and weight, HAL was longer in men than in women (Zhang et al. 2011).

Unlike HAL, the NSA is progressively reduced with age (Houston and Zaleski 1967; Wilson et al. 2009) and is largely dependent of weight-bearing activities. These observations come mainly from studies with individuals with cerebral palsy with reduced or absent weight bearing during development (Houston and Zaleski 1967). Data from modern, historic, and prehistoric human population also show a significant increase in NSA across these populations with the mechanization and sedentary existence (Anderson and Trinkaus 1998). Findings are inconsistent concerning NSA differences between males and females. Nissen et al. (2005) reported a higher NSA in men compared to women, whereas others report no differences in NSA (Gilligan et al. 2013; Nakahara et al. 2011).

Table 2 In vivo studies showing sex and age differences in hip axis length assessed by dual-energy x-ray absorptiometry (*DXA*). This table summarizes findings from studies that observed sex and age differences in hip axis length (*HAL*) assessed by DXA

Authors (year)	Study design	Population description	Number of subjects	Outcomes
Wang et al. (2015)	Longitudinal (7 years of follow-up from premenarche to late adolescence) and cross-sectional study	Sex, 251 white girls; 128 premenopausal mothers; 128 postmenopausal grandmothers Age, girls from premenarche (11.2 ± 0.7 years) to late adolescence (18.3 ± 1.1 years); premenopausal mothers (44.9 ± 4.1 years); postmenopausal grandmothers (70.0 ± 6.3 years) Race/ethnicity, white	507	HAL increased with age from 93.5 ± 6.7 mm (11 years) to 102.8 ± 5.7 mm (18 years) HAL was similar between 18-year-old girls and their premenopausal mothers (102.8 ± 5.7 mm vs.103.9 ± 5.8 mm, respectively); HAL of grandmothers was longer (104.3 ± 5.7 mm) than that of girls and their mothers
Zhang et al. (2011)	Cross-sectional study	Sex, 14,435 women and 4,067 men Age, 15–101 years Race/ethnicity, Chinese	18,502	Peak of HAL was observed at 35–39 years in men and women (112.0 ± 6.2 mm vs. 98.6 ± 5.4 mm, respectively) At age of over 85 years, HAL was 4 % shorter in women and 2 % shorter in men when compared with peak values After adjustment for body height and weight, HAL increased with age after 50 years and 55 years in women and in men, respectively HAL was longer in men than women, ranging from 13.6 % (between peak values) to 15.9 % (≥85 years of age) in

(*continued*)

Table 2 (continued)

Authors (year)	Study design	Population description	Number of subjects	Outcomes
				the same age-matched groups, respectively. The difference between sexes persisted even after adjustment for body height and weight
Leslie et al. (2016)	Longitudinal study (6.2 years of follow-up)	Sex, 4,738 men and 50,420 women Age, $\geq$ 40 years Race/ethnicity, largely of European ancestry (>98 %)	55,158	Mean HAL was significantly greater in subjects with vs. without incident hip fractures (men 123.0 $\pm$ 7.6 vs. 121.3 $\pm$ 7.4 mm, $p = 0.050$; women 106.9 $\pm$ 6.2 vs. 104.6 $\pm$ 6.2 mm, $p < 0.001$)
Nissen et al. (2005)	Cross-sectional study	Sex, 94 males and 155 females Age, 19–79 years Race/ethnicity, Caucasian	249	HAL was longer in men (10.9 $\pm$ 0.7 cm) than in women (9.5 $\pm$ 0.6 cm). Data not adjusted for height

Ethnicity

Most of the studies that have analyzed ethnic and racial differences in bone geometry have been focused on older adults, particularly women. Cummings and colleagues (1994) reported that White women have a longer HAL at the proximal femur than Asian and Black women. During pre- and early perimenopause, Danielson and colleagues (2013) reported less favorable cross-sectional geometry (lower CSA, lower section modulus, and higher buckling ratio) in White women compared to African American and Japanese but better geometry than Chinese women (Danielson et al. 2013). Asian and Black adult men also present a shorter HAL than White men (Cummings et al. 1994; Wang et al. 1997).

After adjustment for tibia length and muscle CSA, Leonard et al. (2010) reported greater bone dimensions, specifically periosteal and endosteal circumferences, and section modulus in Black compared with White children in Tanner stages 1–4 but not in Tanner stage 5 (Leonard et al. 2010). Differences in bone geometry were also reported in 3-year-old children with greater total area, endosteal diameter, tibial diameter, and periosteal circumference in the Black compared to White children of South Africa (Micklesfield et al. 2011).

Mechanical Loading

Bone is a very dynamic tissue and adapts to mechanical loads by changing its material properties, cellular activity, micro-architecture, and geometry. Since physical activity and exercise (structured physical activity) are the most relevant sources of mechanical loading in vivo, many studies have examined their effect on bone. In children, physical activity appears to be a positive predictor of skeletal geometry, including CSA and section modulus of the femoral neck (Forwood et al. 2006). On average, children who spent 40 min of moderate-to-vigorous physical activity per day would be expected to have 3–5% greater femoral neck CSA and section modulus than peers who spent 10 min of physical activity per day (Janz et al. 2007). Moreover, the bone health benefits of physical activity performed during childhood appear to be maintained during late adolescence even after markedly decreased in physical activity levels during puberty (Janz et al. 2014).

Pediatric exercise intervention studies suggest that the positive effects on femur geometry may be sex and maturity dependency (Bradney et al. 1998; Petit et al. 2002). Prepubertal boys submitted to 8 months of 30-min sessions of weight-bearing physical education lessons three times weekly revealed a greater increase in femoral midshaft cortical thickness than control boys with no effects in the femoral neck geometry of prepubertal girls. Interestingly, the differences observed in the femoral midshaft cortical thickness between boys were due to a reduction in the endocortical expansion (rather than periosteal expansion) (Bradney et al. 1998). Observed differences due to exercise intervention have been reported for femoral neck CSA, cortical thickness, and section modulus for girls in early puberty but not in other stages of sexual maturity. Specifically, the femoral neck geometry was greater in the intervention group. The increase in cortical thickness was also due to a decrease in the endosteal expansion in the exercise group (Petit et al. 2002).

Male adult tennis players (29.8 $\pm$ 4.8 years old) who began to play in childhood (starting age 9.8 $\pm$ 3.0 years) showed a greater bone mineral content at the dominant than at the nondominant arm due to an increase in bone size, particularly in total CSA and cortical thickness (Haapasalo et al. 2000). Similar differences were reported for female adult racquet players (age 26.5 $\pm$ 8 and 44.4 $\pm$ 10.5 years old); however the side-to-side differences were higher in females who started playing earlier (10.5 $\pm$ 2.2 years old) than those who began to play latter (26.4 $\pm$ 8.0 years old) (Kontulainen et al. 2002). These between-arm differences provide strong evidence that exercise contributes to bone geometry, since they cannot be a result of sample bias or genetic factors.

In general, these and other studies support the causal role of physical activity and exercise in optimizing bone geometry during the growing years. In middle and old age, the effects are less clear. Due to hormonal changes associated with menopause, most studies have been conducted in postmenopausal women. In these women, the most extensive geometric changes were observed in response to high-impact loading exercise, and training effects primarily influenced the cortical, rather than the trabecular, bone (Hamilton et al. 2010).

The preceding section provided evidence that mechanical loading from impact and muscle forces during physical activity can influence bone geometry. However, it is also possible that bone geometry may affect how forces from physical activity are distributed, which in turn creates adaptive bone mass distribution differences. This effect has most often been studied at the proximal femur. Bone geometry has been shown to moderate the influence of physical activity in vBMD producing different bone mass distribution patterns. However contradictory findings have been reported based on differences between computational (FEA) vs. DXA analysis. Using FEA a high FNAL seemed to favor vBMD, whereas FNW had the opposite effect (Machado et al. 2014): augmented values of these geometric measures appear to promote vBMD at the superolateral compared with the inferomedial neck region. Using DXA analysis a high FNAL and FNW are associated with low aBMD at the femoral neck and especially at superolateral neck. These geometric measures were significant for bone mass distribution only in females (Zymbal et al. 2014b). Again using DXA analysis, Zymbal and colleagues reported that lean soft tissue contributed to the variation of geometric measures of the proximal femur, particularly in men, and independently of vigorous physical activity. In women associations of lean soft tissue and FNAL were mediated by vigorous physical activity (Zymbal et al. 2014a).

Potential Applications: Modeling of Mechanical Loading Scenarios on Bone Strength by QCT-based FEA

Although recent studies suggest the bone is an endocrine organ which plays an important role in energy metabolism, histologically the bone has been regarded as a mineral reserve and structural organ whose primary function is load bearing and motion. In this latter context, the skeleton needs to be resistant to withstand loads and to be lightweight for mobility. These paradoxical functions are met by bone material and structural properties including its geometry; without optimized skeletal geometry, the required lightness could not be achieved without compromising bone resistance.

Physical activity is the major modifiable determinant for optimizing bone function at the level of material and structural properties. Therefore, it is necessary to understand how physical activity contributes to the optimal combination of bone mass and geometry. It is also important to understand the best possible combination of bone mass and geometry to meet the usual loads of daily living and especially of low-trauma falls.

The improved understanding of bone strength in medicine has helped clinicians and therapists to improve the diagnosis and treatment of bone fragility. It is now time to advance this understanding in the public and in sport science and exercise professionals working in schools, clubs, gyms, or health centers.

The QCT-based FEA has been used mainly to analyze changes in bone strength due to age, sex-based differences, treatment with pharmaceuticals (antiresorptive and anabolic treatments), and reduced mechanical loading associated with living in a microgravity environment (astronauts) and bed rest. It is an approach that should

also be used in prevention research. For example, investigating how bone strength can be changed with specific doses of exercise as well as how this change is moderated by sex, age, and race/ethnicity. This is one of the great advantages of QCT-based FEA, the ability to investigate a variety of different mechanical loading scenarios on bone strength via changing the modeling parameters.

Summary Points

- A distinction should be made between diagnosis of osteoporosis (evaluation of aBMD by DXA) and fracture risk assessment.
- There have been many scientific advances in fracture risk prediction beyond aBMD.
- Other factors affecting fracture risk include material properties of the organic matrix, cellular activity, and bone structural properties, namely, micro-architecture (trabecular connectivity and cortical porosity) and macro-architecture (skeletal geometry) which provide dimensional information of whole bone.
- Images obtained by DXA and QCT can be used to evaluate skeletal geometry.
- The geometry of the skeleton includes cross-sectional measures – areas, moments of inertia, radii, circumferences, and cortical thicknesses – and other specific measures of the proximal femur including HAL, NSA, and FNW.
- In general greater cross-sectional measures are associated with a smaller risk of bone fracture, while greater specific measures of the proximal femur (HAL, NSA, and FNW) are associated with greater risk of bone fracture.
- At this time, DXA-derived HAL is the only specific measure of the proximal femur recommended for evaluation of hip fracture risk (and only in postmenopausal women).
- Relationships among cross-sectional measures of bone provide an estimation of the biomechanical parameters of bone strength, i.e., section modulus and bucking ratio.
- Bone strength can only be experimentally evaluated by a mechanical test that determines in ex vivo the ultimate force necessary to fracture a bone.
- In vivo, the estimation of failure loads is only possible using the FEA.
- Features of skeletal geometry are determined by age, sex, ethnicity, and mechanical loads; in turn, the magnitude of mechanical loads on bone is affected by its geometry.
- Physical activity is the most relevant source of mechanical loading in vivo providing crucial adaptations in the geometry of the skeleton.

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Differentiation Biomarkers of Osteoarthritis Determined by Glycoblotting

28

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Abstract

Drastic structural alteration of the cell surface glycans during cell differentiation can provide clinically potential biomarkers. Recently, there have been substantial advances in our understanding of the importance of the posttranslational protein glycosylations in various biological systems. However, we still do not fully understand the significance and mechanism of the glycoform changes during cellular proliferation and/or malignant alterations detected widely in many human diseases progress due to the difficulty of the structural analysis of highly complicated glycan structures. Moreover, the general and conventional procedures for the glycan analysis need extremely tedious and time-consuming multiple processes to enrich whole glycans directly from heterogeneous biological samples such as blood, body fluids, cultured cells, tissue/organ, and so on. As a result, the therapeutic/diagnostic potential of the dynamic disease-specific alterations in the posttranslational glycosylations has not been well exploited with a few notable exceptions. This chapter describes the advantage of the use of a key technology for the glycan-specific enrichment protocol, namely a "glycoblotting method," in the discovery research of new class of human osteoarthritis cartilage-related biomarkers indicating the initiation of chondrocyte cell differentiation in the early stage of the cartilage degradation.

Keywords

Glycomics • Glycoblotting method • Glycoform-focused reverse proteomics and genomics • Cell surface glycoproteins • Osteoarthritis • Chondrocyte differentiation • Cartilage degradation

List of Abbreviations

AQP1	Aquaporin-1
BOA	Benzyloxyamine
CHO	Chinese hamster ovary
ConA	Concanavalin A
DMB	1,2-diamino-4,5-methylene-dioxy-benzene
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase-1
ESCs	Embryonic stem cells
iPSCs	Induced pluripotent stem cells
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
MSCs	Mesenchymal stem cells
NMR	Nuclear magnetic resonance
OA	Osteoarthritis
PCR	Polymerase chain reaction
PNGase F	Peptide *N*-glycosidase F

Key Facts of Glycans as OA Biomarkers

Discovery of highly sensitive biomarkers to assist the development of novel therapeutic reagents of osteoarthritis is strongly needed for improving QOL of patients suffering from osteoarthritis.

Glycoform alteration in the chondrocyte cell surface glycoproteins is associated with a crucial mechanism in the pathogenesis of osteoarthritis.

The glycoblotting is the only method applicable to rapid and large-scale clinical glycomics for various biological samples such as serum, cells, tissue/organs, etc.

By applying glycoblotting-based approach, five new glycoproteins were identified as highly sensitive biomarkers closely related to the pathogenesis of human osteoarthritis.

Definitions of Words and Terms

Glycoblotting method	A new technology to enrich whole glycans released from glycoconjugates based on the specific chemical ligation reactions between carbohydrates and reactive polymers.
Glycoconjugates	Biomolecules modified by glycans through covalent bonds such as glycoproteins, glycosphingolipids, and proteoglycans.
Glycoform-focused reverse proteomics and genomics	The concept toward the discovery of glycoprotein biomarkers starting from the specific glycan structures (glycoforms) revealed by the comprehensive glycomics. Protein and/or peptides carrying focused glycans will be enriched by affinity chromatography and gene expression analysis of the identified glycoproteins can be performed.
Glycomics	A term represents general method glycan analysis to determine sugar composition and sequences attach to the glycoconjugates.
Glycopeptidic epitope	Antigenic determinant elaborated by specific protein glycosylation. Glycosylation often induces site-specific conformational alterations in the peptide epitope during disease progress.

Posttranslational protein glycosylation	One of the most important protein modification processes in Golgi/ER biosynthetic pathways.

Introduction: Importance of Dynamic Protein Glycosylation in Human Chondrocyte Differentiation

Human osteoarthritis (OA) is the most common form of the joint disease that can be characterized by the degeneration of the articular cartilage (Wieland et al. 2005; Clouet et al. 2009; Hunter 2011). Recently, OA is recognized as one of the major chronic diseases that cause frequently disability in older persons, particularly in performing daily activities. Over the next two decades with population aging, it is clear that the prevalence of OA will increase dramatically. Although OA appears to affect more than 100 million individuals all over the world, major pharmacological therapies are currently limited to treating pain, a principal symptom of this disease, generally by using acetaminophen, nonsteroidal anti-inflammatory drugs, cyclooxygenase-2 inhibitors, glucocorticoids, and opioids. Other drug candidates targeting catabolic enzymes or cytokine-activated signaling cascades are also in development while there is a curative method to induce regeneration and repair of cartilage. Considering accumulated evidences that mesenchymal stem cells (MSCs) such as bone marrow, adipose tissue, and synovial fluid have potencies to induce regeneration and chondrogenic differentiation (Sakaguchi et al. 2005; Nöth et al. 2008), reagents can activate the mechanism to initiate differentiation and proliferation of MSCs in vitro and would contribute to repair and regeneration of damaged cartilage. It is important to note that discovery of highly sensitive biomarkers to assist the development of novel therapeutic reagents to induce differentiation of MSCs is strongly needed for improving QOL of patients suffering from OA.

Protein glycosylation is one of the most important posttranslational modifications of proteins, which is considered to affect a variety of biological functions of proteins, including enhancement of protein stability, modulating molecular recognition between receptors and ligands to control cellular adhesion, and intracellular signal transduction (Haltiwanger and Lowe 2004). It has been well documented that glycosylation of proteins greatly contribute to the pathogenesis of many diseases through its dynamic structural alteration (Dube and Bertozzi 2005; Ohtsubo and Marth 2006). One of the characteristics of cartilage is that chondrocytes exist in the extracellular matrix, and one major resident in the chondrocyte cell surface is glycoproteins. Therefore, it was considered that glycoform alteration in the chondrocyte cell surface glycoproteins can be associated with a crucial mechanism in the pathogenesis of OA. To test this hypothesis, our attention was directed to the relationship between changes in the glycan structures attached to the cellular glycoproteins and the pathogenesis of human OA and discovery research of new glycan-related biomarkers indicating chondrocyte cellular differentiation leading to the cartilage degradation.

Unmet Needs in Glycobiology: PCR Cannot Amplify Any Small Oligosaccharide Fragments to Generate Full Length Native Glycoforms

Glycomics is a term defining the sequence identification or profiling of the glycome. Since the glycan biosynthesis is not template driven but subject to multiple sequential and competitive enzymatic pathways, the glycomics differs fundamentally from genomics and proteomics (Fig. 1). At present, there is no PCR-like glycan amplification technology for glycomics. Although proteomics and genomics can use satisfactory amounts of the genomes and proteins of interest by using PCR-based amplification/recombination technology, glycomics need an enrichment process of glycans from highly complicated mixtures such as body fluids, cells, tissues, organs, etc. Major glycan chains attached to the proteins categorized as *N*- and *O*-glycans can be released selectively from the proximal peptide moieties of the core proteins and purified as common samples to be subjected to further analytical processes. If enough amount of glycans or glycoproteins themselves could be isolated directly from heterogeneous biological samples, general analytical technologies such as common NMR, MS, and even X ray crystallography must greatly contribute to accelerating the structural and functional characterization of targeted glycoproteins.

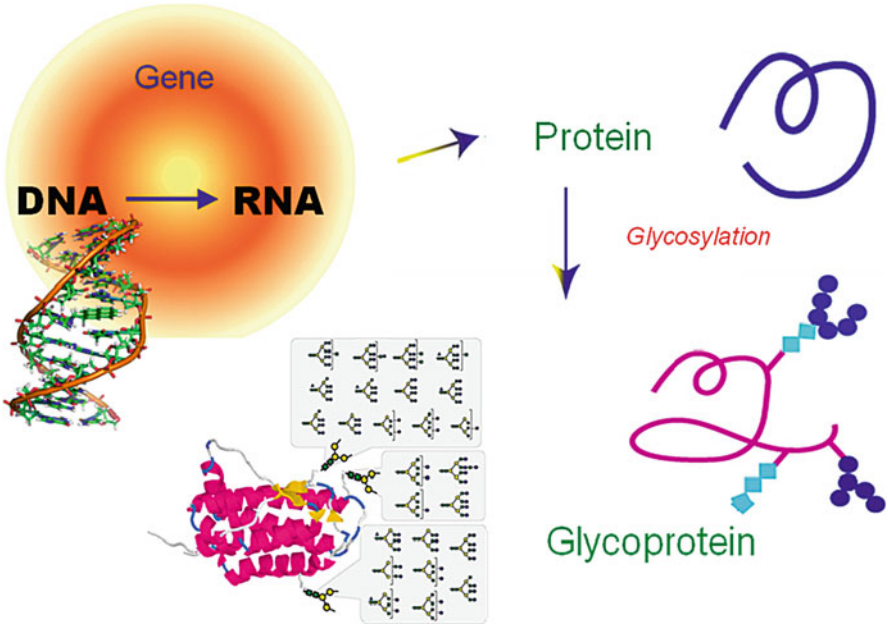

Fig. 1 Biosynthetic pathway of glycoproteins. No one can predict individual glycan structures attached to protein core and their distribution (microheterogeneity). Recombinant human erythropoietin produced by CHO cells displays highly complex *N*-glycan heterogeneity (Original figure made by author)

The bottleneck in the glycomics had long been definitely on the difficulty that the purification of total or even major glycans requires extremely tedious and time-consuming chromatography-based multi-step processes. Since glycans released by enzymatic digestion or chemical manipulation of whole glycoproteins in the biological samples are usually obtained as heterogeneous mixture containing large amounts of impurities such as peptides, nucleotides, lipids, salts, etc. To date the technical problems in the purification processes of glycans make it difficult to achieve high throughput and large-scale glycomics in a quantitative manner. Advent of novel method that allows high-throughput glycomics is strongly required for accelerating our insight into the structural features and molecular basis of functional roles of a variety of glycoconjugates and the discovery research toward disease-specific glycan biomarkers.

Glycoblotting Method: A Standard Method That Allows Rapid and Efficient Glycome Profiling Towards Potential Biomarker Discovery

Recently, we established a standardized protocol for the selective enrichment of glycans from heterogeneous compound mixtures based on the simple chemical ligation reaction (nucleophilic addition reaction) between the reducing carbohydrates and solid materials having specific functional groups that react specifically with compounds carrying an aldehyde/ketone group, namely glycoblotting method (Nishimura et al. 2005). The discovery of *a PCR-like key technology for glycan-specific enrichment protocol* allowed for the first time high throughput and quantitative glycomics (Nishimura 2011). Figure 2 shows the general concept of the glycoblotting-based glycomics (Fig. 2a) and a protocol designated for the structural analysis of the whole protein *N*-glycans and glycans of glycosphingolipids enriched from human brain tissue samples (Fig. 2b).

It was reported that glycoblotting accomplished on the designated bead platform is the only method applicable to large-scale clinical glycomics of human whole serum glycoproteins (96 samples/plate). This protocol required very small amounts of samples (<100 μL/patient) and, when combined with the automated system "SweetBlot^{TM}" and common MALDI-TOFMS, only took 24 h to complete from glycoblotting to the glycoform profiling of 96 human serum samples. Recent studies revealed that dynamic alterations in the human serum glycoform profile represent promising diagnostic biomarkers not only in some cancers such as hepatocellular carcinoma (Miura et al. 2008; Kamiyama et al. 2013; Miyahara et al. 2014), pancreatic cancer (Nouso et al. 2013), renal cell carcinoma (Hatakeyama et al. 2014), and prostate cancer (Ishibashi et al. 2014) but also in various diseases such as ulcerative colitis (Miyahara et al. 2013), nonalcoholic steatohepatitis (Yamasaki et al. 2015), neuronal degenerative diseases (Gizaw et al. 2015, 2016), and diabetic retinopathy (Inafuku et al. 2015, 2016). Versatility of this method is evident because the standard protocol can be applied for the basic glycobiology and biochemistry field as exemplified by obtaining a large avio-*N*-glycome database of egg white samples of Galloanserae to investigate the relationship between the function of

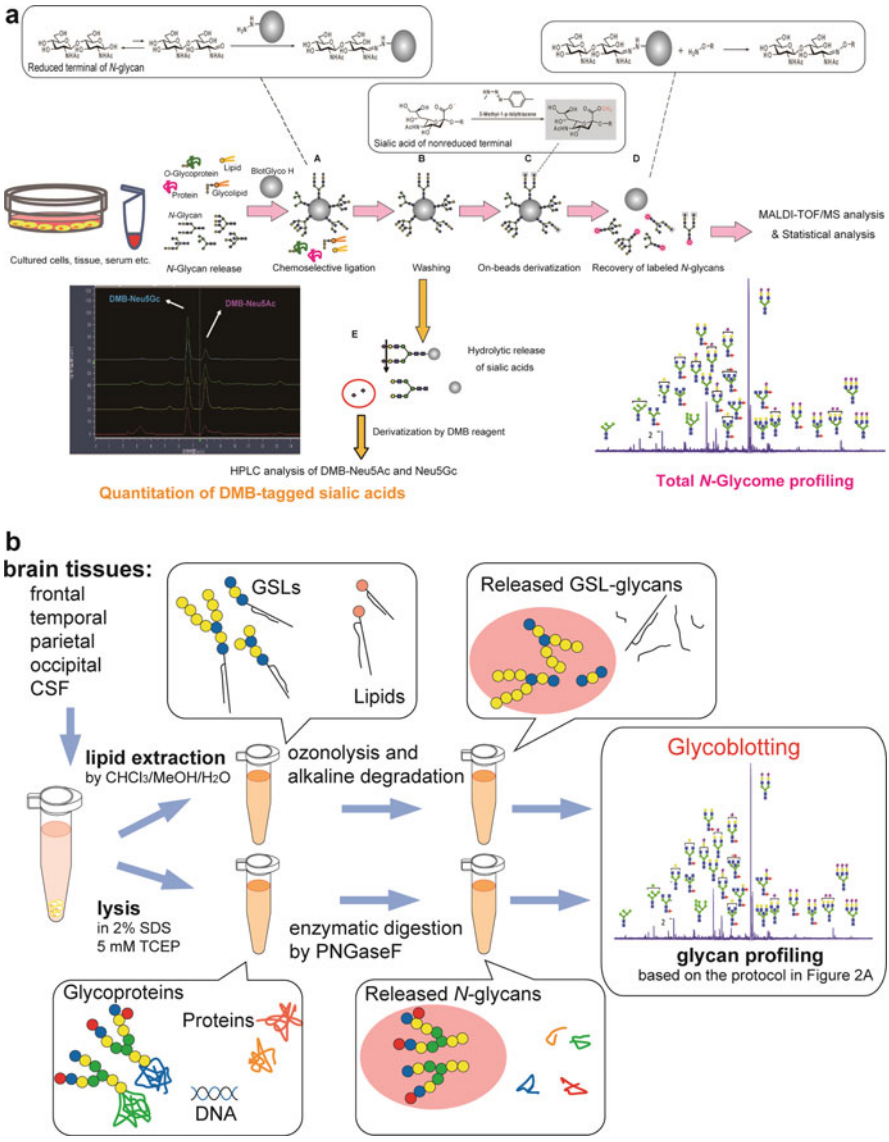

Fig. 2 The glycoblotting method. (**a**) Schematic representation of a standard protocol for the glycoblotting-based *N*-glycan profiling by using BlotGlyco H beads. *A*: Chemoselective ligation of whole *N*-glycans released from glycoproteins of serum, cultured cells, tissue/organ samples by "BlotGlyco" beads, *B*: Washing, *C*: On-bead selective methylation of sialic acids, *D*: *Trans*-iminization by benzyloxiamine to afford BOA-tagged *N*-glycans to be analyzed by MALDI-TOF/MS, and *E*: Selective DMB-labeling of the terminal sialic acids of total *N*-glycans to be employed for the quantitative HPLC analysis of sialic acids derived from the *N*-glycans of total glycoproteins. (**b**) A protocol of the glycoblotting-based glycomics designated for protein *N*-glycans and glycans of glycosphingolipids from human brain tissues (Original figure made by author)

glycan diversity and evolutionary lineage in some avian species (Hirose et al. 2011) and large-scale glycomics of dairy Holstein cow serum samples to discover highly sensitive serum biomarkers to monitor the heat/environmental stresses influencing the metabolic/homeostatic immune balance, performance, and milk productivity of dairy cows (Rehan et al. 2015). Moreover, this technique revealed dynamic *N*-glycan alteration during mammalian cell differentiation and proliferation. Amano et al. demonstrated the merit of glycoblotting-based quantitative glycomics for monitoring dynamic glycoform alteration during mouse P19 cells and embryonic stem cells (ESCs) proliferation and differentiation into cardiomyocytes or neural cells (Amano et al. 2010). It was revealed that the full portrait of *N*-glycan expression at each cell stage allowed for identifying the characteristic glycotypes showing drastic and concerted expression changes during cell differentiation, termed stage specific embryonic glycotypes, indicating for the first time the existence of "threshold" in expression level of the characteristic glycotypes required for initiating individual cell differentiations while functional roles, mechanism, and designated partner molecules remain unknown. As shown in Fig. 3, drastic enhancement of the expression level in various bisecting type *N*-glycans was observed during cell differentiation from P19C6 cells to neural cells. Recently, Terashima et al. demonstrated that murine induced pluripotent stem cells (iPSCs) as well as ESCs exhibited quite similar upregulation in the expression levels of bisect type *N*-glycans during differentiation into the neuronal cells (Terashima et al. 2014).

Entire *N*-glycan Profiling During Chondrogenic Differentiation of ATDC5 Cells by Glycoblotting Method

The comprehensive analysis of *N*-glycan alterations during chondrogenic differentiation of ATDC5 cells, a mouse progenitor cell line, was performed to serve as a common model for studying chondrogenic differentiation after insulin induction (Ishibashi et al. 2014). As shown in Fig. 4a, ATDC5 cells were grown to confluence and formed into cartilage nodules through cellular condensation in the presence of insulin (10 μg/mL). The maturation of cartilage nodules was confirmed by Alcian blue staining as shown in Fig. 4b. Alcian blue positive cells were observed on day 7 and increased in number after day 14. The expression levels of the major cartilage matrix proteins, type II collagen and aggrecan, were clearly increased after day 3 and peaked at day 7–11 (Fig. 4c), indicating that ATDC5 cells are suited model for the discovery study of the mammalian chondrocyte differentiation markers.

Total *N*-glycans of ATDC5 cells with or without induction into chondrocytes were profiled by glycoblotting-based high-throughput MALDI-TOFMS approach (Fig. 5). As summarized in Table 1, 45 kinds of glycoforms were identified and quantified reproducibly. High-mannose type *N*-glycans were the major components with the peak expression twice on day 7 compared with undifferentiated state (Fig. 5b). The expression pattern of high-mannose type *N*-glycan was similar to the results of analysis of chondrogenic differentiation markers (Fig. 4b, c). These results clearly indicated that expression levels of high-mannose type *N*-glycans were

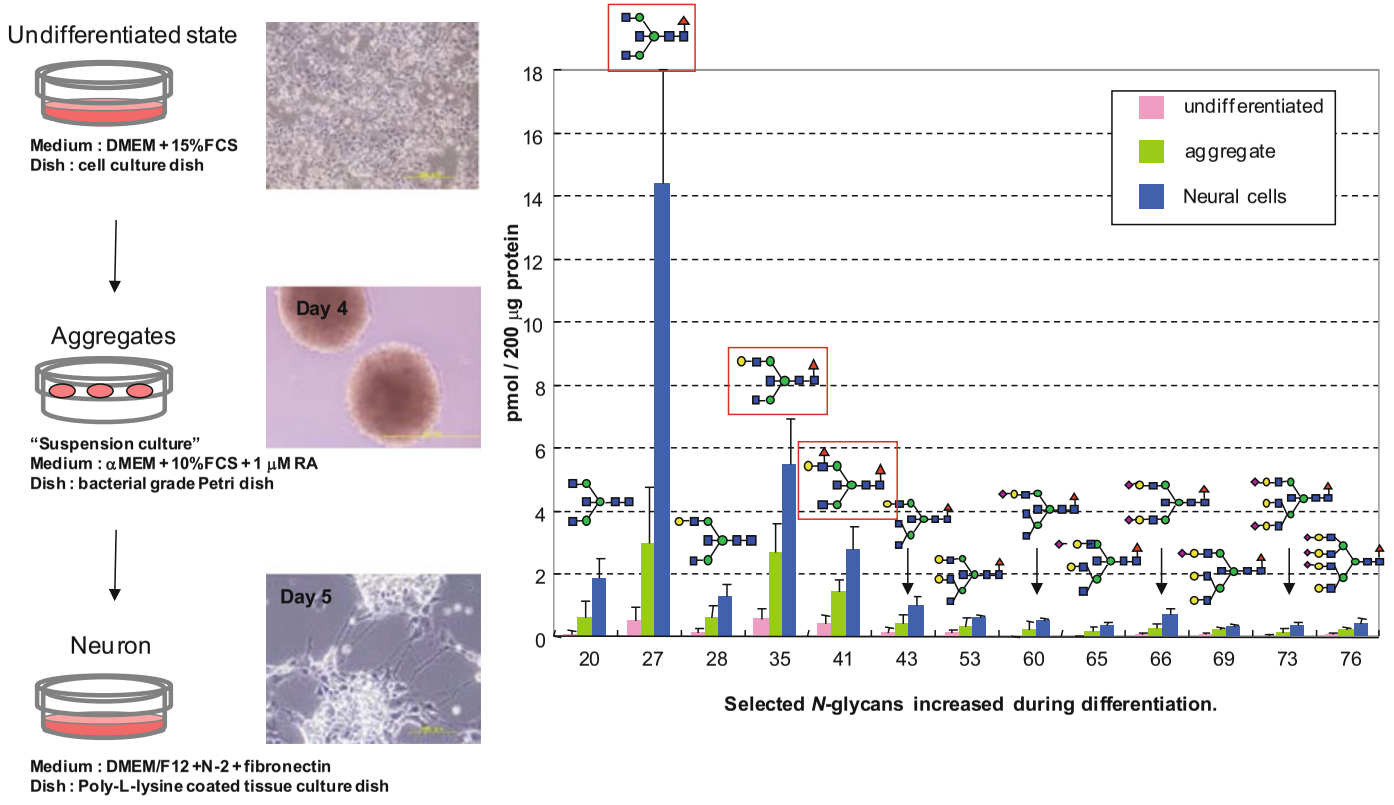

Fig. 3 Differentiation of mouse P19C6 cells to neural cells and *N*-glycan changes monitored by glycoblotting method. P19C6 cells were stimulated by retinoic acid and cells were subjected to the whole *N*-glycan analysis by glycoblotting method. The expression level of various bisecting-type *N*-glycans increased drastically during cell differentiation to neural cells (Amano et al. 2010) (Original figure made by author)

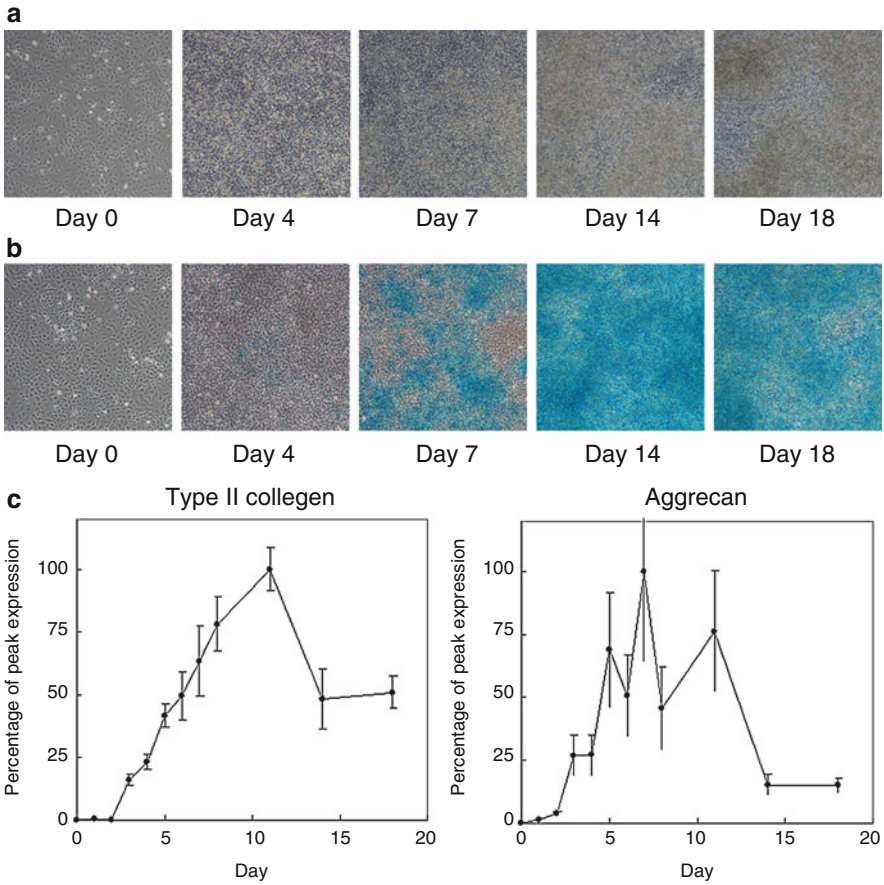

Fig. 4 Chondrogenic differentiation of ATDC5 cells. (**a**) ATDC5 cells formed cartilage nodules through cellular proliferation and condensation after induction with insulin. (**b**) Cultured ATDC5 cells stained with Alcian blue. The Alcian blue positive area was observed on day 7 and increased significantly after day 14. (**c**) Type II collagen and aggrecan expression levels analyzed by qPCR in biological triplicate were increased similarly after the cells reached confluence and peaked at day 7–11 (This figure was cited from Ishihara et al. (2014))

significantly correlated to the chondrogenic differentiation. Figures 5c–g show the expression level of each high-mannose type *N*-glycan structure. The amount of $Hex_5(HexNAc)_2$ (Man5) increased by fivefold throughout the differentiation and retained at the elevated level on mature chondrocytes (Fig. 5c). Interestingly, those of $Hex_6(HexNAc)_2$, $Hex_7(HexNAc)_2$, and $Hex_8(HexNAc)_2$ increased and peaked on day 7 (Fig. 5d–f). On the other hand, $Hex_9(HexNAc)_2$ (Man9) was downregulated significantly during maturation into chondrocytes. These results clearly showed that conversion of $Hex_9(HexNAc)_2$ (Man9) into $Hex_5(HexNAc)_2$ (Man5) was catalyzed by sequential degradation reactions by mannosidases to yield $Hex_5(HexNAc)_2$

Table 1 *N*-glycans detected during chondrogenic differentiation of ATDC5 cells

Peak number	*m/z*	Composition
1	1324.55	Hex2(HexNAc)2dHex1
2	1340.55	Hex3(HexNAc)2
3	1486.61	Hex3(HexNAc)2dHex1
4	1502.60	Hex4(HexNAc)2
5	1543.63	Hex3(HexNAc)3
6	1648.66	Hex4(HexNAc)2dHex1
7	1664.65	Hex5(HexNAc)2
8	1689.69	Hex3(HexNAc)3dHex1
9	1705.68	Hex4(HexNAc)3
10	1746.71	Hex3(HexNAc)4
11	1810.71	Hex5(HexNAc)2dHex1
12	1826.71	Hex6(HexNAc)2
13	1851.74	Hex4(HexNAc)3dHex1
14	1867.73	Hex5(HexNAc)3
15	1892.76	Hex3(HexNAc)4dHex1
16	1908.76	Hex4(HexNAc)4
17	1949.79	Hex3(HexNAc)5
18	1988.76	Hex7(HexNAc)2
19	2010.79	Hex4(HexNAc)3(NeuAc)1
20	2029.79	Hex6(HexNAc)3
21	2054.82	Hex4(HexNAc)4dHex1
22	2070.81	Hex5(HexNAc)4
23	2095.84	Hex3(HexNAc)5dHex1
24	2150.81	Hex8(HexNAc)2
25	2156.85	Hex4(HexNAc)3dHex1(NeuAc)1
26	2172.84	Hex5(HexNAc)3(NeuAc)1
27	2191.84	Hex7(HexNAc)3
28	2216.87	Hex5(HexNAc)4dHex1
29	2257.90	Hex4(HexNAc)5dHex1
30	2298.92	Hex3(HexNAc)6dHex1
31	2312.86	Hex9(HexNAc)2
32	2318.90	Hex5(HexNAc)3dHex1(NeuAc)1
33	2334.90	Hex6(HexNAc)3(NeuAc)1
34	2359.93	Hex4(HexNAc)4dHex1(NeuAc)1
35	2375.92	Hex5(HexNAc)4(NeuAc)1
36	2419.95	Hex5(HexNAc)5dHex1
37	2521.98	Hex5(HexNAc)4dHex1(NeuAc)1
38	2537.98	Hex6(HexNAc)4dHex1
39	2563.01	Hex4(HexNAc)5dHex1(NeuAc)1
40	2681.03	Hex5(HexNAc)4(NeuAc)2
41	2684.03	Hex6(HexNAc)4dHex1(NeuAc)1
42	2827.09	Hex5(HexNAc)4dHex1(NeuAc)2
43	3192.22	Hex6(HexNAc)5dHex1(NeuAc)2

(*continued*)

Table 1 (continued)

Peak number	*m/z*	Composition
44	3351.28	Hex6(HexNAc)5(NeuAc)3
45	3497.34	Hex6(HexNAc)5dHex1(NeuAc)3

Hex hexose, *dHex* deoxyhexose, *HexNAc* *N*-acetylhexosamine, *NeuAc* *N*-acetylneuraminic acid. The experimental masses were used to predict the possible glycans composition reported on http://web.expassy.org/glycomod/and CFG (http://www.functionalglycomics.org)

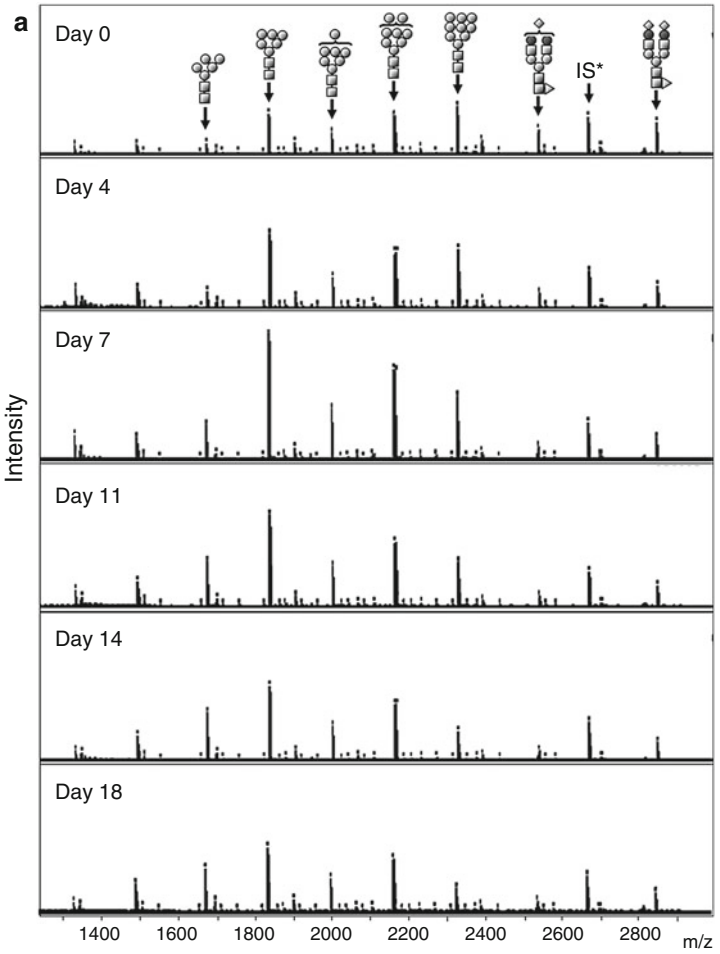

Fig. 5 (continued)

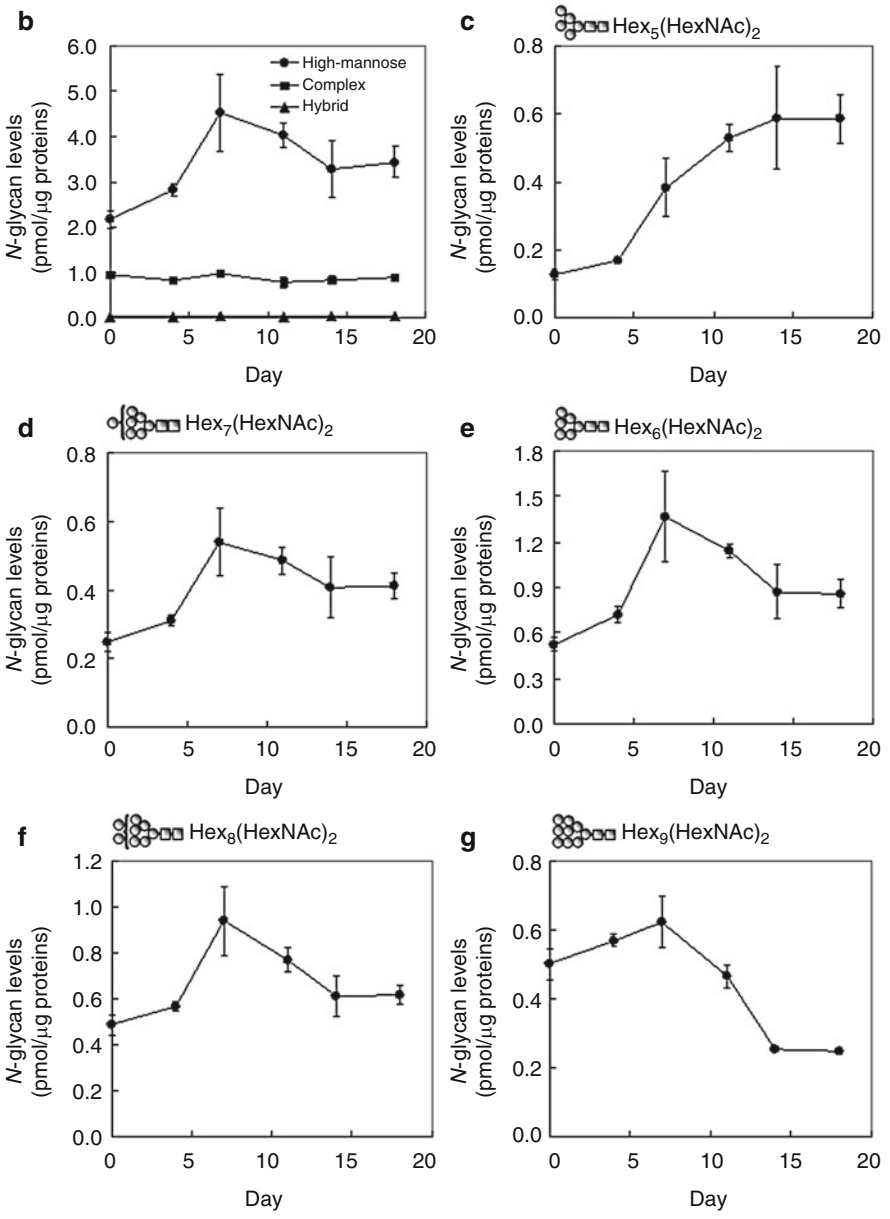

Fig. 5 High-mannose type *N*-glycan expression levels revealed by glycoblotting method during chondrogenic differentiation of ATDC5 cells. (**a**) Dynamic alteration of whole *N*-glycans from day 0 to day 18 after induction with insulin was measured by MALDI-TOFMS in biological triplicate. Forty-five glycoforms shown in Table 1 were profiled. (**b**) Relative expression levels of high-mannose, hybrid, and complex *N*-glycan subtypes. (**c**–**g**) Relative expression levels of each high-mannose type *N*-glycan. The signal intensity was normalized relative to an internal standard (This figure was cited from Ishihara et al. (2014))

(Man5) eventually in mature chondrocytes while other high-mannose type *N*-glycans decreased. It is interesting to note that our previous studies using a conventional HPLC-based glycomics did not show abovementioned dynamic alteration in the individual high-mannose type *N*-glycans in mouse and human OA cartilage (Matsuhashi et al. 2008; Urita et al. 2011).

The gene expression level of glycohydrolases (mannosidases) and glycosyltransferases is related to the biosynthesis of a series of *N*-glycans (Table 2, and Fig. 6). The levels of mRNA for *Ganab* and *Ugcgl 1*, enzymes responsible for

Table 2 Gene expression levels of glycoenzymes during chondrogenic differentiation

Fold change						
No.	Gene symbol	Day 0	Day 3	Day 5	Day 7	Annotation
1	Gcs1	1	1.08	1.10	0.88	–
2	Ganab	1	2.04	1.86	1.52	↑ (upregulated)
	Prkcsh	1	1.28	1.36	1.15	–
3	Ugcgl 1	1	3.06	2.13	1.39	↑ (upregulated)
	Ugcgl 2	1	0.89	1.02	0.82	–
4	Manea	1	1.32	1.41	2.23	↑ (upregulated)
5	Man1b1	1	0.93	0.85	0.78	–
6	Man2c1	1	1.36	1.35	1.28	–
7	Man1a	1	0.46	0.59	0.57	↓ (downregulated)
	Man1a2	1	0.69	0.47	0.29	↓ (downregulated)
	Man1c1	1	0.97	0.75	0.44	↓ (downregulated)
8	Mgat1	1	0.78	0.67	0.64	↓ (downregulated)

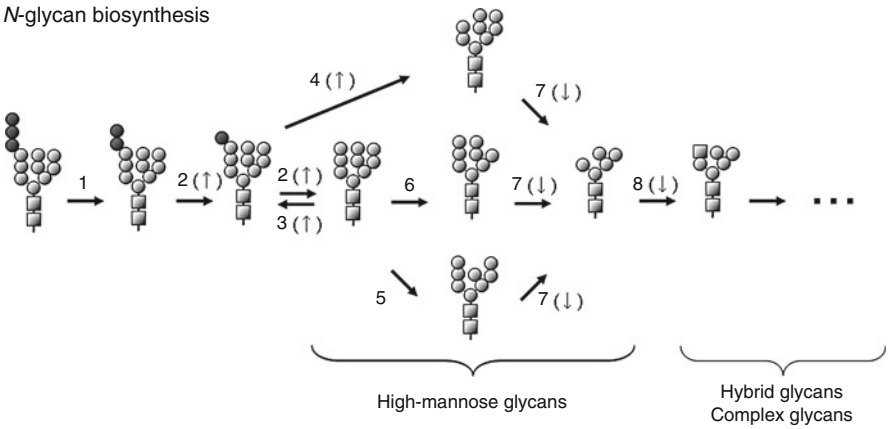

Fig. 6 Biosynthesis of high-mannose type *N*-glycans. The mRNA levels for the glycohydrolases and glycosyltransferases during chondrogenic differentiation analyzed by microarray were shown in Table 2. Up- and downregulated glycoenzymes are indicated by *arrows*, respectively. The numbers in figure correspond to those of each enzyme indicated in Table 2 (This figure was cited from Ishihara et al. (2014))

converting into $Hex_8(HexNAc)_2$, were upregulated and optimal on day 3. However, those of *Man1a* and *Man1c*, which convert $Hex_8(HexNAc)_2$ into $Hex_5(HexNAc)_2$ (Man5), were gradually downregulated. *Mgat1*, which is a key enzyme responsible for switching from high-mannose type *N*-glycans to other hybrid/complex type *N*-glycans, was also downregulated. These results of qPCR analysis demonstrated that both trimming of mannose residues of high-mannose type *N*-glycans and conversion into other *N*-glycan subtypes were suppressed during chondrogenic differentiation following activation of biosynthesis of the high-mannose type *N*-glycans at the early stages of differentiation. This finding was strongly correlated with those of quantitative *N*-glycan analysis as shown in Fig. 4 (Ishibashi et al. 2014).

Glycoproteomic Analysis to Identify Peptides Having High-Mannose Type *N*-glycans

The most important limitation of glycomics is the loss of the information regarding *N*- and *O*-glycosylation sites by glycan-release from the intact glycoproteins, the proximal peptide sequence information involving the individual glycosylation site. For this purpose, the structural analysis of the intact glycoproteins and/or digested glycopeptides is required. Glycoproteomics, a term defining the analyses both of peptide and attached glycan moiety concurrently, are of emerging importance not only in biomarker discovery research but also in regulatory requirements for therapeutic biopharmaceuticals including antibody drugs. It is clear that changes of glycan structures and in site occupancy indicate altered molecular functions and properties of individual glycoprotein as illustrated in Fig. 1, representing glycan heterogeneity of recombinant human erythropoietin.

Even when the target glycoprotein can be isolated successfully by conventional separation protocols, glycopeptides of interest should be enriched and separated from complex mixtures of tryptic peptides for further glycoproteomics commonly based on high performance LC-MS approach (Alley et al. 2009; Liu et al. 2011; Halim et al. 2011). The ion suppression effect by the large excess of nonglycosylated tryptic peptides with much higher ionization potentials often makes identification of the mass signals due to most glycopeptides difficult. The separation methods in combination with modern MS analysis have become one of the most important and indispensable techniques for the practical glycoproteomics. To obtain the information of the glycosylation sites in the intact glycoproteins, we need to select the most suited enrichment method for the characteristic glycoforms of the target glycopeptides (Nishimura 2011). Lectin affinity chromatography is one of the conventional methods to enrich glycopeptides in a glycoform-dependent manner because this procedure can reduce the sample complexity and increase the detection sensitivity of tryptic glycopeptides without loss of the glycan moiety of interest (Uematsu et al. 2005, 2009; Dai et al. 2009). However, it should be noted that the method also has serious disadvantages such as nonspecific binding and multivalency of lectin binding, with the result that a considerable amount of proteins/peptides

enriched by the lectins actually have no designated glycans and linkage that is ascribed to the lectin affinity employed, which require further validation of the glycan structure as well as peptide sequence of candidate proteins by means of MS approach.

To identify glycoproteins displaying unique high-mannose type *N*-glycan profile in chondrogenic differentiation of ATDC5 cells, concanavalin A (ConA) was used as an affinity reagent to selectively recover the glycopeptides bearing high-mannose type *N*-glycans (Kobata and Endo 1992). Following the enrichment of glycopeptides of interest in a crude tryptic digest of the chondrogenic differentiated cells, the ConA-bound fraction was further incubated with peptide-*N*-glycosidase F (PNGase F) and subjected to LC/MS analysis to identify the peptide sequences. The glycosylation sites were assessed by conversion of asparagine into aspartic acid residues at the position of glycan attachment digested by the PNGase. The consensus amino acid sequence known as common *N*-glycosylation sites (Asn-X-Ser/Thr) was also confirmed. As a result, this process gave a list of 246 identified *N*-glycoproteins and 434 *N*-glycopeptides including 460 *N*-glycosylation sites (Ishibashi et al. 2014). Interestingly, the identified glycoproteins were found to involve cartilage-specific extracellular matrix proteins such as aggrecan core protein and collagen α-1(II) chain. The subcellular localization of the identified proteins on the basis of the information from the UniProt database (http://www.uniprot.org/) revealed that 74 proteins (30%) were localized at the plasma membrane and 53 proteins (22%) were localized to the extracellular matrix or were secreted among the identified 246 proteins (Fig. 7). Importantly, glycoform-focused reverse proteomics revealed that many proteins carrying high-mannose type *N*-glycans exhibited concertedly dynamic *N*-glycoform alteration during chondrogenic cells differentiation. It is interesting to note that 52% of the identified glycoproteins were cell surface proteins, which were easily detectable by flow cytometry or immunohistochemistry, suggesting that they may include some potential differentiation markers. These plasma membrane residences might have important roles in maintenance of chondrocytes because the expression levels of most high-mannose type *N*-glycans were downregulated finally both in mouse and human OA cartilage (Matsuhashi et al. 2008; Urita et al. 2011).

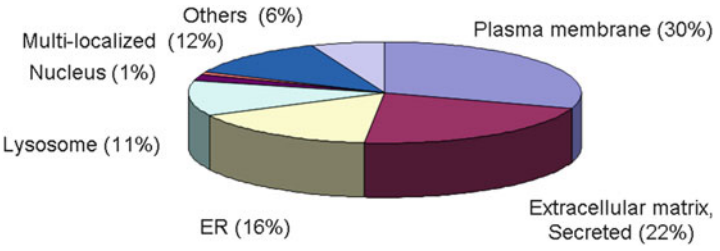

Fig. 7 Subcellular locations of the 246 identified glycoproteins having high-mannose type *N*-glycans. The identified glycoproteins were classified on the basis of the information from the UniProt database (http://www.uniprot.org/) (This figure was cited from Ishihara et al. (2014))

Gene Expression Analysis of the Identified Glycoproteins in Mouse and Human

Quantitative and qualitative alterations of high-mannose type *N*-glycans are significantly correlated to the chondrogenic differentiation. To assess whether the expression of the identified glycoproteins carrying high-mannose type *N*-glycans is regulated at the mRNA level during differentiation, gene expression analysis of the identified proteins was carried out. A total of 246 glycoproteins were preliminary screened by microarray analysis, and genes upregulated at the earlier stages of chondrogenic differentiation compared with type II collagen were selected and focused on cell surface proteins by using the UniProt database. Finally, 15 characteristic genes in ATDC5 cells showing higher expression levels at the earlier stages of chondrogenic differentiation compared with type II collagen were identified by means of the qPCR-based validation as shown in Fig. 8 and Table 3.

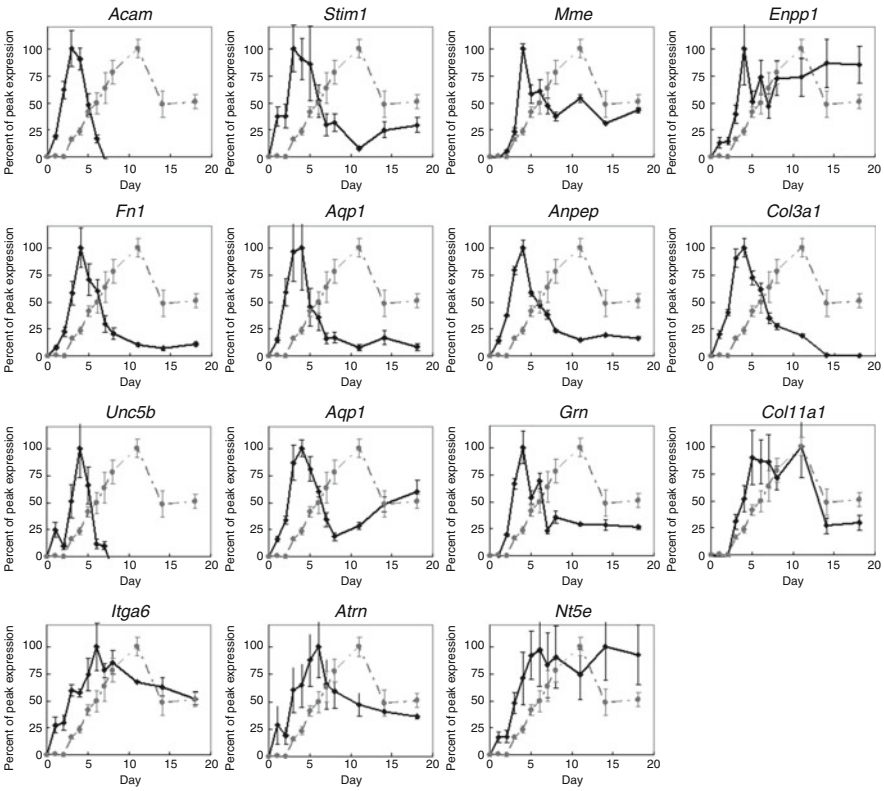

Fig. 8 Gene expression profiles of 15 differentiation marker candidates with increasing transcript levels at the early stages of chondrogenic differentiation in ATDC5 cells. The *solid lines* indicate gene expression profiles of each 15 differentiation marker candidates, and the *dotted line* indicates the gene expression profile of type II collagen. The profiles were performed in biological triplicate (This figure was cited from Ishihara et al. (2014))

Table 3 Fifteen cell surface *N*-glycoproteins showing increased expression during chondrogenic differentiation

Symbol	Swiss-Prot number	Protein name	Sequence	Mascot score[a]	Subcellular location[b]
Nt5e	5NTD_MOUSE	5-nucleotidase	LDNYSTQELGR	59	Plasma membrane
Acam	ACAM_MOUSE	Adipocyte adhesion molecule	HVYNNLTEEQK	87	Plasma membrane
Anpep	AMPN_MOUSE	Aminopeptidase N	FTCNQTTDVIIIHSK	57	Plasma membrane
			KLNYTLK	53	
			LNYTLK	36	
			SGQEDHYWLDVEKNQSAK	68	
Aqp1	AQP1_MOUSE	Aquaporin-1	NQTLVQDNVK	45	Plasma membrane
Atrn	ATRN_MOUSE	Attractin	IDSTGNVTNELR	107	Plasma membrane
Col3a1	CO3A1_MOUSE	Collagen alpha-1(III) chain	LLSSRASQNITYHCK	57	Extracellular matrix, Secreted
Col11a1	COBA1_MOUSE	Collagen alpha-1(XI) chain	VYCNFTAGGETCIYPDK	109	Extracellular matrix, Secreted
			NTSEDILYGNK	45	
Enpp1	ENPP1_MOUSE	Ectonucleotide	VYNGSVPFEER	48	Plasma membrane
		pyrophosphatase/			
		phosphodiesterase family member 1			
Fn1	FINC_MOUSE	Fibronectin	DQCIVDDITYNVNDTFHK	114	Extracellular matrix, Secreted
			HEEGHMLNCTCFGQGR	67	
			LDAPTNLQFVNETDR	121	
			WTPLNSSTIIGYR	81	
			NYTDCTSEGR	71	
Grn	GRN_MOUSE	Granulins Grn	NYTTDLLTK	45	Secreted
Itga6	ITA6_MOUSE	Integrin alpha-6	LWNSTFLEEYSK	77	Plasma membrane
Mme	NEP_MOUSE	Neprilysin	SCINESAIDSR	98	Plasma membrane
			EIANATTKPEDR	72	
Nrp2	NRP2_MOUSE	Neuropilin-2	NFTSPNGTIESPGFPEK	85	Plasma membrane
Stim1	STIM1_MOUSE	Stromal interaction molecule 1	LAVTNTTMTGTVLK	117	Plasma membrane
Unc5b	UNC5B_MOUSE	Netrin receptor UNC5B	LSDTANYTCVAK	55	Plasma membrane

[a] $P < 0.05$ by Mascot Search

[b] Subcellular localizations of the identified proteins using information from the UniProt database (http://www.uniprot.org/)

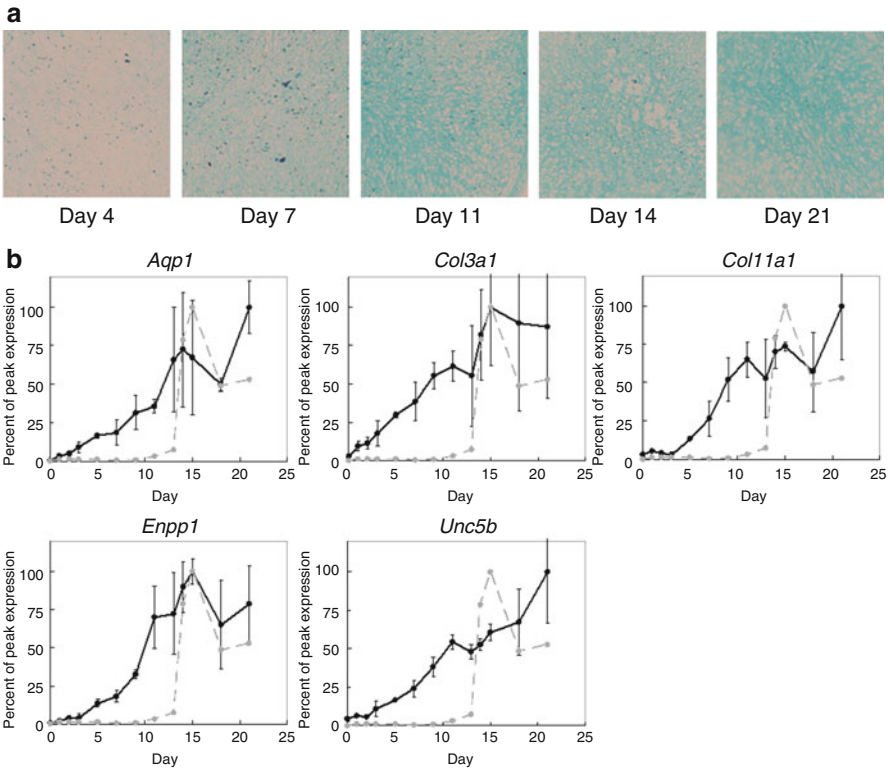

Fig. 9 Chondrogenic differentiation of human MSCs and gene expression profiles of five differentiation marker candidates. (**a**) Alcian blue staining of the human MSCs differentiated by induction after TGF-β3. (**b**) Profiles of gene expression levels of five differentiation marker genes during differentiation in comparison with type II collagen. The profiles were performed in biological triplicate. The *solid lines* indicate gene expression profiles of each of the five differentiation marker genes, and the *dotted line* indicates the gene expression profile of type II collagen (This figure was cited from Ishihara et al. (2014))

To test the feasibility of 15 differentiation marker candidates for human chondrogenic differentiation, gene expression analysis was performed in a human MSC model. Since MSCs have the ability to differentiate into cells of the chondrogenic lineage, human MSCs were cultured and differentiated into chondrocytes. As shown in Fig. 9a, Alcian blue positive cells on extracellular matrices were observed after day 7 of induction and the expression of the major cartilage matrix proteins type II collagen was also confirmed in an induction-dependent manner as well as in mouse ATDC5 by qPCR. In case for the human chondrogenesis model, five out of the 15 genes – *Aqp1, Col3a1, Col11a1, Enpp1,* and *Unc5b* – showed distinct upregulation of the expression at the mRNA level in the earlier stage of differentiation when compared with type II collagen (Fig. 9b). These results clearly indicated that glycoproteins such as aquaporin-1 (AQP1), collagens, ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1), and

netrin receptor produced by these five genes (*Aqp1, Col3a1, Col11a1, Enpp1*, and *Unc5b*) can be highly sensitive markers during the initial stage of human chondrocyte differentiation.

It seems likely that collagen α-1(III) and collagen α-1(XI) are well-known cartilage collagens. Collagen α-1(III) is expressed in the superficial and deepest area of articular cartilage along with type I collagen, in which the deepest zone contains ellipsoidal chondrocytes that synthesize lubricin and types I, II, and III collagen fibers. Collagen α-1(XI) exists in transitional and deep area of articular cartilage (Clouet et al. 2009). ENPP1 is an enzymatic generator of pyrophosphate in differentiated chondrocytes to control extracellular pyrophosphate levels for appropriate mineral homeostasis in cartilage. It is noteworthy that a dysregulated increase in pyrophosphate elaboration in chondrocytes appeared to promote calcium pyrophosphate dihydrate crystal deposition in aging and OA cartilage (Johnson et al. 1999). AQP1 is a well-characterized membrane glycoprotein that belongs to the aquaporin family. AQP1-mediated plasma membrane water permeability plays a crucial role in chondrocyte migration and adhesion (Liang et al. 2008). Moreover, cell adhesion to type II collagen-coated plates was known to be significantly reduced by AQP1 deletion. Netrin receptor is known to be highly expressed in the brain and has functional roles in axon growth of neurons and angiogenesis. It is also expressed at lower levels in developing cartilage. It was reported that the level of netrin receptor transcript increases in mouse embryos from day 10.5 in the cartilaginous primordia of many bones and cartilage (Przyborski et al. 1998) while its function during cartilage development remains unclear. Given the facts that all these glycoproteins found in this study are present in human cartilage and may be related closely to the pathogenesis of OA, they are strongly expected to become biomarkers and therapeutic targets as well as chondrocyte differentiation markers. Recently, it was demonstrated that posttranslational protein glycosylation can generate novel disease-relevant glycopeptidic epitopes as potential molecular targets of antibody drugs (Rangappa et al. 2016).

Potential Application of Glycomics to Prognosis, Other Disease Conditions

Recently, glycomics has contributed extensively to discovery research of prognostic biomarkers for various cancerous diseases. Our challenge based on glycoblotting technology allowed for rapid and large-scale glycomics and glycoproteomics toward discovery of highly sensitive and dynamic biomarkers not only in cancers but also in various diseases. Versatility of this methodology is evident because five glycoproteins discovered by the present approach become more sensitive biomarkers than type II collagen known as a common chondrocyte differentiation marker. It is interesting to note that these glycoproteins may have the potentials to be OA biomarkers. To assess the feasibility of these novel OA biomarker candidates, quantitative validation using body fluids is under way.

Summary Points

This chapter focuses on the novel glycomics approach to discovery of chondrogenic differentiation markers.

Dynamic structural alteration of the cell surface glycans during cell differentiation can provide clinically potential biomarkers.

However, the extremely tedious and time-consuming multiple processes to purify whole glycans from heterogeneous biological samples had long made high-throughput glycomics difficult.

Glycoblotting method allows for rapid and efficient glycan enrichment by chemoselective reaction between carbohydrates and solid materials having specific functional groups.

Merit of the glycoblotting method is demonstrated by combining this technology and glycoform-focused proteomics and genomics approach.

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Raman Spectroscopy as a Biomarker-Investigative Tool in Bone Metabolism

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Contents

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Abstract

Bone is a living material with a composite structure that endures long and repetitive shocks caused by walking or jumping. Currently, clinical imaging modalities do not completely characterize the quality of bone. Additionally, these approaches are inadequate for detecting the earliest disease stages, for determining the age, or for verifying the treatment impact. Raman spectroscopy is an easy technique that provides a fingerprint of the chemical and structural composition of bone. Thus, it allows the determination of biomarkers for the quantification of bone quality and to qualify the evolution with physiological changes and diseases as in osteoporosis and osteogenesis imperfecta.

Keywords

Bone biomarkers • Bone quality • Mineralization • Crystallinity • Collagen maturity • Osteogenesis imperfecta • Osteoporosis • Raman spectroscopy

List of Abbreviations

BMD	Bone mineral density
BP	Bisphosphonate
FWMH	Full width at maximum height
GAG	Glycosaminoglycan
IR	Infrared spectroscopy
MicroCT	Micro-computed tomography
NIR	Near infrared
PCA	Principal component analysis
PG	Proteoglycan
RS	Raman spectroscopy

Key Facts of Bone Metabolism

- The metabolism of bone, which is also known as bone remodeling, is a physiological and continuous process that enables the bone to achieve its remarkable mechanical properties. Each year, 10% of the skeleton of an adult is remodeled. Three types of cells are involved in this process.
  (i) Osteocytes are the most abundant type of cells in bone, and their role is to maintain bone tissue, to feel the mechanical solicitation, and to initiate remodeling if necessary.
  (ii) When remodeling is initiated, osteoclasts migrate to the site to be resorbed to digest the organic portions of bone and dissolve the mineral portions. The resorption phase lasts between 2 and 3 weeks.
  (iii) After a lapse in time (reversal phase), osteoblasts form an organic matrix that will then mineralize around the osteoblasts that then differentiate into osteocytes. This formation phase lasts approximately 3 months.
- Osteoporosis is a disease of aging that resulting from an imbalance between bone formation and resorption, promoting resorption that results in a decrease in bone

mass. In the USA, 70% of women above 80 years old are suffering from the disease.
- Osteogenesis imperfecta is a genetic bone disease associated with increased bone fragility due to a defective collagen matrix and impaired mineralization. In the USA, there are 20,000–50,000 patients suffering from the disease.
- Therapies with bisphosphonates are used for osteoporosis as well as for osteogenesis imperfecta to effectively prevent fractures and increase bone mineral density.

Introduction

Bone is a living material with a composite structure. It combines the elasticity of an organic matrix with the strength of mineral components. This composition enables bone to be efficient and light. Furthermore, it allows the bone to easily restore itself in cases of an injury or to endure long and repetitive shocks caused by walking or jumping. Bone has remarkable mechanical properties because of its composite structure and its multiscale organization. Bone has different composition levels that range from the Angström to millimeter scale (Rho et al. 1998). The smaller levels influence the upper levels.

The correlation of mechanical properties between the tissue level (micrometer level) and the whole bone level has been found (Bi et al. 2011; Gallant et al. 2013). It is important to determine the right criterion to assess the bone quality from a lower scale. Indeed, the bone mineral density has been proven to be insufficient to fully characterize bone (Gamsjaeger et al. 2010). The quantity and distribution of bone are not enough. Additionally, bone quality must be taken into account to quantify the mechanical properties of bone (Kohn et al. 2008). Over the last 20 years, two definitions of bone quality have been used. The first definition accounts for bone properties that influence its biomechanical properties. The second definition accounts for bone fracturing factors, which are independent from the bone mass or quantity (Hernandez and Keaveny 2006). Therefore, bone quality takes into consideration the compositional properties of bone and its architecture. This enables bone to attain required mechanical properties and, thus, to fulfill its biological functions. The mineral and organic composition and arrangement determine the bone quality (Morris and Mandair 2011). Additionally, some authors include the remodeling dynamic (McCreadie et al. 2006).

Bone is a material that remodels itself using the resorption and formation cycles. If the equilibrium between the two phases is disturbed or if one phase is not correctly executed, the quality of bone can be affected. This can cause osteoporosis or other metabolic bone diseases. Biomarkers, which are used to quantify or to qualify the disease severity (Gamsjaeger et al. 2014a) and to help with etiology, are the key elements.

Raman spectroscopy is a technique that provides an access to chemical and organizational bone properties on a micrometer scale (Bazin et al. 2009). The

chemical and organizational bone properties are considered to be biomarkers of bone quality (Morris and Mandair 2011).

The purpose of the defined biomarkers is to predict the mechanical properties at the whole bone level based on micrometer scale measurements (Kim et al. 2012) and to understand the influence of the smaller structure on the upper (Mandair and Morris 2015; Ojanen et al. 2015; Yerramshetty and Akkus 2008). The correlation between mechanical and chemical properties at a micrometer level corroborates the utility of biomarkers investigated using Raman spectroscopy (Hammond et al. 2014; Imbert et al. 2014).

Bone Structure

The hierarchical structure of bone has been highlighted by many publications that accurately detailed a multiscale organization of bone (Rho et al. 1998). At a macroscopic scale, the two types of bone are observed: the trabecular bone (cancellous or spongy bone) and the cortical bone (compact bone) (Fig. 1). The former is composed of trabeculae and is primarily found in epiphysis where the stress is multidirectional. However, the latter is very thick in the diaphysis because of its high mechanical (compressive) strength. The cortical bone microstructure consists of cylindrical units, osteons (100–500 μm), that are parallel to bone axis and are separated by interstitial tissue (partial osteons due to the secondary remodeling and

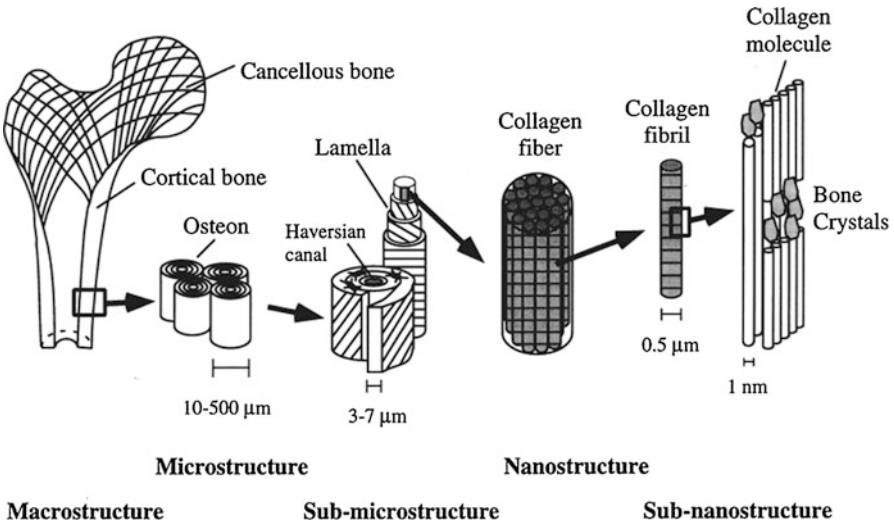

Fig. 1 Hierarchical structural organization of bone (Reprinted authorized by Rho et al. 1998). Hierarchical structural organization of bone, from bigger to smaller structures: cortical and cancellous bone; osteons with the Haversian systems; lamellae; collagen, and mineral platelets

primary bone). At the sub-microscopic level, the osteons are organized in concentric lamella ranging from the Havers canal in the center to the cement line at the periphery. The Havers canals are lined with nerves and vessels that supply cells with nutrients. The Volkmann canals are oriented perpendicular to the Havers canals and connect them together.

To understand the unique mechanical properties of bone, it is important to visualize its heterogeneous composition at the nanometer scale. Bone is a composite material that is made of an organic matrix and inorganic mineral crystals. The organic matrix primarily consists of type I collagen fibers that are organized in fibrils. The mineral crystals are platelets of carbonated hydroxyapatite with a nanometer size (50 × 25 × 3 nm) and are oriented with their long axis parallel to collagen molecules (300 nm) (Fig. 2). The cross-links between collagen molecules stabilize the structure. The mineral part of the bone represents 60–70% of the material weight. The rest are proteins, primarily type I collagen (90% of the proteins), cells, and water.

Because the apatite crystals are less elastic and more brittle than collagen fibers, the bone strength is induced by the mineral part, while the tissue elasticity is characterized by the organic part (Buchwald et al. 2012a; Kozielski et al. 2011).

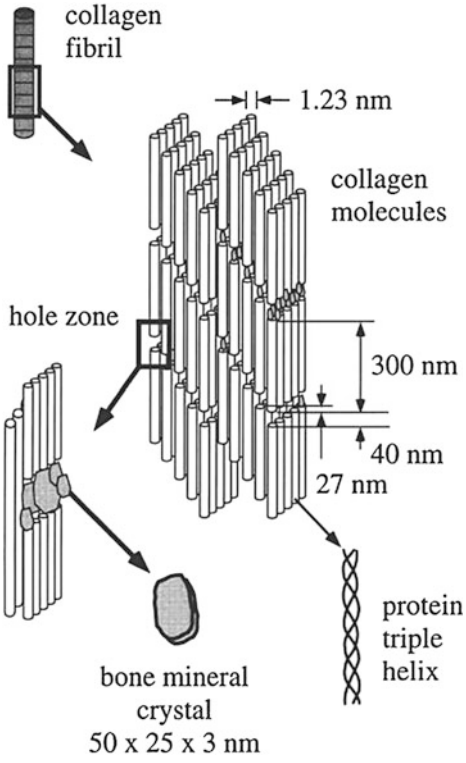

Fig. 2 The structure at a nanometer scale (Reprinted authorized by Rho et al. 1998). The structure of collagen fibrils and platelets of carbonated hydroxyapatite

Raman Principle and Technological Solutions

Currently, the clinical imaging modalities for measuring bone mass and density, such as dual energy X-ray absorptiometry (DXA) or bone mineral content (BMD), do not completely characterize the bone quality (Hernandez and Keaveny 2006). Additionally, they are inadequate for detecting the earliest disease stages, for determining aging, or for verifying the treatment. Vibrational spectroscopies offer several advantages for biological investigation because these techniques provide quantitative information on both the organic matrix and mineral phases of bone. Raman and infrared spectroscopies as screening tools are appealing for developing medical diagnosis to compare normal and pathological tissue and to study disease progression.

Raman spectroscopy is a light scattering technique that measures and quantifies energetic changes in light. The incident photons, generated using a laser, interact with molecules present in a sample. The amount of energy either lost or gained by a photon is characteristic of the nature of each bond (only 1×10^{-7} of the scattered light is Raman). Thus, the change in wavelength (shift) of scattered photon provides chemical and structural information, referred to as fingerprint. These shifts, expressed in wavenumber units (cm^{-1}), correspond to the vibrational energy of molecules. Not all vibrations are observable using Raman spectroscopy (depends on the symmetry of molecules). However, the result is sufficient to precisely identify different molecules that are present in the sample, and the intensity of the peaks can be used to quantify their concentration (Bazin et al. 2009; Nyman et al. 2011). Figure 3 illustrates the information that Raman spectroscopy can provide.

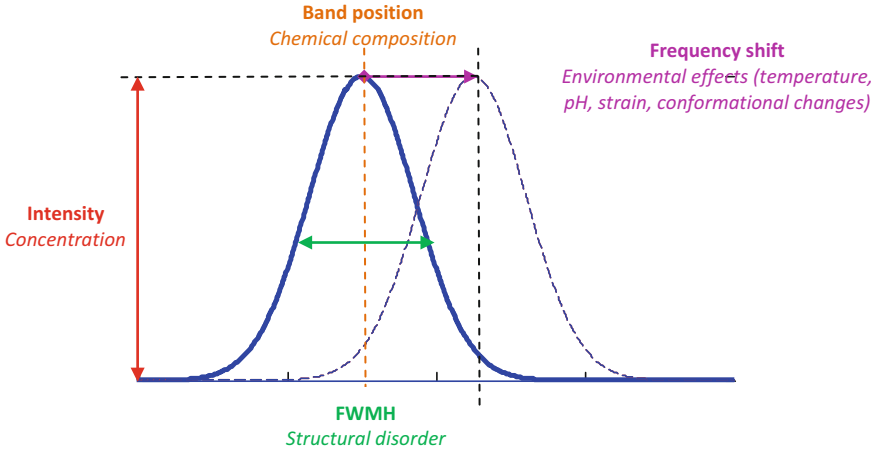

Fig. 3 Information provided by Raman spectroscopy. Raman spectrum provides a fingerprint of molecules and bonds that are present in the sample: (i) Band positions are characteristic of the chemical composition. (ii) Frequency shifts are induced by the structure of the molecules and their local environment. (iii) Intensity primarily corresponds to the concentration of molecule but can also be sensitive to laser polarization. (iv) Linewidth (FWMH) reflects the crystal structure

The infrared (IR) and Raman spectroscopic (RS) techniques provide complementary information on the quantity and quality of bone composition. Infrared transmission spectroscopy is based on a different physical principle, where the photons are absorbed by molecular bonds of the sample. Because the physical background is different, some vibrations that are not observable in IR spectra are visible in Raman spectra and vice versa. Because IR spectroscopy is a transmission technique, it requires more sample preparation (embedding and cutting of thin sample sections). Furthermore, IR spectroscopy is sensitive to water, which makes biological investigations difficult. However, Raman spectroscopy has clear advantages:

- RS is relatively insensitive to water and allows measurements on fully hydrated samples with minimal preparation.
- RS is a nondestructive method. Thus, the same sample can be analyzed using several different techniques.
- RS offers micron scale spatial resolution (0.6–1 μm) compared to the IR technique (5–10 μm) and allows to differentiate between various important structures such as cement lines, lamellae and, micro-crack surroundings (Buchwald et al. 2012a; Kazanci et al. 2007).

A recent study (Turunen et al. 2011) compared characterization of maturating rabbit cortical bone using the IR and near-infrared RS techniques. The results demonstrated multiple correlations between compositional parameters calculated from IR and RS. In addition, there were correlations with bone mineral density (BMD) determined using micro-computed tomography.

Bone has a composite structure. Thus, a single point analyzed using Raman micro-spectroscopy does not accurately represent the chemical composition and structure of the whole specimen. Nevertheless, a motorized stage coupled with a Raman microscope allows acquisition of Raman spectra maps of the sample. Therefore, Raman imaging is gaining popularity because it provides spatial information of samples (Kozielski et al. 2011; Timlin et al. 2000).

Raman signal depends on the composition of samples and is sensitive to the local orientation of fibers or crystals in relation to polarization of incident and scattered light (Goodyear et al. 2009).

Figure 4 describes the structure of collagen molecules. In collagen, the C = O bonds are perpendicular to the collagen molecular axis. However, the C-N bonds are either perpendicular or parallel to this axis. The amide I band is attributed primarily to the C = O stretching vibration. The band is more intense when the polarization of incident light is perpendicular to the collagen fibers. The amide III band is attributed to C-N vibrations and shows two different vibrational modes for perpendicular and parallel orientations. In apatite crystals, the C-axis, parallel to the collagen fibers, is coupled with ν_1 phosphate vibrations. Therefore, the phosphate ν_1 band intensity is more intense when the polarization of incident light is parallel to the collagen fibers. However, the phosphate ν_2 and ν_4 bands are less sensitive to the orientation effects (Buchwald et al. 2012a; Kozielski et al. 2011). Therefore, Raman spectral imaging can provide much information about the composition and organization of bone

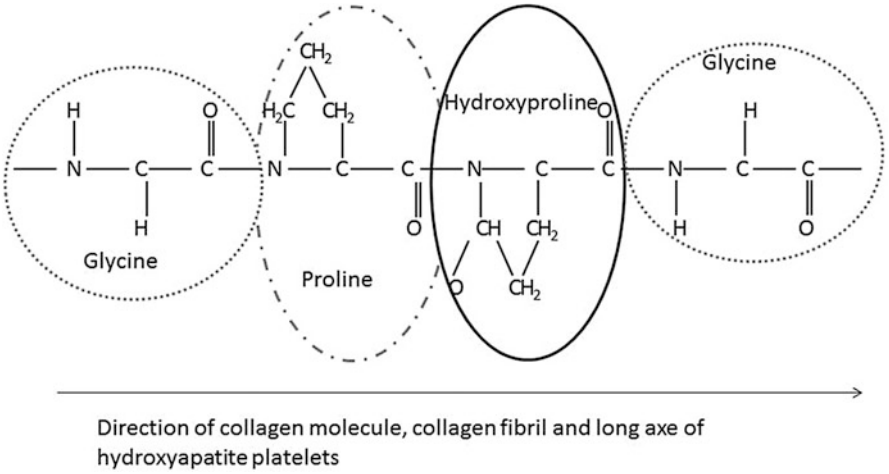

Fig. 4 Sketch of the smallest collagen motif. Structure of the smallest collagen molecule motif. (i) C = O bonds are perpendicular to the collagen molecular axis. (ii) C-N bonds are either parallel to the collagen molecular axis or perpendicular to it in the proline and hydroxyproline groups

tissue. The mineral and organic composition of bone tissue can be determined from bands that are not sensitive to the polarization direction. However, the orientation of collagen fibers can be estimated using bands that are orientation dependent (Kazanci et al. 2006, 2007).

The development of noninvasive diagnostics tools is a major goal today, and Raman spectroscopy has recently seen major advances in the area of in situ characterization for biological studies. An intravital Raman spectroscopy study allows the monitoring of the mineral and organic composition of bone tissue in vivo (Penel et al. 2005). Another study focused on a new technique in which a pulse laser probe recorded the Raman spectrum of bone underneath the skin (Draper et al. 2005). Currently, new probes are being developed to perform Raman arthrospectroscopy (Esmonde-White et al. 2011).

Bone Spectra

Experimental Preparation

Raman spectroscopy does not require complicated sample preparation. The analysis can be performed on fresh, frozen, fixed, or embedded samples with thicknesses from submicron scale to that of intact bone (Timlin et al. 2000). Nevertheless, fixative solutions and embedding media have significant effects on the composition and physicochemical properties of bone, thus altering the Raman spectra (Yeni et al. 2006). These techniques may require the characterization with identical

parameters of solutions and media separately to examine the overlaps between the spectra that can subsequently be subtracted from the bone spectra.

Because flat surfaces reflect more Raman scattering light, it is recommended to polish the analyzed surface (Shen et al. 2010). Raman spectra of bone can be recorded under dry or physiological conditions (Bart et al. 2014; Hammond et al. 2014; Ramasamy and Akkus 2007; Yao et al. 2013; Yerramshetty et al. 2009; 2006).

The anatomical location of bone can also have a significant effect. The analysis of bands on cortical and trabecular bones showed that cortical bone has compositional characteristics of older bone, which is possibly due to the higher bone turnover that has been traditionally reported to be in the trabecular compartment (Goodyear et al. 2009). On long bones, it has been confirmed that the chemical composition of bone changes along the length of bones and across the cortex from the periostea to the endostea (Buckley et al. 2014). On a lower scale, osteonal tissue, interstitial tissue, cement lines, and lamellae can be analyzed individually (Kazanci et al. 2006).

The interference of background fluorescence obscures prominent Raman bands of mineral and matrix components of bone tissue. Although fluorescence can be dealt with by using a variety of techniques, such as the utilization of a confocal configuration or photo bleaching (Golcuk et al. 2006), the choice of an appropriate wavelength is a compromise between longer wavelengths (NIR) that reduce the protein fluorescence and a shorter excitation that can improve the sensitivity (Raman scattering intensity is inversely proportional to the fourth order of the excitation wavelength). Utilization of near-infrared excitation at 785 nm remains the most commonly used wavelength because it minimizes the fluorescence.

Preprocessing

Currently, basic preprocessing consists of (i) removing cosmic ray artifacts, (ii) removing noisy spectra using a wavelet technique, if necessary, (iii) filtering the data using a multipoint linear moving average, (iv) subtracting the underlying background signal using an iteratively fitted polynomial (Lieber and Mahadevan-Jansen 2003), and (v) performing curve fitting or deconvolution to calculate the peak intensities and positions. Preprocessing is often performed using custom written procedures in Matlab (the Mathworks Inc., MA, USA). Then, spectra are analyzed using different methods. Macroscopic information can be obtained from the direct comparison of spectra, and more accurate results can be derived by comparing the peak intensities and the position of the bands (the shift and width).

Without calibration of the intensity axis (Mandair and Morris 2015; Morris and Mandair 2011), peak intensities depend on the Raman scattering efficiency, laser power, optical components in the Raman system, characteristics of the sample (irregularity, grain size, degree of polishing, refractive index of the surface), and variations in the distance from the objective to the sample. Accordingly, Raman peak ratios are usually used rather than peak intensities to detect differences in bone tissue composition (Nyman et al. 2011). Regarding the choice of comparing either the

intensities or the areas of the peaks, some authors advocate that using peak intensities causes less error because of the smaller influence of the baseline (Gamulin et al. 2013), whereas some researchers have found no differences (Goodyear et al. 2009).

Assignments

Figure 5 shows a typical Raman spectrum of bone tissue with labels for the major bands that correspond to the mineral and organic constituents. Table 1 lists the positions of all of the relevant Raman vibrational bands in the bone spectra (Bansil et al. 1978; Gamulin et al. 2013; Kazanci et al. 2007; Mandair and Morris 2015; Pinheiro et al. 2014.

The most prominent band that is associated with the mineral component of bone is the phosphate ν_1 symmetric stretch (P-O stretch) at $\approx$ 960 cm^{-1}. Other phosphate bands, the ν_2 and ν_4 modes, appear at $\approx$ 430 and $\approx$ 590 cm^{-1}, respectively. The ν_3 mode at $\approx$ 1045 cm^{-1} is broad and overlaps other bands; thus, it is not pertinent.

The mineral bone component is also represented by carbonate ν_1 symmetric stretching (C-O stretch) at $\approx$ 1070 cm^{-1}.

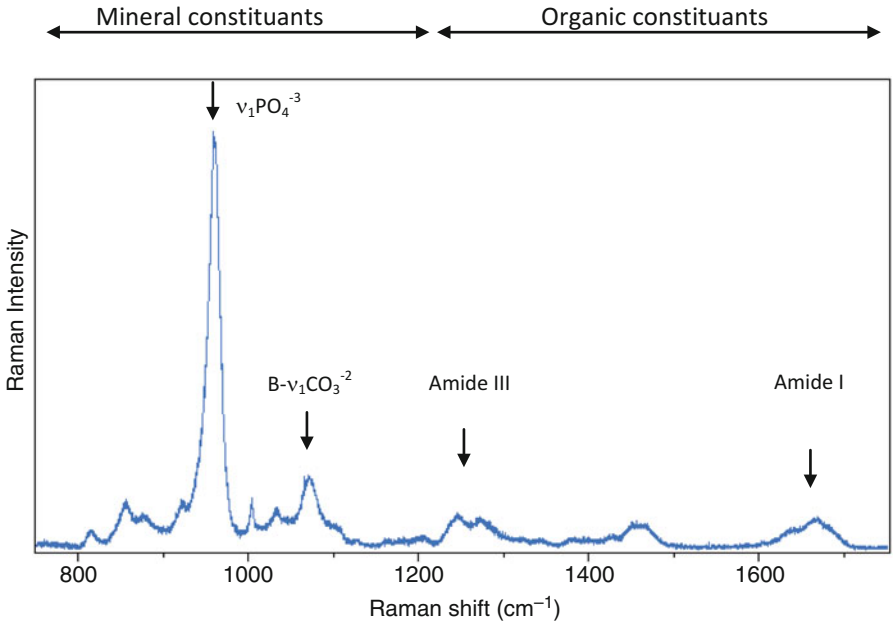

Fig. 5 Typical Raman spectrum of bone tissue. Typical Raman spectrum of bone tissue after baselining and filtering, showing the main bands between 750 and 1800 cm^{-1}: the 960 cm^{-1} band corresponds to phosphate, the 1075 cm^{-1} band corresponds to the type B carbonate, the large 1250 cm^{-1} band corresponds to the amide III band, and the large 1667 cm^{-1} band corresponds to the amide I band

Table 1 Raman peak positions and bands assignments. Raman peak positions and bands assignments *s* strong, *m* medium, *w* weak, *sh* shoulder, *br* broad, *v* very (Bansil et al. 1978; Gamulin et al. 2013; Kazanci et al. 2007; Mandair and Morris 2015; Pinheiro et al. 2014)

Raman shift (cm^{-1}) ($\approx$)		Assignments
430	s	P-O stretching of phosphate ($\nu_2PO_4^{-3}$)
590	s	P-O stretching of phosphate ($\nu_4PO_4^{-3}$)
815		C-C stretching of backbone
855	m	C-C stretching of proline
875	m	C-C stretching of hydroxyproline
920	sh	C-C stretching of proline
960	vs	P-O symmetric stretching of phosphate ($\nu_1PO_4^{-3}$)
1003	m	C-C Phenylalanine ring breathing (aromatic ring)
1045		Asymmetric stretching of phosphate ($\nu_3PO_4^{-3}$)
1060	br	Proteoglycan
1070	s, br	C-O symmetric in plane stretching of B-type carbonate ($\nu_1CO_3^{-2}$)
1075	w, br	Asymmetric stretching of phosphate ($\nu_3PO_4^{-3}$)
1103	w	A-type carbonate ($\nu_1CO_3^{-2}$)
1077		O-P-O asymmetric stretching of phosphate ($\nu_3PO_4^{-3}$)
1246	br	C-N-H stretching of amide III (β-helix conformation and random coil)
1272		Amide III (α-helix conformation)
1298		Lipids (δ = CH)
1375		Proteoglycan (CH_3 glycosaminoglycans)
1458	br	δCH_2 bending mode in proteins and lipids
1460		$\delta CH_2/CH_3$ deformation
1667	s, br	C = O stretch of amide I (α-helix conformation)
1690	sh, br	C = O stretch of amide I (β-sheet conformation)

Important matrix bands include the C-C stretches of proline and hydroxyproline at $\approx$ 855 cm^{-1} and $\approx$ 875 cm^{-1}, respectively, which are specific to collagen, and the CH_2 wagging mode at $\approx$ 1450 cm^{-1}, which is a general marker for protein content. The vibrations for the collagen backbone include the amide III (C-N stretches and N-H bends) and amide I (C = O stretch) bands, which reach maximum intensities at $\approx$ 1250 cm^{-1} and $\approx$ 1660 cm^{-1}, respectively.

Biomarkers

It is important to define the criterion to quantify the quality of bone; thus, the utilization of a biomarker is a method to determine the influence of the mineral and organic portions of bone on its mechanical properties. Biomarkers for bone turnover and bone resorption are recommended to be included in observational and prospective clinical trials according to the International Osteoporosis Foundation and International Federation of Clinical Chemistry and Laboratory Medicine (Gong

et al. 2014). In this chapter, four biomarkers were chosen to qualify bone: three feature the impact of the mineral and one highlights the contribution of collagen cross-linking. The mechanical properties are impacted by three features of bone minerals: the crystallinity, the mineral to matrix ratio (Freeman et al. 2001), and the carbonate substitution (Carretta et al. 2015). These biomarkers are all ratios except for the crystallinity; thus, if both values increase, the ratios do not vary.

Mineral to Matrix Ratio

The mineral to matrix ratio is a biomarker that quantifies the degree of mineralization of bone. As the ratio becomes larger, the mineral content in bone becomes higher. The net mineralization can be determined using ash weight measurements (McCreadie et al. 2006). The ratio can be linked to the calcium content that is measured by quantitative back-scattered electron microscopy with a correlation coefficient of 0.75 (Roschger et al. 2014). The mineral to matrix ratio is not a marker of the differences of substitution in the crystals.

Because the Raman confocal microscopy polarization can influence the signal, it is important to take this into account in the selection of the band for the calculation of this ratio. In prior studies, the phosphatase $\nu_1PO_4^{3-}$ ($\approx$960 cm^{-1}) and collagen amide I ($\approx$1677 cm^{-1}) bands were used to compute this ratio (Timlin et al. 1999), but they were strongly dependent on the orientation. (Gamsjaeger et al. 2010; Kazanci et al. 2006; Ramasamy and Akkus 2007; Roschger et al. 2014). It is recommended thus either to add a depolarizer or to use another band that is not sensitive to an orientation effect, such as the phosphatase $\nu_2PO_4^{3-}$ band ($\approx$438 cm^{-1}), the collagen amide III band ($\approx$1256 cm^{-1}), the phosphatase $\nu_4PO_4^{3-}$ band ($\approx$589 cm^{-1}), or the collagen amide III ($\approx$1256 cm^{-1}) band (Kazanci et al. 2006). Some studies have shown that the mineral to matrix ratio is often calculated using the phosphatase $\nu_1PO_4^{3-}$ ($\approx$960 cm^{-1}) to the CH_2 wagging band ($\approx$1450 cm^{-1}) (Burket et al. 2011, 2013; Carden et al. 2003; Kim et al. 2012; Yao et al. 2013; Yeni et al. 2006).

The mineral to matrix ratio is dependent on the origin of the bone in the skeleton, for example, either from the femoral head or femoral metaphysis. The ratio is higher in the metaphysis than in the epiphysis of a human femur (Buckley et al. 2014). Nevertheless, even though the site has an influence, on a mouse sample, there was no difference between the anterior and posterior quadrants of the femur (Ramasamy and Akkus 2007).

Crystallinity

Crystallinity is an indication of the size of the hydroxyapatite crystals along their long axes direction (Pinheiro et al. 2014) with a very high correlation between the biomarker and the c length (R = 99,2%) (Morris and Mandair 2011). It is also considered to be a biomarker of mineral maturation (Yamamoto et al. 2012). It

depends on the disorder and the strain that the mineral lattice experiences (Timlin et al. 2000). The usual method for determining the crystallinity is the inverse of the full width at maximum height (1/FWMH) of the ν_1 phosphate peak (Akkus et al. 2004; Awonusi et al. 2007; Bi et al. 2011; Burket et al. 2011; Carretta et al. 2015; Freeman et al. 2001; Goodyear et al. 2009; Imbert et al. 2014; Kavukcuoglu et al. 2009; Kim et al. 2012; Meganck et al. 2013; Newman et al. 2014; Nyman et al. 2011; Ojanen et al. 2015; Orkoula et al. 2012; Silva et al. 2006; Turunen et al. 2011; Yavorskyy et al. 2008; Yerramshetty et al. 2009). However, some authors also measure this biomarker with the position of the ν_1 phosphate peak (Pinheiro et al. 2014; Timlin et al. 1999).

Carbonate Substitution Rate

The carbonate to phosphate ratio is a biomarker that measures the amount of carbonate substitution in the hydroxyapatite lattice. Indeed, human bone mineral differs from synthetic hydroxyapatite in that its lattice is not pure hydroxyapatite but has ions substituted primarily with carbonate. There are two types of carbonate substitutions: A-type, which is the substitution of a carbonate for a hydroxyl group, and B-type, which is a carbonate for phosphate substitution and is the most visible substitution in Raman analysis with a specific Raman shift at 1071 cm^{-1}. Calibration curves of the content of CO_3^{2-} substitution in synthetic hydroxyapatite have been made to determine the substitution rates and their influence on the Raman spectrum of bone and enamel samples (Awonusi et al. 2007; Clasen and Ruyter 1997). The carbonate substitution rate is usually calculated with the ratio of the intensities (respectively the area) of the Raman shift at 1070 cm^{-1} of the type B carbonate substitution on the intensities (respectively the area) of the Raman shift at 960 cm^{-1} of the $\nu_1 PO_4^{3-}$ (Akkus et al. 2004; Bi et al. 2011; Burket et al. 2011; Donnelly et al. 2009; Imbert et al. 2014; Kavukcuoglu et al. 2007; Kim et al. 2012; Kozielski et al. 2011; Orkoula et al. 2012; Penel et al. 2005; Ramasamy and Akkus 2007; Roschger et al. 2014; Silva et al. 2006) (respectively Gentleman et al. 2009; Hammond et al. 2014; Kohn et al. 2008; McCreadie et al. 2006; Newman et al. 2014; Tarnowski et al. 2002; Turunen et al. 2011; Wallace et al. 2009; Yao et al. 2013).

Collagen Maturity and Cross-Linking

The above biomarkers mainly describe the state of the hydroxyapatite mineral in the bone. However, collagen also must be qualified. Because collagen organizes itself in highly structured fibrils with specific cross-links, quantifying its maturation is important. Collagen cross-linking evolves with the age of the protein fibrils, which has an impact on the biomechanical properties of collagen and on bone (Bailey et al. 1998; Knott and Bailey 1998). The relative pyridinoline compounds (major trivalent collagen cross-link) qualify the quality of collagen. This is calculated as the

ratio of the peak heights or areas of different parts of the amide I band, which is typically the ratio of the peak at 1660 cm^{-1} to the peak at 1690 cm^{-1} (Bart et al. 2014; Gamsjaeger et al. 2014a; Goodyear et al. 2009; Kohn et al. 2008; Orkoula et al. 2012; Pinheiro et al. 2014; Turunen et al. 2011; Wallace et al. 2009). Nalla et al. observed a good correlation with aging using the 1610 to 1655 cm^{-1} ratio because the peak at 1655 cm^{-1} increases in intensity with age. This is consistent with an increase in the nonreducible cross-linking content (Nalla et al. 2006). Pinheiro et al. used the ratio of collagen type II to type I (854 cm^{-1}/881 cm^{-1}) to qualify this quality and the cross-linking: the more nonreducible cross-links there are, the older and less elastic the bone will be (Pinheiro et al. 2014). Thus, a smaller ratio is preferable.

Other Biomarkers

The orientation of collagen fibers can be estimated using ratios that are orientation dependent ($\nu_1PO_4^{3-}$/amide I) by comparing with ratios that are not orientation dependent ($\nu_2PO_4^{3-}$/amide III, $\nu_4PO_4^{3-}$/amide III) in spongy bone tissue (Buchwald et al. 2012a; Kozielski et al. 2011).

Proteoglycans (PG) are noncollagenous proteins, and the ratio of the PG ($\approx$1375 cm^{-1}) to the amide III band allows the characterization of the relative PG content in bone and cartilage (Gamsjaeger et al. 2014a).

Biomarkers for Investigating Bone Metabolism

Table 2 provides the research articles in which Raman biomarkers have been used to characterize physiological changes, such as growth, exercise, aging, and diseases for different bone types.

Table 3 is a summary of the articles that are listed in Table 2 to show the evolution of biomarkers as a function of physiological changes and diseases according to the literature. In this systematic bibliography, all studies did not use all of the biomarkers. However, the table is a summary of all conclusions drawn with respect to the general trends followed by the biomarkers.

Potential Applications to Prognosis, Other Diseases, or Conditions

Other Bone Studies

Biomarkers are also used to determine if the bone structure is altered during experiments. For example, we want to determine if the synchrotron modifies the mineral or the organic portion of a bone (Gupta et al. 2006), if sterilization impairs

Table 2 Biomarkers and literature. Summary of acquisition parameters and calculation methods of biomarkers ratios can either be intensity (I), area (A), and/or position (P)

Bone type and specificity	Laser wavelength (nm)	Ratios	Mineralization	Collagen maturity	Key words	References
Rat, wet	532	I & P	960 cm^{-1}/1663 cm^{-1}		Physiochemical properties; Mineral crystals; Aging	Akkus et al. 2004
Hydroxyapatite artificial	785	P			Carbonated apatite	Awonusi et al. 2007
Mouse OI model, submerged in PBS	660	I			Physiochemical properties; Crystallinity; Osteogenesis imperfecta	Bart et al. 2014
Mouse, dehydrated and embedded	785	I	960 cm^{-1}/ 1665–1250–856 cm^{-1}		Nanoindentation; Micro CT; Fracture risk	Bi et al. 2011
Human	785	A	v_2/Amide III, v_4/Amide III	v_1/Amide I (fibers orientation)	Spongy bone; Fibers orientation; Mapping; Polarization	Buchwald et al. 2012a
Human	785	A	960 cm^{-1}/1244 cm^{-1} 960 cm^{-1}/1268 cm^{-1}	1268 cm^{-1}/ 1244 cm^{-1} (disorder)	Osteoarthrosis; Subchondral bone; Spongy bone	Buchwald et al. 2012b
Human	830	I	960 c m^{-1}/Mea n(830–900) cm^{-1}		Long bone; Water; Mineral; Collagen and GAG content	Buckley et al. 2014
Baboon, dry	785	I	965 cm^{-1}/1450 cm^{-1}		Aging; Primate; Osteon; Nanoindentation; Tissue properties	Burket et al. 2011
Ovine, dry	785	I	965 cm^{-1}/1451 cm^{-1}		Osteoporosis; Bisphosphonate; Cancellous bone; Mechanical properties	Burket et al. 2013

(*continued*)

Table 2 (continued)

Bone type and specificity	Laser wavelength (nm)	Ratios	Mineralization	Collagen maturity	Key words	References
Bovine	785	I	959 cm^{-1}/1450 cm^{-1}		Biomechanic; Fracture; Nanoindentation	Carden et al. 2003
Bovine	532	I	960 cm^{-1}/1260 cm^{-1}	1665 cm^{-1}/ 1637 cm^{-1}	Biomechanic; Finite element method	Carretta et al. 2013a
Human	532	I	960 cm^{-1}/1660 cm^{-1}	1665 cm^{-1}/ 1637 cm^{-1}	Biomechanic; Finite element method; Osteoporosis	Carretta et al. 2013b
Human	532	I	v_1/Amide I	1663 cm^{-1}/ 1632 cm^{-1}	Trabecular bone; Strain; Toughness; microCT; Mechanical properties; PCA	Carretta et al. 2015
Rat	785	I	960 cm^{-1}/1675 cm^{-1}		Aging; Nanoindentation; Biomechanic	Donnelly et al. 2009
Horse, hydrated in PBS	785	A			Imaging; Double notch; Bone mechanic	Dooley et al. 2009
Mouse, dry	514	P			Aging; Fluoride treatment; Apatite	Freeman et al. 2001
Mouse, dehydrated and embedded	785	A			Bone maturation; Composition; Orientation	Gamsjaeger et al. 2010
Human, dry	785	A	(410–460) cm^{-1}/ (1215–1300) cm^{-1}	1660 cm^{-1}/ (1620–1700) cm^{-1}	Bone material properties; Normative data	Gamsjaeger et al. 2014a
Human	785	A	(410–460) cm^{-1}/ (1620–1700) cm^{-1}		Proteoglycan; Cartilage; Collagen	Gasmjaeger et al., 2014b

Rat	1064	I	v_1/Amide I		Bone healing; Osteonecrosis; PCA	Gamulin et al. 2013
Bovine, w/o photobleaching	532	P & I			Photobleaching	Golcuk et al. 2006
Mouse, hydrated in PBS	785	I	589 cm^{-1}/1260 cm^{-1}	1660 cm^{-1}/ 1690 cm^{-1}	Bone composition; Mechanical properties	Goodyear et al. 2009
Bovine	532	P & I			Deformation mechanisms; Micromechanics of bone	Gupta et al. 2006
Rat, hydrated in PBS	660	A	(930–980) cm^{-1}/ (15 50–1720) cm^{-1}		Diabetes; Nanoindentation; Collagen	Hammond et al. 2014
Human, dry	785	I	961 cm^{-1}/1667 cm^{-1}		Nanoindentation; Mechanical properties; Minerality	Imbert et al. 2014
Mouse, dehydrated and embedded	785	I	961 cm^{-1}/1667 cm^{-1}		Nanoindentation; Mechanical properties; Composition	Kavukcuoglu et al. 2007
Mouse	785	I	961 cm^{-1}/1667 cm^{-1}		Nanoindentation; Mechanical properties; Chemistry of bone	Kavukcuoglu et al. 2009
Human, dehydrated and embedded	532	I	438 or 589 cm^{-1}/1256 cm^{-1}		Orientation; Composition; Mapping; Osteon	Kazanci et al. 2006
Human, dehydrated and embedded	532	A	438 or 589 cm^{-1}/1256 cm^{-1}		Orientation; Composition; Imaging	Kazanci et al. 2007
Human	830		960 cm^{-1}/1660 cm^{-1} 960 cm^{-1}/920 cm^{-1} 960 cm^{-1}/(885 + 870) cm^{-1}		Osteoarthrosis; Subchondral bone; pQCT; PCA	Kerns et al. 2014

(*continued*)

Table 2 (continued)

Bone type and specificity	Laser wavelength (nm)	Ratios	Mineralization	Collagen maturity	Key words	References
Rat	785	I	961 cm^{-1}/1450 cm^{-1}		Nanoindentation; Composite beam theory; Vitamin D	Kim et al. 2012
Human	785	I	961 cm^{-1}/1450 cm^{-1}		Nanoindentation; Osteoporosis; Trabecular bone	Kim et al. 2014
Mouse	785	A	958 cm^{-1}/ (hydroxyproline + proline)	1660 cm^{-1}/ 1690 cm^{-1}	Mechanical loading; Mechanical properties; Mineral; Collagen	Kohn et al. 2008
Human	785	I	v_2/Amide III, v_4Amide III	v_1/Amide I (fibers orientation)	Spongy bone; Fibers orientation; Mapping; Polarization	Kozielski et al. 2011
Human, dehydrated and embedded	785	A	(903–991) cm^{-1}/ (1616–1720) cm^{-1}		Osteoporosis; w/o fracture; Women	McCreadie et al. 2006
Mouse, kept moist in PBS	785	I & A	960 cm^{-1}/(851 + 873 + 917) cm^{-1} (A) 960 cm^{-1}/ 1445 cm^{-1} (I) 960 cm^{-1}/ amide I (A)	1660 cm^{-1}/ 1690 cm^{-1} after five-peak fit	Osteogenesis imperfecta; Bisphosphonates; Fracture repair	Meganck et al. 2013
Human	244	A		1610 cm^{-1}/ 1655 cm^{-1}	Aging; Fracture toughness; Mechanical properties	Nalla et al. 2006
Rat	660	A	960 cm^{-1}/1660 cm^{-1}		Mechanical properties; Kidney disease	Newman et al. 2014
Human, dry	785	I			Review; Comparison of 15 peak ratios (Osteonal/ interstitial tissue; transverse/ longitudinal cuts; w/o embedded, dehydrated)	Nyman et al. 2011

Human	785	I	958 cm^{-1}/1447 cm^{-1}		Aging; Trabecular bone; Nanoindentation	Ojanen et al. 2015
Rat	1064	I	959 cm^{-1}/(855 + 877 + 922) cm^{-1}	1660 cm^{-1}/ 1685 cm^{-1}	Osteoporosis; Collagen; Cross-links; Mineral	Orkoula et al. 2012
Rabbit, intravital	633				Intravital; Apatic biomaterials; Assignments comparison	Penel et al. 2005
Rat	785	I	1077 cm^{-1}/854 cm^{-1}	1660 cm^{-1}/ 1670 cm^{-1}	Bone repair; Biomaterial; Laser and LED Phototherapies	Pinheiro et al. 2014
Mouse, wet	633		960 cm^{-1}/1450 cm^{-1}		Bone adaptation; Collagen orientation; Microtesting; Mineral density	Ramasamy and Akkus 2007
Human, dehydrated and embedded	785	A	(410–460) cm^{-1}/(1215–130) cm^{-1}		Calcium content; Quantitative back-scattered electron microscopy	Roschger et al. 2014
Rat	633	I	960 cm^{-1}/1451 cm^{-1}		Osteoporosis; Spinal cord injury	Shen et al. 2010
Mouse, dry	532	A	2946 cm^{-1}/960 cm^{-1}		Bone strength; Collagen organization; Aging	Silva et al. 2006
Mouse	785	A	957 cm^{-1}/1665 cm^{-1}		Mineralization; Mouse calvaria; Multivariate analysis	Tarnowski et al. 2002
Bovine	785				Fatigue loading; Raman imaging; Spectral shifts of phosphate Vi	Timlin et al. 2000
Rabbit	785	I & A	958 cm^{-1}/(917 + 873 + 851) cm^{-1} 958 cm^{-1}/1271 cm^{-1} 958 cm^{-1}/1447 cm^{-1}	1660 cm^{-1}/ 1690 cm^{-1}	Maturating cortical bone; Comparison RS/FT-IR	Turunen et al. 2011
Mouse	785	A	960 cm^{-1}/1667 cm^{-1}	1660 cm^{-1}/ 1690 cm^{-1}	MicroCT; Mechanical properties; PCR; Biglycan deficiency	Wallace et al. 2009

(*continued*)

Table 2 (continued)

Bone type and specificity	Laser wavelength (nm)	Ratios	Mineralization	Collagen maturity	Key words	References
Rat	780		v_1/Amide I, v_1/Amide III, v_1/CH		Bone allograft; Sterilization; Quality assessment	Yamamoto et al. 2012
Mouse, wet	633	A	960 cm^{-1}/1450 cm^{-1}		Osteogenesis imperfecta; Mechanical properties; Gender dependence	Yao et al. 2013
Mouse, dehydrated and embedded	633	I	960 cm^{-1}/1450 cm^{-1} 960 cm^{-1}/1667 cm^{-1}		Fixation; Embedding medium	Yeni et al. 2006
Human, wet	633		960 cm^{-1}/1450 cm^{-1}		Aging; Mineralization; Crystallinity; Carbonation	Yerramshetty et al. 2006
Human, wet					Osteoporosis; Strength; Crystallinity; Mechanical properties	Yerramshetty and Akkus 2008

Table 3 Biomarkers and particularity. Evolution of biomarkers with physiological changes and diseases; References in bold are studies with human subject

Particularity	Mineral to matrix ratio	Carbonate to phosphate substitution	Crystallinity	Collagen maturity, quantity of irreductible cross-links	References
Physiological development					
Growth	↗	↗	↗		Burket et al. 2011; Donnelly et al. 2009; Gamsjaeger et al. 2014a; Tarnowski et al. 2002; Turunen et al. 2011
Exercise	↗	↘			Kohn et al. 2008; Shen et al. 2010
Aging	↗	↗	↗	↗	Akkus et al. 2004; Burket et al. 2011; Carretta et al., 2013a; Donnelly et al. 2009; Morris and Mandair 2011; Nalla et al. 2006; Ojanen et al. 2015; Yerramshetty et al. 2006
Bone diseases					
Osteoporosis	↘	↗	↗	↗	Burket et al. 2013; Carretta et al., 2013b; Freeman et al. 2001; Gong et al. 2014; Kavukcuoglu et al. 2007; Kim et al. 2014; McCreadie et al. 2006; Morris and Mandair 2011; Orkoula et al. 2012; Shen et al. 2010; Silva et al. 2006

(*continued*)

Table 3 (continued)

Particularity	Mineral to matrix ratio	Carbonate to phosphate substitution	Crystallinity	Collagen maturity, quantity of irreductible cross-links	References
Osteogenesis imperfecta with BP	↗	≈	↘	≈	Imbert et al. 2014; Meganck et al. 2013
Osteogenesis imperfecta without BP	↘		↘	↘	Bart et al. 2014; Meganck et al. 2013; Silva et al. 2006; Yao et al. 2013
Congenital contractural arachnodactyly		↘			Kavukcuoglu et al. 2007
No osteocalcin		↘	↗		Kavukcuoglu et al. 2009
Other disease					
Diabetes mellitus	↗			↗	Hammond et al. 2014
Kidney disease	≈	≈	≈	≈	Newman et al. 2014
Byglycan deficiency	↗		↗	↘	Wallace et al. 2009

bone properties (Yamamoto et al. 2012), if lasers have a positive impact on bone grafts (Pinheiro et al. 2014), or if healing has been completed (Gamulin et al. 2013). Using biomarkers can also be a method to determine if structures are altered by measurements, e.g., if laser energy damages the collagen and mineral organization (Gamsjaeger et al. 2014a; Kozielski et al. 2011).

Researchers have also used biomarkers to follow-up and quantify bone formation in experiments with osteoblasts (Gentleman et al. 2009; Wang et al. 2009).

Joint Analysis and Other Tissue

Osteoarthritis is a bone disease and a joint disease. Thus, Raman spectroscopy can provide insight into the disease, and the technique can provide information on the synovial fluid, on the cartilage, and on subchondral bone (Buchwald et al. 2012b; Esmonde-White et al. 2011; Gamsjaeger et al. 2014b; Kerns et al. 2014; Yavorskyy et al. 2008).

Skin has also been studied using Raman spectroscopy with other biomarkers that are more focused on collagen properties; however, the principle remains the same (Zhao et al. 2007).

Biomechanical Properties and Mechanical Testing

In a mouse model, the Raman composition parameters correlate with the mechanical properties at the tissue level: the mineral to matrix ratio correlated with the nanoindentation modulus and hardness (Bi et al. 2011; Kim et al. 2014). At the whole bone level, the mineral to matrix ratio was also correlated with the post-yield deflection (a measure of bone brittleness) (Bi et al. 2011).

The biomarkers are influenced by the mechanical load applied to the bone samples during the experiments, as Carden et al. showed in their experiments that included Raman imaging at different places next to an indent from the indentation spot (Carden et al. 2003). Indeed, Raman shifts vary in their frequencies and intensities as the mineral lattice is distorted and as the ion spacing is changed with the load. Moreover, the organic bands are also changed as the cross-linking is degraded (Morris and Mandair 2011). The same phenomenon has been observed in bone with microdamage that is related to fatigue stimulation (Timlin et al. 1999).

The crystallinity has been linked to mechanical properties, such as degradation rate, fatigue resistant time, and tissue level strength and stiffness, in human cortical bone (Yerramshetty and Akkus 2008). However, according to Morris and Mandair, the strongest predictors of the mechanical properties of bone are the mineral to matrix ratio and the carbonate substitution ratio (Akkus et al. 2004; Morris and Mandair 2011).

It is also possible to correlate those biomarkers with the fracture parameters because the ultimate strain and the post-yield work as efficiently as the collagen quality and as the carbonate substitution biomarkers to distinguish osteoporotic donors (Carretta et al. 2013b). Carretta et al. used the same biomarkers to predict the local ultimate strain and toughness of trabecular bone (Carretta et al. 2015).

Dooley et al. used the Raman shifts of the $\nu_1 PO_4^{3-}$ band to determine a mapping distribution of the stress in strained and failed regions of bone (Dooley et al. 2009).

Summary Points

- This chapter focused on the relevance of the Raman Spectroscopy technique to investigate the biomarkers for bone metabolism.
- Bone has a multilayer composite structure, which combines the elasticity of an organic matrix with the strength of mineral components.
- Raman spectroscopy provides fingerprint information on the chemical and structural organization of bone.
- Biomarkers are determined mainly as ratios of selected bands that represent mineral, matrix, and carbonate contents.
- The biomarkers reviewed here were used to assess the bone quality and to follow the changes in bone due to the physiological environment (sport, weightlessness, etc.), diseases (osteogenesis imperfecta, osteoporosis, etc.…), or treatment effects (bisphosphonate use).

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Quantitative Ultrasound as a Biomarker Tool in Newborn Infants for the Bone

30

Paola Gaio, Marco Daverio, Margherita Fantinato, and Giovanna Verlato

Contents

Abstract

Early nutrition and adequate growth can influence future adult health. Nonetheless, recommended nutrient intakes, which can affect growth and bone health, are rarely achieved in preterm infants during the first weeks of life. Peak fetal accretion of bone growth occurs during the last trimester of gestation, and preterm infants are exposed to a higher risk of developing metabolic bone disease with an increased bone fragility, a higher fracture risk, and a long-term reduced linear growth and childhood height. MBD has consequences either in the medium or long term. In the medium term, fractures are reported in 10% of VLBWI, and in the long term, children who were born prematurely had a decreased weight and height at the age of 7–8 years, and compared to controls they had a lower bone mineral content. Monitoring bone growth has become mandatory in neonatology. Quantitative ultrasound technique is an inexpensive, portable, noninvasive, and

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radiation-free method of evaluating bone status and can be applied to monitor premature bone health.

Most of the studies, by applying QUS, report a positive correlation of bone status with gestational age but an inverse correlation with postconceptional age. Former preterm has lower bone growth at term compared to term infants suggesting that perinatal factors after birth are still limiting preterm bone health.

There are no established guidelines on the timing of monitoring bone disease in preterms, and different monitoring protocols are suggested.

QUS, though it is necessary to establish universal normative values, could be an easy, painless, and rapidly performing method to monitor patient bone health from birth till infanthood. Further studies are necessary to evaluate the influence of perinatal factors on long-term bone health.

Keywords

Bone development • Early nutrition • Metabolic bone disease • Neonatology • Preterm newborns • Quantitative ultrasound

List of Abbreviations

ALP	Alkaline phosphatase
BPD	Bronchopulmonary dysplasia
BTT	Bone transmission time
BW	Birth weight
CRIB	Clinical risk index for babies
DEXA	Dual-energy X-ray absorptiometry
ELBWI	Extremely low birth weight infant
GA	Gestational age
mc-SOS	Metacarpal speed of sound
mc-BTT	Metacarpal bone transmission time
MBD	Metabolic bone disease
NEC	Necrotizing enterocolitis
QUS	Quantitative ultrasound
TPN	Total parenteral nutrition
VLBWI	Very low birth weight infant

Key Fact

- Survival in newborns with birth weight $<$1,000 g or $<$1,500 g is increasing, but after birth they turn away from their in utero growth trajectory.
- Preterm infants develop bone growth impairment when compared to term infants, leading to an increased risk of metabolic bone disease.
- Metabolic bone disease is related to long-term consequences such as high risk of fractures, shorter final height, and lower peak bone mass.

- In preterm infants screening tests for detection of bone disease are difficult to apply since these patients are severely ill; they need to be cared with caution and hardly moved out of neonatal intensive care units.
- Quantitative ultrasound technique is an inexpensive, portable, noninvasive, and radiation-free method of evaluating bone status and can be easily applied to monitor premature bone health during hospitalization.
- Quantitative ultrasound technique can be used during follow-up visits to monitor bone growth in former preterm infants.

Definition of Words and Terms

BPD	Infant who necessitates treatment with oxygen >21% for at least 28 days after birth stratified for severity at 36 weeks of GA in preterm less than 32 weeks of age or at 28–56 days in preterm ≥32 weeks of GA.
Extremely low birth weight infant	Preterm infant born less than 1,000 g.
Full enteral feeding	Achievement of an amount of complete nutritional enteral feeding at a rate of at least 120–150 mL/kg/die that allows complete weaning from parenteral nutrition.
Metabolic bone disease	A skeletal disorder characterized by low bone mass with microarchitectural deterioration (osteopenia) and demineralization (osteomalacia) of bone tissue with a consequent increase in bone fragility and susceptibility to fractures.
Necrotizing enterocolitis	Inflammatory-ischemic necrosis of the intestinal mucosa associated with the presence of gas into the intestinal wall and portal venous system. Bell stage classification defines its gravity.
Parenteral nutrition	The intravenous administration of macro- and micronutrients in the elemental formula directly disposable to the cell bypassing the usual process of eating and digestion.
Peak bone mass	The amount of body tissue present at the end of skeletal maturation that takes place at the end of puberty.
Preterm	Neonate born before 37 weeks of gestational age.
Quantitative ultrasound	Ionizing radiation-free method that measures broadband ultrasound attenuation through a specific bone site.
Very low birth weight infant	Preterm infant born less than 1,500 g.

Introduction

In the last two decades, quantitative ultrasound (QUS) technique has been used worldwide for the assessment of bone status both in adult (Foldes et al. 1995; Baran 1995) and in the pediatric population (Fewtrell et al. 2008; Mcdevitt et al. 2007; Scattolin et al. 2013; Betto et al. 2014).

It was first described in the late 80s as a tool to evaluate fracture risk and bone status in osteoporotic patients (Njeh et al. 1997). Later this method was transferred to the pediatric population. In particular it found a field of application in chronically ill children and in neonates. The aim was to elaborate a nonionizing, portable, and low-cost technique that could be comparable to dual-energy X-ray absorptiometry (DEXA).

Technically QUS has many advantages: it does not involve ionizing radiation and it is easily accessible and portable. Particularly in critically ill patients, it can be performed bedside, and it doesn't require patients to lay still so that sedation can be avoided in the pediatric and neonatal population (Table 1).

QUS measurements are based upon transmission of an ultrasound beam through a specific body site emitted and received by the two instrument probes. The software elaborates two parameters: speed of sound (SOS, m/s) and bone transmission time (BTT, sec). SOS depends both on bone and soft tissue characteristics, while BTT is independent of the amount of soft tissue and reflects bone mineralization and architecture (Rubinacci et al. 2003). QUS allows not only measures at peripheral skeleton of mineral status but also of other bone properties such as cortical thickness, elasticity, geometry, and porosity. In literature different bone sites has been used to evaluate bone status. In particular for newborns, tibia (Liao et al. 2005; Fewtrell et al. 2008), humerus (Rubinacci et al. 2003), and metacarpus (Ritschl et al. 2005; Scattolin et al. 2013; Betto et al. 2014) evaluations have been described.

Metabolic Bone Disease in Preterm Infants

In human embryos the process of ossification starts within the first weeks of gestation (primary ossification centers in the femurs and humerus can already be identified at 6 weeks of gestational age (GA)). This process during pregnancy is determined by the balance of deposition and remodeling of both organic matrix and mineral components of the bone (McDevitt and Ahmed 2007). These processes

Table 1 Pros and cons of quantitative ultrasound technique in preterm infants

Pros	Cons
Performed bedside	Lack of standard charts
No ionizing radiation	No univocal parameters of bone disease
No need for sedation	No univocal body site evaluation
Low-cost technique	
Reproducible	

allow fetal bone structure to gain shape and reach the correct average. Nonetheless bone growth and development occur mostly in the third trimester of gestation: at least 80% of calcium and phosphorus deposition is reached between 25 weeks of gestation and term (Bishop and Fewtrell 2003).

There are many factors that can influence bone development during pregnancy, fetal, placental, and maternal. The fetus has a high rate of mineral accretion; serum levels of phosphate, calcium, and calcitonin are high, while PTH and vitamin D metabolites are low (McDevitt and Ahmed 2007).

During pregnancy, calcium and phosphorus are actively transferred to the fetus, with maximum accretion rate reached between 32 and 36 weeks of gestation: 100–130 mg/kg/day for calcium and 60–70 mg/kg/day for phosphorus (Ziegler et al. 1976). Bone development does not depend anyway solely upon mineral deposition. It is in fact also determined by balancing the processes of deposition and reabsorption of organic matrix performed by osteoblastic and osteoclastic cells. This can be enhanced by mechanical stimulation of fetal movements against uterine walls and also by placental supply of estrogen and other hormones. Maternal factors influencing bone status in fetuses and newborns are many; at present there is still a lack of knowledge upon this matter and some issues are still controversial. Maternal habits such as diet, smoke, sun exposure, physical activity during pregnancy, and maternal body mass index can contribute to determine bone accretion. Some studies evaluated the role of maternal vitamin D levels: low levels of maternal 25-OH-vitamin D in the third trimester seem to correlate with lower mineral density in offspring (Lawlor et al. 2013). Factors influencing bone growth during pregnancy are reported in Fig. 1. Since preterm infants and in particular those born extremely premature such as very low-birth-weight infants (VLBWIs) and extremely low-birth-weight infants (ELBWIs) lose the chance to complete fetal development, it is easily predictable that this population is at high risk for impaired bone status.

At birth there is a sudden interruption of placental supply in macro- and micronutrients. Even if it is well established that for a preterm infant, the goal in neonatology is to reach the same growth rate of a fetus of the same GA, in clinical practice this often fails. Preterm infants usually receive parenteral nutrition that due to solubility limitations cannot fulfill the need in calcium and phosphorus supplementations. Moreover critically ill patients often have high energy requirements that cannot be guaranteed because of signs of intolerance or necessity for fluid restriction.

Optimizing nutrition in preterm population is often a challenge for neonatologists. A wide literature has investigated what are the right intakes for preemies, for macronutrients, minerals, and oligoelements. Aggett et al. defined the nutritional needs as “The corresponding dietary requirement would be the intake sufficient to meet the physiological requirement. Ideally this should be achieved without extreme homeostatic processes and excessive depletion or surplus in bodily depots” (Aggett et al. 1997). Adequate intakes have positive effects both in the short- and long-term period, improving somatic growth including bone health, but also reducing morbidities and promoting better neurological development.

In 2006 Ehrenkranz et al. showed that in a population of ELBWI, a better growth rate in the first month of life was associated with better Bayley index score at 18–22

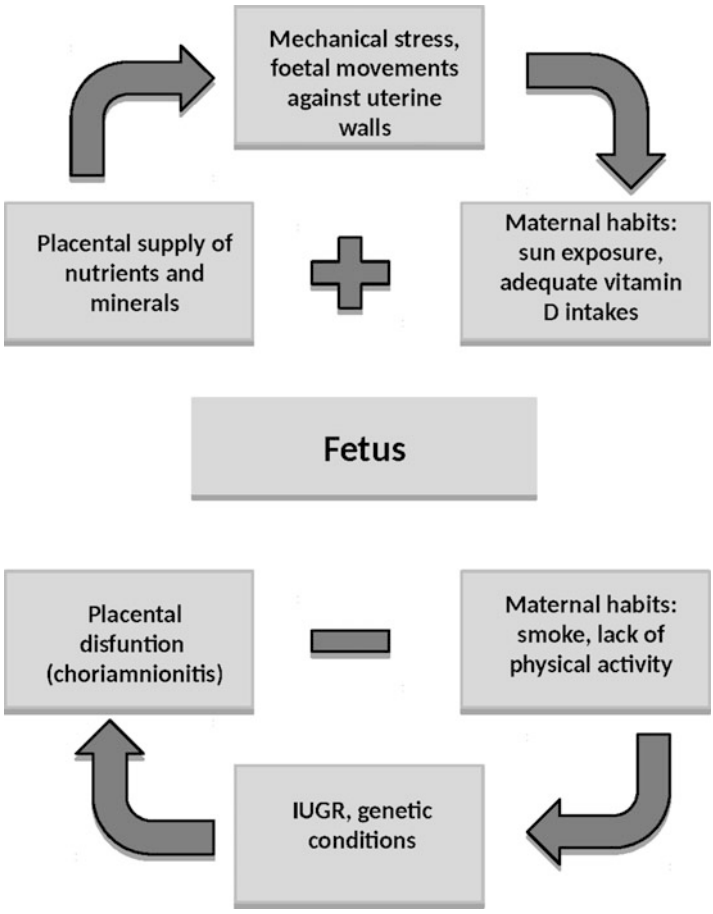

Fig. 1 Factors influencing bone growth during pregnancy

months of corrected age (Ehrenkranz et al. 2006). These findings were confirmed in another study published in 2009 by Stephens et al.: they found that both energy and protein intakes in the first week of life were positively correlated with Bayley index score at 18 months of corrected age (Stephens et al. 2009).

Higher nutritional intakes were also associated with better bone development. Meneghelli et al. in 234 preterm infants born less than 1,250 g showed that newborns with low energy intake in the first week of life (<70 kcal/kg/day) and low serum phosphate level (<1.4 mmol/L) at 21 days had lower mc-BTT at 36 weeks of GA (Meneghelli et al. 2016).

Assumptions for energy intakes are based upon studies evaluating healthy fetuses of the same GA. At least 100 Kcal/kg/die are needed when adequate protein intakes are fulfilled to meet the needs of extremely preterm infants (Agostoni et al. 2010). The range of energy intakes for these preterm infants should be considered from

110 to 135 kcal/kg/day for enterally fed infants and from 95 to 125 kcal/kg/day when infants are fed intravenously (Senterre et al. 2014).

Amino acids should be started intravenously early after birth, ideally within the first 24 h. A starting dose of 1.5–2 g/kg/day seems to be recommended with a subsequent increase to a maximum of 4 g/kg/day (Ehrenkranz 2007; Simmer 2007). This average should be reached within the first days of life with specific parenteral amino acid solutions that are targeted upon the needs of preterm neonates. When intravenous intakes reduce and enteral nutrition raises the same average of amino intakes (4 g/kg/day) should be guaranteed either with preterm formula milk or with fortified human milk. To do so, milk fortification should be started when oral intakes reach 80–100 ml/kg/day (Agostoni et al. 2010).

Adequate calcium and phosphorus homeostasis is crucial for a healthy bone growth. Mineral supplementation should be started immediately after birth with intravenous solutions. For calcium intake should be started at 1 mmol/kg/day raising up to 1.6–2.5 mmol/kg/day. For phosphorus intake should be started at 1 mmol/kg/day raising up to 1.6–2.5 mmol/kg/day (Mimouni et al. 2014). The goal is to modulate intakes to keep serum levels between 2 and 2.5 mmol/L for calcium and between 1.8 and 2.6 mmol/L for phosphorus. When preterm infants are fed enterally, the rate of intestinal absorption for each single nutrient should be kept in consideration. Adequate calcium and phosphorus intakes should be granted with fortified human milk or preterm formula or either with oral supplementations. The adequate oral mineral intakes have been discussed. In 2010 ESPGHAN committee indicated a calcium intake of 120–140 mg/kg/day and a phosphorus intake of 60–90 mg/kg/day (Agostoni et al. 2010). In 2013 AAP statement suggested calcium intake of 150–220 mg/kg/day and a phosphorus intake of 70–140 mg/kg/day (Table 2; Abrams and Committee on Nutrition 2013).

Nutrition, though the most significant, is not the only determinant factor for bone development and growth in these severely ill infants. It is in fact also known that many drugs administered to preterm infants, such as steroids and diuretics, can negatively influence bone development. In the last decade, the use of "long-term courses" of steroids in preterm infants has been significantly diminished because of the risk of poor neurodevelopmental outcome. For this reason at present the use of steroids is limited to those patients with severe bronchopulmonary dysplasia (BPD) in the aim to reduce their need for ventilation support or oxygen supplementation. Eelloo et al. in 2008 performed bone densitometry in 51 former preterm 5–8-year-old children, showing that among them, those diagnosed with chronic lung disease

Table 2 Energy and nutrient intake goals in VLBWI (Abrams and Committee on Nutrition 2013; Agostoni et al. 2010; Mimouni et al. 2014; Senterre et al. 2014)

	Parenteral nutrition	Enteral nutrition
Energy (Kcal/kg/day)	95–125	115–140
Amino acids (g/kg/day)	3.5–4	4–4.5
Calcium (mg/kg/day)	60–100	100–220
Phosphorus (mg/kg/day)	50–90	60–140

who received postnatal steroids have reduced bone mineral density (Eelloo et al. 2008). Nonetheless, this study has many limitations: patients with chronic lung disease were significantly smaller than controls and recruitments consented to enroll only 30% of possible patients. In a recent study, Jensen et al. conducted a retrospective evaluation to describe the prevalence of metabolic bone disease (MBD) in a population of VLBWI diagnosed with BPD. MBD is a skeletal disorder characterized by low bone mass with microarchitectural deterioration (osteopenia) and demineralization (osteomalacia) of bone tissue with a consequent increase in bone fragility and susceptibility to fractures. They found that 58% of patients had mild MBD and 31% had severe MBD. In the same study, they also showed that steroids and furosemide were more likely used in those patients who developed severe bone disease (Jensen et al. 2016).

Again, these severely ill infants with prolonged mechanical ventilation and possible sedation lay immobile for long periods of time so that the antigravitational movements that could promote bone matrix deposition are absent. Erdem et al. evaluated the changes in bone mineral density and anthropometric indices of ELBW preterm infants undergoing daily physical activity (Erdem et al. 2015). In this small randomized controlled trial, patients who underwent daily physical activity showed increasing tibial SOS values after birth, while patients treated with standard care showed decreasing tibial SOS values. Treated patients showed also improved anthropometric growth. Other studies showed similar results in small groups of VLBWI (Moyer-Mileur et al. 2000; Chen et al. 2010). A Cochrane review published in 2014 widely review the literature published upon this matter (Schulzke et al. 2014). Authors concluded that "Current evidence does not support the routine use of physical activity programs in preterm infants." In particular they pointed out the need for further larger trials to assess also possible harm of this practice and evaluate long-term outcomes. For these considerations it seems to be cautious to individualize the care of every single patient, evaluating the real need for sedation and curarization, possibly reserving physical activity approach for more stable patients. Factors related to MBD in preterm infants are reported in Fig. 2.

For all these reasons, preterm infants are at high risk to develop both impaired mineralization and organic bone matrix deposition. This condition is called MBD of prematurity. It has been estimated that MBD occurs in 55% of preterm infants born less than 1000 g (ELBWI) and in 23% of those born less than 1,500 g (VLBWI) (Dabezies and Warren 1997).

The real prevalence of this condition is anyway still difficult to define because of the different methods to screen infants at risk and the difficulty in interpreting screening results.

This condition occurs between the 6th and 12th postnatal week. Usually it is clinically silent in the neonatal period or in some severe cases determines bone fractures. Since in most cases MBD is clinically silent, many authors have researched biochemical or instrumental markers that could help identify among this patient population those who develop MBD. Considering QUS methods, the presence of MBD could be defined by mc-BTT value <2SD of mean value for age, weight, and length, but to date no univocal consensus exists upon this matter.

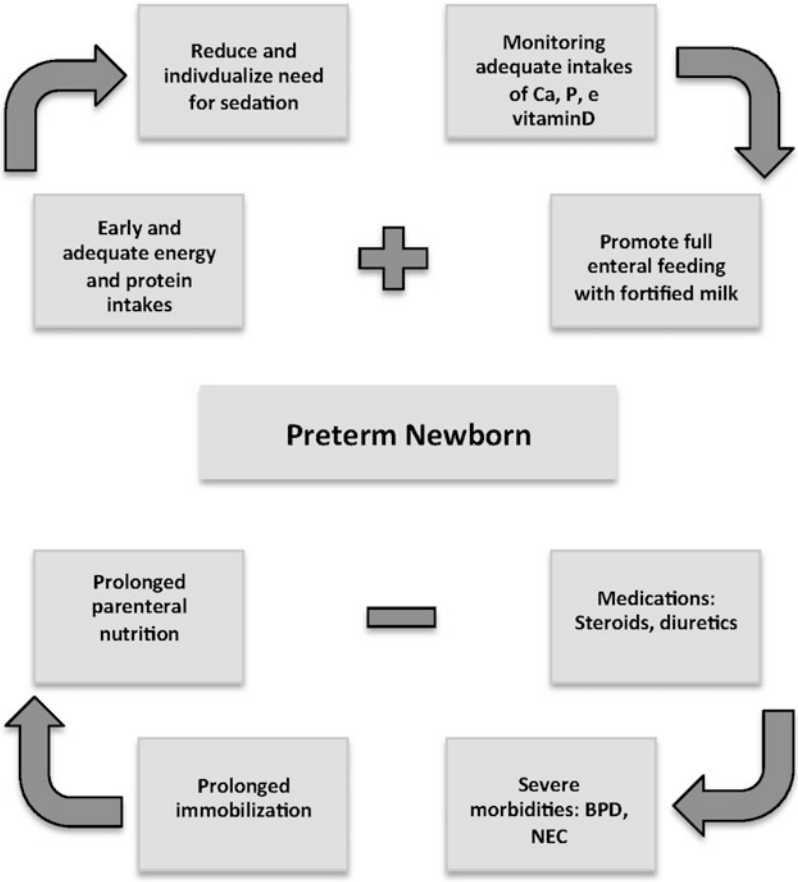

Fig. 2 Factors influencing bone growth during the neonatal period

MBD-Related Morbidities and Screening

MBD has consequences either in the medium- and long-term period. In the medium term, fractures are reported in up to 10% of VLBWI (Dabezies and Warren 1997), but population-based studies in neonatology are lacking so the real incidence could be underestimated. In the long term, children who were born prematurely have a decreased weight and height at the age of 7–8 years, and compared to controls they had a lower lumbar bone mineral content and bone mineral density (Bowden et al. 1999; Chan et al. 2008). Boys born at term have, at prepubertal examinations, greater bone size and mass than children born preterm (AbouSamra et al. 2009). In a cohort of 200 newborns randomized in four different nutritional regimens, anthropometry, bone mineral content, and bone area using DEXA and markers of bone turnover were assessed after 20 years. They found that infants born preterm had

Table 3 Possible short- and long-term consequences of metabolic bone disease

Fractures
Lower bone content and mass
Possible decreased peak bone mass
Reduced final height

lower height SD scores, higher body mass index SD scores, and lower lumbar spine BMD SD score when compared with controls and that these deficits were more marked in former preterm infants with BW <1,250 g (Fewtrell et al. 2009; Table 3).

It has also been hypothesized that alterations in terms of mineral content and bone density observed in children and young adults, as the result of early suboptimal bone growth, can promote the development of osteoporosis in advanced age (Hernandez et al. 2003; Javaid and Cooper 2002); in fact, the bone mass of an individual in later adult life depends on the peak obtained during skeletal growth and the subsequent rate of bone loss (Javaid and Cooper 2002), but peak bone mass was indicated as a more powerful predictor of osteoporosis (Hernandez et al. 2003). Therefore there might be an intrauterine "programming" also for osteoporosis, according to the hypothesis of the early origin of adult diseases (Barker et al. 1995). At present low birth weight is not reported to be a risk factor for fractures in adults, but further studies are needed to explore these important topics (Cooper et al. 2001).

For the above mentioned reasons, **monitoring bone growth** is mandatory, but it is not completely clear who are the most "high-risk patients". Some authors reported that newborns <27 GA are at higher risk of MBD (Tomlinson et al. 2006) and MBD was reported in 30% in ELBWI by means of radiological exam (Viswanathan et al. 2014). QUS measurements are normal at birth, and changes related to MBD develop around 4–12 weeks suggesting that bone growth was normal during fetal life and that it was altered after preterm birth. Factors in intrauterine life can also expose preterms to the risk of developing MBD. Severe demineralization is reported in newborns of mother with chorioamnionitis and in fetuses with intrauterine growth retardation suggesting that altered placental function could limit nutrient transfer (Holland et al. 1990). Nonetheless it is recognized that prematurity per se is the most important risk factor for developing MBD. Several studies have investigated this topic and suggested that the following conditions increase the risk of MBD: infants <28 weeks of GA (Tomlinson et al. 2006), VLBWI (Harrison and Gibson 2013), BW <800 g (Mitchell et al. 2009), prolonged total parenteral nutrition (TPN) >3–4 weeks (Tomlinson et al. 2006), or even TPN >14 days (McDevitt et al. 2007; Fig. 3).

The American Academy of Pediatrics in a recent clinical report (Abrams and Committee of Nutrition 2013) indicates the following conditions as high-risk factors for the development of rickets: born at <27 weeks of GA, BW <1,000 g, long-term parenteral nutrition (4–5 weeks), severe bronchopulmonary dysplasia with the use of loop diuretics (e.g., furosemide and fluid restriction, long-term steroid use), history of necrotizing enterocolitis (NEC), failure to tolerate formulas or human milk fortifiers with high mineral content. The AAP also suggests to perform biochemical testing after 4–5 weeks after birth in VLBWI.

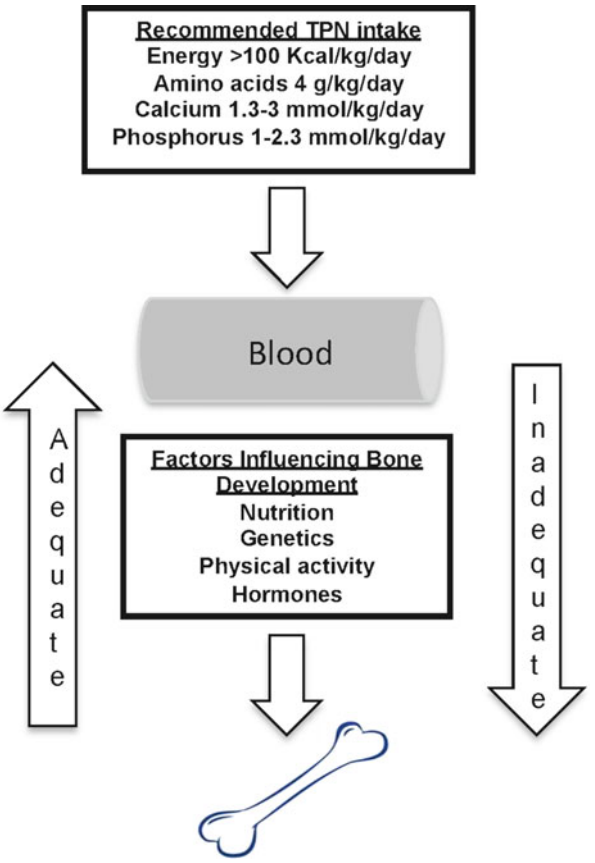

Fig. 3 Nutritional and other factors influencing bone development

QUS as a Tool for Monitoring Bone Disease

Previously X-ray was the only device available for detecting osteopenia but now also DEXA and QUS are available. The presence of MBD in preterm infants is reported when the bone trabeculae appear "eroded" in radiographs. But in standard radiographs, the bone changes of MBD are a late sign as these appear only when 20–30% of bone mineral content is lost. To the present day, DEXA is the most extensively used method for the assessment of bone mineralization (Mussolino et al. 1998), and in some cases DEXA has also been used in newborns to measure bone mineral content (Sievänen et al. 1999). Nonetheless DEXA has some important limitations related to radiation dose and need of sedation to limit artifacts related to movement.

We have previously reported the advantages of QUS technique (inexpensive, portable, noninvasive, and radiation-free method) of evaluating bone status. A limit of QUS is that although several studies have been performed, they enrolled a

relatively small number of patients; the measurements were done in different bone segments with a lack of validated normative values for preterm populations.

Ritschl et al. (2005) analyzed within 24 h after birth 132 Caucasians appropriate for GA preterm newborns (range 23–37 weeks) up to the age of 14 months. They provided GA reference curves of mc-SOS and mc-BTT assuming that measurements performed within 24 h of birth reflect bone status corresponding to the duration of gestation. In addition they measured term infants within 24 h and up to 18 months to create the postnatal reference curves. They found that QUS parameters have a steep increase in the last trimester of gestation but that after birth preterm infants have a decrease in SOS with an earlier and deeper nadir compared to term infants and a slower BTT increase reaching values of term infants only around 6 months of life.

Another study evaluated prospectively 172 preterm and term infants (23–42 weeks) by ultrasound in four places on long bones, and though they found an inter-patient high variability of QUS measurements to define normal values, they found a good day-to-day reproducibility; interestingly bone quality at term could be predicted by QUS development within the first 3 weeks of life (Rack et al. 2012). Other studies have provided reference ranges for tibial SOS in preterms within 96 h or within 1 week from birth (Littner et al. 2003; Chen et al. 2012).

Due to the presence of different techniques, the definition of MBD is not uniform, Altuncu et al. defined low bone density when tibial QUS was $\leq$2 Z score and at risk of osteopenia if tibial Z score was $\leq$1; instead Pereira-da-Silva et al. defined low bone strength as the condition when SOS was $<10^{th}$ percentile of the reference value obtained in Portugal (Altuncu et al. 2007; Pereira-da-Silva et al. 2011).

Other studies applied QUS to monitor MBD and searched for correlations with markers of bone disease. Most of the studies, by applying QUS, found correlations at birth with GA and BW of preterm newborns but an inverse correlation with postconceptional age (Mitchell et al. 2009; Nemet et al. 2001; Rack et al. 2012; Rubinacci et al. 2003; Tomlinson et al. 2006) suggesting a reduced bone mineralization and bone growth rate in this population after birth.

Altuncu's study found that tibial Z score in former preterm infants at term was significantly lower than their term counterparts (Altuncu et al. 2007). They found that preterms with alkaline phosphatase (ALP) $\geq$900 IU/L had lower tibial Z score at term corrected age. In their study 17% of preterm infants had low bone density for chronological age.

Tomlinson found that median SOS standard deviation score fell in all newborns from birth to 35–37 weeks of GA (Tomlinson et al. 2006). The median Z score was at birth 0 and at 35–37 weeks -2, and this fall was higher in those preterms of 24–27 weeks of GA. This study also evidenced that peak ALP was negatively correlated with tibial SOS (not with SOS Z score) at the end of the study and inversely correlated also with clinical risk index for babies (CRIB) score and length of parenteral nutrition. Other studies applying bone ultrasound reported a reduction in tibial SOS in former preterm infants at term corrected age (McDevitt et al. 2005) compared to term counterparts (Nemet et al. 2001; Rack et al. 2012; Rubinacci et al. 2003) especially in VLBWI (Nemet et al. 2001), and interestingly changes at

term could be predicted by early QUS examination (Rack et al. 2012). QUS results correlate with early clinical variables (such as peak ALP, CRIB, TPN) suggesting that bone growth is conditioned by early life factors.

Other studies investigated the role of nutrition on bone development monitored by QUS. Scattolin et al. found in a group of 105 preterms that early higher amino acid intakes and energy intakes positively correlated with mc-BTT at 36 weeks of GA suggesting that early nutritional intervention could have an impact on bone growth at least at medium term (Scattolin et al. 2013).

A recent study evaluated the possible influences of an early aggressive parenteral nutrition (with sooner provision of amino acids and higher energy intakes) on growth and bone health in preterm newborns of birth weight <1,250 g through anthropometric, clinical, biochemical, and QUS assessment (Meneghelli et al. 2016). Preterm newborns with total energy average in the first week ≥70 kcal/kg per day had a better mc-BTT at 21 days than those with an energy <70 kcal/kg per day ($P = 0.000$). Infants with serum phosphate on day 21 <1.4 mmol/L and mean total energy intake in the first week of life <70 kcal/kg per day showed lower mc-BTT values at 36 weeks of GA confirming that nutrition in early weeks of life can have influences on bone growth at least until discharge.

Markers of bone status have been studied. *ALP is suggestive, also if not proof, of rickets at very high levels (>1000 IU/L)* (Abrams and Committee on Nutrition 2013) but also at lower levels as preterm with ALP >900 IU/L (Altuncu et al. 2007) had lower QUS Z score and was all either osteopenic or at risk of osteopenia when ALP was >800 IU/L (Mitchell et al. 2009). Others reported that peak ALP inversely correlated with QUS (Tomlinson et al. 2006; Nemet et al. 2001; Rack et al. 2012) though not all preterms with QUS measurements <2 Z score had elevated ALP. Specificity improves when ALP is used in combination with serum phosphorus levels. ALP >900 IU/L with serum phosphorus levels <5.6 mg/dL (<1.8 mmol/L) yields 100% sensitivity with 70% specificity (Backström et al. 2000). These results confirm the observations of Figueras-Aloy et al. (2014) where the best correlation with bone mass density (a bone status index assessed by dual-energy X-ray absorptiometry) is obtained by combining ALP (>500 IU/L) and phosphate plasma levels with a cutoff of 4.5 mg/dL (1.45 mmol/L) to differentiate mild from severe MBD. Although ALP is the most used marker of MBD, the cutoff used to define the MBD varies widely between studies, from 300 IU/L (upper limit of the neonatal normal range) to values of 900 IU/L (Backström et al. 2000; Tinnion and Embleton 2012; Hung et al. 2011). It is also known that ALP shows a peak level of 400–800 IU/L after 4–6 weeks of life in preterms and then decreases in infants that do not present bone disease. Other studies did not find a significant correlation between ALP and QUS parameters suggesting that ALP cannot be the solely parameter to detect MBD (Scattolin et al. 2013; Meneghelli et al. 2016; Faerk et al. 2002). Though several studies explored these topics, there are no established guidelines on the timing of monitoring, and different monitoring protocols are suggested (Harrison and Gibson 2013; Mitchell et al. 2009). A recent survey aimed to assess screening, diagnostic, and treatment practices in US neonatal intensive care units and found that ALP

(99.4%), serum phosphate (92.6%), and/or serum calcium (88%) were most used by responders (Kelly et al. 2014).

Recently the American Academy of Pediatrics suggested in VLBWI to start monitoring ALP and P at 4–6 weeks after birth and continue biweekly till its reduction and having verified the absence of risk factors and on full feeds of mineral-fortified human milk or preterm formula. In the case of ALP >800–1000 IU/L, maximizing mineral intakes such as phosphorus supplementation (if phosphorus <4 mg/dL) and instrumental evaluation should be performed. In VLBWI exclusively breastfed, a follow-up serum ALP at 2–4 weeks after discharge from the hospital may be considered.

We think that QUS technique could help monitoring former preterms during hospitalization and after discharge.

Potential Applications to Prognosis, Other Diseases, or Conditions

We have explained the reasons why QUS could be applied in neonatology in preterm and term infants.

QUS can be applied to monitor bone growth in patients suffering from particular diseases such as patients suffering from renal diseases, gastrointestinal diseases like short bowel syndrome after important intestinal resections due to NEC, volvulus, or abdominal malformations.

It can be applied to monitor changes in bone status in high-risk patients (treated with diuretics, steroids) when repeated measurements are used in a single patient. It could monitor bone changes related to therapy (calcium phosphorus and 25-OH-vitamin D or 1-25-OH-vitamin D supplementation).

Summary Points

- With the improvement in neonatal care, more ELBWIs are surviving, but they continue to have significant morbidities such as MBD that can have consequences either in the medium- and long-term period.
- Preterm infants <27 weeks of GA with BW <1,000 g with prolonged parenteral nutrition (>4 weeks), with severe BPD (use of diuretics, steroids), and with history of NEC are the most at risk of developing MBD.
- Former VLBWI should be monitored after discharge even if there is no consensus yet on the optimal screening tests, treatment, and prevention of MBD.
- Most of the studies, by applying QUS, report a positive correlation of bone status with GA but an inverse correlation with postconceptional age.
- Former preterms have lower bone growth at term compared to term infants suggesting that perinatal factors after birth are still limiting preterm bone health.

- QUS, though it is necessary to establish universal normative values, could be an easy, painless, and rapidly performing method to monitor patient bone health from birth till infanthood.
- Further studies are necessary to study the influence of perinatal factors on long-term bone health.

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Part IV

Specific Diseases and Conditions

Bone Markers in Rett Syndrome

31

Gitte Roende and Jens-Erik Beck Jensen

Contents

Abstract

Rett syndrome (RTT) is a severe neurodevelopmental disease affecting primarily girls because of a mutation of the gene methyl-CPG-binding protein 2, *MECP2,* located at the X chromosome. The hallmarks of RTT are global developmental delay, regression of mile stones as partial or complete loss of purposeful hand skills and acquired spoken language/babbling, gait abnormalities, and stereotypic hand movements. Girls with RTT are often growth retarded, have low bone mass, and increased occurrence of low-energy fractures. Recent studies of biochemical

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bone markers, e.g., osteocalcin, bone-specific alkaline phosphatase, N-terminal propeptides of collagen type 1, and C-terminal telopeptide (crosslinks), have shown reduced bone formation in the context of reduced/normal bone resorption in RTT patients compared to sex-, age-, and pubertal-matched healthy controls. This deviation of growth and bone formation is apparent from early age but without association to low bone mass or low-energy fractures. As for healthy children, patients with RTT have higher levels of bone markers in early childhood and prepubertal years followed by an overall decrease through puberty and by age. It is unknown whether there is a slight increase in biochemical bone markers during early-mid puberty. Patients with RTT do not have skewed levels of sex hormones, growth hormones, or thyroid hormones. Vitamin D levels are often in the range of insufficiency. No association to specific *MECP2* mutations has been reported, but it is possible that *MECP2* exerts a general influence on growth and bone formation. However, the specific relation to the osteoblast function and activity and the interplay with the osteoclasts remain to be elucidated. In support of a low bone turnover, phenotype in RTT is the result from a histomorphometric study of bone biopsies in RTT showing reduced bone volume and low bone formation rate. Studies of *MECP2* null mice also report abnormal bone development with reduced osteoblast number, osteoblast dysmorphology, growth plate abnormalities, reduced bone volume, and reduced bone strength. As this deviation of bone development is potentially reversible in *MECP2* null mice, it is important to continue research of bone metabolism in order to optimize possibilities of prevention and treatment of bone pathology in RTT.

Keywords

Rett syndrome • *Methyl-CPG-binding protein 2* • Bone mass • Growth • Bone formation • Bone resorption • N-terminal propeptides of collagen type 1 • C-terminal telopeptide crosslinks • Osteocalcin • Bone-specific alkaline phosphatase

List of Abbreviations

B-ALP	Bone-specific alkaline phosphatase
CTX	C-terminal telopeptide crosslinks
DNA	Deoxyribonucleic acid
DXA	Dual energy X-ray absorptiometry
GH	Growth hormone
MECP2	The gene *methyl-CPG-binding protein 2*
MeCP2	The protein methyl-CPG-binding protein 2
OC	Osteocalcin
P1CP	Type 1 procollagen carboxyterminal propeptide
P1NP	N-terminal propeptides of collagen type 1
PTH	Parathyroid hormone
RNA	Ribonucleic acid
RTT	Rett syndrome
TSH T3 and T4	Thyroid hormones
Vitamin D	25-Hydroxy vitamin D2 and D3

Keys Facts

Methyl-CPG-binding protein 2 (*MECP2)*	*MECP2* is a gene located at the long arm of the X chromosome, Xq28. Each human nuclear cell has 46 chromosomes (of packed DNA), 44 autosomes, and 2 sex chromosomes, XX (female) or XY (male). Each chromosome consists of a long arm, q and a short arm, p and harbors our gene pool. A gene can undergo transcription and translation into a protein.
Methyl-CPG-binding protein 2 (MeCP2)	MeCP2, the protein product of *MECP2*, has an N-terminal and a C-terminal at each end of the protein and other important domains as the methyl CpG binding domain (allowing the protein to bind to DNA), the transcription repression domain (regulates gene transcription), and the nuclear localization signal area (allows transport of MeCP2 to the nucleus). MeCP2 is abundantly expressed in all cells but has a critical involvement in the maturing, maintenance, and function of CNS neurons. Mutations in the *MECP2* render an unstable MeCp2 protein as evidenced by low brain weight, small neurons, and decreased formation of neuron dendrites and synapses (communication points between neurons) in most parts of the brain.
Mutations of the *MECP2*	Today, more than 1000 different *MECP2* mutations have been discovered (RettBASE: IRSF MECP2 Variation Database, http://mecp2.chw.edu.au). They comprise for the most part of single base pair (the DNA building blocks) substitutions (65–70%), C-terminal deletions (10%), and complex rearrangements within the *MECP2* gene (6%).
X inactivation	X inactivation in females is a mechanism of dosage compensation that equalizes the expression of X-linked genes between the two sexes. Early in the

	female embryogenesis one of the two X chromosomes becomes inactivated in every somatic cell, leaving the descendent cells to have the same X chromosome inactivated. Normally, the two different cell populations of activated X chromosomes are equally presented, and this seems overall to be the case in Rett syndrome as well; that is, both the X chromosome with the mutated *MECP2* gene and the X chromosome with the nonmutated *MECP2* gene is equally expressed in the cells.
Dual energy X-ray absorptiometry (DXA)	DXA is a widely applied method for assessment of bone mass guided by international established standards in adults and to a less degree in adolescents and children. An X-ray source produces two beams with different energy levels, which pass through soft tissue and bone with different attenuation. A detector measures the transmitted X-rays and by imaging software a density based gray tone image is produced, by which measurements of bone area (cm), bone mineral content (g), and bone mineral density (g/cm^2) can be calculated. Thus, DXA do not provide a true volumetric density and report relative lower areal BMD for smaller bones and relative higher areal BMD for larger bones, which is important to notice when interpreting bone mass measures in growth retarded patients with Rett syndrome.
Fractures in Rett syndrome	Low-energy fractures (e.g., spontaneous fractures, no known trauma, or falls within one's height) are frequently occurring in Rett syndrome in the range of 12–40% of RTT populations. Patients with Rett syndrome sustain fractures from early childhood. Low-energy fractures are significantly associated with reduced mobility and lack of ambulation.

Clinical appearance and behavior in Rett syndrome	Girls with Rett syndrome as a group are without dysmorphic syndrome features. In the first years of life, they seem rather passive in their motor achievements, placid, and easy to take care of. Later in infancy, during the regression period they may appear autistic in their behavior with screaming tantrums. Later in childhood, most girls with Rett syndrome are sociable, appear at ease, adapt within frames of a structured everyday life, and are very good at giving strong intense eye contact.
Mobility in Rett syndrome	Gait impairment is part of the diagnostic criteria for Rett syndrome but varies greatly among girls with Rett syndrome. In general, girls with Rett syndrome have difficulties in initiating movements and require patient supportive helpers in order to perform motor tasks. Overall, the major part of girls with Rett syndrome are able to sit independently and nearly half of the population of Rett syndrome girls are able to stand and walk independently. Motor achievement declines with age, if every day motor activities are not sufficiently performed.
Puberty	Sexual development has traditionally been estimated by Tanner stages 1–5 regarding breast development and pubic hair in young females, as described by JM Tanner 1962. Breast development at stage 2 defines the beginning of puberty.

Definition of Words and Terms

Biochemical bone markers	A group of molecules formed in the process of bone formation, degradation, and turnover, which can be measured in blood or urine.
Bisphosphonate	A group of medical drugs consisting of carbon-substituted analogs of pyrophosphate that are potent inhibitors of osteoclast-mediated bone resorption.

Bone formation	The creation of bone.
Bone resorption	The degradation of bone.
Cohort study	The study of an outcome or disease-free study population identified by the exposure or event of interest and followed in time until the disease or outcome of interest occurs.
Cortical bone	The compact bone forming the outer shell of bone.
Cross-sectional study	An observational study of data collected from a population at one specific point in time. The analysis of data can, for instance, compare different population groups at a single point in time.
DNA	Deoxyribonucleic acid is a double strand molecule that carries most of the genetic information used in the development, functioning, and reproduction of all cells and is the main constituent of the chromosomes in the nucleus of the cells.
Gene	A part of the DNA molecule which make up the basic hereditary unit. The genes control the traits seen in off springs.
Gene mutation	A change in the DNA sequence of a gene, by which the sequence differs from what is normally seen.
Histomorphometry	The quantitative study of the microscopic organization and structure of a tissue (as bone).
Knockout mouse model	A knockout mouse is a genetically modified mouse in which researchers have inactivated, or "knocked out," an existing gene by replacing it or disrupting it with an artificial piece of DNA.
MECP2 null mice	A genetically modified mice with no Mecp2 gene product (mRNA or protein) detected in tissues.
Osteoblast	The bone forming cell.
Osteoclast	The bone degrading cell.
Phenotype	The set of observable characteristics of an individual resulting from the interaction of its genes with the environment.
Postnatal	After birth.
Scoliosis	Sideways curvature of the spine.
Syndrome	A combination of signs and symptoms that appear together and characterize a disease or a medical condition.
Trabecular bone	The cancellous woven bone forming the interior scaffold of bone.
Transcription	The process by which the information in a strand of DNA is copied into a new molecule of messenger RNA (mRNA), which is essential for the translation of genetic information into the working proteins.

Introduction

Rett syndrome (RTT) is a severe neurodevelopmental disease affecting primarily girls. It was first recognized by Dr. A. Rett when he saw two girls with wringing hand movements sitting next to each other in his pediatric clinic in Vienna. The first clinical descriptions of girls with RTT were published in German in 1966 (Rett 1966) and later, in 1983, more clinical cases were described in English by Dr. B. Hagberg (Hagberg et al. 1983), at which point the disorder became known worldwide.

RTT is diagnosed according to internationally accepted clinical criteria (Neul et al. 2010).

In more than 90% of the population of RTT girls with classic symptoms, a de novo mutation of the gene methyl-CPG-binding protein 2 (*MECP2)* can be shown (Amir et al. 1999; Percy et al. 2007, 2010). *MECP2* is located at the X chromosome, Xq28, and undergoes X chromosome inactivation in all cells in a random manner (Amir et al. 2000; Chahrour and Zoghbi 2007).

The product of the *MECP2* gene, the protein methyl-CPG-binding protein 2 (MeCP2), is ubiquitously found throughout the body and is involved in transcriptional silencing of target genes, but the knowledge of important target genes is still sparse (Chahrour and Zoghbi 2007). In the central nervous system the MeCP2 is involved in maturing, maintenance, and function of the neurons in the brain (Chahrour and Zoghbi 2007).

It is estimated that RTT will occur in 1:10,000–20,000 live female births worldwide (Percy et al. 2007).

The clinical picture of RTT can present in a typical form, Table 1, or in an atypical form, Table 2, giving a broad spectrum of symptoms including mental retardation, diminished motor skills and locomotion, epileptic seizures, breathing abnormalities and other autonomic dysfunctions, movement disorders, and stereotyped hand movements (Hagberg 2002; Julu et al. 1997). The hallmarks of neurological progression in RTT are described in Table 3. However, in spite of a strong neurological phenotype, RTT is a multisystem disorder and recently focus has been oriented towards bone health, bone mass, and fracture occurrence in RTT.

Most patients with RTT are growth retarded (Schultz et al. 1993; Tarquinio et al. 2012), have low bone mass (Jefferson et al. 2011; Motil et al. 2008; Roende et al. 2011a), and an increased risk of low-energy fractures (Downs et al. 2008; Roende et al. 2011b). The etiology of affected bone health in RTT has not been fully described, but studies of bone metabolism in patients with RTT (Motil et al. 2014; Roende et al. 2014) has provided a greater knowledge of bone development in girls with RTT.

Bone Development in Patients with RTT

Several studies have described low bone mass in patients with RTT, primarily by methods using dual energy X-ray absorptiometry (Cepollaro et al. 2001; Haas et al. 1997; Jefferson et al. 2011, 2015; Motil et al. 2006, 2008; Roende

Table 1 Revised diagnostic criteria for typical RTT (Neul et al. 2010)

Consider diagnosis when postnatal deceleration of head growth	
Required symptoms	1. A period of regression followed by recovery or stabilization
	2. All main criteria and all exclusion criteria
	3. Supportive criteria are not required but often present (Table 2)
Main criteria	1. Partial or complete loss of acquired purposeful hand skills
	2. Partial or complete loss of acquired spoken language (babbling)
	3. Gait abnormalities: Impaired (dyspractic) or absence of ability
	4. Stereotypic hand movements such as wringing/squeezing, clapping/tapping, mouthing, washing/rubbing automatisms
Exclusion criteria	1. Brain injury secondary to trauma (peri- or postnatal), neurometabolic disease, or severe infection that causes neurological disease
	2. Grossly abnormal psychomotor development in the first 6 months

Table 2 Revised diagnostic criteria for atypical RTT (Neul et al. 2010)

Consider diagnosis when postnatal deceleration of head growth	
Required symptoms	1. A period of regression followed by recovery or stabilization
	2. At least 2–4 main criteria
	3. 5 out of 11 supportive criteria
Main criteria	1. Partial or complete loss of acquired purposeful hand skills
	2. Partial or complete loss of acquired spoken language (babbling)
	3. Gait abnormalities: Impaired (dyspractic) or absence of ability
	4. Stereotypic hand movements such as wringing/squeezing, clapping/tapping, mouthing, washing/rubbing automatisms
Supportive criteria	1. Breathing disturbances when awake
	2. Bruxism when awake
	3. Impaired sleep pattern
	4. Abnormal muscle tone
	5. Peripheral vasomotor disturbances
	6. Scoliosis/kyphosis
	7. Growth retardation
	8. Small cold hands and feet
	9. Inappropriate laughing/screaming spells
	10. Diminished response to pain
	11. Intense eye communication – “eye pointing”

et al. 2011a; Shapiro et al. 2010) or ultrasound (Cepollaro et al. 2001; Gonnelli et al. 2008; Zysman et al. 2006). Reduced bone mass has been found in various parts of human RTT bones, eg., by total body measurements (Haas et al. 1997; Jefferson et al. 2011; Motil et al. 2006, 2008), of the lumbar spine and femoral neck (Haas et al. 1997; Jefferson et al. 2011, 2015; Roende et al. 2011a; Shapiro et al. 2010), of the radius, tibia, calcaneus, and proximal phalanges (Cepollaro et al. 2001; Gonnelli et al. 2008; Zysman et al. 2006).

Table 3 Hallmarks of the progression of symptoms in typical RTT (Hagberg 2002)

Stage	Symptom development
1: Early onset stagnation	A normal prenatal and perinatal history. From 5 to 6 till 18 months of age there is a delay in development, especially in terms of postural delay (ability to sit and stand unsupported and rise to standing)
2: Developmental regression	From 1 till 3–4 years of age there is loss of acquired skills as to fine motor control, babbling/pronouncing words, and active playing. RTT girls withdraw from social communication, but often eye contact is preserved. Mental deficiency is evident. Often irregular breathing patterns emerge and stereotypical hand movements slowly evolve. This period has a variable length from weeks till months
3: Pseudostationary period	There is a stabilization of social and communicative abilities. Intense eye gazing is characteristic. Fine motor and language skills are often still reduced according to preregression skills, but ambulatory ability is apparently preserved/not worsened. There is prominent hand apraxia/dyspraxia and stereotypical hand movements dominate. Often increasing muscular tone develops. Epileptic seizures may develop. Scoliosis often progress. Signs of low bone mass may be evident with increased fracture occurrence
Stage 4 of late motor deterioration is now believed to reflect nonuse of motor abilities and is no longer considered a true hallmark of RTT	

Apart from low bone mass patients with RTT also have smaller bone size with reduced body height, lumbar height and width, in the order of 10% (Roende et al. 2011a).

It seems as bone development is affected from early age in RTT patients (Jefferson et al. 2015; Motil et al. 2014; Roende et al. 2014). Studies in both humans and mice have broadened our knowledge on the pathology of RTT bones. A study of histomorphometry on bone biopsies in girls with RTT has shown reduced cancellous bone volume and low bone formation rate (Budden and Gunness 2003). In support of an abnormal bone development in RTT patients, studies on *MECP2* null mice have reported a bone phenotype of growth plate abnormalities, reduced cortical and trabecular bone volume (O'Connor et al. 2009), and reduced bone strength (Kamal et al. 2015) as well as reduced osteoblast number, unaffected osteoclast number, and osteoblast dysmorphology and dysfunctions (Blue et al. 2015).

Overview of Biochemical Bone Marker Studies in RTT

Application of biochemical bone marker analyses has provided a mean of characterizing bone metabolism in RTT patients, as bone biopsies are difficult to obtain both in clinical practice and in clinical research settings.

Overall, bone metabolism can be characterized by biochemical markers of bone formation, resorption, and turnover (Jürimäe 2010; Szulc et al. 2000; Tables 4 and 5). The majority of studies in RTT patients have described only markers of bone

Table 4 Bone formation and turnover markers studied in RTT patients

Name	Function
N-terminal pro-peptides of collagen type 1 (P1NP)[a]	Collagen constitutes the major part (90 %) of structural protein of the bone matrix. P1NP is cleaved from procollagen from the amino-terminal during synthesis of collagen type 1 and thus released into the extracellular matrix. A part of P1NP is incorporated into the matrix and is released upon bone resorption, but the major part of P1NP is released into the circulation and thus reflects bone formation
Type 1 procollagen carboxyterminal propeptide (PICP)[a]	During the extracellular generation of type 1 collagen also the carboxy-terminal peptides of procollagen are cleaved, whereby PICP – a soluble protein – is removed into circulation, thus considered to be a marker of bone formation. However, PICP is considered a less precise bone marker compared to P1NP
Osteocalcin (OC)[b]	OC is synthesized by mature osteoblasts and represents the largest part of non-collagen protein in bone matrix. Yet, the exact physiological function of OC is unknown, but it is partly incorporated into the matrix (50 %) and partly released into circulation (50 %). OC binds to hydroxyapatite and thus is thought to be involved in bone resorption, by which it is released into circulation. However, circulating OC is also regarded as a specific marker of bone formation as it is being increasingly synthesized with increasing mineralization and by the more mature osteoblast during osteoblastogenesis. Therefore, OC can be considered a marker of bone turnover
Bone-specific alkaline phosphatase (B-ALP)[c]	B-ALP is produced by osteoblasts as a tetramer, initially bound to the outer surface membrane. It is subsequently enzymatically released into circulation as a soluble and as an insoluble form. Although the precise physiological function of B-ALP is not known, it is believed to be involved in the mineralization process and bone formation by catalyzing the process of release of phosphate, which is essential for bone mineralization

References
[a]Brandt et al. (2001)
[b]Gundberg (2001)
[c]Demers (2001)

Table 5 Bone resorption marker studied in RTT patients (Leary 2001)

Name	Function
C-terminal telopeptide crosslinks (CTX)	CTX are degrading products of mature type 1 collagen and is regarded as a specific marker of bone resorption. It is released into the circulation upon osteoclast activity with secretion of acid and hydrolytic enzymes performing degradation of bone into molecular fragments

formation (Cepollaro et al. 2001; Gonnelli et al. 2008; Hofstaetter et al. 2010; Motil et al. 2006).

Cepollaro et al. analyzed total alkaline phosphatase, bone-specific alkaline phosphatase, type 1 procollagen carboxyterminal propeptide, and osteocalcin in 82 RTT patients 2–21 years old and 82 age-matched controls. They did not find significant differences between the groups (Cepollaro et al. 2001).

Gonelli et al. analyzed bone-specific alkaline phosphatase in 109 RTT patients 3–25 years old and 101 age-matched controls but found no significant group difference (Gonnelli et al. 2008).

Motil et al. also analyzed bone-specific alkaline phosphatase and osteocalcin in 10 patients with RTT and 10 healthy age- and pubertal-matched controls aged 2.5–13 years without showing significant differences between the groups (Motil et al. 2006).

Hofstaetter et al. reported a 5.3-fold increase in osteocalcin, but bone-specific alkaline phosphatase was within reference range in one 17-years-old female with typical RTT (Hofstaetter et al. 2010).

These insignificant results may be explained by few enrolled patients or crude whole-group analyses, as it is important to adjust for age specific and pubertal related changes in biochemical bone markers (Szulc et al. 2000). It is well known in healthy children that biochemical bone markers decrease by age, increase at (early) puberty, and decline after puberty (Eastell 2005; Jürimäe 2010; Rauchenzauner et al. 2007; Tuchman et al. 2008; van Coeverden et al. 2002; Walsh et al. 2009).

More recent studies have addressed both biochemical markers of bone formation, resorption, mineralization, and turnover in large groups of RTT patients including children, young people, and adults. These studies have reported significant differences between RTT patient and age-matched references or age- and pubertal-matched controls (Motil et al. 2014; Roende et al. 2014).

Motil et al. analyzed bone metabolism in 50 girls with RTT and compared levels of osteocalcin, bone-specific alkaline phosphatase, and C-telopeptide with age-matched references. They found significant lower levels of osteocalcin and for most patients significant higher levels of bone-specific alkaline phosphatase in different age groups of RTT patients aged 1–38 years. They did not find significant altered levels of C-telopeptide in RTT patients compared to reference levels (Motil et al. 2014).

Roende et al. studied 61 girls and women with RTT and 122 well-matched controls and found significant decreased levels of osteocalcin, N-terminal propeptides of collagen type 1, and C-terminal telopeptide crosslinks in children and young girls with RTT, whereas women with RTT had biochemical bone marker values at the same level as controls. Bone-specific alkaline phosphatase was reduced in children and young girls, but not significantly, and in adults, bone-specific alkaline phosphatase was higher in RTT women compared to controls (Roende et al. 2014).

As in healthy children, biochemical bone markers in children and young girls with RTT decrease by age, Fig. 1a–d (Roende et al. 2014). Whether there is also a small peak of bone marker levels in puberty during the growth spurt in a RTT population remains to be studied prospectively in a large cohort of pubertal RTT

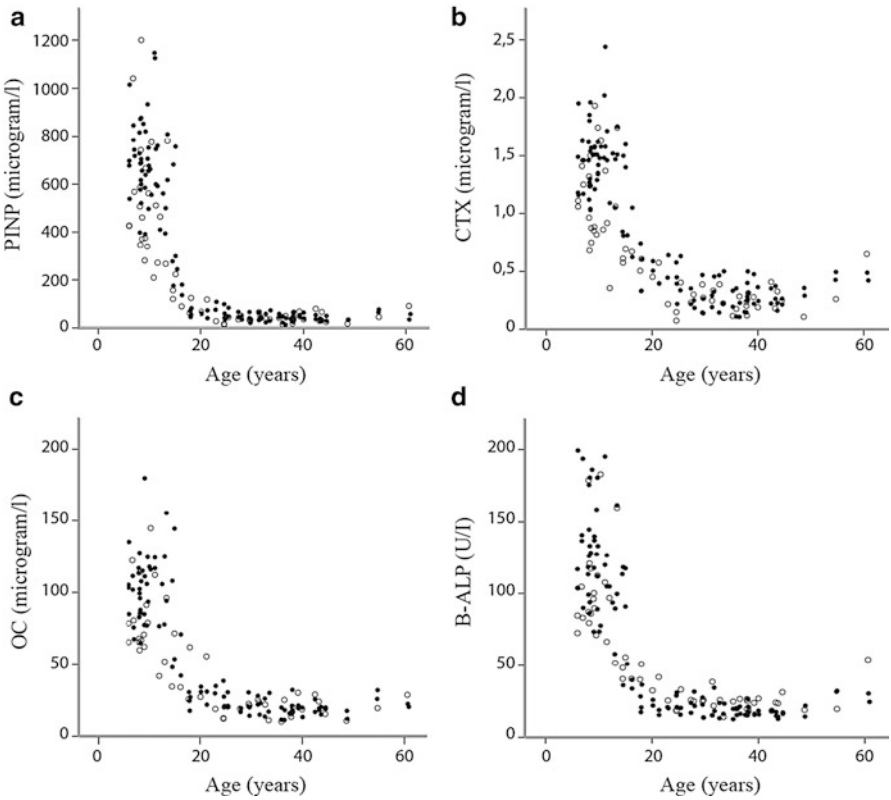

Fig. 1 (**a**–**d**) Shows scatter plots of biochemical bone markers related to age of $n_{\text{patients}}|n_{\text{controls}}$, *white fill* = patients, *black fill* = controls. **a**: P1NP (μg/l) 59|118, **b**: CTX (μg/l) 59|118, **c**: OC (μg/l) 51|102 and **d**: B-ALP (U/l) 57|114 (Reference: Roende G, Petersen J, Ravn K et al. Low bone turnover phenotype in Rett syndrome: results of biochemical bone marker analysis. Pediatr Res. 2014;75(4):551–558. Reprinted with permission)

girls. Because of cross-sectional designs recent studies have not been able to address this question. Adult women with RTT seem to have levels of biochemical bone markers reflecting values in healthy women, and therefore do not seem to be at increased risk of osteoporosis due to increased bone resorption (Roende et al. 2014).

Associations of Biochemical Bone Markers and Other Clinical Parameters of Bone Health in RTT

Biochemical bone formation markers and bone resorption markers are positively associated in patients with RTT (Motil et al. 2014; Roende et al. 2014), as expected, as RTT patients form and develop bones that resemble healthy bones. Therefore, the

overall model of dependent dual actions of osteoblasts and osteoclasts seem to apply for RTT patients as well. The challenge is to explain how and why bone development deviates in RTT patients.

Even though patients with RTT have low bone mass, the biochemical bone markers are not associated with bone mass in the RTT populations (Motil et al. 2014; Roende et al. 2014). This lack of association has also been shown in healthy children (van der Sluis et al. 2002). This may in part be explained by the more dynamic changes of bone marker levels as opposed to the more slow changes in bone mass evaluated by DXA during bone development in children.

In general, patients with RTT do not seem to have skewed levels of hormones affecting bone health, eg., thyroid hormones (TSH, T3, and T4) and parathyroid hormone (PTH) (Motil et al. 2014; Roende et al. 2014) and growth hormone (GH) (Huppke et al. 2001). However, low levels of Vitamin D (25-Hydroxy vitamin D2 and D3) is often measured in patients with RTT, probably due to low diet intake and reduced sun exposure (Motil et al. 2011), but associations to biochemical bone marker levels have not been shown (Roende et al. 2014). Nor have associations between inflammatory markers and biochemical bone markers been shown (Motil et al. 2014).

Furthermore, biochemical bone markers have not been associated to *MECP2* mutation groups, walking ability, or previous low-energy fractures (Roende et al. 2014).

In patients with cerebral palsy and motor deficiencies, no significant differences in biochemical bone marker levels between CP patients and healthy controls have been found (Chen et al. 2011). This supports the hypothesis of an overall genetic influence by *MECP2* rather than compromised mobility in patients with RTT as an important factor in explaining deviating bone development in RTT patients.

Interpretation of Results from Biochemical Bone Marker Studies in RTT

Studies of biochemical bone markers are strongly dependent on timely sampling as diurnal variations occur (Szulc et al. 2000). Furthermore, bone marker levels depend on sex, age, pubertal stage, ethnicity, and nutritional status, which should be accounted for when analyzing biochemical bone markers (Szulc et al. 2000).

As patients with RTT are found worldwide (Percy et al. 2007), are almost only girls/women (Percy et al. 2007), can be aged up to 60 years (Roende et al. 2011b), show pubertal development (Killian et al. 2014; Knight et al. 2013), are often growth retarded (Schultz et al. 1993; Tarquinio et al. 2012) and small in stature (Roende et al. 2011a), and may have affected nutritional status (Motil et al. 2012), several pitfalls can occur. Therefore, strict protocols with proper references are needed when measuring and analyzing biochemical bone markers in RTT patients.

Biochemical bone markers can be interpreted as a surrogate of bone metabolism of the whole body, which is an advantage when studying bone metabolism in children where multiple actions of bone modeling occur (longitudinal growth and change in size and shape) (Jürimäe 2010). However, studies of detailed actions of osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells) as provided by bone biopsies cannot be studied by levels of biochemical bone markers in blood (Jürimäe 2010).

Even though RTT patients often develop some degree of scoliosis, only few undergo surgery (Anderson et al. 2014) where bone biopsies could be harvested on permission. Only one histomorphometric study of iliac crest bone biopsies in a few patients with RTT has been published. However, results from this study showed low bone volume and reduced bone formation rate (Budden and Gunness 2003) in accordance with the results of the biochemical bone marker studies in RTT (Motil et al. 2014; Roende et al. 2014).

Taken together, studies of RTT patients have shown a low bone metabolism in childhood from early age compared to healthy controls, and the pattern of bone turnover seem to be that of a low rate of bone formation in the context of normal/reduced bone resorption (Motil et al. 2014; Roende et al. 2014). As RTT patients turn older, the bone turnover becomes more normalized with levels of bone formation and resorption markers resembling reference/control values (Roende et al. 2014).

Furthermore, biochemical bone markers in part reflect longitudinal bone growth, as studies in healthy children have reported associations between biochemical bone marker levels and growth velocity (height velocity), although less pronounced for girls than for boys (Szulc et al. 2000; Tuchman et al. 2008).

Because of the cross-sectional design of recent studies the association of growth velocity and biochemical bone marker levels in young RTT patients have not been elucidated (Motil et al. 2014; Roende et al. 2014).

Currently, known biochemical bone markers are not specific markers for either bone mineralization or longitudinal bone growth (Jürimäe 2010).

In RTT patients, dual explanations of deviating bone development may be possible. That is, biochemical bone marker levels reflect an overall low bone formation potential in this patient group with short stature and decreased general growth. Further, the cause of a low bone turnover state in RTT may differ throughout childhood and young adulthood (Roende et al. 2014).

The general picture of a low bone formation phenotype in RTT, evidenced by smaller bones and lower bone mass compared to healthy controls (Roende et al. 2011a), is in accordance with findings of slightly smaller organs, hands, and feet for age and height in RTT (Armstrong et al. 1999; Leonard et al. 1999; Schultz et al. 1993). Possibly, this picture could be explained by an overall influence of *MECP2* on growth and bone formation in particular, but the specific link to osteoblast function and activity and the interplay with osteoclasts has not yet been elucidated (Roende et al. 2014). Strong, specific, consistent risk factors (e.g., reduced mobility level, a diagnosis of epilepsy, treatment with antiepileptic drugs, and low vitamin D levels) of low bone mass have not been found in RTT patients (Roende et al. 2011a). This suggests that these possible risk factors may be

considered as (important) modifiers of bone formation and strength rather than actual causes of a low bone formation in RTT.

Clinical Implications and Potential Applications to Prognosis, Other Diseases, or Conditions

As biochemical bone markers are not being used as routine measurements in the evaluation of bone health in apparently normal children or other pediatric groups with chronic diseases (Jürimäe 2010; Szulc et al. 2000), a routine measurement is not relevant for RTT patients either with our current level of knowledge of bone development in RTT children.

One of the major limiting factors for the clinical use of biochemical bone markers in RTT is the lack of proper reference material and the lack of established indications (Jürimäe 2010; Szulc et al. 2000).

However, recent studies of biochemical bone markers in RTT (Motil et al. 2014; Roende et al. 2014) have rendered important knowledge to assist clinical decisions in the treatment or prevention of low bone mass in RTT. In a balanced low turnover state of bone metabolism, antiresorptive treatment seems less useful by further decreasing activities of the osteoblasts and the osteoclasts. This warrants a critical approach to the use of bisphosphonate treatment of young RTT patients (Motil et al. 2014; Roende et al. 2014).

On the contrary, individual initiatives to strengthen bone health in RTT patients should be enforced, that is, enhancing mobility and daily weight bearing activity (Jefferson et al. 2015; Roende et al. 2011a) and securing a proper diet rich in calories and proteins, with supplementation of vitamin D, calcium, and phosphorous when needed (Motil et al. 2011, 2012).

Future Studies of Biochemical Bone Markers and Bone Metabolism in RTT

Future well-designed prospective longitudinal studies of biochemical bone markers are very much needed to examine the course of bone markers during puberty and to examine the association to growth velocity and bone mineral accrual in the RTT population (Roende et al. 2014).

The roles of biochemical bone markers in the treatment and prevention of fractures in RTT are other important longitudinal studies of interest.

As recent studies of a functional knockout mouse model of RTT have shown potential reversible alterations in cortical bone material and biomechanical properties (by postnatal activation of the *MECP2* gene) (Kamal et al. 2015), and as gene-based therapy studies in RTT mice models are performed (Gadalla et al. 2013; Garg et al. 2013), it is important to continuously study possible bone metabolism activators and regulators in RTT.

Summary Points

- Rett syndrome is a neurodevelopmental disease affecting mostly girls.
- The cause is often a mutation in the gene *methyl-CPG binding protein 2, MECP2,* located at the X chromosome.
- Girls with Rett syndrome are often growth retarded and have low bone mass and an increased risk of low-energy fractures.
- Bone development is deviating from early childhood.
- Studies of biochemical bone markers show reduced bone formation in the context of normal/reduced bone resorption.
- Low bone turnover in Rett syndrome is not associated to bone mass measures, fractures, specific *MECP2* mutations, or a skewed endocrinological status in cross-sectional studies.
- *MECP2* null mice have a similar bone phenotype with low bone formation rate, reduced cortical and trabecular bone volume, and reduced bone strength.
- Bone biopsies from girls with Rett syndrome have shown reduced bone volume and bone formation rate.
- It is a hypothesis that *MECP2* may have an overall influence on growth and bone formation without a known link to osteoblast and osteoclast maturation and functioning at present.
- Treatment initiatives reinforcing bone formation and strength are important in girls with Rett syndrome.

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Use of Bone Biomarkers After Weight Loss: Example of Bariatric Surgery

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Contents

Abstract

The prevalence of obesity has increased around the world, and the frequency of bariatric surgery, one of the therapeutic alternatives for specific cases, has also increased progressively. Despite the beneficial effects of bariatric surgery on various obesity-related comorbidities, the most significant postoperative weight losses have been associated with negative skeletal repercussions. In this context, serum bone health markers could provide early warning to the higher skeletal risk. In this chapter we will discuss the mechanisms involved in the

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relationship between obesity, its treatment with bariatric surgery, and bone metabolism, in addition to how changes in this relationship influence the main bone biomarkers.

Keywords
Obesity • Bariatric surgery • Biological markers • Bone remodeling

List of Abbreviations

ALP	Total alkaline phosphatase
BSAP	Bone-specific alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenetic proteins
BTM	Bone turnover markers
CTX	Collagen-type I C-telopeptide
DAN	Differential screening-selected gene aberrative in neuroblastoma
ICTP	Carboxy-terminal telopeptide of type I collagen
IGF-1	Insulin growth factor 1
LRP5	Low-density lipoprotein receptor-related protein 5
LRP6	Low-density lipoprotein receptor-related protein 6
NIH	National Institutes of Health
NTX	Collagen-type I N-telopeptide
OPG	Osteoprotegerin
PTH	Parathyroid hormone
PICP	Carboxy-terminal procollagen propeptides of collagen type I
PINP	Procollagen type I amino-terminal propeptide
PIIINP	Amino-terminal procollagen propeptides of collagen type III
RANK	Receptor that activates the nuclear factor kappa B
RANKL	RANK ligand
RYGB	Gastroplasty with Roux-en-Y gastric bypass
TPH1	1-Tryptophan hydroxylase
TRAP5b	Tartrate-resistant acid phosphatase isoenzyme 5b
WHO	World Health Organization
1,25OHD	1,25-Hydroxy-vitamin D
25OHD	25-Hydroxy-vitamin D

Key Facts of *Bariatric Surgery*

- Obesity can be associated with a number of diseases, such as diabetes mellitus, dyslipidemia, and high blood pressure.
- Bariatric surgery is a therapeutic option for certain cases of obesity.

- Bariatric surgery has been associated with a reduction in metabolic disorders, cardiovascular events, and mortality.
- Negative repercussions of bariatric surgery on bone have been reported.
- Bone health follow-up is necessary after bariatric surgery.

Definitions of Words and Terms

Adipokines	Proteins produced by adipocytes that have local and systemic activity.
Adipocytes	Cells that are part of the adipose tissue.
Roux-en-Y gastric bypass	Surgery that treats obesity by reducing the volume of the stomach and bypassing the duodenum and proximal jejunum.
Bariatric surgery	Also known as gastroplasty, it helps obese individuals lose weight by reducing the volume of the stomach in addition to other procedures.
Sclerostin	Protein produced by osteocytes that could have a negative impact on bone formation.
Bone turnover markers	Biochemical markers of bone metabolism that reflect bone formation and resorption processes.
Obesity	Excessive accumulation of body fat.
Osteomalacia	Poor bone tissue mineralization.
Osteopenia	Low bone mineral density that precedes osteoporosis.
Osteoporosis	Low bone mineral density associated with high risk of fractures.

Introduction

Obesity is a very prevalent condition with dramatic repercussions to the patients. It is considered a public health problem in many countries. Bariatric surgery is a therapeutic alternative for approaching severe obesity, and its frequency has increased in the last years (Buchwald and Oien 2013). However, the fast weight loss associated with the procedure may have negative bone repercussions (Yu 2014; Liu et al. 2015). In this situation, monitoring the serum levels of bone health markers may allow early detection of changes in bone metabolism, warning about the greater skeletal risk. Hence, it is important to discuss the mechanisms involved in the relationship between obesity, its treatment with bariatric surgery, and bone metabolism, as well as the way in which changes in this relationship influence the main bone biomarkers.

Obesity and Bariatric Surgery

Obesity constitutes a metabolic disease influenced by behavioral, endocrine, and genetic factors (WHO 2000), reflecting increased consumption associated with reduced caloric expenditure, with consequent body fat accumulation (WHO 2000).

The proportions of the disease have grown: between 1980 and 2013, the number of overweight or obese people increased from 857 million to 2.1 billion, with a higher increase between 1992 and 2002 and in 20–40-year-olds (Ng et al. 2014). Currently, about 30% of the world population has excess weight (Ng et al. 2014), and obesity is considered a true pandemic (Swinburn et al. 2011).

Many comorbidities that affect quality of life and life expectancy have been associated with obesity (Mechanick et al. 2012). Thus, global goals have been established to control the increase in the prevalence of the disease until the year 2025 (WHO 2013), monitoring the frequency of excess weight (Gortmaker et al. 2011) and stimulating therapeutic actions.

The multifactorial character of obesity implies a multidisciplinary approach that aims to change behavior and includes incentives for the practice of physical activity, in addition to nutritional, psychological, and pharmacological interventions (Jensen et al. 2014), which enable a loss ranging from 5% to 10% of the total body weight (Ryan et al. 2010). However, many patients do not adhere to this treatment strategy, and many regain the lost weight up to 2 years after the initial approach (Ryan et al. 2010), making conventional therapies tiring and sometimes inefficient. In this context bariatric surgery has been used as a therapeutic option for obese patients whose conventional treatments were not completely satisfying, promoting significant and long-lasting weight loss (Mechanick et al. 2013). Moreover, the procedure has been associated with a reduction in metabolic comorbidities, cardiovascular events, and mortality (Arterburn and Courcoulas 2014).

Bariatric surgery can be classified as restrictive, malabsorptive, or mixed, depending on the anatomical and functional changes they make. The mixed techniques, such as gastroplasty with Roux-en-Y gastric bypass (RYGB), combine gastric volume reduction with some degree of intestinal malabsorption (Capella and Capella 1996), presenting good long-term results with respect to weight loss and maintenance. Additionally, they promote neural and hormonal changes that contribute to satiety and increase the rate of comorbidity remission, improving quality of life (Martins 2005). Therefore, RYGB is currently the most popular bariatric surgery technique (Yu 2014).

Surgery candidates must meet specific indication criteria for the surgery established by the National Institutes of Health (NIH), such as age between 16 and 60 years and body mass index (BMI) higher than 40 kg/m^2, or BMI between 35 and 40 kg/m^2 along with some obesity-related comorbidity. Other factors to consider are failure of conventional methods to promote weight loss or the absence of endocrine-related obesity (NIH 1991; Mechanick et al. 2013).

Despite the benefits of bariatric surgery, there are risks associated with the procedure (Arterburn and Courcoulas 2014), such as the deleterious effects on bone metabolism (Yu 2014).

Metabolism and Bone Markers

Bone tissue consists of a protein matrix, minerals, water, and cells. Despite its inert aspect, it is constantly being remodeled by osteoblasts, cells responsible for bone formation and mineralization, and osteoclasts, cells responsible for bone resorption (Novack and Teitelbaum 2008). Bone formation and resorption are kept in strict balance. The process of remodeling is orchestrated by osteoblasts and regulated by many hormones and cytokines (Molina 2007).

The importance of controlling the remodeling process performed by cells of the osteoblastic line encourages the investigation of the pertinent signaling pathways. In this context, the relevance of the signaling cascade *Wnt/β*-catenin stands out. This cascade has a role in bone homeostasis by differentiating these cells (Nusse 2005) and regulating the transcription of important proteins for its function, known as bone morphogenetic proteins (BMP) (Zhang et al. 2013). Receptors present in the cell membrane of osteoblast precursors [LRP5 and LRP6 (low-density lipoprotein receptor-related protein)] are activated when they bind to the *Wnt* protein, stabilizing *β*-catenin and regulating the gene transcription that promotes bone formation (Fig. 1) (Nusse 2005; Lewiecki 2011).

It is further speculated that bone formation can occur independently from the Wnt/*β*-catenin pathway, with serotonin playing an important role in this process. The LRP5 receptor appears to inhibit the expression of 1-tryptophan hydroxylase (TPH1), biosynthetic enzyme that limits the speed in which serotonin is expressed

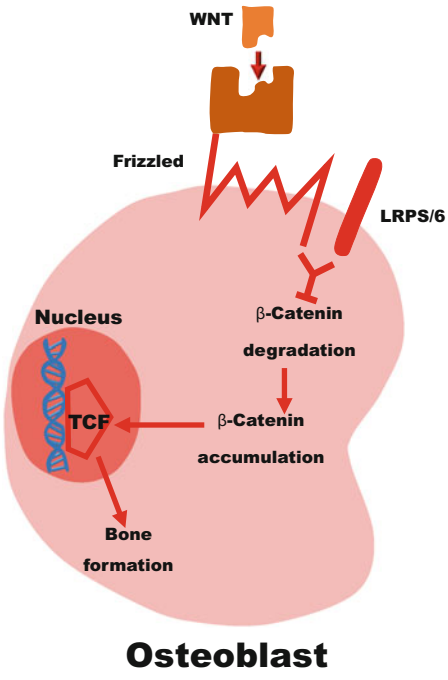

Fig. 1 Representative scheme of the signaling pathway *Wnt/β*-catenin (Sources: Nusse 2005; ten Dijke et al. 2008; Lewiecki 2011)

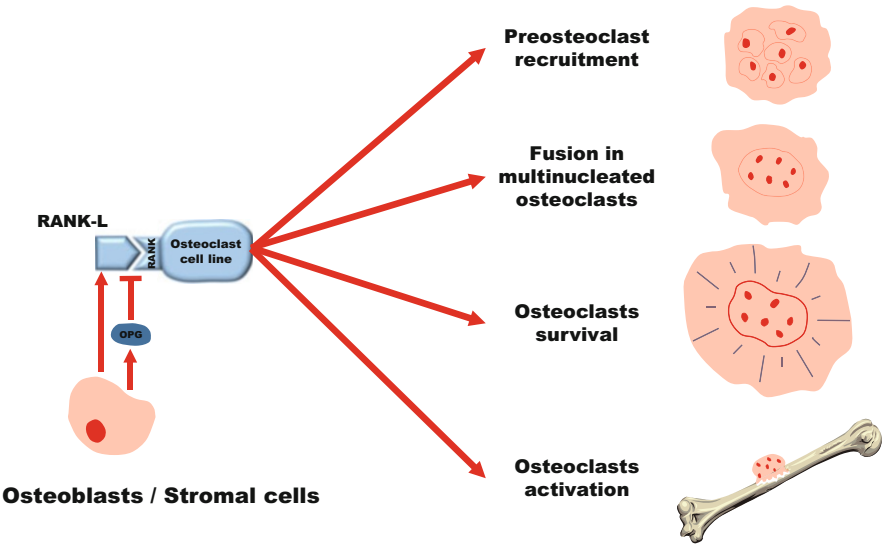

Fig. 2 Representative scheme of the regulation axis RANKL/RANK/OPG (Source: Kearns et al. 2008)

in duodenal enterochromaffin cells. Thus, a reduction in the blood serotonin levels of LRP5-deficient rats regulates bone metabolism, reducing bone formation (Yadav et al. 2008).

Remodeling is still intrinsically related to the regulation axis known as RANKL/RANK/OPG (Fig. 2). Functioning of this axis is based on the expression of the receptor that activates the nuclear factor kappa B (RANK) in osteoclast cell membrane and on the production of RANK (RANKL) ligand and osteoprotegerin (OPG) by osteoblasts. In vivo, the main RANKL function is the regulation of bone resorption and calcium metabolism by osteoclastogenesis and the stimulation of mature osteoclast activity (Kearns et al. 2008; Li et al. 2000). Activation of the RANKL/RANK system induces a cascade that stimulates osteoclast differentiation (Ferrer Cañabate et al. 2002) from monocytes and macrophages, possibly stemming from myeloid precursors (bone marrow – hematopoietic series) (Bonewald and Johnson 2008).

Osteoblasts also synthesize OPG, a soluble protein that acts as a competitive receptor that binds to RANKL; prevents its binding to RANK; blocks osteoclast genesis, maturation, and action; and controls bone loss (Bezerra et al. 2005). High levels of RANKL promote osteoclastogenesis, while high expression of OPG inhibits this process or even promotes osteoclast apoptosis.

Parathyroid hormone (PTH) and calcitonin are among the main hormones involved in bone remodeling. The main physiological role of PTH is to maintain plasma calcium homeostasis. It increases osteoclastic bone degradation, releasing calcium from the bone matrix (Molina 2007). This hormone also acts on the kidneys,

Table 1 Principal hormones specifically related to bone health

Hormonal factors
25-Hydroxy-vitamin D (25OHD)
Parathyroid hormone (PTH)
Insulin growth factor 1 (IGF-1)
Calcitonin

Sources: Molina 2007; Bonjour et al. 2014

increasing calcium resorption and inorganic phosphate excretion; in the intestines, it indirectly increases the absorption of dietary calcium by activating vitamin D (Molina 2007). In the kidneys PTH stimulates the conversion of 25-hydroxy-vitamin D (25OHD) to its active form, 1,25-hydroxy-vitamin D (1,25OHD), which stimulates calcium absorption by enterocytes and resorption in distal renal tubules and inhibits the transcription and secretion of PTH (Molina 2007). However, calcitonin action is opposite that of PTH, inhibiting bone resorption by directly acting on osteoclast mobility, differentiation, and function and increasing urinary calcium excretion by inhibiting calcium resorption by the renal tubules (Molina 2007).

Unbalanced bone remodeling may result in many metabolic bone diseases, such as osteopenia and osteoporosis. Both are diagnosed by densitometry, which measures bone mineral density (BMD). This test is reliable and very popular, but it is only useful when the disease is already present. On the other hand, the measurement of serum markers related to bone health could provide earlier detection of bone metabolism changes, allowing preventive interventions. These markers could be divided into: (I) specific bone-related hormonal factors and (II) bone turnover markers (BTM) (Bonjour et al. 2014). The former (Table 1) reflect the nutritional supply of calcium, vitamin D, and proteins (Bonjour et al. 2014). BTM reflect bone cell activity and can be divided into (i) bone formation markers, (ii) bone resorption markers, and (iii) proteins that regulate osteoclast activity (Table 2) (Sousa et al. 2015).

Obesity and Bone Metabolism

Bone and adipose tissues seem to have a strict but yet unclear relationship. Although excess weight is related to a number of comorbidities, obesity has been considered a protective factor against the loss of BMD and fractures (Kirchengast et al. 2001; Knoke and Barrett-Connor 2003), except in older adults and menopausal women (Compston et al. 2011; Armstrong et al. 2012). Indeed, positive correlations between BMI and BMD in the lumbar spine and hips have been reported (Papapietro et al. 2013).

The high percentage of body fat may increase the aromatization of androgens into estrogens, benefiting bone mass (Trémollières et al. 1993; Ricci et al. 2001). Furthermore, higher amounts of adipose tissue are related to high insulin levels or high insulin growth factor 1 (IGF-1) action, together with direct activation of bone formation (Reid 2006). In addition to these, other hormones and cytokines produced by adipocytes could affect bone metabolism (Yamauchi et al. 2001; Reid 2006; Cao 2011; Aguirre et al. 2014).

Table 2 Principal bone turnover markers

Bone formation markers	Bone resorption markers	Regulatory proteins of osteoclastic activity
Alkaline phosphatase	CTX	Osteoprotegerin
Bone alkaline phosphatase	NTX	RANKL
Osteocalcin	ICTP	BMP
PINP	Hydroxyproline	
PICP	Pyridinoline	
PIIINP	Deoxypyridinoline	
	TRAP5b	

BMP bone morphogenetic protein, *CTX* cross-linked C-terminal telopeptides of type I collagen, *ICTP* carboxy-terminal telopeptide of type I collagen, *NTX* N-terminal telopeptide of type I collagen, *PICP* carboxy-terminal procollagen propeptides of collagen type I, *PINP* amino-terminal procollagen propeptides of collagen type I, *PIIINP* amino-terminal procollagen propeptides of collagen type III, *RANKL* receptor activator of nuclear factor NF-κB ligand, *TRAP5b* tartrate-resistant acid phosphatase isoenzyme 5b (Source: Sousa et al. 2015)

Bone Metabolism and Markers after Bariatric Surgery

Despite the improvement on body weight and the metabolic syndrome, and the reduction of mortality promoted by bariatric surgery (Liu et al. 2015), evidence suggests that this procedure causes significant bone mass loss (Rodríguez-Carmona et al. 2014), which can persist for years (Yu 2014). In fact, bariatric surgery has been associated with BMD reduction and high risk of fractures (Viégas et al. 2010). Papapietro et al. (2013) reported BMD reduction in 44.7% of their patients 34 months after the surgery. Low BMD seems to be related to intense weight loss (Fleischer et al. 2008) and, specifically, to less fat-free mass (Tsiftsis et al. 2009).

Although measuring BMD may be useful to quantify bone mass after bariatric surgery, detection of bone involvement by this diagnostic method is usually late. In this sense, detection of changes in serum biomarkers related to bone metabolism (Table 3) could be used as early indication of bone involvement.

High BTM levels have been found after bariatric surgery (Hage and El-Hajj Fuleihan 2014). In a prospective study with 73 individuals submitted to RYGB, a significant increase in the levels of the bone resorption marker collagen-type I N-telopeptide (NTX) 3 and 18 months after surgery evidenced high remodeling (Bruno et al. 2010). This finding was corroborated by Monteiro et al. (2009) and Tsiftsis et al. (2009), who also found high serum levels of another bone resorption marker, the collagen-type I C-telopeptide (CTX), 1 year after biliopancreatic diversion (Monteiro et al. 2009; Tsiftsis et al. 2009). Yu et al. (2014) also reported a CTX increase in excess of 200% associated with low BMD in women 6 months after RYGB (Yu et al. 2014). Similarly, Elias et al. (2014) reported high CTX and low BMD 18 months after RYGB (Elias et al. 2014). Muschitz et al. (2015) found high CTX levels 1 month after RYGB, which continued high 2 years after the surgery

Table 3 Principal serum biomarkers related to bone metabolism, which can change significantly after bariatric surgery

Bone formation markers	Bone resorption markers	Other factors (hormonal/biochemical)
Alkaline phosphatase	CTX	Adiponectin
Bone alkaline phosphatase	NTX	Calcium
Osteocalcin	Pyridinoline	Sclerostin
PINP	Deoxypyridinoline	Ghrelin
		IGF-1
		Leptin
		Parathyroid hormone
		25-Hydroxy-vitamin D

CTX cross-linked C-terminal telopeptides of type I collagen, *IGF-1* insulin growth factor 1, *NTX* N-terminal telopeptide of type I collagen, *PINP* amino-terminal procollagen propeptides of collagen type I (Sources: Hage and El-Hajj Fuleihan 2014; Muschitz et al. 2015)

(Muschitz et al. 2015). In fact, bariatric surgery is associated with high CTX and NTX, a negative bone remodeling balance (Viégas et al. 2010).

In addition to the resorption markers, bone formation markers also seem to change after these surgeries. Giusti et al. (2005) studied premenopausal women submitted to restrictive bariatric surgery and found a significant reduction in the serum levels of total alkaline phosphatase (ALP) 2 years after surgery (Giusti et al. 2005). In contrast, Tsiftsis et al. (2009) studied women of childbearing age submitted to biliopancreatic diversion and found a significant increase in total ALP 12 months after surgery (Tsiftsis et al. 2009). Likewise, Santos et al. (2012) found higher serum levels of total ALP in women submitted to RYGB than in controls (Santos et al. 2012). On the other hand, Vasconcelos et al. (2010) and Casagrande et al. (2012) assessed women submitted to RYGB and found no difference in total serum ALP 12 months after surgery (Vasconcelos et al. 2010; Casagrande et al. 2012).

Currently, total serum ALP has been replaced by its bone fraction, bone-specific alkaline phosphatase (BSAP), a more specific marker of bone formation that suggests bone anabolism when elevated (Miyazaki et al. 2004). Bruno et al. (2010) found high BSAP 6 and 18 months after RYGB (Bruno et al. 2010). In an earlier study, our group did not find a significant change in the level of this marker as late as 6 months after RYGB (Biagioni et al. 2014).

Osteocalcin, another bone formation marker, also seems to increase after bariatric surgery. Tsiftsis et al. (2009) reported that women of childbearing age submitted to biliopancreatic diversion experienced a significant osteocalcin increase 12 months after surgery (Tsiftsis et al. 2009). Likewise, Bruno et al. (2010) found high osteocalcin levels 6 and 18 months after RYGB (Bruno et al. 2010).

Another bone formation marker, procollagen type 1 amino-terminal propeptide (P1NP), reached peak levels 12 months after surgery in 52 premenopausal women (Muschitz et al. 2015).

Many reasons could explain the effect of bariatric surgery on bone. Apparently, the occurrence and/or intensity of these changes depend on the type of surgery

(Yu 2014). Bone loss did not occur in women who underwent vertical banded gastroplasty, suggesting that malabsorptive techniques may be more strongly related to bone loss (Elias et al. 2014). In this sense, relative malabsorption of dietary nutrients and negative energy balance could change the levels of certain hormones, thereby having an etiological role in bone loss.

Giusti et al. (2005) found low serum calcium levels in women of childbearing age 2 years after restrictive surgery (Giusti et al. 2005), corroborating Bruno et al. (2010), who also found low calcium levels in men and women 18 months after RYGB (Bruno et al. 2010). This reduction in serum calcium level could stimulate PTH secretion, mobilizing bone calcium with consequent bone demineralization (Mason 2000; Molina 2007). However, other studies did not find significant changes in the serum calcium levels of males and females submitted to RYGB (Goode et al. 2004; Fleischer et al. 2008).

Besides calcium, changes in the levels of 25OHD, another factor associated with PTH secretion (Mason 2000; Molina 2007), have been found in obese individuals submitted to bariatric surgery (Slater et al. 2004; Coates et al. 2004; Viégas et al. 2010; Santos et al. 2012; Biagioni et al. 2014). Hypovitaminosis D has been associated with obesity even before RYGB and may be related to many factors, such as low availability of the vitamin stored in the form of vitamin D3 in adipose tissue or low hepatic hydroxylation of the previtamin due to steatosis, a common condition in obese individuals (Holick and Chen 2008). Another factor that would predispose to vitamin D deficiency is low exposure to sunlight due to a sedentary lifestyle or the reclusive nature of these individuals (Brzozowska et al. 2013). After surgery, malabsorption becomes associated with the condition. Generally, it is believed that 50% of the patients submitted to malabsorptive bariatric surgery have vitamin D lack 2 years after surgery, and the percentage increases to 65% 4 years after surgery (Slater et al. 2004; Bloomberg et al. 2005). Carlin et al. (2006) observed low 25OHD and high PTH after surgery, suggesting that the procedure may worsen pre-existing deficiency (Carlin et al. 2006; Casagrande et al. 2012). Indeed, high serum levels of PTH (Viégas et al. 2010) associated with 25OHD deficiency/insufficiency and bone demineralization (Goode et al. 2004; Johnson et al. 2006; Santos et al. 2012) have been reported in patients submitted to RYGB. In a prospective study of the metabolic effects of RYGB, Youssef et al. (2007) found high PTH in 53.3% and 25OHD deficiency in 20.2% of the patients 2 years after surgery (Youssef et al. 2007). Likewise, Santos et al. (2012) found high PTH associated with 25OHD deficiency/insufficiency in 41.7% of the patients submitted to RYGB 3 years after surgery (Santos et al. 2012).

However, changes in some remodeling markers and associated bone losses have been reported regardless of 25OHD and PTH levels (Pugnale et al. 2003), suggesting that bone loss could be promoted by other mechanisms. Biagioni et al. (2014) found that bone resorption increased in obese women of childbearing age submitted to RYGB, evidenced by high serum CTX levels 6 months after surgery. However, PTH and 25OHD levels did not change significantly (Biagioni et al. 2014). Additionally, another study compared two groups, one taking orlistat and sibutramine and another submitted to vertical banded gastroplasty, and found high serum osteocalcin levels

12 months later in both groups (Guney et al. 2003). However, the risk of femur fracture increased only in the operated group, suggesting higher bone involvement in patients with faster weight loss (Guney et al. 2003). Indeed, this higher and faster weight loss has been associated with a proportional BMD reduction (Salamone et al. 1999; Collazo-Clavell et al. 2004; Coates et al. 2004; Fleischer et al. 2008): a body weight loss of 10% would be accompanied by a 1–2% bone mass loss in many body sites (Salamone et al. 1999).

Hence, in addition to nutritional changes, the lower bone load promoted by weight loss could explain the increased bone resorption. It has been suggested that higher skeletal load could be an effective mechanism to avoid bone loss and increase bone formation (Schoenau 2006). The mechanostat theory proposed by Frost describes the intrinsic relationship between body mass and bone mass, postulating that bone mass is influenced by the maximum increase of muscle forces during growth or in response to higher body load (Frost 1987). Thus, the intense weight loss promoted by bariatric surgery would reduce the traction force on the skeleton considerably, negatively impacting bone turnover.

The ability of bone tissue to change its mass and structure in response to mechanical loads depends on the mechanosensitivity of osteocytes and on mechanosensing mediated by the lacuno-canalicular porosity (Burger and Klein-Nulend 1999; Schoenau 2006). In this context, the important role of a protein called sclerostin emerges. This protein is closely related to how bones respond to mechanical loads, acting as a natural modulator of osteoblast function and enabling its use as a bone resorption marker (Bellido et al. 2005). Sclerostin, codified by the SOST gene, is expressed exclusively by osteocytes, the most numerous cells in bone tissue. Initially described by Balemans et al. (2001), sclerostin negatively impacts bone formation by inhibiting terminal osteoblast differentiation and promoting osteoblast apoptosis (Balemans et al. 2001; Brunkow et al. 2001; Robling et al. 2008). Sclerostin binds to the receptor LRP5/6 in the cell membrane of these cells, blocking the *Wnt/β*-catenin signaling pathway and inhibiting the transcription of osteogenic genes (Van Bezooijen et al. 2007; Moester et al. 2010; Lewiecki 2011) (Fig. 3). Additionally, the similarity between sclerostin and differential screening-selected gene aberrative in neuroblastoma (DAN), a family of glycoproteins capable of antagonizing BMP, enables the former to antagonize the latter (Liu et al. 2011). Van Bezooijen et al. (2004) found that sclerostin inhibits the deposition of calcium on rat bone (Van Bezooijen et al. 2004). Other animal studies found that this protein acts as a mechanosensor and that its synthesis and expression reduce in response to mechanical load and high PTH levels (Keller and Kneissel 2005; Robling et al. 2008). Sclerostin levels increase during weight loss or inactivity, leading to bone catabolism (Robling et al. 2008; Bloomfield 2010). These findings suggest that sclerostin deactivation can induce positive bone balance (Van Bezooijen et al. 2004).

Studies that assessed sclerostin levels during weight loss are scarce, and the mechanisms involved in this process remain controversial. Armamento-Villareal et al. (2012) found that spontaneous weight loss in obese individuals would be associated with high sclerostin levels, suggesting an inhibitory role on bone

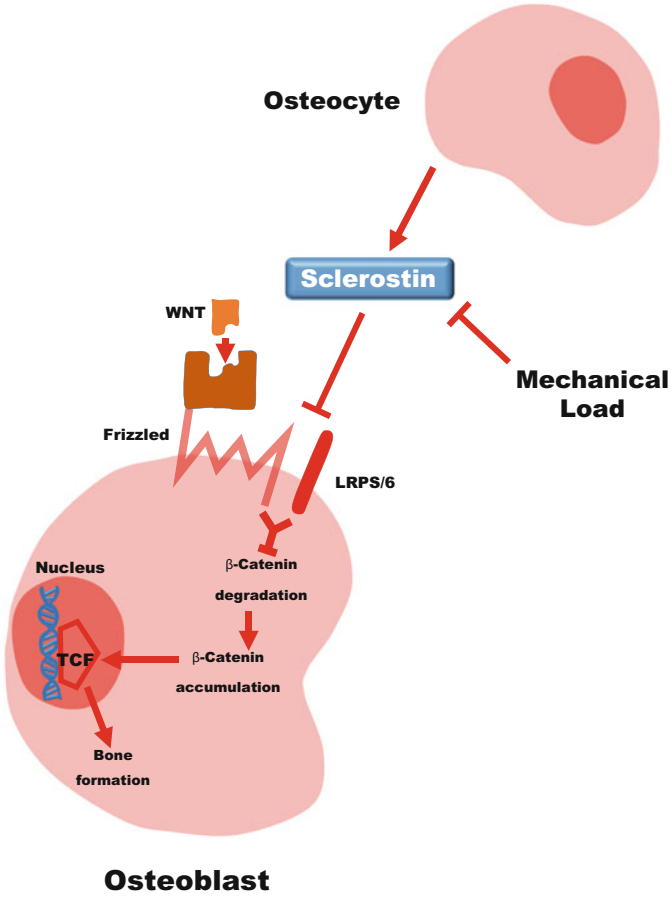

Fig. 3 Representative scheme of the sclerostin action on the signaling pathway Wnt/β-catenin. LRP5/6 (low-density lipoprotein receptor-related protein 5/6) (Sources: Nusse 2005; ten Dijke et al. 2008; Lewiecki 2011)

formation and consequent tissue loss (Armamento-Villareal et al. 2012). Grethen et al. (2012) did not find differences in the serum levels of sclerostin in patients submitted to RYGB and normal weight women (Grethen et al. 2012). On the other hand, Muschitz et al. (2015) found high sclerostin levels over 2 years of RYGB follow-up (Muschitz et al. 2015).

The theory that drastic weight loss reduces the mechanical load on the skeleton and the role of sclerostin in this process seems plausible to explain bone mass loss after bariatric surgery, but persistent low bone mass and high levels of bone markers years after surgery when body weight is finally stable suggest that these are not the only mechanisms involved (Yu 2014).

In this context, changes in the levels of substances produced by the adipose tissue, namely, adipokines, could be involved in the bone loss process. Throughout

the remodeling process, the balance between formation and resorption conducted by osteoblastic and osteoclastic cell lines, respectively, is maintained by the RANK/RANKL/OPG regulation axis (Bonewald and Johnson 2008). An unbalance in this axis promotes osteoclastogenesis, increasing bone resorption, or osteoclast apoptosis, preventing bone loss and stimulating formation (Zhang et al. 2013). Excess adipose tissue has been associated with an unbalanced RANK/RANKL/OPG axis and consequent bone mass change. Likewise, bone loss and body composition changes induced by bariatric surgery would be related to bone loss. Bone mass loss is associated with changes in the levels of certain adipokines, such as adiponectin and leptin (Vilarrasa et al. 2009; Cao 2011; Aguirre et al. 2014). Studies have found that high osteoclastic activity and bone resorption are positively associated with high RANKL levels (Eghbali-Fatourechi et al. 2003). However, in obese rats submitted to RYGB, Pérez-Castrillón et al. (2014) found a significant reduction in RANKL levels and high leptin levels, suggesting that this adipokine could have inhibited marker expression (Pérez-Castrillón et al. 2014). In humans 6 months after RYGB, Gannagé-Yared et al. (2008) found low leptin levels and high adiponectin levels despite unchanged osteoprotegerin (OPG) levels (Gannagé-Yared et al. 2008).

Potential Prognostic Uses and Other Diseases or Conditions

Evidence suggests that bariatric surgery induces intense bone mass loss (Rodríguez-Carmona et al. 2014), predisposing individuals to osteomalacia, osteopenia, and osteoporosis (Collazo-Clavell et al. 2004; Yu 2014; Liu et al. 2015), conditions associated with higher fracture risk and, consequently, higher morbidity and mortality.

Osteoporosis and osteopenia are diagnosed by a bone densitometry test, which detects established bone loss by measuring BMD. The prevention of these conditions, especially after bariatric surgery, which involves dramatic dietary, nutritional, hormonal, and physical changes, can be achieved by monitoring biochemical bone markers, which reveal the effects of surgery on bone metabolism relatively quickly (Bonjour et al. 2014). In this sense, postoperative monitoring of the serum levels of these markers could reveal skeletal risk, enabling the introduction of curative measures before bone disease is fully established.

Conclusions

With the growing global prevalence of excess weight, the number of bariatric surgeries is expected to increase proportionally. Despite its beneficial effects on a number of obesity-related comorbidities, some authors reported that the procedure has negative skeletal repercussions. However, the exact nature of these repercussions and the mechanisms by which the hormonal and structural changes promoted by the procedure influence bone remodeling have not been entirely clarified. Hence, clinical

assays with large samples, long follow-ups, and careful designs are necessary to elucidate the mechanisms involved in bone metabolism changes and to establish strategies that prevent bone loss and promote health after surgery. In this sense, bone markers could be useful tools for the early detection of these changes.

Summary Points

- The prevalence of obesity has increased.
- The frequency of bariatric surgery has increased.
- Bariatric surgery has been associated with negative bone tissue repercussions.
- Some bone health-related markers change soon after bariatric surgery.
- Bone markers could be used for detecting surgery-related bone changes early.

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Bone Biomarkers in HIV

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Abstract

HIV infection is associated with higher rates of low bone density and fragility fracture, occurring at an earlier age and to a greater extent with ageing in those with HIV, compared to the general population. These outcomes result from a combination of direct HIV effect on bone turnover, the effects of immune pertubation, as well as the inflammatory effect caused by HIV viraemia. Additionally those with HIV have higher rates of lifestyle risk factors as well as higher rates of co-morbidities that increase the biomarkers of bone formation and resorption, and correlate with increased bone turnover and low bone density.

Treatment with antiretroviral therapy, particularly with nucleoside analogues such tenofovir disoproxil fumarate, and protease inhibitor therapy, results in increased bone turnover and lower bone density. These changes are most prominent in the first year of antiretroviral therapy, and then tend to plateau. Changes in bone biomarkers predict the changes in bone turnover and bone density with antiretroviral therapy. Bone biomarkers and bone mineral density have been demonstrated to improve with changes to components of antiretroviral regimens.

Bone biomarkers have been used for research purposes in predicting and monitoring bone turnover and bone density changes in HIV. However the use of bone biomarkers is not yet developed to routine clinical application in HIV management.

Keywords

HIV • Antiretroviral therapy • Bone biomarkers • Osteoporosis • Fracture

List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
ALP	Alkaline phosphatase
ART	Antiretroviral therapy
BAP	Bone-specific alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
CTX	Carboxyterminal collagen telopeptides
DPD	Deoxypyridinoline
DXA	Dual-energy x-ray absorptiometry
FRAX	Fracture Risk Assessment Tool
GAG	Glycosaminoglycan
HIV	Human immunodeficiency virus
HPRO	Hydroxyproline
ICTP	Carboxyterminal cross-linked telopeptide of type I collagen
IL6	Interleukin 6
INSTI	Integrase strand transfer inhibitor
IRIS	Immune inflammatory reconstitution syndrome
MACS	Multicenter AIDS cohort study
NICM	Noninfectious comorbidities
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitor

NNRTI	Non-nucleoside reverse transcriptase inhibitor
NTX	Amino-terminal collagen telopeptides
OM	Osteomalacia
OPG	Osteoprotegerin
PI/rPI	Protease inhibitor/ritonavir-boosted protease inhibitor
PICP	C-terminal carboxyterminal peptide of type 1 collagen
PINP	N-terminal carboxyterminal peptide of type 1 collagen
PYD	Pyridinoline
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RR	Relative risk
START	Strategic timing of antiretroviral treatment
TAF	Tenofovir alafenamide
TBS	Trabecular bone score
TDF	Tenofovir disoproxil fumarate
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAP	Tartrate-resistant acid phosphatase
TRACP5b	Tartrate-resistant acid phosphatase isoform 5b
VACS	Veterans Aging Cohort Study
VPR	Viral protein of regulation

Introduction

Human immunodeficiency virus (HIV) infection impacts on many organ systems in the body, including the bone. The changes found in the bone with HIV are reflected by the changes in bone turnover and thus bone biomarkers (McComsey et al. 2010; Camozzi et al. 2009; Mondy et al. 2003, Haskelberg et al. 2011). A distinguishing feature of HIV infection is the confluence of HIV viremia and immune perturbation, the effects of antiretroviral therapy (ART), and the higher levels of comorbidities and lifestyle risk factors that lead to an increase in the markers of bone resorption and formation. The changes in bone activity in HIV, as well as the effects of HIV treatment and comorbidities, result in increased rates of low bone density, osteopenia, osteoporosis, osteomalacia, osteonecrosis, and fragility fracture – compared to the general population (Cazanave et al. 2008; Harris and Brown 2012; Morse et al. 2007; Prieto-Alhambra et al. 2014; Womack et al. 2011; Triant et al. 2008; Shiau et al. 2013).

In a meta-analysis of 11 studies examining the prevalence of low bone mineral density (BMD) in 884 patients with HIV and 657 HIV-negative controls, low BMD (T-score ≤ -1.0) in those with HIV was much higher – 6.4-fold and osteoporosis 3.7-fold higher – than in aged matched HIV-negative controls (Brown and Qaqish 2006). In the US Veterans Aging Cohort Study (VACS), after adjustment for demographics, comorbidities, and lifestyle risk factors, fracture risk in those with HIV was 24% higher than in HIV uninfected (although attenuated to 10% higher by adjusting for body mass index (BMI)) (Womack et al. 2011).

Other studies have shown fracture rates in HIV-infected patients are 30–70% higher than in matched HIV-negative controls (Shiau et al. 2013; Güerri-Fernandez et al. 2013). A large Danish case-control study showed overall fracture rates in those with HIV infection to be threefold higher and hip and spine fractures ninefold higher than in the general population (Prieto-Alhambra et al. 2014).

Characteristically, low bone density in HIV occurs at an earlier age than in the general population, as a feature of "premature aging" where comorbidities in patients with chronic HIV infection such as cardiovascular disease, neurocognitive disorders, and frailty occur more frequently and a decade or two earlier than expected (Deeks and Phillips 2009; Guaraldi et al. 2011). There is however not complete agreement about the validity of accelerated aging in HIV (Althoff et al. 2014). Additionally, there is concern that as the cohort of HIV-infected patients ages, the morbidity associated with low BMD and fracture will escalate, particularly as the gap in risk of low BMD, osteoporosis, and fracture between HIV infected and those without HIV that widens with age (Triant et al. 2008) (Fig. 1).

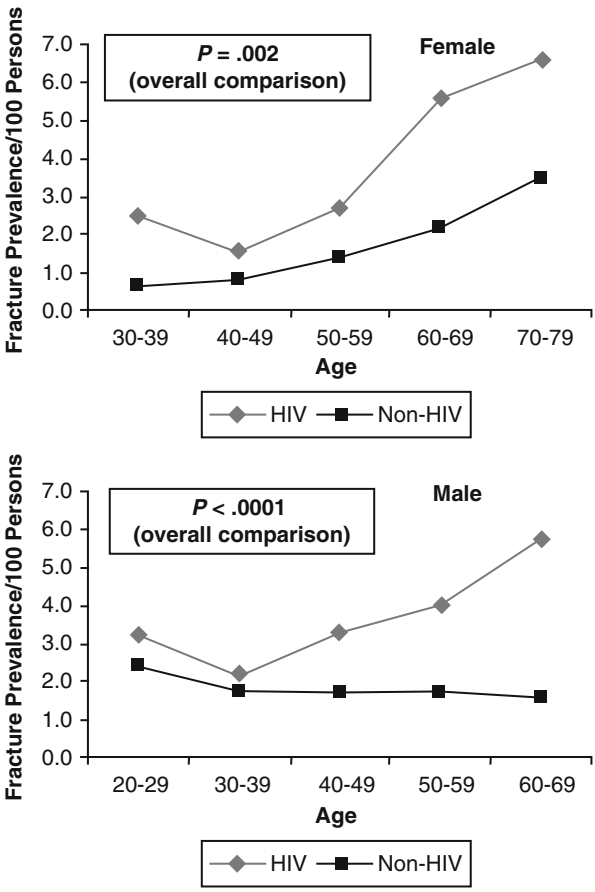

Fig. 1 Comparison of fracture prevalence in human immunodeficiency virus (*HIV*)–infected vs non-HIV-infected patients according to gender and age group ((Triant et al. 2008). Copyright Endocrine Society 2008)

Measuring Bone in HIV

Direct measurement by histological examination of bone biopsy at the iliac crest is the gold standard for measurement of bone turnover. This is however an invasive procedure and is not a practical option for routine clinical care. Surrogate markers of bone formation and resorption are thus utilized.

Dual-energy x-ray absorptiometry (DXA) scan is the gold standard in measurement of bone density. For patients aged $\geq$50 years, T-score bone loss (standardized to peak bone mass aged 30 year) of > -1 SD and < -2.5 SD is classified as osteopenia, and > -2.5 SD loss is osteoporosis. In patients $<$50 year, Z scores (matched for age and gender) more accurately assess low bone density. While DXA gives a good indication of bone density, it is not sensitive enough to provide information on pathogenesis or recent changes in bone turnover. Additionally, it does not completely describe bone strength and thus does not totally correlate with risk of fracture.

An innovative research tool provided by DXA is the trabecular bone score (TBS) (Silva et al. 2014). TBS is a gray-level textural metric that can be extracted from the two-dimensional lumbar spine DXA image. TBS is related to bone microarchitecture and provides skeletal information not captured by the standard BMD measurement. An elevated TBS value correlates with better skeletal microstructure; a low TBS value correlates with weaker skeletal microstructure. Lumbar spine TBS has been evaluated in cross-sectional and longitudinal studies. TBS is associated with fracture risk in individuals with conditions related to reduced bone mass or bone quality and is a reliable marker of anti-osteoporotic drug efficacy. Based on these data, lumbar spine TBS holds promise as an emerging technology that could well become a valuable clinical tool in the diagnosis of osteoporosis and in fracture risk assessment.

The Fracture Risk Assessment Tool (FRAX®) provides FRAX scores that are country-specific predictors of future fracture based on patient demographics and personal and family history of fracture, with or without hip DXA score. A percentage risk $>$15% spine and $>$3% hip fracture over 10 years warrants medical intervention. General population country-based FRAX scores underestimate fracture risk in HIV, and even when HIV-adjusted FRAX scores are used, there is evidence that actual fracture risk is greater in the HIV population (Yin et al. 2015).

Biomarkers are biochemical markers of bone turnover and are measured in plasma or urine. They can measure bone formation (bone-specific alkaline phosphatase (BAP), C- and N-terminal carboxyterminal peptide of type 1 collagen (PICP, PINP), and osteocalcin (OC)) or bone resorption (tartrate-resistant acid phosphatase (TRAP), hydroxyproline (HPro), urinary pyridinoline (Pyd) and deoxypyridinoline (Dpd), amino-terminal, and carboxyterminal collagen telopeptides (CTx, NTx)). Biomarkers can be enzymes (BAP, TRAP), collagen derived (PICP, PINP, HPro, Pyd, Dpd, CTx, NTx), or non-collagen (OC).

The qualities of useful biomarkers include bone specificity, stability, long half-life, and ease of measurement. The bone is constantly remodeling with a balance between bone formation and resorption in the healthy individual. The levels of

biomarkers can fluctuate on a daily basis, particularly with urine-based testing in comparison to serum-based testing. Some of the variation can be reduced with fasting, morning specimen collection (biomarkers are highest early in the morning and osteoclastic activity can be reduced with meals). The fluctuations in levels need to be considered when assessing sequential patient results – a significant result is defined at 2.8 times the biological variation (Seibel 2005). Biomarkers, apart from alkaline phosphatase (ALP), are renally excreted, and thus levels of biomarkers are usually elevated with impairment in renal function.

There can be other factors that impact on biomarkers and may affect results of biomarker measurement. For example, after fracture, biomarkers can be elevated for up to 6 months. There are also other high-turnover states where biomarkers may be abnormally high such as Paget's disease, hyperparathyroidism, hyperthyroidism, malignancy such as myeloma, and chronic kidney disease with renal failure.

Biomarkers are useful in measuring the dynamic process of bone turnover and remodeling, in differentiating disease processes, and in monitoring responses to therapy. However, the use of biomarkers in the clinic has been limited by a number of factors, including intra- and inter-patient variability, difficulties in measuring biomarkers that may require specific conditions due to diurnal variation, and interlaboratory variability in measurement, and there is ongoing research on the utilization of biomarkers as prognostic markers for BMD changes.

Biomarkers have been used extensively alongside DXA in HIV for research purposes, mainly to assess changes that occur with the use of various ART drugs (Haskelberg et al. 2011). In the non-HIV setting, biomarkers have been shown to have prognostic value in predicting fracture risk in untreated osteoporosis (Burch et al. 2014). Bone turnover has been much less studied in older men, and the decisions on which biomarkers are optimal require further research. Nevertheless in older men and women, higher levels of biomarkers have been associated with increased fracture risk, irrespective of BMD. The Dubbo case-control study of elderly men in their early 70s demonstrated that biomarkers predicted fracture risk, irrespective of BMD (Meier et al. 2005). High osteoclastic carboxyterminal cross-linked telopeptide of type I collagen (S-ICTP) levels and low femoral neck BMD together increased incident fracture risk tenfold. A prospective Swedish study in 1040 women showed elevated levels of S-TRACP5b and S-CTX-I, and urinary osteocalcin were associated with increased risk of vertebral but not hip fracture for up to 10 years (Ivaska et al. 2010).

Pathogenesis of Bone Disease in HIV

The impact on bone turnover in HIV infection is partly due to the direct effects of the HIV virus and inflammation in the individual, but also attributable to the higher rates of traditional risk factors for low bone density: increased smoking, alcohol and drug

consumption, hepatitis C co-infection, low BMI, low vitamin D levels, hypogonadism, diabetes, renal insufficiency, and greater numbers of other comorbidities (McComsey et al. 2010).

Vitamin D insufficiency (<30 ng/ml) and deficiency (<10 ng/ml) have been found to be more prevalent in HIV-infected patients (Cervero et al. 2012; Allavena et al. 2012). Serum vitamin D concentration showed a positive correlation with bone mineral density (BMD) and a negative correlation between serum osteocalcin and urinary pyridinium cross-links (Madeddu et al. 2004). Both ART and HIV infection itself affect the bone and vitamin D metabolism. Many studies detected similar levels of vitamin D and its metabolites in both osteopenic and non-osteopenic HIV-infected patients, suggesting that vitamin D was not predictive of bone metabolic disorders (Ramayo et al. 2005).

HIV infection results in a pro-inflammatory state due to cytokine activation provoked by HIV viremia. The increased inflammation that occurs with HIV infection increases the risk of comorbidities in infected patients (Deeks et al. 2013). Even in patients who are treated with ART, there is ongoing inflammation from low-level HIV viremia arising from HIV activation in long-lived reservoir cells. As with other pro-inflammatory conditions such as rheumatoid arthritis, cytokine production results in increased bone resorption. Higher levels of TNFα found in HIV infection lead to an upregulation of receptor activator of nuclear factor kappa-B ligand (RANKL) which induces osteoclastic activity (Gibellini et al. 2007). Additionally, RANKL, a cytokine member of the tumor necrosis factor receptor/tumor necrosis factor (TNFR/TNF) family, is released from T cells and osteoblasts and stimulates the differentiation of osteoclast precursors into mature osteoclasts (Khosla 2001). Higher-serum RANKL levels have been found to be associated with low spinal BMD. There are higher levels of the cytokine IL6 in patients with HIV, even with ART-induced virological suppression (Brown et al. 2011). IL6 has been associated with increased osteoclastic activity.

HIV has been found to directly impact on bone formation and turnover. HIV viral proteins increase osteoclastic activity as well as having an apoptotic effect on osteoblasts. In vitro, HIV, via gp120 interaction with cell membranes results in TNFα-mediated induction of osteoblast apoptosis. HIV gp120 leads to the upregulation of the receptor of activated RANKL in PBMCs and increased osteoclastic activity (Gibellini et al. 2008). The viral protein p55gag suppresses osteoblastic activity and promotes osteoblast apoptosis. HIV viral protein of regulation (Vpr) increased RANKL expression in PBMCs in conjunction with endogenous glucocorticoids and synergistically with exogenous glucocorticoids. High levels of HIV RNA increase TNFα expression resulting in increased RANKL expression that leads to increased osteoclastic activity (Fakruddin and Laurence 2005). B-cell lymphocytes secrete OPG as a result of interaction with T-cell lymphocytes via the CD40 ligand. The immune dysregulation that occurs in HIV infection disrupts this B-cell T-cell interaction, reduces B-cell OPG production, and increases RANKL (Titanji et al. 2014).

The Impact of HIV Treatment

Advances in treatment of HIV infection with ART have transformed the prognosis in the majority of those with HIV. In contrast to the 1980s when HIV/AIDS was first recognized as a fatal illness, HIV for the majority of patients is now treated as a chronically manageable condition. This change in prognosis has shifted the focus to managing the long-term consequences of HIV infection and the impacts of HIV treatment.

Evidence from large-scale clinical trials (START, TEMPRANO) supports the initiation of ART not only with more advanced HIV infection but also for those patients at earlier stages of infection with normal immunity (The INSIGHT START Study Group 2015; The TEMPRANO ANRS 12136 Study Group 2015). Thus HIV treatment guidelines have shifted to recommend treating with ART, where feasible, all patients with HIV as soon as they are diagnosed (Department of Health and Human Services 2016). ART consists classically of three drugs from two different classes. There is a backbone of two nucleoside/nucleotide reverse transcriptase (NRTI) inhibitors and an anchor drug that is usually a non-nucleoside reverse transcriptase inhibitor (NNRTI), a ritonavir-boosted protease inhibitor (rPI), or an integrase strand transfer inhibitor (INSTI). The NRTI backbone is composed in most cases of either Truvada (tenofovir-emtricitabine) or Kivexa (abacavir-lamivudine). INSTIs, because of their excellent efficacy and tolerability, are increasingly used as the anchor drug in ART and indeed are the recommended drug of choice in HIV treatment guidelines (Department of Health and Human Services 2016).

ART these days is highly effective in achieving ongoing HIV virological suppression, improving CD4 T lymphocyte count, and maintaining health, enabling most patients to live a close to normal lifespan with a chronically manageable condition. Treatment requires a high level of adherence on an ongoing basis – treatment is lifelong. ART is effective and well tolerated, and ongoing HIV treatment is associated with a significant reduction in mortality and morbidity from AIDS-defining conditions as well as serious non-AIDS conditions such as cardiovascular, liver, kidney disease and cancers (The Strategies for Management of Antiretroviral Therapy (SMART) Study Group). However, particular drugs used in ART may impact detrimentally on comorbidities (e.g., association of abacavir with increased risk of myocardial infarct, association of tenofovir with increased risk of renal and bone disease), and the choice of drugs used in ART is affected by their potential impact on comorbidities, which are more common in patients with HIV than in the general population. This effect of ART on comorbidities also applies to bone disease in HIV where the initiation of ART, irrespective of drug regimen, results in reduction of BMD, although some drugs are more implicated than others.

In addition to the impact of HIV, ART also adversely affects bone turnover and bone density, particularly in the first year following commencement of therapy (Tebas et al. 2000). Irrespective of ART regimen used, there is a 2–6% loss of BMD in the first year of commencing ART (Piso et al. 2011). The question of why BMD loss and increased biomarkers occur with initiation of ART has not been fully explained. One postulated theory is that these changes are manifestations of an

immune inflammatory reconstitution syndrome (IRIS) phenomenon, with rapid bone turnover and increased fracture risk soon after initiating therapy (Nguyen and Reveille 2009; Lawson and Walker-Bone 2012). The initiation of ART results in immune reconstitution and an increased T lymphocyte population that promotes osteoclastic activity. Certainly a meta-analysis of three studies showed that lower CD4 T lymphocyte count at initiation of ART results in greater loss of BMD (Grant et al. 2013). However, increased biomarkers and lower BMD occur in all HIV patients initiating therapy, even with normal immunity. Interestingly, with ART, there is virological suppression, immune restoration, reduced inflammation, and gain in body mass – all factors which would tend to improve bone turnover and BMD – perhaps explaining the leveling off of the early bone loss that occurs with ART initiation. Bolland has postulated that changes in BMD with ART initiation could mostly be accounted for by lower BMI in HIV-positive patients (Bolland et al. 2015). While lower BMI has been associated with lower BMD, there is a definite impact from ART initiation. Other predictors of greater BMD loss were older age, higher plasma HIV RNA, and use of tenofovir or PIs (McComsey et al. 2010; Harris and Brown 2012; Brown and Qaqish 2006). Prospective studies have shown that the greatest decline in BMD occurs in the first 24 weeks following initiation of ART, with a partial recovery in BMD from 24 to 48 weeks. Biomarkers also increase with initiation of ART, reaching a maximum at 24 weeks and decreasing or plateauing thereafter, but remaining at a higher level than prior to the initiation of ART. Long-term follow-up of HIV-positive and HIV-negative cohorts indicates comparatively ongoing greater BMD loss in the HIV-positive group (Grant et al. 2015).

Increases in biomarkers with therapy have not been associated with increased serum levels of RANKL and OPG. A study of HIV patients heavily pretreated with ART who had low BMD showed an association of low BMD with high OPG and lysylpyridinoline/creatinine ratio (Dpd) values (Seminari et al. 2005).

There is evidence for a plateauing of BMD after 48 weeks, although the longer-term implications of the increased bone loss with ART have not yet been fully determined, given the long period to the development of clinical complications of fracture. Because treatment of HIV infection requires a high level of adherence to lifelong therapy, the implications of potential detrimental effects of long-term ART on bone turnover and BMD are of concern to those treating HIV, particularly as the cohort of HIV patient age.

The adverse impact on bone with ART occurs despite general health improvement, as well as weight gain and reduction in general morbidity and mortality. The changes found with ART do vary depending on the particular therapy as well as the individual's underlying risk factors.

Specific HIV Drugs and Their Impact on BMD and Biomarkers

The drug most associated with lowering bone density is the nucleotide analogue tenofovir disoproxil fumarate (TDF), a widely used component of effective ART

regimens. TDF affects gene expression in osteoblasts, which results in impairment of osteoblast functioning (Grigsby et al. 2010).

A large-scale treatment naïve placebo-controlled study randomizing HIV patients to TDF or stavudine with efavirenz and lamivudine, with DXA follow-up, showed significantly lower BMD in the TDF arm at 48 weeks, although the differences between the arms narrowed over 144 weeks (Gallant et al. 2004). In the ASSERT study, patients were randomized to efavirenz with tenofovir-emtricitabine (Truvada) or abacavir-lamivudine (Kivexa) (Stellbrink et al. 2010). The study was powered for a BMD end point at 96 weeks. Biomarkers were significantly increased at week 24, and bone formation markers (OC, PINP, BAP) but not bone resorption (CTx) at week 48, as well as BMD at week 96, were significantly decreased in the Truvada arm.

Interestingly baseline BMD in the untreated HIV population with median age in mid-30s was significantly lower than in the general population. The use of TDF has also been associated with increased fracture risk (RR 1.12/year of TDF exposure) in a large retrospective cohort (Bedimo et al. 2012).

Another mechanism of TDF impact on bone is that high-level exposure to TDF can lead to proximal tubular toxicity and hypophosphatemia (Fanconi's syndrome) leading to low bone density (Mathew and Knaus 2006). Other drugs used in HIV which can cause phosphate wasting and Fanconi's syndrome are adefovir (structurally related to tenofovir) and no longer used therapeutically in HIV, but used to treat hepatitis B infection at lower dosage; additionally foscarnet, an anti-herpes drug, used to treat cytomegalovirus-induced retinitis found in severely immunosuppressed patients. To address the issue of TDF renal and bone toxicity, the next-generation nucleotide analogue tenofovir prodrug, tenofovir alafenamide (TAF), has been developed. TAF has a predilection for hematopoietic cells where it concentrates, thus focusing on cells affected by HIV and also enabling much lower doses of drug to achieve therapeutic levels and treatment efficacy. Less tenofovir is available in the circulation (90% reduction) and there is significantly lower exposure to the bone. The postulated benefits of TAF versus TDF in terms of bone turnover, biomarkers, and BMD have been confirmed in studies (Mills et al. 2015; Wohl et al. 2015).

One solution to tenofovir bone toxicity is to switch therapy in virologically controlled patients. The STEAL study randomized patients suppressed on ART to a co-formulated backbone of tenofovir-emtricitabine or abacavir-lamivudine. The study aimed to examine outcomes of virological suppression and major adverse events. There was significantly greater loss of BMD in the tenofovir-emtricitabine arm and no difference in fracture at 96 weeks. Biomarkers measured were PINP, BALP, and CTx, in addition to OPG and RANKL (Haskelberg et al. 2012) (Fig. 4, Table 1).

The protease inhibitor (PI) class of compounds have also been implicated in low bone density, particularly in earlier cross-sectional studies (Duvivier et al. 2009; Womack et al. 2011). However, the evidence is conflicting, and longitudinal studies have not supported the association of PIs with low bone density. One longitudinal study showed an association between low BMD over 72 weeks with general risk factors and HIV, rather than ART and particularly PI therapy (Mondy et al. 2003). However, in this study patients enrolled already had extensive ART experience. Biomarkers tended to remain stable over the 72-week observation period.

Table 1 Switching HIV antiretroviral therapy: effect on bone biomarkers and bone mineral density (Haskelberg et al. 2012)

	Mean change from baseline to week 48						Mean change from baseline to week 96*					
Variable	ABC-3TC	TDF-FTC	Mean difference (95% CI)	*P*	ABC-3TC (mean %)	TDF-FTC (mean %)	ABC-3TC	TDF-FTC	Mean difference (95% CI)	*P*	ABC-3TC (mean %)	TDF-FTC (mean %)
BMD												
Right hip (g/cm^2)	−0.006	−0.013	0.007 (−0.005–0.019)	0.256	−0.6	−1.2	0.004	−0.007	0.011 (0.003–0.019)	0.006	0.4	−0.6
Spine (g/cm^2)	0.005	−0.016	0.021 (0.011–0.030)	<0.001	0.5	−1.2	0.008	−0.005	0.013 (0.002–0.025)	0.017	0.8	−0.3
Bone resorption												
βCTx (ng/L)	7.3	89.8	−82.5 (−120.2 to −44.8)	<0.001	27.5	68.4	10.9	72.5	−61.6 (−97.4 to −25.7)	0.001	31.7	65.4
Bone formation												
BALP (μg/L)	−3.7	0.2	−3.9 (−6.5 to −1.4)	0.002	132.9	121.8	−3.1	−0.7	−2.5 (−5.0 to 0.1)	0.060	149	135.5
P1NP (μg/L)	−7.3	4.2	−11.6 (−15.7 to −7.4)	<0.001	−8.4	16.1	−8.4	−0.5	−7.9 (−12.9 to −2.9)	0.002	−8.0	8.6
Bone regulation												
OPG (pmol/L)	−0.2	0.0	−0.1 (−0.4 to 0.0)	0.074	−1.4	6.0	−0.2	0.1	−0.3 (−0.6 to 0.0)	0.077	0.5	7.7
RANKL (pmol/L)	−0.1	−0.1	0.02 (−0.04 to 0.09)	0.448	−27.0	−29.3	−0.1	−0.1	0.0 (−0.1 to 0.1)	0.695	−24.1	13.4
Ten−year fracture risk according to FRAX® scores computed with BMD												
Major OP fracture risk	ND	ND	ND	ND	ND	ND	−0.3	−0.2	−0.1 (−0.3 to 0.1)	0.460	ND	ND
Hip fracture risk	ND	ND	ND	ND	ND	ND	−0.2	−0.1	−0.1 (−0.2 to 0.1)	0.361	ND	ND

(*continued*)

Table 1 (continued)

Variable	Mean change from baseline to week 48						Mean change from baseline to week 96*					
	ABC-3TC	TDF-FTC	Mean difference (95% CI)	*P*	ABC-3TC (mean %)	TDF-FTC (mean %)	ABC-3TC	TDF-FTC	Mean difference (95% CI)	*P*	ABC-3TC (mean %)	TDF-FTC (mean %)
Clinical implication												
Reaching NOF criteria (%)[a]	ND	ND	ND	ND	ND	ND	0.6	2.5	−1.8 (−4.6 to 0.9)	0.371	ND	ND
Low femoral BMD (%)[b]	ND	ND	ND	ND	ND	ND	3.8	8.7	−4.8 (−10.4 to 0.4)	0.06	ND	ND
Low spine BMD (%)[b]	ND	ND	ND	ND	ND	ND	3.8	7.5	−3.6 (−8.7 to 1.4)	0.125	ND	ND

Note. Abbreviations: *ABC-3TC* abacavir-lamivudine, *BALP* bone-specific alkaline phosphatase (*$n = 270$), *βCTx* C-terminal cross-linking telopeptide of type 1 collagen (*$n = 281$), *BMD* bone mineral density, *CI* confidence interval, *ND* not done, *NOF* National Osteoporosis Foundation, *OP* osteoporotic, *OPG* osteoprotegerin (*$n = 270$), *P1NP* procollagen type 1 N-terminal propeptide (*$n = 281$), *RANKL* Receptor Activator of Nuclear Factor Kappa Ligand (*$n = 270$), *TDF-FTC* tenofovir-emtricitabine

[a]Proportions of participants above the thresholds recommended for antiresorptive therapy according to US NOF guidelines

[b]Low BMD defined as T-score < -1

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PIs have also been associated with low bone density via effects on vitamin D metabolism. Additionally, PIs are usually boosted with ritonavir (rPIs), which is a powerful inhibitor of CYP 450 3A4 pathway, and corticosteroid exposure can be markedly increased when coadministered with rPIs, leading in some cases to Cushing's syndrome and steroid-induced low bone density.

Efavirenz, a non-nucleoside reverse transcriptase inhibitor, interrupts the pathway for vitamin D synthesis (Brown and McComsey 2010) Efavirenz induces the cytochrome Cyp3A4 which results in increased conversion of 25(OH)D to 24,25-dihydroxyvitamin D, which is an inactive metabolite.

INSTIs appear to have less negative impact on bone. The RADAR study randomized treatment naïve HIV patients to rPI darunavir/ritonavir plus INSTI raltegravir or Truvada (Bedimo et al. 2014). Biomarker (PINP and CTx) changes at 16 weeks predicted changes in BMD at 48 weeks. Biomarkers increased in both arms but significantly more in the Truvada arm (BMD decrease was higher in the Truvada arm). A bone substudy of the NEAT study – a study with the same treatment arms as RADAR – measured biomarkers (OC, BAP, PINP, CTx, OP) at baseline and week 48 (Bernadino et al. 2015). Baseline biomarkers predicted BMD $\geq$ 5% at weeks 48 and 96. Biomarkers increase at week 48 was significantly lower in the raltegravir arm at week 48 (Figs. 2 and 3).

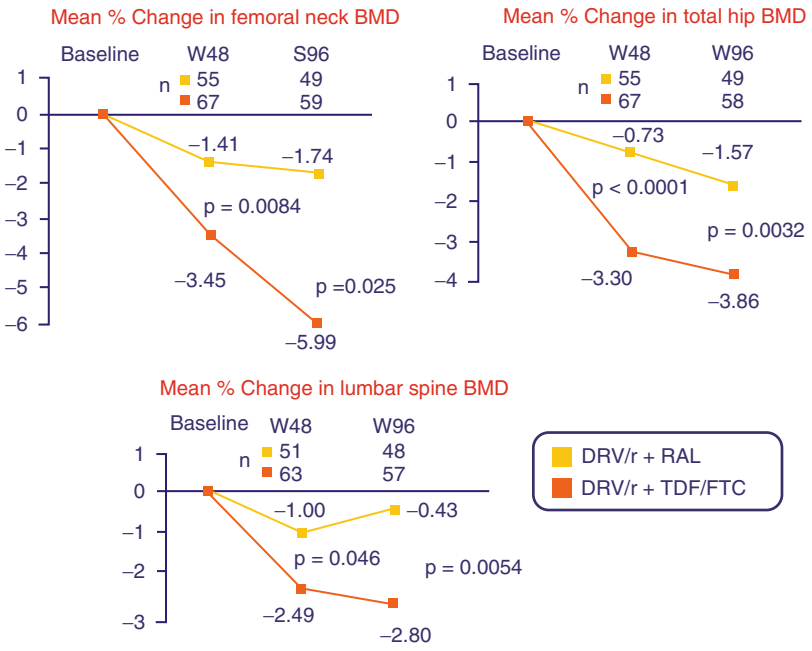

Fig. 2 NEAT 001/ANRS 143 Study; DRV/r + RAL vs DRV/r + TDF-FTC; BMD changes (Bernadino et al. 2015). BMD falls with ART initiation but the degree of BMD loss depends on particular ART therapy – TDF more severe

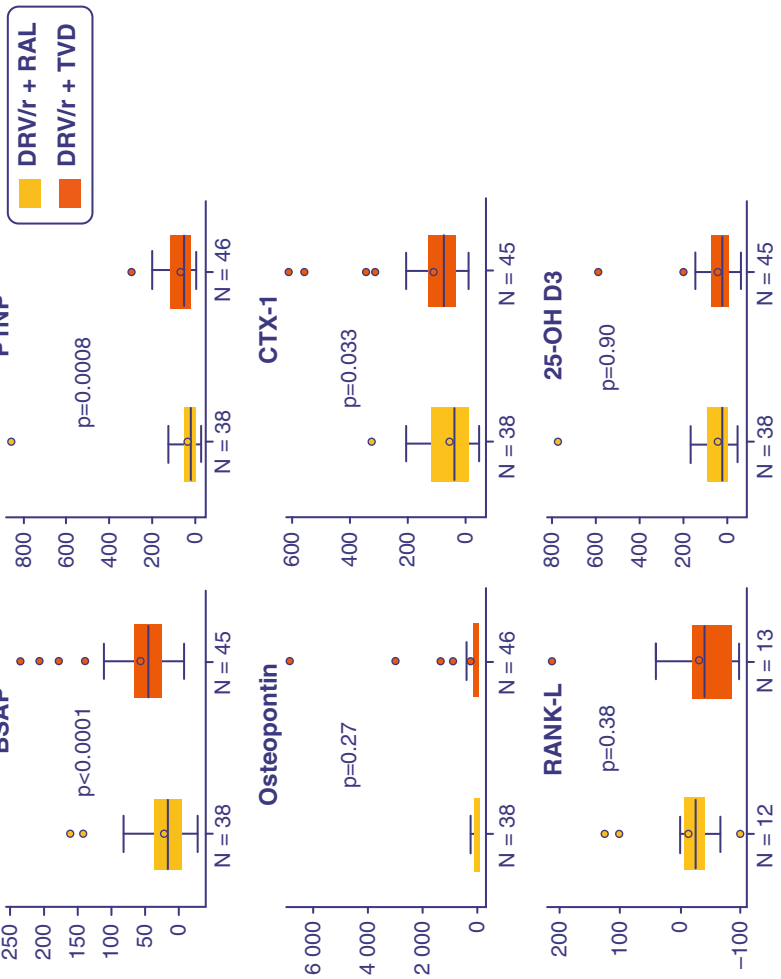

Fig. 3 NEAT 001/ANRS 143 Study: median percentage changes in biomarkers at W48 (Bernadino et al. 2015). Changes in biomarkers reflect BMD changes

In the single-study treatment, naïve HIV patients were randomized to Atripla (efavirenz/tenofovir/emtricitabine) versus INSTI dolutegravir + Kivexa. Biomarkers (CTx, osteocalcin, BSAP, P1NP) increased in both arms over 144 weeks, peaking at week 48 or 96, but biomarker increase was greater in the Atripla arm (Fig. 4). Of interest is that only biomarkers and not BMD were measured in this study that aimed to enroll the entire cohort, rather than a subgroup that attends tertiary centers that have access to DXA. A study switching patients from tenofovir to the INSTI raltegravir in patients also taking rPIs showed increases in BMD and reduction in biomarkers (NTx, BAP, OC) over 24 and 48 weeks (Bloch et al. 2014). A substudy of second-line therapy which examined BMD changes over 48 weeks in patients who failed their first-line ART regimen and was randomized to switch to rPI lopinavir/ritonavir and either INSTI raltegravir or two NRTIs showed significantly less BMD loss in the raltegravir arm (Martin et al. 2013).

HIV Prevention

The prevention of HIV transmission has been hampered by the lack of an effective prophylactic vaccine. Various measures have been and continue to be applied such as condom use, male circumcision, diagnosis and treatment of sexually transmitted infections, early treatment of HIV infection, and use of ART in pregnancy. As part of a range of measures, an increasingly utilized tool for prevention of HIV infection at the individual and community level is the provision of preexposure prophylaxis (PrEP), which involves in HIV-negative individuals the daily use of Truvada. Truvada concentrates exceptionally well in the genital tissues, acting as a barrier to HIV acquisition when appropriately taken by high-risk individuals. The implications of long-term PrEP on bone turnover and BMD in those without HIV are still to be determined. ATN 110 is a safety study of 200 young men (18–22 years) who have sex with men (MSM) commencing PrEP. BMD by Z-score was below normal levels at baseline, and there was a correlation at 48 weeks between tenofovir drug levels and BMD decline (−1.5% hip BMD decline in those with therapeutic levels of tenofovir versus 1.5% hip BMD increase in those with zero drug levels on dried blood spot) (Mulligan et al. 2015). A San Francisco-based placebo-controlled PrEP study using TDF showed 10% of MSM participating had low BMD at baseline. There was a statistically significant but clinically modest reduction in BMD in patients taking TDF at 48 weeks (Liu et al. 2011). The use of DXA has not been widespread in the rollout of PrEP. Biomarker measurements are in their infancy, even in PrEP research studies. IPERGAY was a study that examined “on demand” (two doses of Truvada prior to and after an at risk sexual exposure) rather than continuous PrEP, thus providing for reduced Truvada exposure. This study was mainly aimed at efficacy and did not examine bone markers (Molina et al. 2015). A currently enrolling PrEP adherence study (CCTG 595) will examine in a substudy of 50 participants the impact on biomarkers (PINP, CTX) as well as PTH and vitamin D, of open-label administration of vitamin D 4000 IU/day from week 24 to 48.

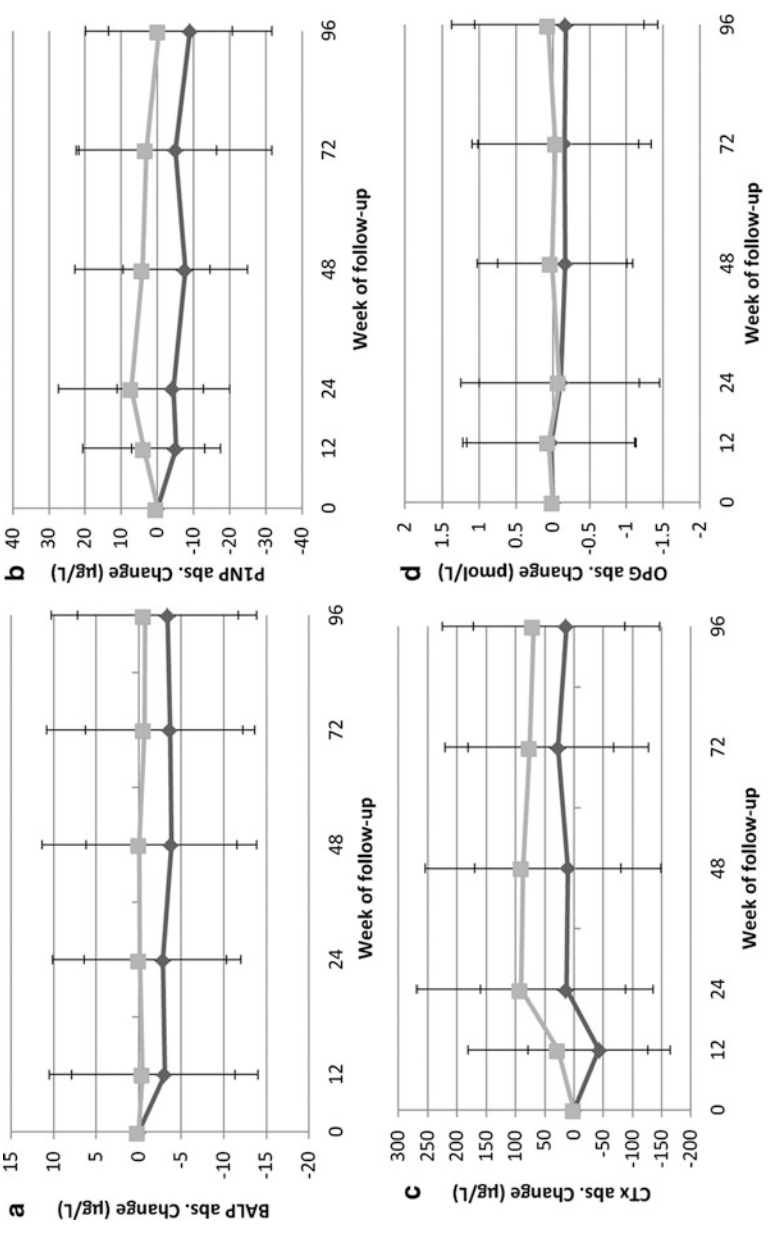

Fig. 4 Switching HIV antiretroviral therapy: bone biomarker changes seen at 12 weeks and precede bone density changes (Haskelberg et al. 2012)

There will be a great need for markers to predict which patients require more comprehensive follow-up of bone turnover and bone density as PrEP becomes more widely available, particularly as PrEP will not be confined to the young adult population.

Body Composition, Body Fat, and Biomarkers

One of the complications of HIV therapy has been lipodystrophy – a syndrome of central or visceral fat accumulation (lipohypertrophy), peripheral subcutaneous fat wasting (lipoatrophy), lipomata, and dyslipidemia (Carr et al. 1998). Thymidine nucleoside analogue drugs (stavudine and zidovudine) have been most associated with lipoatrophy and have been replaced by drugs with lower toxicity. However, older patients with extensive treatment experience may show the disfiguring signs of lipoatrophy that have not been clinically resolved with changes to ART that do not cause this complication. Lipohypertrophy has been most associated with PIs. Both lipoatrophy and lipohypertrophy have been predictors for low BMD (Huang et al. 2001). In a cross-sectional study of 331 ART-naïve patients, aged mid-30s with HIV about to commence therapy, lower BMD was associated with lower lean mass, higher adiponectin, and lower OPG, but not HIV disease variables or inflammatory markers (IL6, CRP) (Brown et al. 2013). They concluded that low adiponectin and higher OPG were protective in untreated HIV.

Special Populations

Early HIV

Primary HIV infection (PHI) or HIV seroconversion occurs 2–8 weeks following acquisition of HIV and is characterized in most patients by a severe flu-like illness similar to mononucleosis. This early symptomatic stage of HIV infection is associated with significant HIV viremia and CD4 T lymphocyte depletion, followed by a recovery and steady state achieved at approximately 6 months. During PHI, the inflammatory state results in major cytokine release and impact on bone turnover and biomarkers, as demonstrated in a subset of 52 patients with PHI in the Multicenter AIDS Cohort Study (MACS) cohort (Slama 2015).

Women

Women are often underrepresented in HIV clinical research. Women who are postmenopausal are at great risk than men for low bone density and fragility fracture. This is due to the accelerated bone turnover and bone loss that occurs with declining estrogen levels at menopause, so that instead of the gradual 1% annual loss of bone mass that occurs after the mid-30 years, there is approximately 3% annual bone loss during menopause. This degree of bone loss is mirrored by the bone loss

encountered in the first year of initiating some ART medications such as tenofovir. Thus for women entering menopause and commencing certain ART regimens, there is a double risk of low bone density.

Longitudinal changes in BMD in premenopausal and postmenopausal HIV-infected and HIV-uninfected women were compared in two separate cohorts (Yin et al. 2010, 2012). In the premenopausal period, annual percent decrease in BMD was not different in HIV+ and HIV- women at the lumbar spine (LS) ($-0.8 \pm 0.2\%$ vs. $-0.4 \pm 0.2\%$, $p = 0.20$) and remained similar after adjustment for traditional risk factors. Among HIV+ women, bone loss was associated with vitamin D deficiency and opiate use but not with antiretroviral exposure.

In the postmenopausal period, annualized rates of bone loss adjusted for baseline BMD were higher in HIV+ women by 2.4-fold at the LS (-1.2 vs. -0.5%, $P < 0.0009$). In multivariate models, containing traditional risk factors for osteoporosis, HIV status remained associated with bone loss. Among women on HAART, tenofovir (TDF)-containing regimens were associated with greater bone loss at the spine.

Children

Children and adolescents are at the peak stage in life of bone formation. Those infected with HIV, particularly those taking ART, have lower bone density than HIV-negative controls (Mora et al. 2001). Biomarkers are similarly higher in HIV-infected children (Aurpibul and Puthanakit 2015). The peak bone mass in early adulthood is determined by bone growth in childhood, and there is concern that HIV as well as ART, in particular tenofovir, will result in reduced peak bone mass and subsequent higher risk of osteopenia and osteoporosis (Gafni et al. 2006).

Older Patients/Advanced Patients

Studies in older patients ($\geq$50 year in the CHAMPS study and $\geq$55 years in another study) enrolling cohorts of HIV-positive and HIV-negative men reveal higher levels of low BMD in the HIV-positive group. Biomarkers did not differ between the groups (Sharma et al. 2010).

Osteoporosis and Other Organ Diseases

Osteoporosis may cluster to other noninfective comorbidities (NICM) depicting a polypathology syndrome. This association between bone disease and other NICM may not simply represent an epidemiological overlap between multiple age-related conditions, but rather display a common pathogenetic link. For example, there is an association between osteoporosis and atherosclerosis, showing an intriguing dual effect on vascular and bone health in the general population. In the general

population, individuals with more severe osteoporosis harbor more extensive atherosclerosis and suffer greater cardiovascular events (Schulz et al. 2004; Bellasi et al. 2014).

A cohort of 636 consecutive HIV-infected patients had simultaneous assessment of coronary artery calcium score (CAC) with cardiac CT as well as lumbar and femoral BMD by DXA. After adjusting for age, sex, and traditional and HIV-specific risk factors, patients with CAC had a twofold risk of low femoral BMD (OR: 2.33; 95% CI: 1.09–4.99; $p = 0.02$). CAC was found to be independently associated with low femoral BMD in HIV-infected patients (Bellasi et al. 2014).

Treatment of Osteoporosis

Treating low BMD. Patients with history of fragility fracture, osteoporosis, or osteopenia with 10-year FRAX risk score of > =20% for spine and > =3% for hip should be considered for treatment.

An algorithm for the management of antiretroviral therapy in HIV-infected patients at risk of bone disease has recently been published (Fig. 5).

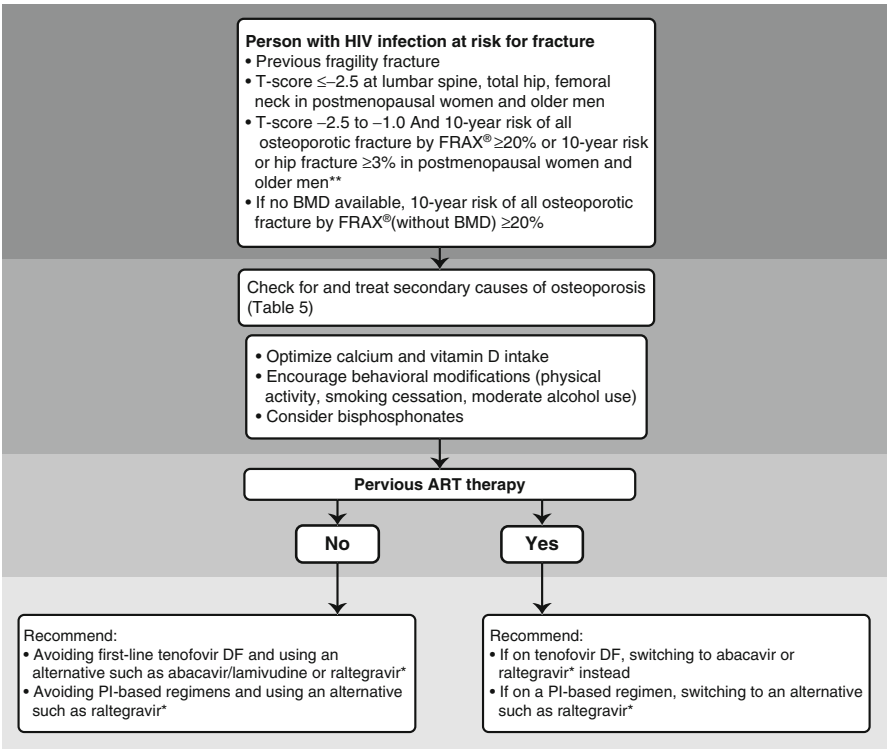

Fig. 5 Recommendations for management of HIV-infected patients at risk of fracture (Brown 2015)

Bisphosphonates have been demonstrated to be effective in treatment of osteoporosis in patients with HIV. Bisphosphonates bind to bone matrix and inhibit osteoclast activity; thus they are antiresorptive agents. Bisphosphonates induce the secretion of OPG by osteoblasts, which bind to RANKL and inhibit osteoclast activity. Osteoblasts also secrete RANKL, which interacts with RANK, found on the surface of osteoclasts, and result in osteoclast differentiation and proliferation. There is a balance of OPG and RANKL, expressed in a ratio and examined in clinical studies. In HIV, one of the mechanisms of bone loss is believed to be the imbalance of OPG:RANKL (Natsag et al. 2015) Bisphosphonate use in HIV has been shown to reduce the levels of biomarkers such as NTX [77]. There is a reduction of bone resorption markers of >40% within 3 months of starting bisphosphonate therapy. This is followed by a reduction in bone formation markers over the next 6–12 months.

In a study that examined treatment of HIV-infected patients on ART who were randomized to receive either alendronate with vitamin D and calcium supplementation or placebo with vitamin D and calcium, those receiving alendronate had significantly greater improvement in BMD at 48 weeks (Guaraldi et al. 2004). High-baseline CTx, sTNFR2, or low vitamin D in patients taking alendronate predicted greatest BMD response at week 48.

The BMD benefits of treatment of HIV-infected patients with annual intravenous zoledronate were demonstrated in a randomized placebo-controlled 2-year study (Bolland et al. 2007). Urinary NTx decreased by 60% at 3 months in the zoledronate treatment arm. Longer-term follow-up revealed ongoing BMD benefits of therapy after 5 years (Grey et al. 2012).

Denosumab, a monoclonal antibody against RANKL, is an approved therapy for osteoporosis administered subcutaneously six monthly. Denosumab produces a very rapid fall in resorption markers, which remain suppressed, with a slower fall in formation markers. The RANKL/RANK interaction is important for the immune system as well as in dendritic T-cell interaction, and interference may impair protection against infection (Cummings et al. 2009). There are to date no studies reporting on safety and efficacy of denosumab in patients with HIV.

Experience in the use of teriparatide, a human recombinant parathyroid hormone which stimulates new bone formation, is currently limited in HIV patients (Wheeler et al. 2015). Teriparatide is most effective in improving trabecular bone formation and thus is potentially useful for spinal osteoporosis and vertebral fracture, with limited effectiveness at the hip and limbs. Measuring biomarkers, P1NP should double within the first month of treatment and continue to increase over the first 6 months.

There are well-designed studies examining changes in bone density and biomarkers with switching ART in HIV. Reasons for switching therapy in HIV are now rarely due to virological failure. This is mostly due to improved adherence in patients using drugs with high levels of efficacy and tolerability. In developed countries large numbers of patients now manage their HIV with a single-tablet co-formulated

regimen. Data from recent studies such as START and TEMPRANO have supported the initiation of therapy in all patients with HIV, as soon as they are diagnosed, irrespective of their CD4 T lymphocyte count or HIV viremia and clinical status (The INSIGHT START Study Group 2015; The TEMPRANO ANRS 12136 Study Group 2015). Thus patients who are asymptomatic and commencing ART have a lower tolerance for drug toxicities. The major reason for switching ART these days is due to tolerability issues associated with the current ART regimen. Another reason for switching is regimen simplification with the availability of single-tablet co-formulated regimens.

The cohorts of patients with HIV are aging, and in most clinics in developed countries, over 50% of patients are over 50 years old. With aging there are increasing comorbidities, which in HIV occur, in larger numbers and at an earlier age than in the general non-HIV-infected population.

Osteonecrosis in HIV

The risk of developing osteonecrosis in HIV is approximately 100-fold compared to the general population. The most common site is femoral head (>75%), but other potential sites are the knee, ankle, shoulder, and small bones of the hands or feet. The main predisposing factors in the general population are prolonged corticosteroid use and excessive alcohol intake. These factors also apply in HIV. Patients treated with ART that includes rPI therapy have risks due to cyp3A4 inhibition by ritonavir, resulting in the accumulation of corticosteroids from, e.g., inhaled fluticasone or corticosteroid joint injections (Samaras et al. 2005).

Osteomalacia

Osteomalacia refers to a condition characterized by defective bone mineralization, typically due to inadequate vitamin D, calcium, or phosphate. In HIV-infected patients, osteomalacia has been associated with tenofovir use (Mateo et al. 2014). These patients typically present with diffuse bone pain, especially in the lower limbs due to multiple stress fractures, and difficulty ambulating. Osteomalacia in HIV is associated with greatly increased levels of biomarkers BAP and OC. A dramatic clinical and laboratory improvement is observed with the discontinuation of the drug confirming the diagnosis of osteomalacia. Of particular concern from a clinical point of view is that pseudofractures in osteomalacia can be misdiagnosed as disseminated bone malignancy, because the whole-body scintigraphy, in the late stage of OM, shows a bone pattern of diffuse or focal tracer's uptake that is similar to bone metastases (De Socio et al. 2012).

Although bone damage is common in HIV patients, alternative causes, including drug toxicity, must be considered. In particular, clinicians should be aware of TDF-induced osteomalacia.

Conclusions

HIV infection has transformed into a chronically manageable condition due to the development of efficacious and well-tolerated ART, used as lifelong therapy. Patients with HIV however experience higher levels of bone turnover with elevated biomarkers and lower BMD, resulting in greater risk of osteopenia, osteoporosis, and fragility fracture. Biomarkers precede and inversely mirror the changes in BMD. In the HIV-infected cohort, multiple factors converge in elevated risk of bone disease: the direct as well as inflammatory effects of HIV, immune dysregulation, the toxicity of ART, and the greater prevalence of lifestyle risk factors and comorbidities.

The use of biomarkers in HIV is largely confined to research and is yet to move into the therapeutic arena. As greater understanding of the role of biomarkers in assessing and monitoring bone disease in HIV develops, it is likely that biomarkers will play an increasing role in HIV management of bone disease. The ultimate goal is to be able to select the patients at highest risk for fracture and the use of biomarkers in conjunction with DXA and FRAX scoring to better identify and offer intervention and treatment to those patients with greatest fracture risk.

Summary Points

- HIV is associated with increased bone turnover, low bone density, and increased fracture risk.
- HIV viremia, immune dysregulation, antiretroviral therapy, greater risk of comorbidities, and lifestyle factors contribute to increased bone turnover and bone disease in HIV.
- Osteopenia, osteoporosis, and fracture occur more frequently in HIV than in the general population, and the difference widens with increasing age.
- Bone formation and resorption biomarkers are significantly increased within 12–24 weeks of initiating antiretroviral therapy.
- Initiation of any antiretroviral regimen for HIV increases bone turnover and reduces bone density 2–6%, particularly in the first year of therapy, then leveling after this.
- Certain antiretrovirals, particularly tenofovir disoproxil fumarate, have been more implicated in bone turnover and bone density changes.
- Switching antiretrovirals or specific therapy with anti-osteoporosis agents can improve bone turnover and bone density.
- Bone biomarkers are used in HIV research and additionally may have a role in monitoring responses to therapy for bone disease.

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Bone Biomarkers in Intrauterine Growth Restriction

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Contents

Abstract

Bone tissue is subject to remodeling during the lifetime of an individual. Through a continuous remodeling cycle, old bone is resorbed by osteoclasts with the formation of cavities that are subsequently filled by osteoblasts, which induce bone formation. Fetal life is associated with a high rate of skeletal growth and intense bone modeling activity. Both fetal and neonatal calcium and bone metabolism are uniquely adapted to meet the specific needs of these developmental periods. The fetus must actively receive sufficient calcium across the placenta to meet the large demands of the rapidly mineralizing skeleton, whereas the neonate must quickly adjust to loss of placental calcium transport, while continuing to undergo rapid skeletal growth. Biochemical markers of bone turnover are reliable indices for measuring changes of bone formation and resorption, reflecting the dynamics of bone metabolism at the cellular level. Due to limitations in the application of bone densitometry during the perinatal period, bone biomarkers are effective alternatives to estimate bone turnover. There is considerable evidence that impaired fetal skeletal growth predisposes to late-onset disorders and

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an accelerated rate of bone loss during later life. As for other adult diseases, intrauterine growth restriction (IUGR) is considered a risk factor for altered bone growth and osteoporosis development. This notion appears to be confirmed by animal data. However, this is less clear in human IUGR neonates. Some studies show a relationship of fetal growth with bone mineral density (BMD), whereas others do not. Similarly, reports determining bone biomarkers provide evidence of unaltered bone metabolism in IUGR fetuses/neonates, although data are not consistent.

Keywords
Intrauterine growth restriction • Fetus • Neonate • Osteoporosis • Bone turnover • Biochemical markers

List of Abbreviations

AGA	Appropriate for gestational age
ALP	Alkaline phosphatase
BALP	Bone-specific alkaline phosphatase
BMC	Bone mineral content
BMD	Bone mineral density
Glu-OC	Undercarboxylated osteocalcin
ICTP	Cross-linked carboxyl terminal telopeptide of type I collagen
IUGR	Intrauterine growth restriction
NTx	N-telopeptide of type 1 collagen
OC	Osteocalcin
OPG	Osteoprotegerin
PICP	Carboxy-terminal propeptide of type I collagen
PINP	Amino-terminal propeptide of type I collagen
PTH	Parathormone
RANKL	Receptor activator of nuclear factor-kB ligand
SGA	Small for gestational age

Key Facts of Bone Turnover in the Fetus/Neonate

- The most prominent bone morphological changes during human life occur in the fetal and neonatal periods.
- Linear growth in the fetus is high during the second trimester of pregnancy, but drops sharply after the 30th week of gestation.
- Fetal bone mineralization and ossification increase toward term.
- Accumulating evidence suggests that fetal bone growth and mineralization are directly affected by fetal growth patterns.
- The results of human reports do not support a constant relationship between fetal growth and bone mineral density.

- Studies investigating the effect of IUGR on bone biomarkers demonstrate conflicting findings, probably related to different causes and/or inadequate definition of IUGR.

Definition of Words and Terms

Appropriate for gestational age (AGA)	Fetuses/neonates with a birth weight between the 10th and 90th percentile for the corresponding gestational age.
Intrauterine growth restriction (IUGR)	The failure of the fetus to achieve his/her intrinsic growth potential, as a consequence of anatomical and/or functional disorders in the feto-placental-maternal unit, resulting to increased morbidity and mortality in intra- and extrauterine life.
Small for gestational age (SGA)	Fetuses/neonates with a birth weight below the 10th percentile for the corresponding gestational age.

Introduction

Throughout life, bone is constantly resorbed and new bone is formed. Both modeling and remodeling occur during skeletal growth. Bone modeling results in linear growth, while remodeling allows expansion of bone circumference and mineral deposition (Camozzi et al. 2007). Biochemical markers provide a dynamic view of the remodeling process, which covers rate of bone growth and turnover (Camozzi et al. 2007). The most prominent bone morphological changes during human life occur in the fetal and neonatal periods (Bhandari et al. 1999). Linear growth in the fetus is high during the second trimester of pregnancy, but drops sharply after the 30th week of gestation (Tanner 1989), whereas fetal bone mineralization and ossification increase toward term (Largo et al. 1980). Due to limitations in the use of bone densitometry during the perinatal period, biochemical markers of bone turnover provide an excellent alternative to examine the state of the skeleton (Bhandari et al. 1999).

Little is known about the dynamics of intrauterine bone formation and resorption, resulting in either normal or low bone mass at birth (Harrast and Kalkwarf 1998). In this regard, of particular importance is the influence of intrauterine growth restriction (IUGR), taken its reported association with low bone mass in infancy (Namgung and Tsang 2000, 2003; Chunga Vega et al. 1996; Beltrand et al. 2008), shorter stature during childhood (Largo et al. 1980), and increased susceptibility for later development of chronic degenerative diseases, including osteoporosis (Cooper et al. 2002; Gale et al. 2001). Interestingly, low placental volume, as usually occurs in IUGR, has

been demonstrated to be a reliable marker of a reduced postnatal skeletal size and increased risk of later fracture (Holroyd et al. 2012).

The growth of the skeleton starts in the first fetal weeks and is completed at the end of teenage. Genetic, environmental, and endocrine factors influence the extent of growth. However, a proportion of the variance of bone mineral content (BMC) found in the general population cannot be explained by genetic factors or childhood environment (Cadogan et al. 1997). Epidemiological studies suggest that part of this residual variation might be explained by the fetal growth pattern (Cooper et al. 1995). In this respect, IUGR is considered an important determinant of BMC at birth and an independent predictor of fetal bone mineralization even when birth weight is maintained within the normal range (Beltrand et al. 2008). Experimental data indicate that IUGR is associated with reduced BMD at birth and programs reduced cortical BMC, dimensions, and strength (Lanham et al. 2008; Cooper et al. 1997; Oliver et al. 2007). Interestingly, the findings of a recent study revealed a negative long-term effect of IUGR in postnatal skeletal growth, mineral content, and bone strength in weanling and adult female rats (Chen et al. 2013). However, the results of human reports do not support a constant relationship between fetal growth and BMD (Namgung et al. 1993; Akcakus et al. 2007). Furthermore, studies investigating the effect of IUGR on bone biomarkers demonstrate conflicting findings (Namgung et al. 1993; Briana et al. 2008, 2009).

This chapter considers published data regarding biochemical markers of bone turnover and overall bone metabolism in IUGR fetuses/neonates.

Biochemical Markers of Bone Turnover in the Fetus/Neonate

Assessment of bone health in neonates is important, since early events in life may predispose the adult to degenerative diseases (McDevitt and Ahmed 2007). Information on bone metabolism can be attained by blood and urine laboratory tests. Recently developed bone-specific markers are categorized by bone remodeling process, i.e., bone formation and resorption (Wada et al. 2009). Several biochemical markers of bone turnover have extensively been used in studies investigating the state of the skeleton, as well as bone metabolism in the fetus/neonate.

Serum total alkaline phosphatase (ALP) is a nonspecific indicator of osteoblastic activity; thus, levels of the bone isoenzyme [bone-specific alkaline phosphatase (BALP)] are measured in order to improve specificity (Okesina et al. 1995). In the perinatal period, BALP is a reliable marker of bone formation (Uemura et al. 2002), unlike osteocalcin (OC), which, due to placental clearance, is possibly not suitable for evaluating bone formation in the fetus/neonate (Rodin et al. 1989). In contrast, in cord and early neonatal blood, undercarboxylated osteocalcin (Glu-OC) is the major component of OC (Shimizu et al. 2002) and is considered a valid marker of bone formation, involved in the regulation of matrix mineralization (Verhaeghe et al. 1995).

Quantification of urine cross-linked N-telopeptide of type I collagen (NTx) (which is a direct product of osteoclastic proteolysis) has been widely used as one

of the most responsive and specific indicators of the bone catabolism process in infants and children (Bollen and Eyre 1994; Lapillonne et al. 2002). Moreover, serum NTx is useful in evaluating bone resorption (Scariano et al. 1998), especially in pregnancy (Kaji et al. 2007) and probably in the perinatal period, since its levels are not influenced by the dynamic changes of renal function during the abovementioned periods (Wilkins 1992).

The system of osteoprotegerin (OPG)/receptor activator of nuclear factor-kB ligand (RANKL)/receptor activator of nuclear factor-kB (RANK) is the dominant, final regulator of osteoclastogenesis and bone resorption (Khosla 2001). RANKL, a member of the tumor necrosis factor (TNF) family, produced by osteoblastic lineage cells and activated T lymphocytes, is the key factor for osteoclast differentiation, activation, and survival, resulting in bone resorption and calcium mobilization from the bones (Lacey et al. 1998). OPG, a TNF-like protein secreted by osteoblasts, is capable of blocking the binding between RANKL and its receptor RANK, thus inhibiting osteoclast formation and bone resorption (Lacey et al. 1998; Simonet et al. 1997).

Data on collagen turnover in the fetus are scarce. In theory, collagen biosynthesis and degradation may be altered in conditions affecting fetal growth. Major changes in collagen metabolism have been reported to take place, depending on growth, development, and aging (Prockop et al. 1979). Type I collagen is present as a major component of the extracellular matrix in most human tissues and as the only collagen type in mineralized bone (Prockop et al. 1979). The turnover of collagen is reflected in blood by markers of its synthesis and degradation. The markers of synthesis include the carboxy-terminal propeptide of type I procollagen (PICP) and the N-terminal propeptide of type III procollagen (PIIINP), whereas cross-linked telopeptide of type I collagen (ICTP) is a marker of collagen degradation. Circulating PICP and ICTP concentrations are considered to be reliable biochemical markers of bone formation and resorption, respectively (Ogueh et al. 1998).

Proteins synthesized by the group of wingless (Wnt) genes are key mediators of osteoblastogenesis and govern the formation of the fetal skeleton (Miller 2002). Wnt signaling is modulated by several negative regulators. The best studied of these is Dickkopf-1 (DKK-1), a cysteine-rich protein, which disrupts the Wnt cascade, resulting in the inhibition of osteoblast differentiation. Deletion of a single allele of DKK-1 increases bone mass in mice (Morvan et al. 2006). Increased DKK-1 levels may lead to enhanced osteoblast-dependent osteoclastogenesis (Fujita and Janz 2007). Additionally, circulating DKK-1 levels have been reported to be sensitive enough to reflect its expression in bone microenvironment (Fujita and Janz 2007).

Biochemical Markers of Bone Turnover in IUGR

While alterations in bone formation and resorption, by using biochemical markers, have been reported in normal fetuses/neonates, there is little published data on the effects of IUGR on fetal/neonatal bone turnover.

Previous studies have shown that being born small for gestational age (SGA) was associated with decreased bone formation, possibly due to impaired transplacental

mineral supply from the mother to the fetus. BMC was lower in SGA infants at birth and was associated with a decrease in plasma OC levels, suggesting that fetal mineralization is affected by the fetal growth pattern (Namgung et al. 1993; Akcakus et al. 2007; Namgung and Tsang 2000). Similarly, OC levels were 20% lower in IUGR neonates than in age- or weight-matched newborns (Verhaeghe et al. 1995). Another report, by investigating amniotic fluid concentrations of the collagen markers of bone formation and degradation, respectively, PICP and ICTP showed a reduction in bone formation, but no alteration in bone catabolism, when fetal growth was compromised (Harrast and Kalkwarf 1998). In contrast, similar serum concentrations of PICP and ICTP have been previously reported in term or near-term infants (Namgung et al. 1996; Hytinantti et al. 2000). The authors concluded that reduced BMC in SGA infants is predominantly related to a lower supply of minerals rather than defective regulation of bone collagen type I metabolism (Namgung et al. 1996). However, a contrary aspect has been also reported, suggesting that very active bone formation and resorption of type I collagen significantly increase with fetal growth in both preterm and full-term infants (Nakano et al. 2006). Similarly, Kajantie et al. showed a positive correlation between cord plasma concentrations of amino-terminal propeptide of type I collagen (PINP, marker of type I collagen synthesis), as well as ICTP and birth weight in preterm infants before 32 weeks of gestation (Kajantie et al. 2001).

More recent studies investigated biochemical markers of bone turnover in well-defined IUGR fetuses/neonates. Low birth weight does not necessarily equate to decreased fetal growth, since infants can be SGA, as a result of individual normal genetic variation. Thus, the use of customized birth weight standards that are adjusted for significant determinants of birth weight is considered more appropriate for identifying subjects with true restricted fetal growth, which are at risk for experiencing short- and long-term adverse outcomes. In the above studies, IUGR subjects were defined based on customized fetal growth estimation, adjusting for maternal and fetal characteristics, which allowed precise evaluation of fetal growth restriction by identifying newborns who have failed to reach their genetic potential of growth (Mongelli and Gardosi 1995).

More specifically, the results of a recent study (Gourgiotis et al. 2012) indicated that type I collagen turnover, as expressed by elevated fetal PICP, as well as fetal and neonatal ICTP levels, is enhanced in IUGR fetuses/neonates. The procollagen gene is expressed in the proliferative phase of bone formation, which results in the production of the characteristic osteoblast extracellular matrix (Lian and Stein 1992). Although SGA infants present with decreased BMC, their bone speed of sound – a biophysical property of bone, reflecting bone protein matrix – is higher (Littner et al. 2005). Thus, it may be speculated that the IUGR state probably influences BMD and bone protein matrix in opposite directions. Therefore, the osteoblast cell function in the proliferative phase of bone formation may be enhanced, reflecting activation of procollagen gene expression and procollagen synthesis. Nevertheless, one cannot rule out the possibility that IUGR may have a diverse effect on the renal clearance of these markers (Schreuder et al. 2006).

In contrast, no significant differences in markers of bone formation (BALP, ALP, OC) and resorption (parathormone [PTH], NTx), as well as levels of calcium and

phosphorus between IUGR and appropriate for gestational age (AGA) infants at term have been documented in a previous report (Briana et al. 2008). However, osteocalcin, as already mentioned, may not be a reliable marker of bone formation during the perinatal period (Rodin et al. 1989). In accordance with these results, no significant differences in the serum marker of bone resorption (ICTP) were previously reported between IUGR fetuses and controls (Harrast and Kalkwarf 1998; Namgung and Tsang 2000). Another possible explanation for these findings may relate to the fact that IUGR fetuses of this cohort presented with asymmetric growth restriction, i.e., body weight was much more reduced than body length (Brodsky and Christou 2004). Placental weight, and therefore placental exchange area, is correlated with fetal weight rather than length, whereas skeletal mass is related to length (Brodsky and Christou 2004). Consequently, calcium supply should be sufficient for the relatively unaffected skeletal mass in IUGR, as also indicated by the similar serum calcium concentrations between IUGR cases and AGA controls. It could also be speculated that the effects of major factors which program bone growth in utero may become apparent later in life (Cooper et al. 1997; Engelbregt et al. 2004). Evidence suggests that birth weight predicts the basal levels of growth hormone and cortisol, which determine the bone loss rate in adulthood (Fall et al. 1998). These data are compatible with the hypothesis that IUGR alters the sensitivity of the growth plate to growth hormone and cortisol, predisposing to reduced peak skeletal size and mineralization later in life. Therefore, it could be speculated that skeletal mass may not be already impaired in asymmetric IUGR fetuses and neonates, but the effect of IUGR may become apparent in adulthood (Briana et al. 2008).

Following this publication, another study, by investigating the dominant component of OC in fetal and neonatal blood (Glu-OC) in well-documented IUGR subjects, did not indicate lower bone formation in IUGRs (Briana et al. 2012).

The incidence of neonatal hypocalcaemia is reportedly increased in IUGR, but it has been hypothesized that this may be associated with birth asphyxia rather than IUGR per se (Tsang et al. 1975). Accordingly, placental calcium pump is documented to be activated in asymmetric IUGR, consistent with an increased transplacental calcium transport, which may be secondary to increased fetal calcium demand (Strid et al. 2003). In support of this finding, the same study demonstrated lower DKK-1 concentrations in IUGR fetuses. Wnt signaling increases bone mass via a number of mechanisms, including renewal of stem cells, stimulation of pre-osteoblast replication, induction of osteoblastogenesis, and inhibition of osteoblast and osteocyte apoptosis (Krishnan et al. 2006). DKK-1 interacts with Wnt co-receptors, resulting in the inhibition of osteoblast synthesis and bone formation (Qiang et al. 2008). Therefore, the downregulation of DKK-1 concentrations in IUGR probably represents a compensatory mechanism, which favors the formation of mineralized bone. It may be speculated that this mechanism could contribute to normal bone formation in the IUGR fetus and neonate (Briana et al. 2012).

The identification of the osteoclastogenesis inducer, RANKL, its cognate receptor RANK, and its decoy receptor OPG has contributed to the great advance in the understanding of the molecular mechanisms involved in the normal physiology of the skeleton, especially during pregnancy and the perinatal period. Another study, by

determining the dominant regulators of bone resorption in characteristic IUGR subjects, indicated that the low bone mass in IUGR may not be associated with higher resorption rates, since circulating concentrations of OPG and serum RANKL, probably reflecting cellular ones, were similar between IUGR cases and AGA controls (Briana et al. 2009). Interestingly, Tenta et al. recently revealed a remarkable upregulation of the OPG/RANKL ratio in SGA neonates, pointing out the role of bone turnover in compensating for the delayed neonatal growth (Tenta et al. 2013).

Considerable interest has recently been focused on the role of adipocytokines, such as leptin, adiponectin, and the newly identified resistin, visfatin, and apelin in the complex control of metabolism, energy homeostasis, bone biology, and, as far as the fetus is concerned, overall fetal growth and development (Magni et al. 2010; Briana and Malamitsi-Puchner 2009). Furthermore, a differential regulation of adipocytokines in the IUGR state has been demonstrated (Briana and Malamitsi-Puchner 2009), and fetal leptin dysregulation in IUGR has been proposed to be predictive of adult disease occurrence, including osteoporosis (Alexe et al. 2006). A huge amount of data has documented the effect of leptin on bone modeling and remodeling processes (Alexe et al. 2006). The findings of a recent study (Briana et al. 2014) indicated a negative correlation between fetal/neonatal resistin concentrations and respective concentrations of both sRANKL and NTx (established markers of bone resorption) in both IUGR and AGA groups. Furthermore, in the IUGR group, the data suggest a positive association of both fetal visfatin and apelin concentrations with respective concentrations of BALP (a reliable marker of bone formation). Thus, it may be hypothesized that all three studied adipocytokines may exert a protective effect on bone metabolism, either by inhibiting bone resorption or promoting bone formation in both normal and IUGR pregnancies (Briana et al. 2014).

Taken together, accumulative evidence suggests that fetal bone growth and mineralization are directly affected by fetal growth patterns. IUGR has been identified as an independent risk factor for adverse fetal bone growth, shorter stature in childhood, and increased susceptibility to later osteoporosis development. However, published data on biochemical markers of bone turnover revealed conflicting results, which may probably be related to different causes and/or inadequate definition of IUGR. Further investigations into the programming of adult bone health may lead to the prevention of adult bone diseases, in particular disorders of bone strength, such as osteoporosis. This is of major public health relevance, as nutritional interventions at critical developmental periods in the future may be able to ameliorate diseases with developmental origins.

Summary Points

- Bone tissue is subject to remodeling during the lifetime of an individual.
- Fetal life is associated with a high rate of skeletal growth and intense bone modeling activity.
- Both fetal and neonatal calcium and bone metabolism are uniquely adapted to meet the specific needs of these developmental periods.

- Biochemical markers of bone turnover are reliable indices for measuring changes of bone formation and resorption, reflecting the dynamics of bone metabolism at the cellular level.
- Due to limitations in the application of bone densitometry during the perinatal period, bone biomarkers are effective alternatives to estimate bone turnover.
- Considerable evidence suggests that impaired fetal skeletal growth predisposes to late-onset disorders and an accelerated rate of bone loss during later life.
- Intrauterine growth restriction is considered a risk factor for altered bone growth and osteoporosis development.
- Some studies show a relationship of fetal growth with bone mineral density, whereas others do not.
- Reports determining bone biomarkers provide evidence of unaltered bone metabolism in IUGR fetuses/neonates, although data are not consistent.

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Bone Disease of Prematurity: Calcium and Phosphorus as Urinary Biomarkers

35

Eveline Staub

Contents

Abstracts

Urinary calcium and phosphorus have been used for the past decades as biomarkers for osteopenia of prematurity. As opposed to the actual diagnosis of decreased bone mineral content, studies have mainly focused on detecting the early phase of mineral deficiency, where bone demineralization might not yet be manifest in radiological images or, worse and as a late complication, by pathological bone fractures. Two methods of urinary mineral markers are used for this

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purpose: pure urine concentration of calcium and phosphorus or urine calcium/creatinine and phosphorus/creatinine ratios. Urinary calcium and phosphorus concentrations in spot urine samples have been used to guide individualized supplementation over and above the standardized fortified preterm nutrition to achieve bone mineral accretion rates similar to in utero conditions. The idea behind this concept, referred to as slight surplus supply, is that the simultaneous low-level excretion of calcium and phosphorus provides sufficient supply to guarantee adequate bone mineralization, independent of age, weight, and urine volume. Potential pitfalls include the immaturity of the kidneys in preterm infants as well as drugs and kidney diseases, both of which can alter the pattern of mineral excretion without accurately reflecting serum levels. With the other method of urinary mineral assessment, calcium/creatinine and phosphorus/creatinine ratios take into account volume-induced concentration changes. The measurement of excretion of any urine metabolite expressed as ratios over creatinine is standard practice in adult and pediatric nephrology. However, the creatinine excretion in preterm infants does not necessarily only express urine concentration but is possibly dependent on gestational and postnatal age, type of nutrition and renal function. The establishment of reference ranges for calcium/creatinine and phosphorus/creatinine ratios in preterm infants has been attempted but is subject to local nutritional practices and has not been tested against bone mineral content or in the context of individualized mineral supplementation. The existing literature does not answer the question of the superiority of either of the two methods in detecting osteopenia of prematurity. For guidance in individualized calcium and phosphorus supplementation, the concept of slight surplus supply has been shown to work in improving bone mineral accretion (despite methodological shortcomings), while the application of the mineral/creatinine ratios for this purpose is still lacking.

Keywords

Osteopenia • Rickets • Metabolic bone disease • Bone metabolism • Nutrition • Supplementation • Calcium • Phosphorus • Mineral • Premature • Spot urine • Excretion

List of Abbreviations

ALP	Alkaline phosphatase
Ca	Calcium
Crea	Creatinine
DEXA	Dual energy X-ray absorptiometry
DPA	Double photon absorptiometry
LBW	Low birth weight
P	Phosphorus
SPA	Single photon absorptiometry
VLBW	Very low birth weight

Key Facts of Osteopenia of Prematurity

- The simultaneous presence of sufficient amounts of the two minerals calcium and phosphorus is required for the adequate mineralization (i.e., hardening) of developing bones in children.
- Babies born as early as 4 months before term and with birth weights well below 1.5 kg do not receive the full quantity of calcium and phosphorus via the placenta from their mothers compared to babies born at term.
- Breast milk and regular infant formula do not contain enough calcium and phosphorus for the large amounts that preterm babies require during the first postnatal weeks and months, where their bones grow and mineralize at a fast rate.
- In the absence of sufficient calcium and phosphorus supply, preterm babies may develop osteopenia of prematurity, a condition with brittle bones and reduced growth, and fractures of the ribs or long bones of the limbs as potential complications.
- Expressed breast milk for preterm infants is fortified with human milk fortifier, containing additional calories, protein, and fat and, to prevent osteopenia, extra calcium and phosphorus. If fed artificial formula milk, products tailored to the needs of preterm babies are used.
- Even this extra mineral supply is sometimes not enough when babies are very ill, receive specific drugs, or are immobilized in their incubator for extended periods of time, which is why calcium and phosphorus above and beyond the standard may be needed.
- Too much calcium and/or phosphorus can be harmful for the kidneys, interfere with digestion, or even cause toxic symptoms. Hence careful monitoring of calcium and phosphorus via the excretion in urine has been adopted as a strategy for guiding supplementation.

Apart from preventing severe complications of osteopenia (such as fractures) in the short term, a carefully tailored supplementation of calcium and phosphorus should aim for adequate growth and bone mineralization, so that former preterm babies grow to an average height and have strong bones when they reach adulthood.

Definitions of Words and Terms

Bone mineral content	A measure of bone mass, which is a combination of bone density and bone size and representing the total of mineral contained in bone in grams. Even though bone mineral content is a good surrogate marker for bone stability, the size dependency means that a shorter subject will have lower bone

	mineral content compared to a subject of longer length, even if the (shorter) bones are completely healthy. Bone mineral density is a dependent factor of bone mineral content and is defined as bone mineral content per specific volume of bone (expressed as g/cm^2).
Calcipenia	A state of calcium deficiency.
Calciuria	Excretion of calcium in urine.
Corticosteroids	A class of drugs mimicking the action of steroid hormones produced in the adrenal glands, such as Prednisone, Dexamethasone, and Betamethasone. Their main action is the reduction of inflammation, boosting of stress response, and regulation of various body salts.
Crystal hydroxyl apatite	The crystalized form of calcium and phosphorus together with hydroxy groups (groups consisting of oxygen and hydrogen atoms) is called crystal hydroxyl apatite ($Ca_{10}[PO_4]_6[OH]_2$) and constitutes the mineralized hard part of the bone.
Diuretics	A group of drugs increasing the urine production and urine flow in the kidney. Administration of diuretics such as Furosemide promotes the loss of water, salt, and minerals from the body. They are generally used in any disease where excess body fluid causes problems, such as heart failure, chronic lung problems, high blood pressure, and kidney failure.
Dolichocephaly	Deviation of the skull shape with elongation of the diameter from the front to the back of the head and flattening on both sides, resulting in a long oval head shape with narrow face. This skull shape is commonly observed in very preterm infants and hypothesized to be a result of the reduced firmness of the skull bones during a time of inappropriate bone mineralization after preterm birth.
Double photon absorptiometry	A beam with a combination of two radioactive particles (photons) of two different energy levels is sent through tissue of the

	forearm. Soft tissue absorbs more of one energy level, while bone absorbs more of the other. The difference in transmission of the two energy levels provides a more accurate estimation of bone density than single photon absorptiometry, because it accounts better for the soft tissue component.
Dual energy X-ray absorptiometry	This technique works with the same principle as the double photon absorptiometry, but the beam of photons originates from an X-ray source rather than radioactive source. Absorption of the two different energies at prespecified body sites (most commonly the wrist, lumbar spine, or hip) is measured by a detector at the opposite side of the beam and bone density calculated from the differing absorption rates of the two photon beams.
Gestational age	As per definition of the American Association of Pediatrics on age terminology during the perinatal period (Engle et al. 2004), gestational age is defined as "the time (in weeks) elapsed between the first day of the last menstrual period and the day of delivery". This definition is traditionally used to determine the expected due date, which is 40 weeks from the first day of the last menstrual period, and to describe the age of the baby at delivery.
In utero	From Latin "inside the womb", generally referring to the infant or processes during pregnancy.
LBW infant	Low birth weight infant, defined by a birth weight of less than 2000 g.
Methylxanthines	A group of drugs including caffeine, theophylline, and aminophylline, exercising stimulating action on a number of tissues. In neonatology, caffeine and theophylline are used for their stimulating effect on the respiratory centre in the brain stem to prevent and treat apnea of prematurity, where the preterm infant stops breathing for up to 20 s due to the immaturity of the

	respiratory centre, often accompanied by reduced levels of oxygen in the blood (hypoxia) and slowing of the heart rate (bradycardia).
Multicomponent human milk fortifier	Commercially available powder or liquid containing carbohydrates, protein, fat, minerals, and vitamin. Added to expressed breast milk, milk fortifiers provide preterm babies with supplemental substrates to cater for increased requirements in times of accelerated growth after preterm birth.
Nephrocalcinosis	Diffuse calcium deposits in the tissue of the kidney, indicating a surplus of calcium excretion. Most commonly seen in ultrasound examinations of the kidneys as brighter than average ultrasound signal in the inner (medullary) layer of the kidneys, in later stages also diffuse throughout the kidney tissue and may cause clumps of calcium deposits. Nephrocalcinosis in preterm infants stems from increased calcium excretion resulting in precipitates in the layer of the tubules due to their immaturity.
Parathyroid hormone	A hormone produced in the parathyroid gland adjacent to the thyroid gland. The main effect is the regulation of serum calcium and phosphorus level by controlling absorption in gut and bone, and excretion in the kidneys.
Phosphaturia	Excretion of phosphorus in urine.
Phospopenia	A state of phosphorus deficiency.
Postnatal age	As per definition of the American Association of Pediatrics on age terminology during the perinatal period (Engle et al. 2004), postnatal age is defined as “the time elapsed after birth”, irresponsible of gestational age at birth. To account for prematurity, postmenstrual age rather than postnatal age is often used in neonatology. Postmenstrual age is defined as “the time elapsed between the first day of the last menstrual period and birth (gestational

	age) plus the time elapsed after birth (postnatal age)". For example, a preterm baby born at 24 weeks at 6 weeks after birth has a postmenstrual age of 30 weeks, while a term baby born at 40 weeks at 6 weeks after birth has a postmenstrual age of 46 weeks, even though the postnatal ages of the two babies are the same.
Prematurity	A normal pregnancy lasts 40 weeks from the first day of the last menstrual period to the day of delivery. Babies born before 37 completed weeks of the pregnancy (or gestation) are by definition premature or preterm. Babies born before 32 or 28 weeks gestation are called very preterm or extremely preterm babies, respectively. As with the categories of birth weights, these categories of prematurity help in the comparison of complications and outcomes, because babies born very early have generally more severe complications and worse outcomes than babies born closer to term.
Renal tubules	The kidney is composed of thousands of nephrons, consisting of the glomerulus (the filtering unit, where the blood is filtered in a small cluster of blood vessels) and the tubule (along which the filtered liquid passes). During the passage through the tubules, various substances are excreted into and reabsorbed from the filtered liquid, eventually forming the urine.
ROC curve	ROC stands for receiver operator characteristics. The ROC curve represents a statistic tool to assess the accuracy of a diagnostic test at different cut-off values. On the curve, the truly positive results (or the so-called sensitivity) of a diagnostic test is plotted against the falsely negative results of the same test (1-specificity) and allows us to estimate at which value of a test result the best compromise between the two can be found, i.e., at which value we can be reasonably certain that the result reflects the truth (presence or absence of a disease).

Single photon absorptiometry	A single beam of radioactive particles (photons) is sent through the tissue of the forearm, where it passes through soft tissue and bone. A detector on the opposite side measures the absorption of the beam after having passed through tissue. The density of the bone is correlated with the amount of particles absorbed through the arm: the more particles are absorbed, the thicker the bone and vice versa.
Tibial speed of sound	Ultrasound waves are emitted from an ultrasound probe through tissue of the shin and reflected at various rates at the interfaces of tissue components. Fatty tissue, muscle tissue, and bone tissue influence the speed at which the sound travels through them by their physical properties. The ultrasound probe then also serves as a detector for the reflected sound, extrapolating the density of individual tissues. Focused on the sound echo reflected by the tibial (shin) bone, the magnitude of the tibial speed of sound serves as a measure for bone density.
Transplacental	From Latin "across the placenta", meaning by way of connection through the placenta, the unit connecting mother and fetus during the pregnancy.
VLBW infant	Very low birth weight infant defined by a birth weight of less than 1500 g. Many studies on preterm infants use the classification of birth weights into LBW and VLBW to facilitate comparisons between groups of infants with equal risk profiles (as smaller infants are at higher risks for severe complications and long-term sequelae).

Introduction

Osteopenia of Prematurity

The vast majority (60–80%) of total body calcium (Ca) and phosphorus (P) is transferred via the placenta from mother to fetus during the last trimester of pregnancy, i.e., from week 28 to term (40 weeks gestation). This parallels an

exponential increase in fetal bone mineralization, where Ca and P are built into the bone matrix in the form of crystal apatite ($Ca_{10}[PO_4]_6[OH]_2$) (Rigo et al. 2000). The trans placental mineral transport is active in nature against a concentration gradient, because fetuses maintain a higher blood concentration of Ca and P than their mothers in order to account for the extraordinary high requirements during the rapid skeletal development (Kovacs 2014; Namgung and Tsang 2012). Preterm babies born as early as 24 weeks gestation (i.e., up to 4 months before term) miss out on these important events. Additional unfavorable postnatal circumstances such as insufficient nutritional supply, severe illness, and relative immobilization do not allow for a mineral accretion rate similar to in utero conditions. The result is suboptimal bone mineralization, a condition which has been variably named osteopenia of prematurity, metabolic bone disease of prematurity, bone mineral deficiency of prematurity, or rickets of prematurity (Rehman and Narchi 2015). Short term consequences are thinning of the bone and eventually pathological fractures (Fig. 1). But the condition may well have long-term implications. The phenotypical dolichocephalic skull flattening of preterm babies has been attributed to decreased bone mineral content (Pohlandt 1994a) (Fig. 2), and the associated misshaped orbits and eyeballs could be at least partly responsible for myopia later in life (Pohlandt 1994b). Development of dental enamel might be impaired and

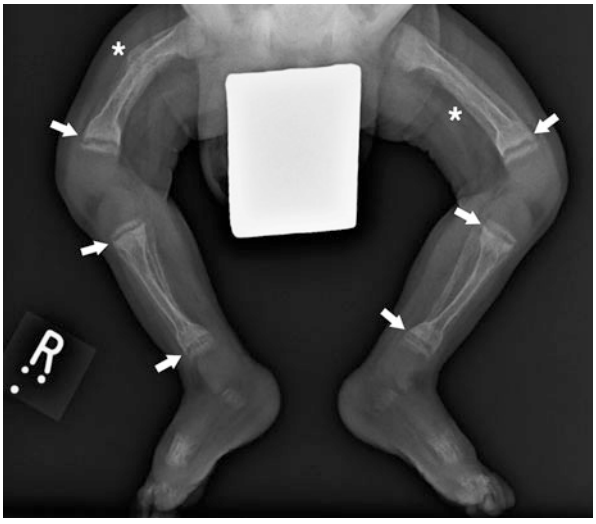

Fig. 1 Severe osteopenia of prematurity. X-ray of the lower limbs in preterm boy born at 24 weeks gestation, postmenstrual age of 44 weeks at the time of imaging study. After repeated episodes of feeding intolerance and severe lung disease with extended periods of mechanical ventilation and sedation led to poor nutritional intake with mineral deficiency, the patient suffered from severe osteopenia of prematurity. The X-ray shows highly demineralized bone structure. Metaphyseal regions are cupped and frayed (white arrows). Bilateral femoral fractures of different ages (white asterisks) (With kind permission of the parents)

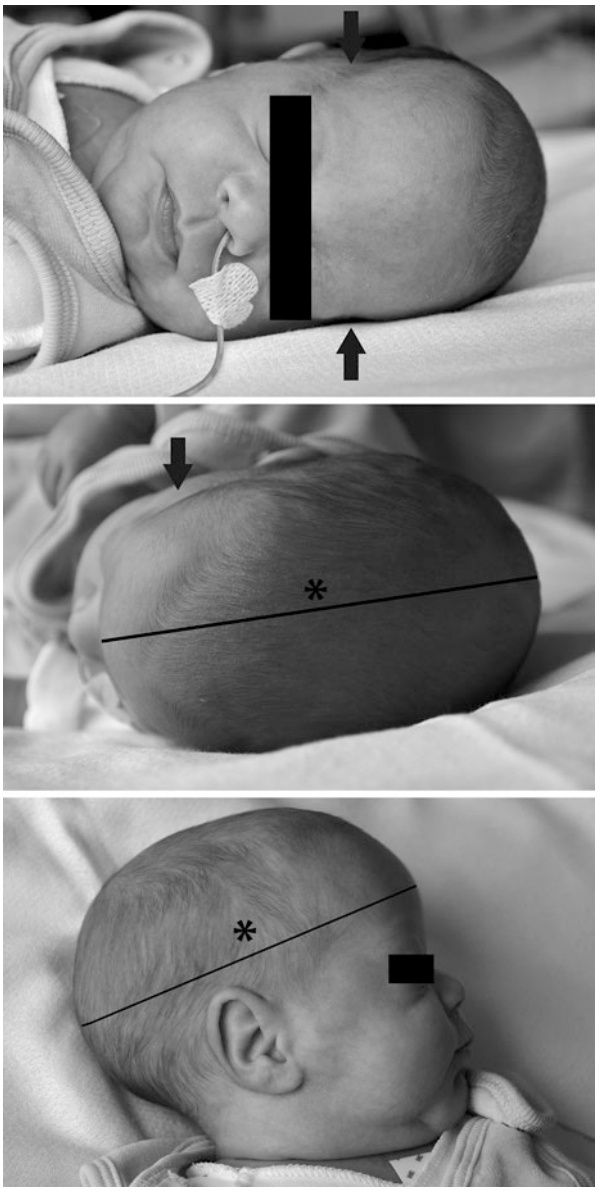

Fig. 2 Dolichocephalic skull shape. This boy (born at 27 weeks gestation, at postmenstrual age 36 weeks) exhibits features typically seen in dolichocephalic skull shape of preterm infants, with narrow face, flattening of the temporal bones (black arrow heads), and long fronto-occipital diameter (*) (With kind permission of the parents)

predispose preterm infants to tooth decay (Grahnen et al. 1974). Importantly, osteopenia of prematurity impairs longitudinal growth, possibly beyond adolescent age. Various authors debate whether or not this results in reduced final length and/or decreased peak bone mass in former preterm infants (Chan et al. 2008; Peralta-Carcelen et al. 2000; Weiler et al. 2002).

Nutritional Support/Factors Influencing Bone Mineralization Ex Utero

Breast milk is usually well equipped with all macro- and micronutrients for optimal growth and development of the term infant and provides invaluable immunological protection for immature babies. But breast milk does not provide enough Ca and P to sustain the very rapid postnatal growth rates of preterm and low birth weight (LBW) babies. Multicomponent human milk fortifiers added to expressed breast milk have been shown to improve weight gain, linear and head growth (Kuschel and Harding 2004). Nutritional guidelines recommend 120–200 mg/kg/d of Ca and 60–140 mg/kg/d of P intake in stable growing preterm infants (Agostoni et al. 2010; Mimouni et al. 2014). However, these references do not account for the variability in growth rates (which determine Ca and P requirements) and intestinal absorption of Ca and P. Conditional to sufficient supply of Vitamin D, newborns absorb Ca contained in breast milk much better than from infant formula (Mimouni et al. 2014). Drugs frequently used during the newborn period such as methylxanthines, diuretics, and corticosteroids influence Ca and P excretion and alter mineral requirements (Gimpel et al. 2010; Zanardo et al. 1995; Cranefield et al. 2004). Additionally, babies born prematurely suffer from reduced physical activity in the incubator and lack the mechanical strain against the uterine wall which normally stimulates bone formation and growth during pregnancy (Miller 2003). Severe respiratory illness requiring mechanical ventilation and subsequently sedation aggravate the reduced bone loading. The summary of the factors influencing bone mineralization begs for individualized monitoring and adjustment of mineral supplementation in the preterm infant. This is even more true in the face of the risks presented by undifferentiated and unbalanced Ca and P supplementation: nephrocalcinosis, decreased intestinal iron absorption, increased fecal viscosity through over supplementation with Ca (Hallberg 1998; Quinlan et al. 1995; Schell-Feith et al. 2010), and neurological symptoms as signs of a high and unbalanced P intake (Greer 1989).

Issues of Diagnosing Osteopenia and Monitoring Supplementation

Methods for accurately diagnosing mineral deficiency in preterm infants are few and far between. Radiological evidence of demineralized bone with conventional X-ray technique develops only after substantial loss of bone mineral content and is considered a late sign of osteopenia of prematurity (Fig. 1) (Masel et al. 1982). Single and double photon absorptiometry (SPA and DPA) as well as dual-energy X-ray absorptiometry (DEXA) have been evaluated for assessement of bone mineral content in small infants (Kalkwarf et al. 2014; Pohlandt 1994c). They involve ionizing radiation and bear technical inaccuracies in subjects with small weights and sizes. They are in general not readily available diagnostic tools in neonatal intensive care units, and transport of the tiny preterm infants to an imaging facility is resource intensive and risky. Quantitative ultrasound such as tibial speed of sound

shows promises in diagnosing bone mineral deficiency as a noninvasive bedside tool and includes the possibility of longitudinal investigation (Fewtrell et al. 2008). However, this technique has not been validated for monitoring and guiding mineral supplementation.

Various serum parameters of bone metabolism have been proposed for the purpose of diagnosing metabolic bone disease of prematurity and monitoring supplementation.

Serum Ca represents less than 1% of total body Ca but is tightly regulated due to its importance for various physiological processes (such as muscle contractility, neuronal transmission, and cell signaling). Control of serum Ca levels is through hormonal regulation by parathyroid hormone, Vitamin D, and calcitonin, which influence intestinal absorption, renal reabsorption or excretion, and release from bone mass into the extracellular space (Kovacs 2014; Brown 2007) (Fig. 3). Even when lacking adequate enteral supply, serum Ca levels will be kept at a remarkably constant level over a long period of time by mobilizing Ca from, and at the expense of, bone matrix (Harrison and Gibson 2013; Namgung and Tsang 2012). In the context of metabolic

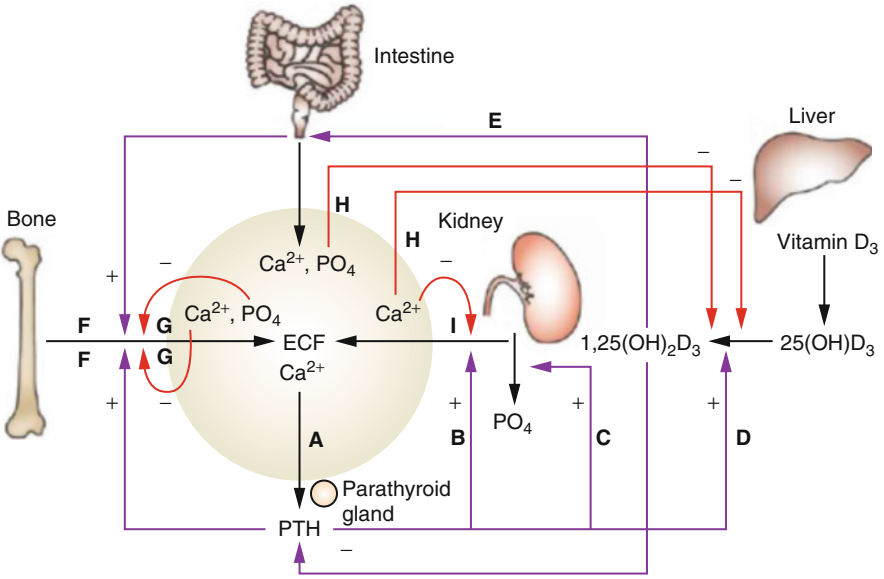

Fig. 3 Regulation of calcium homeostasis. A decrease in calcium (Ca^{2+}, abbreviated Ca) levels in the extracellular fluid (ECF) stimulates the parathyroid gland to excrete parathyroid hormone(PTH) (A) which in turn increases the reabsorption of Ca from the renal distal tubules (B) and excretion of phosphorus (PO_4, abbreviated P) in urine, (C) and promotes synthesis of 1,25-Dihydroxy-Vitamin D_3 ($1,nOH)_2D_3$) (D). Vitamin D then increases intestinal absorption of Ca and P (E) and, together with PHT, release of Ca and P from bone (F). Elevation of Ca in the ECF inhibits reabsorption of Ca (and P) from bone (G) and hydroxylation of 1,25-Hydroxy-Vitamin D_3 (H). High Ca levels also inhibit Ca reabsorption in distal tubule of the kidney (I). Purple arrows indicate actions increasing Ca levels, red arrows indicate actions decreasing Ca levels (Figure adapted from Brown 2007, with permission from the publishers)

bone disease of prematurity, this means that low levels of serum Ca indicate an advanced stage of osteopenia. However, in many cases low serum Ca is an expression of other regulatory problems such as hyperparathyroidism or vitamin D metabolism disorders rather than inappropriate Ca supply with subsequent osteopenia; low serum Ca levels generally warrant further investigations (Cho et al. 2015).

P is more widely distributed in non-osseous tissue than Ca: approximately 15% of total body P is found in the extracellular space in an inorganic form or as part of structural macromolecules involved in energy metabolism, DNA synthesis, and intracellular signaling. Regulation of serum P is mainly through the kidneys, where a high fraction is reabsorbed via tubules under tight control by parathyroid hormone, fibroblast growth factor 23, and Vitamin D (Fig. 4) (Torres et al. 2007). In

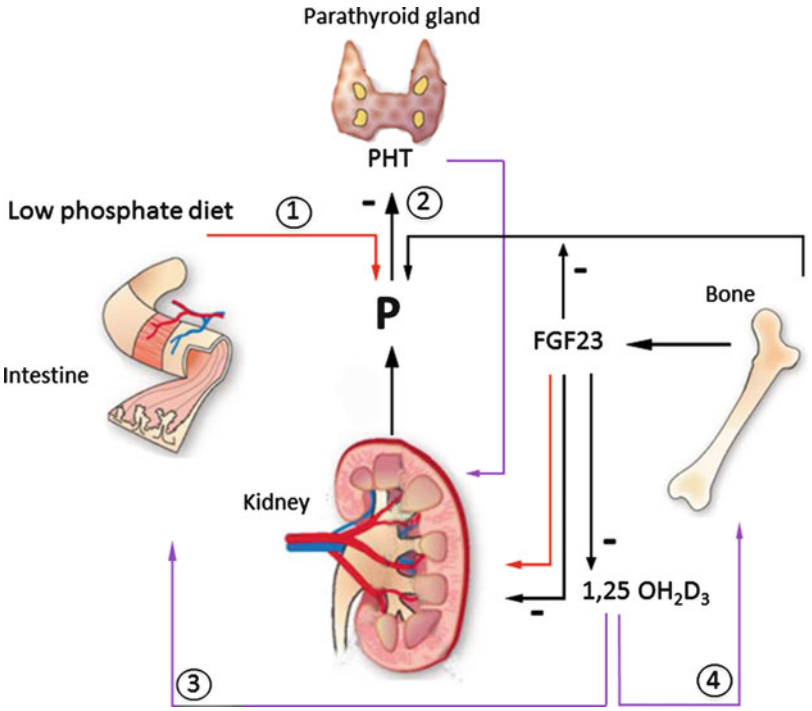

Fig. 4 Regulation of phosphorus homeostasis. A decrease in phosphorus (P) levels (for example through low dietary intake (1)) triggers a down regulation of parathyroid hormone (PTH) from the parathyroid gland (2). This results in a decreased P excretion in the kidneys and, consequently, higher extracellular P levels. At the same time, increased 1,25-Dihydroxy-Vitamin D_3 boosts reabsorption of P from the intestines (3) and, through osteoclast activation, P release from bone (4). Fibroblast growth factor 23 (FGF23) from osteocytes acts as regulating factor of those three mechanisms by preventing excessive renal absorption of phosphorus, by down regulating Vitamin D synthesis and by modulating osteoclast activity, therefor preventing excessive levels of P and potential toxic hyperphosphataemia. Purple arrows indicate actions increasing P levels, red arrows indicate actions decreasing P levels (Figure adapted from Torres et al. 2007, with permission from the publishers)

the case of inadequate P intake, as seen in preterm infants fed unfortified P-poor human milk, renal excretion decreases and renal vitamin D production stimulates bone resorption in order to allocate a sufficient amount of P for intracellular metabolism (Namgung and Tsang 2012). Even though low levels of serum P are a better indicator for metabolic bone disease of prematurity than serum Ca, monitoring of serum levels may not be sensitive enough to prevent osteopenia and monitor mineral supplementation (Harrison and Gibson 2013).

Alkaline phosphatase (ALP) is a membrane bound enzyme in osteoblasts involved in bone mineralization. Although also produced in liver and intestine, 90% of ALP in infants is of bone origin and, in the absence of relevant cholestatic liver disease, is considered a marker for bone turnover in newborns (Hung et al. 2011). Lack of mineral supply resulting in increased bone resorption can result in increased ALP, and ALP greater than two to three times the upper limit of normal is considered by some authors as an indicator for osteopenia in preterm infants (Hung et al. 2011; Kovar et al. 1982). However, ALP also rises during increased bone mineral accretion and accelerated growth in the weeks following preterm birth (Kovar and Mayne 1989). In fact, ALP poorly correlates with bone mineral content measured by DEXA (Faerk et al. 2002), which is why ALP as an isolated marker is not useful for diagnosing, monitoring, or preventing metabolic bone disease of prematurity.

A few other biomarkers such as osteocalcin and pro c-type natriuretic peptide have been evaluated for the purpose of monitoring bone health and growth, but they are in general not readily available as diagnostic tests, and the evidence remains limited (Kilpelainen et al. 2012; Pittard et al. 1992). Overall, repetitive testing of serum parameters does not agree well with restrictive policies of blood sampling in small preterm infants, important to avoid repetitive painful procedures and accentuation of anemia. Consequently, monitoring of urinary Ca and P has been explored as an easy tool for diagnosing mineral deficiency and monitoring supplementation accordingly.

Measurement of Calciuria and Phosphaturia

Testing of Spot Urine Samples in Preterm Infants

The standard of practice for measurement of renal excretion of any substance is the collection of urine over 24 h with subsequent analysis of the substance in relation to urine concentration (to account for circadian variation of fluid intake and excretion). Most stable growing preterm infants receive a constant daily fluid intake either through continuous parenteral nutrition or through feedings at two to three hourly intervals. With this steady hydration status in mind and in order to avoid cumbersome 24 h urine collection, spot urine samples have been shown to be valid predictors for 24 h secretion of specific substances (Trotter et al. 1996). Consequently, repetitive measurements of Ca and P in spot urine samples were adopted as

noninvasive diagnostic tests to detect insufficient mineral supply early and monitor supplementation (Aladangady et al. 2004; Pohlandt 1994c). Over the past decades, two methods of urinary Ca and P measurements have been used, evaluated, and discussed for this purpose in preterm infants: excretion of Ca and P by measuring their pure concentrations in urine (in mmol/l or mg/l) or urinary Ca/creatinine (Crea) and P/Crea ratios (in mol/mol or gram/gram).

Urinary Excretion of Ca and P

The measurement of pure Ca and P concentration in urine is based on the concept named slight surplus supply, introduced and tested by Pohlandt and colleagues (Pohlandt 1994c; Trotter and Pohlandt 2002). According to this concept, a slight surplus of serum Ca and P allows for a slight excretion in urine. Graphically speaking, Ca and P over and above the physiological requirements swap over into the urine in the same way water flows over the top of a container when full. The simultaneous excretion of a low level of Ca and P then indirectly proves that sufficient serum levels are present, independent of age and weight of the patient.

The type of nutrition plays a pivotal role for these inferences. The bone mineral hydroxyl apatite ($Ca_{10}[PO_4]_6[OH]_2$) consists of Ca and P in a molar ratio of 1.7. Since intestinal Ca absorption is naturally less efficient than P absorption, enteral supply of Ca and P should meet a ratio up to 2.0 in order to provide enough of both minerals for appropriate bone accretion (Agostoni et al. 2010). Human breast milk is naturally low in P, and Ca is readily absorbed from the gut. If administered unfortified, breast milk will drive preterm infants with rapid growth rates into a phosphopenic state where serum and, consequently, urine P are low. Serum Ca in this case will be normal, but urinary Ca excretion is increased since the lack of P does not allow for the Ca to be built into bone matrix. This creates a relative Ca excess, leading to increased excretion in urine. In contrast, cow's milk based formula milk provides more P than Ca, and a poorer intestinal Ca absorption compared to human breast milk. Serum P will remain normal in this case, as will serum Ca for reasons of maintaining crucial physiological processes. Parathyroid hormone will be activated during states of low Ca supply and minimize excretion via urine. Simultaneously, it will mobilize Ca and P from bone. Excess P will be excreted into urine. The constellation of hypocalciuria and hyperphosphaturia with normal serum Ca and normal to elevated P levels are typically found in preterm infants fed standard cow's milk formula (Schanler and Garza 1988; Schilling et al. 1982).

Breast milk fortifiers and formula tailored to the needs of preterm infants are designed to cater for these differences in mineral supply and are supposed to provide sufficient Ca and P in an appropriate ratio to achieve bone accretion similar to the in utero rate (Kuschel and Harding 2004). Standardized supplementation over and above the mineral content provided in fortified breast milk and preterm formula has not been shown to improve bone mineral content or prevent secondary complications from osteopenia, such as dolichocephalic head shape and myopia (Carroll

et al. 2011). However, as outlined above, individual needs and high inter- and intraindividual variations in intestinal absorption (Trotter and Pohlandt 2002) may not be met by standard recommendations, which is why additional mineral supplementation tailored to the individual and with monitoring of urinary Ca and P was introduced by Pohlandt over 20 years ago (Pohlandt 1994c). In this trial, preterm infants received Ca and P supplements in addition to standard preterm nutrition (either fortified breast milk or preterm formula). The hypothesis was that the intrauterine mineral accretion rate (4.5 mg/cm/100 g weight gain) would be achieved when supplements were sufficient to allow for a simultaneous slight spill of Ca and P into urine (1–2 mmol/l each). The results provided evidence that bone mineral accretion is indeed close to in utero levels. The trial displays methodological flaws, since it was not carried out in a prospective or randomized fashion and lacks a control group but, to date, remains the only outcome-driven study of individualized supplementation.

Despite the compelling simplicity of the concept, a few pitfalls need to be kept in mind, as urinary concentrations of 1–2 mmol/l of either mineral do not necessarily signal a truly sufficient supply. Importantly, only the simultaneous excretion of both minerals indicates an adequate quantity for bone mineralization. An excessive concentration of either only Ca or P warrants further investigations to exclude other reasons for urinary mineral loss. The slight surplus supply concept presumes normal renal function, i.e., capacity of glomerular filtration and tubular absorption comparable to at least a mature newborn. However, both functions are subject to maturation with increasing gestational age. Very preterm and extremely low birth weight infants excrete significantly more sodium at earlier gestational ages due to tubular immaturity (Al-Dahhan et al. 1983). The hypernatriuria is associated with an increased Ca excretion (Giapros et al. 2007), which is then not necessarily proof of sufficient Ca supply. Furthermore, drugs typically used in this very preterm population such as methylxanthines for apnea of prematurity, diuretics such as furosemide and corticosteroids for chronic lung disease of prematurity, increase Ca excretion considerably (Cranefield et al. 2004; Gimpel et al. 2010; Zanardo et al. 1995). Furthermore, a decreased tubular threshold for P in preterm infants leads to reduced reabsorption resulting in an excess phosphaturia even in the presence of P deficiency (Mihatsch et al. 1996). Apart from immaturity, tubular damage from drug toxicity or hypoxic-ischemic events presents another reason for tubular dysfunction (Tugay et al. 2006; Kaur et al. 2011). Excessive phosphaturia should prompt investigation of serum P levels to detect the requirement for even greater P supplements. In these cases, serum levels of P rather than urinary excretion appear to be the more helpful parameter to guide supplementation. However, there is no outcome-based study or other evidence for this recommendation. In the case of increased Ca excretion due to renal immaturity or drugs, control of Ca supplementation becomes even more difficult, because serum Ca will not decline until very late in the depletion of bone matrix and cannot be used as a parameter for over or under supply. Considering that one or multiple of above situations influencing phosphaturia and/or calciuria are present in the vast majority of the preterm population, who are at risk for osteopenia of prematurity, the

question of the utility and validity of the concept of slight surplus supply to guide individualized mineral supplementation remains unanswered.

Urinary Ca and P Crea Ratios

In contrast to the idea behind the slight surplus supply, measurement of mineral/Crea ratios is based on the concept that the concentration of any excreted substance is inversely proportional to urine volume: the smaller the amount of urine produced, the more concentrated are the renally excreted substances. Therefore, mineral/Crea ratio corrects for volume-induced concentration changes. Theoretically, the ratios should be superior to pure urinary mineral concentrations and have been the standard of diagnosis in spot urine samples in pediatric and adult nephrology for a long time (Matos et al. 1997; Nordin 1959; Sargent et al. 1993). Attempts have been undertaken to establish reference ranges for Ca/Crea and P/Crea ratios in preterm infants according to gestational and postnatal age, as arguably Ca and P excretion is different in this population compared to more mature newborns and children (Aladangady et al. 2004). This study found various factors affecting mineral excretion: parenteral nutrition increased Ca/Crea ratio and decreased P/Crea ratio. Increased postnatal age and administration of furosemide increased Ca excretion, and gestational age increased P excretion. However, these biochemical data have been criticized as mere description of biochemical variables reflecting nutritional intake and the age of the infant and having no correlation to this presumed reference, with bone mineral content as the important outcome (Pohlandt and Mihatsch 2004). No other evidence has since been published to further prove or disprove the value of mineral/Crea ratios. In contrast, the usefulness of the correction for volume-induced concentration changes has been challenged. Firstly, stable growing preterm infants receiving feeds at very frequent intervals should not experience major circadian variations in urinary concentration of minerals (Trotter et al. 1996). Secondly, Crea concentration in urine cannot impartially be assumed as a function of urine volume output without taking other factors into consideration. Crea excretion is age dependent and depends on both postnatal age and gestational age (Sonntag et al. 1996; Rassin et al. 1986). Expression of excretion of any substance in relation to Crea could show an age dependency, which is possibly only true for Ca excretion, but not for the substance in question (Applegarth and Ross 1975). In order to correctly assess urinary excretion of any substance by means of Crea ratios, reference ranges for Crea excretion at any given gestational or postnatal age would need to be established first. In fact, some evidence even suggests that urinary substance/Crea ratios do not adequately reflect the actual daily excretion of the substance in question (Boehm et al. 1998) and that there is no association of Ca/Crea and P/Crea ratio with postnatal or gestational age (Staub et al. 2014). Additionally, the amount and type of dietary protein influence urinary Crea, with whey- and casein-based infant formula stimulating a greater excretion of Crea than breast milk (Rassin et al. 1986). The same study did not find an effect of the daily fluid volume on Crea excretion. All of these data together question the usefulness of mineral/Crea ratios in spot urine

samples for assessment and monitoring of osteopenia of prematurity and respective mineral supplementation. Contrary to the pure urinary concentrations of Ca and P, no study so far has been undertaken to put the values in correlation of bone mineral content.

Comparison of the Two Methods in the Absence of Established Criterion Standard

When contemplating the potentially confounding issues surrounding the two methods of assessment of calciuria and phosphaturia, one major point is the inability of either method to actually diagnose metabolic bone disease of prematurity. The existing studies either merely report the amount of mineral excretion in association with various biochemical and physiological parameters, and are influenced by local nutritional practices (Aladangady et al. 2004; Karlen et al. 1985; Giapros et al. 2007), or use Ca and P excretion to guide supplementation in order to prevent or improve osteopenia of prematurity (Pohlandt 1994c; Carroll et al. 2011). In this context, a comparison of the two methods showed substantial agreement between urinary P concentration and urinary P/Crea ratio, but only moderate agreement for Ca in the intention to supplement the minerals (Staub et al. 2014) (Fig. 5). The study applied hypothetical cutoff values from other studies reporting urinary mineral excretion in similar cohorts. However, no formal diagnosis of osteopenia was made, and no endpoint measuring bone mineral content or accretion rate could be assessed. Therefore, the two methods of urine screening could not be verified against a valid standard, as is not available in this context. No study has determined the cutoff values of either method to correlate with a substantial mineral deficiency and/or the absence or presence of osteopenia of prematurity in a prospective study or any other fashion. Consequently, the classic diagnostic accuracy paradigm with reporting of test sensitivity and specificity cannot be applied. Much in the same line of reasoning, it does not make sense to shift the cutoff values for both screening methods in the comparative study of Staub et al. in order to optimize their agreement. This kind of ROC (receiver-operator-characteristics) analysis would require uplifting either of the two methods to the reference standard, which for reasons stated earlier, is not appropriate. Quantitative ultrasound (tibial speed of sound) or DEXA have previously been used to diagnose and follow up bone (de)mineralization in premature infants (Chan et al. 2008; Fewtrell et al. 2008). These diagnostic tools could be used in a clinical test validation (Reitsma et al. 2009). Therein, test results (in this case either urinary mineral concentration or mineral/Crea ratios) are correlated with bone mineral content measured by ultrasound or DEXA as the clinically relevant outcome. The validation then consists in evaluating whether the test results are meaningful, i.e., how well calciuria and phosphaturia measure what they are supposed to measure, in this case excessive loss of minerals from bone matrix or appropriate mineral supplementation to prevent the latter.

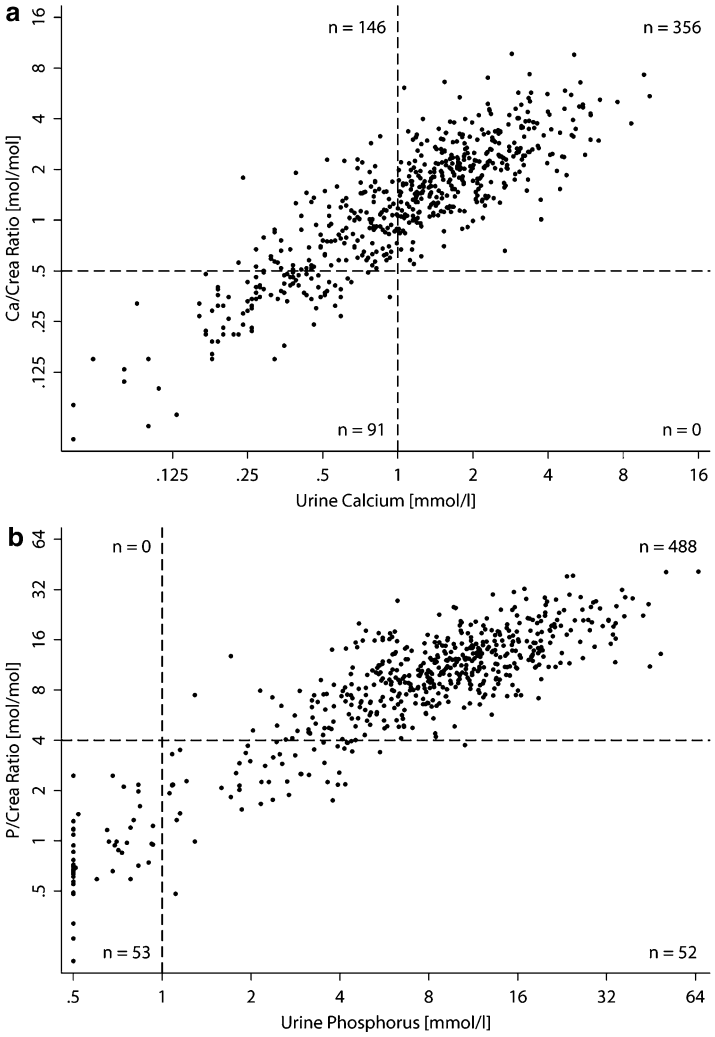

Fig. 5 Comparison of urinary mineral concentration vs mineral/creatinine ratios in the intention to supplement. The intention to supplement calcium (Ca) and phosphorus (P) compared in a study population of 230 preterm babies <32 weeks gestation and/or <1500 g birth weight, depending on screening method in spot urine samples. **a.** Log-log scatterplots of urinary calcium concentration versus urinary Ca/creatinine (Crea) ratio. Dashed lines indicate cutoff values for supplementation: 1 mmol/l for urinary Ca concentration, 0.5 mol/mol for urinary Ca/Crea ratio. Agreement between the two methods was poor (Cohen's $\hat{\kappa} = 0.43$, 95% CI 0.36–0.49) **b.** Log-log scatterplot of urinary P concentration versus urinary P/Crea ratio. Dashed lines indicate cutoff values for supplementation: 1 mmol/l for urinary P concentration, 4 mol/mol for urinary P/Crea ratio. Agreement between the two methods was moderate (Cohen's $\hat{\kappa} = 0.63$, 95% CI 0.54–0.72) (Figure and data from Staub et al. 2014, with permission from the publishers)

Potential Applications of Urinary Ca and P to Prognosis of Osteopenia of Prematurity and Other Prognostic Value of Urinary Ca and P for Osteopenia of Prematurity

Sufficient Ca and P supply, in the correct ratio, allows for adequate bone mineralization in preterm infants and can prevent severe complications of osteopenia of prematurity, such as fractures. These are rarely seen any more since introduction of standardized fortification of milk plus/minus additional mineral supplementation. However, the lag in bone mineralization compared to the standard of the in utero accretion rate may have consequences far beyond the neonatal age. Indeed, multiple studies have shown that former preterm infants show lesser bone mineralization than their term born counterparts during childhood and into adolescence (Chan et al. 2008; Peralta-Carcelen et al. 2000; Weiler et al. 2002). Since appropriate bone mineralization is also a prerequisite for adequate bone growth, height is affected by early osteopenia. The same studies also show significantly reduced lengths in the former preterm and VLBW infants. However, it remains unclear whether reduced bone mass is related to reduced length or in fact is a long-term consequence of osteopenia of prematurity independent of reduced growth following prematurity. Equally, there is not yet a conclusive answer whether early mineral deficit and suboptimal bone mineralization negatively influence peak bone mass at the time around the completion of skeletal growth and, consequently, risk of osteoporosis in adulthood. A systematic review has suggested that higher birth weight was associated with higher bone mineral content of the spine and hip in adulthood (Baird et al. 2011). But the influence of early nutrition and growth has not been systematically assessed nor the association between urinary Ca and P excretion with later bone mineral content. In fact, there is no evidence regarding the predictive or prognostic value of any biomarkers for bone health in preterm infants in relation to their growth and long-term consequences. Urinary Ca has been shown to negatively correlate with bone mass in young adult girls (Matkovic et al. 1995), and postmenopausal women with osteoporosis have been found to have an increased renal Ca and P excretion compared to women with normal bone mineral density (Murad et al. 2012). Applied to preterm infants, the degree of calciuria (and/or phosphaturia) has never been examined as possible prognostic factors for bone mineral content in childhood or adulthood, therefore presenting an opportunity for prospective research.

Applications of Urinary Ca and P to Other Diseases and Conditions in Pediatric Patients

A comprehensive review of the applications of urinary Ca and P as biomarkers, even when limited to the pediatric patient, is well beyond the scope of this chapter. They are often used in the diagnostics of a variety of diseases and conditions. Urinary Ca excretion is commonly evaluated in the workup of nephrolithiasis (kidney stones), which frequently consist of Ca oxalate or Ca phosphate precipitates (Tasian and

Copelovitch 2014). An array of conditions are associated with hypercalciuria, such as renal tubular dysfunction, endocrine disturbances, bone metabolism disorders, and drugs and other primary or secondary causes of excessive Ca excretion (Gillespie and Stapleton 2004). Equally, abnormalities in P excretion are not a unique entity but the result of different conditions. The renal clearance of P depends on serum P levels and the tubular reabsorption capacity. The latter is influenced, for example, by the volume of urinary flow, hormone levels (PTH), or dysfunction of the renal proximal tubules, as seen in congenital or acquired Fanconi syndrome (Imel and Carpenter 2015). As in the case of osteopenia of prematurity, Ca and P excretion are important parameters for bone metabolism in patients of any age (Bonjour 2011). Abnormalities in calciuria and phosphaturia should always prompt further investigations with regards to bone health.

Summary Points

- Preterm babies born prior to the third trimester of pregnancy miss the majority of transplacental Ca and P transfer.
- Inadequate nutritional calcium and phospophorus supply, immature renal function, medication, and immobility present a combination of factors that put the preterm baby at risk of osteopenia of prematurity.
- Urinary calcium and phosphorus excretion are used to monitor and guide individual mineral supplementation above the standard intake through fortified breast milk or preterm formula milk but are not suited to actually diagnose osteopenia of prematurity.
- The principle of slight surplus supply considers the simultaneous urinary excretion of calcium and phosphorus above a specific threshold as proof of sufficient serum levels to allow for bone accretion rates similar to the in utero standard.
- Calcium or phosphorus to creatinine ratios account for volume-induced changes of mineral excretion but might not correctly assess the actual excretion of calcium or phosphorus due to the immaturity of the kidneys and other factors influencing creatinine excretion itself.
- Both monitoring methods lack an outcome-driven test validation to prove appropriate bone mineralization under guided calcium and phosphorus supplementation.
- Urinary calcium and phosphorus excretion have not been assessed as prognostic factors for bone mineralization beyond the neonatal age in former preterm infants.

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Bone Biomarkers in Gestational Hypertension

36

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Contents

Abstract

The skeletal system undergoes a continuous process of remodeling throughout life, which involves a delicate balance between bone resorption due to osteoclastic activation and new bone formation due to osteoblastic activity. Biochemical markers of bone turnover are reliable indices for measuring changes of bone formation and resorption, reflecting the dynamics of bone metabolism at the cellular level. During normal pregnancy, major changes occur in maternal calcium homeostasis and bone metabolism, in order to fulfill the calcium demand of the fetus for skeletal growth and mineralization. Fetal calcium requirements can be met by calcium mobilization from the maternal skeleton through hormone-mediated adjustment of maternal calcium metabolism. Due to limitations in the application of bone densitometry during pregnancy, biochemical markers are effective alternatives to estimate bone turnover. The diphasic changes in maternal bone histology (temporary loss of cancellous bone in early pregnancy restored by term gestation) are consistent with corresponding blood biochemistry changes: increased bone resorption markers in the first trimester, followed by elevated bone

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formation markers at term. Maternal bone turnover during pregnancy is reportedly enhanced in hypertensive disorders of pregnancy, including preeclampsia (PE) and pregnancy-induced hypertension (PIH), although data are not consistent. Biochemical markers provide evidence for increased maternal bone turnover in PE, probably leading to a further reduction in maternal bone mineral density (BMD). In contrast, in PIH, data are scarce and do not support considerable changes in maternal bone metabolism.

Keywords

Pregnancy • Preeclampsia • Pregnancy-induced hypertension • Bone turnover • Biochemical markers • Bone mineral density

List of Abbreviations

ALP	Alkaline phosphatase
BALP	Bone-specific alkaline phosphatase
BMD	Bone mineral density
BMI	Body mass index
DPD	Deoxypyridinoline
ICTP	Cross-linked carboxyl-terminal telopeptide of type I collagen
IL-6	Interleukin-6
NTx	*N*-telopeptide of type I collagen
OC	Osteocalcin
OPG	Osteoprotegerin
PE	Preeclampsia
PICP	Carboxyl-terminal pro-peptide of type I collagen
PIH	Pregnancy-induced hypertension
PTH	Parathormone
RANKL	Receptor activator of nuclear factor-kB ligand
TGF-β	Transforming growth factor-β
TNF-alpha	Tumor necrosis factor-alpha

Key Facts of Bone Turnover in Pregnancy

- Pregnancy is a high bone turnover state inducing dynamic changes in maternal bone and calcium metabolism.
- Maternal bone turnover is stimulated by the increased placental transfer of calcium to meet fetal demands for ossification and general metabolism.
- Bone turnover, as measured by biochemical markers, increases during normal pregnancy, resulting in a reversible bone loss and a subsequent reduction in bone mineral density.
- An altered maternal bone metabolism, probably leading to a further reduction of bone mineral density, has been demonstrated in pregnancies complicated by gestational hypertensive disorders, i.e., preeclampsia and pregnancy-induced hypertension.

- Biochemical markers of bone metabolism suggest increased bone turnover in preeclampsia, while in pregnancy-induced hypertension, data do not support significant changes in maternal bone metabolism.

Definitions of Words and Terms

PE Blood pressure augmentation after 20 weeks of gestation to >140/90 mmHg on at least two occasions 6 h apart in a previously normotensive woman, combined with proteinuria defined as protein dip stick ≥1+ in ≥2 midstream urine samples 6 h apart or a 24-h urine excretion of ≥0.3 g protein in the absence of urinary infection

PIH New-onset hypertension after 20 weeks of gestation with systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg without any systemic features of preeclampsia and normal blood pressure levels within 3 months postpartum

Introduction

Bone tissue is subject to remodeling throughout the lifetime of an individual, depending on a delicate balance between resorption due to osteoclastic activation and new bone formation due to osteoblastic activity (Raisz 1999). Pregnancy is a high bone turnover state inducing dynamic changes in maternal bone and calcium metabolism (Pitkin 1985). Maternal bone turnover is stimulated by the increased placental transfer of calcium to meet fetal demands for ossification and general metabolism, which far exceeds maternal intestinal absorption (Ogueh et al. 2000). It is, however, uncertain to what extent the bone is mobilized during pregnancy to provide calcium for the fetus and whether this is of such an extent as to cause osteoporosis (Khovidhunkit and Epstein 1996).

Biochemical markers of bone turnover provide a dynamic view of the remodeling process, which covers rate of turnover and pathogenesis, and may improve fracture risk prediction (Camozzi et al. 2007). Changes of bone remodeling markers reflect bone growth and bone turnover (Bhandari et al. 1999). Information on bone metabolism can be attained by blood and urine laboratory tests. Recently developed bone-specific markers are categorized by bone remodeling process, i.e., bone formation and resorption (Wada et al. 2009). Due to limitations in the use of bone densitometry during pregnancy and the perinatal period, biochemical markers of bone turnover provide an excellent alternative to examine the state of the skeleton (More et al. 2003).

Accumulating evidence suggests that bone turnover, as measured by biochemical markers, increases during normal pregnancy, resulting in a reversible bone loss and a subsequent reduction in bone mineral density (BMD), independently of prepregnancy bone mass (More et al. 2003; Namgung and Tsang 2003; Yamaga

et al. 1996). Furthermore, an altered maternal bone metabolism, probably leading to a further reduction of BMD, has been demonstrated in pregnancies complicated by gestational hypertensive disorders, including preeclampsia (PE) and pregnancy-induced hypertension (PIH), compared with normal pregnancies (Morikawa et al. 1989; Lalau et al. 1993; Shaarawy et al. 2005; Dorota et al. 2012). PE and PIH are common obstetrical problems that would develop usually during the latter part of pregnancy. PIH is diagnosed when the pregnant woman has new-onset hypertension after 20 weeks of gestation with systolic blood pressure $\geq$140 mmHg and/or diastolic blood pressure $\geq$90 mmHg without any systemic features of preeclampsia and normal blood pressure levels within 3 months postpartum (Brown 1995). PE is defined as blood pressure augmentation after 20 weeks of gestation to >140/90 mmHg on at least two occasions 6 h apart in a previously normotensive woman, combined with proteinuria defined as protein dip stick $\geq$1+ in $\geq$2 midstream urine samples 6 h apart or a 24-h urine excretion of $\geq$0.3 g protein in the absence of urinary infection (Brown 1995).

While the factors that would influence the magnitude of BMD changes in pregnancy have been studied in various settings, few studies have addressed how particular antenatal complications, such as PE and PIH, would affect such changes. Accordingly, while alterations in bone formation and resorption, by using biochemical markers, have been reported in normal pregnancy, there is little published data on the effects of hypertensive disorders of pregnancy on maternal bone biomarkers. This chapter considers published data regarding biochemical markers of bone turnover and overall bone metabolism in gestational hypertensive disorders.

Bone Mineral Density in Gestational Hypertensive Disorders

BMD loss in pregnancy has been directly compared between normotensive women and those with gestational hypertensive disorders in a few studies in the literature. A higher but nonsignificant BMD loss has been reported in patients with PIH, as compared with controls in a longitudinal study (To et al. 2003). In accordance, studies utilizing radiological assessment of BMD and a quantitative ultrasound system demonstrated subtle changes in the bone size and densities, but not a clear-cut difference in BMD of women with PIH (Morikawa et al. 1989) and a greater, but nonstatistically significant, degree of BMD loss in preeclamptic women, as compared with normal controls, respectively (Sowers et al. 2001).

Very recent data showed an obvious increase in weight and body fat percentage, as well as a demonstrable progressive fall in BMD from early to late gestation in normal pregnancies. Univariate analysis documented a higher BMD loss during pregnancy in women with gestational hypertension, but the effects of the presence/absence of hypertensive disorders could no longer be seen on multivariate regression analysis. On the other hand, anthropometric parameters including early pregnancy BMD values and body mass index (BMI), as well as gestational fat accumulation, were more important factors affecting the extent of BMD loss (To and Wong 2011). The incidence of both PIH and PE increases with increasing BMI. Thus, it could be

speculated that basic anthropometric parameters, such as obesity, are more likely to be responsible for the differences in BMD loss observed in pregnancy, rather than the gestational hypertensive condition.

The mechanisms underlying the effect of gestational hypertensive disorders on maternal bone turnover have not been fully investigated.

PE is characterized by endothelial dysfunction related to abnormal trophoblastic invasion and a potentially harmful maternal response to a semi-allogenic fetus that activates inflammatory cells, oxidative stress pathways, and cytokine profiles (Gu et al. 2008). Accumulative data show that altered proinflammatory cytokines could be associated with decreased bone mass, clinically detectable osteoporosis, and greater fracture risk in elderly age groups (McLean 2009). In this respect, the increased proinflammatory cytokines in PE have been suggested to stimulate osteoclasts and, subsequently, induce bone resorption (Shaarawy et al. 2005; Anim-Nyame et al. 2002). It has also been hypothesized that the generalized endothelial dysfunction characterizing PE could possibly involve the bone marrow microcirculation, leading to increased osteoclastic activity.

Furthermore, the higher bone turnover in PE has been attributed to the increased parathormone (PTH) and decreased 1,25(OH) vitamin D levels, leading to reduced calcium excretion and plasma ionized calcium levels (Sanchez-Ramos et al. 1991; Graves et al. 1994). Similarly, disturbances of calcium-regulating hormones have been demonstrated in PIH (Lalau et al. 1993). The relationship between the parathyroid hormone-calcitriol axis and the development of hypertension in pregnancy has been the subject of several experimental and observational studies (Sanchez-Ramos et al. 1991; Graves et al. 1994). Increased PTH concentrations have repeatedly been reported at the time of PE, as well as several weeks prior to the onset of PE or PIH (Brown 1995; Wang et al. 1995).

Biochemical Markers of Bone Turnover in Preeclampsia and Pregnancy-Induced Hypertension

Older studies suggest alterations of bone metabolism in hypertensive versus normotensive pregnancies, proposing that markers of bone turnover are in favor of bone formation in normal pregnancies and in at least the mild form of PE (Shaarawy et al. 2005), whereas bone resorption might be increased in established PE (Shaarawy et al. 2005; Anim-Nyame et al. 2001, 2002; Kumtepe et al. 2005).

In this respect, significantly higher concentrations of osteocalcin (OC) and degradation products of type I collagen in preeclamptic patients, as compared with healthy pregnant woman, have been demonstrated, suggesting accelerating bone turnover in PE (Gorzelak et al. 2001). OC is the major non-collagenous protein of bone matrix, which is produced in the bone by activated osteoblasts and is partly delivered to the circulatory system. Although the exact physiological function is still uncertain, circulating levels of OC seem to reliably reflect the rate of bone formation (Gorzelak et al. 2001). Type I collagen accounts for more than 90% of the organic matrix of the bone and is synthesized primarily in the bone. During renewal of the

skeleton, type I collagen is degraded and small peptide fragments are excreted into the bloodstream (Eriksen et al. 1993). The increased concentrations of OC and collagen type I C-telopeptide concentrations in PE in the above study could possibly be attributed to the known changes in calciotropic hormone levels in this group of patients (Gorzelak et al. 2001). Similarly, an earlier report, by investigating circulating OC and urinary deoxypyridinoline (DPD), as biochemical markers of bone formation and bone resorption, respectively, documented increased bone turnover in PE, as compared with normotensive pregnancy (Kumtepe et al. 2005).

Several growth factors have been implicated in the regulation of bone remodeling. Transforming growth factor-β (TGF-β) seems to exert a pivotal role in bone formation, by stimulating collagen type I synthesis, mitogenesis, alkaline phosphatase (ALP) activity, osteoblast chemotaxis, proliferation, and differentiation. It is produced in response to factors that stimulate bone resorption, and its release is mediated by osteoclasts (Mundy 1991). TGF-β acts directly on osteoblasts to regulate bone remodeling, structure, and biomechanical properties and is actively involved in coupling bone formation to previous bone resorption. Increased TGF-β concentrations have been observed in patients with PE, probably representing a response compensating bone resorption processes in patients with PE (Djurovic et al. 1997). Anim-Nyame et al. carried out a study to test the hypothesis that the lower serum levels of calcium and hypocalcuria seen in PE are due to decreased bone metabolism (Anim-Nyame et al. 2001). Thus, they measured circulating concentrations of the cross-linked carboxyl-terminal telopeptide of type I collagen (ICTP), a marker of bone resorption, and the carboxyl-terminal pro-peptide of type I collagen (PICP), a marker of bone formation in women with normal pregnancy and PE. ICTP and PICP have been shown to correlate with bone histomorphometry (Eriksen et al. 1993). Contrary to their hypothesis, the authors demonstrated an increase in overall bone turnover in PE, within the constraints of usage of ICTP as a marker of bone formation and PICP as a marker of bone resorption. The authors hypothesize that the increased bone metabolism in PE is the result of osteoclastic activation by a factor released during the development of the disease. PE is characterized by increased production of proinflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Greer et al. 1994). Furthermore, oxidative stress seems to be associated with the generalized endothelial dysfunction of the disorder (Hubel 1999). Reactive oxygen species, IL-6, and TNF-α stimulate osteoclastic activity leading to bone resorption in animal models (Roodman 1993).

Following this publication, the same group conducted another study to test the hypothesis that the increased bone turnover observed in established PE occurs earlier in pregnancy prior to the presence of clinically evident disease (Anim-Nyame et al. 2002). The authors evaluated circulating levels of ICTP and PICP in serial blood samples obtained between 16 and 36 weeks of gestation from a group of pregnant women who eventually developed PE and from a group of women with normal pregnancy outcome. Their data showed that bone metabolism in pregnancies destined to develop PE increases with gestation, but exceeds that of a normal pregnancy at 36 weeks of gestation, suggesting that the increase in bone turnover

is greater in PE, compared with normal pregnancy, but only when the disease is clinically evident. However, no correlation was found among ICTP and PICP and blood pressure in the PE group, suggesting that the increased bone metabolism is not due to the hypertensive component of PE.

The above results were consistent with the observation that PICP and a breakdown product of type III collagen increase progressively during normal pregnancy, while the third-trimester cross-sectional values for a subgroup of women with severe PE are elevated when superimposed on the normal pregnant data (Puistola et al. 1993). The authors attributed the higher levels of collagen markers in PE to generalized tissue breakdown. However, PICP predominantly derives from bone formation, and, therefore, the upregulation in its circulating levels is consistent with an increase in bone turnover in PE (Ross and Knowlton 1998).

Furthermore, Shaarawy et al. demonstrated significantly decreased maternal serum concentrations of OC and PICP in severe PE, whereas maternal urinary levels of *N*-telopeptide of type I collagen (NTx) (a marker of bone resorption) were significantly increased, compared with the corresponding levels of controls (Shaarawy et al. 2005). NTx is a type I collagen that accounts for more than 90% of the organic matrix of the bone and is synthesized primarily in the bone. During remodeling of the skeleton, type I collagen is degraded, and small peptide fragments are excreted into the bloodstream (Mori 2004). The coupling index of markers of bone turnover in normal pregnancy or mild PE in the above study was in favor of bone formation, whereas in severe PE the markers suggested marked bone resorption. The authors conclude that increased bone resorption and decreased bone formation occur in PE.

Finally, a study indicated no significant differences in biochemical markers of bone turnover [bone-specific alkaline phosphatase (BALP, a sensitive marker of bone formation), ALP, OC, NTx] in a population comprising both mothers with PE and PIH (Briana et al. 2008). BALP is a glycosyl-phosphatidyl-inositol-anchored ectoenzyme present on the membrane of osteoblastic cells. The presence of ALP on the cell membrane is required for bone mineralization (Low 1987). A possible explanation for the lack of differences in biochemical markers of bone turnover in our study may rely on the fact that altered bone metabolism in PE is probably associated with the endothelial dysfunction of the bone marrow microcirculation and not with the hypertensive component of the disease.

On the other hand, a recent study documented higher serum NTx levels in women with PE, compared with controls at delivery, while the levels of serum BALP did not differ between groups. The authors conclude that biochemical markers of bone resorption are greater in PE, compared with normal pregnancy, only when the disease becomes clinically evident (at delivery) (Kumar et al. 2012). The results of this report do not indicate any correlation between bone biomarkers and blood pressure, suggesting that increased bone resorption is not due to the hypertensive component of PE.

Other studies have focused on newer markers of bone turnover. Morphogenesis and remodeling of the bone entail the synthesis of bone matrix by osteoblasts and the coordinated resorption by osteoclasts (Roodman 1999). The OPG/RANKL system

has a well-defined central role in bone resorption (Walsh and Choi 2003). Osteoprotegerin (OPG), a member of the TNF receptor family, has been identified as a key factor in inhibiting bone resorption (Simonet et al. 1997; Yasuda et al. 1998), by acting as a decoy receptor for the receptor activator of nuclear factor-kB ligand (RANKL). RANKL induces maturation and activation of osteoclasts, thus leading to bone resorption (Leinbbrandt and Penninger 2008).

Maternal circulating OPG levels gradually increase, reaching peak levels immediately before delivery (Uemura et al. 2002; Naylor et al. 2003), whereas circulating levels of RANKL have been documented to decrease during the progress of a normal pregnancy (Naylor et al. 2003). OPG has been implicated in the pathophysiology of progressive atherosclerosis and vascular mortality (Kiechl et al. 2004), and, thus, a role for this cytokine in the pathogenesis of PE has been proposed (Hong et al. 2005). The results of a recent study suggest that women who experienced a preeclamptic pregnancy have higher levels of OPG and a higher OPG/RANKL ratio during the whole peripartum period when compared with controls, with the difference becoming significant after delivery (Vitoratos et al. 2011). The authors speculate that the PE-related increase in OPG levels compared with controls may have a positive effect on the pregnancy-related bone loss (Reid and Holen 2009). In contrast, a study from our group, comprising not only preeclamptic but also women with PIH, did not demonstrate any differences in serum OPG and sRANKL concentrations between women with gestational hypertensive disorders and normal pregnant women (Briana et al. 2009). In another study, the concentrations of sRANKL and OPG were significantly higher in the second trimester of normal pregnancy, when compared with the first and the third trimesters and with nonpregnant controls. The concentrations of OC were increased in the first trimester of normal pregnancy, in comparison with nonpregnant women and with the second and third trimesters of pregnancy. The circulating levels of CrossLaps-degradation products of type I collagen were higher in the second trimester of normal pregnancy, when compared with the first and third trimesters. In PE, serum concentrations of OC and CrossLaps-degradation products of type I collagen were significantly higher, when compared to the third trimester of pregnancy. The authors conclude that bone formation is higher in the first trimester, whereas bone resorption is increased in the second trimester of a normal pregnancy. Moreover, bone turnover is higher in patients with PE, when compared with healthy normotensive pregnant women (Dorota et al. 2012).

Adipocytokines have recently been implicated in the complex control of bone biology (Magni et al. 2010). The findings of another study failed to demonstrate any associations of novel adipocytokines (resistin, apelin, and visfatin) with several biochemical indices of bone turnover in normal pregnancies (Briana et al. 2013). Nevertheless, in gestational hypertensive disorders, maternal apelin concentrations negatively correlated with NTx (an established marker of bone resorption), while maternal visfatin concentrations positively correlated with concentrations of OPG (a main inhibitor of bone catabolism). It could possibly be speculated that both apelin and visfatin may inhibit bone resorption in pregnancies complicated by PE or PIH.

Finally, a very recent study attempts to clarify the possible impact of PE on bone metabolism, by determining sclerostin, a new marker of bone metabolism, which is known to have an inhibitory effect on bone formation, as well as other older bone biomarkers (Wild et al. 2014). The results revealed no significant differences in serum levels of sclerostin, ICTP, and PICP between women with PE and normotensive pregnant women. However, significantly lower serum calcium and 25-hydroxyvitamin D levels were observed in PE. The authors conclude that women with PE show no signs of high bone turnover and may not be at higher risk of developing osteoporosis later in life.

Taken together, the data published in the literature favor the assumption that the actual differences in BMD loss between women with gestational hypertensive disorders and those who remain normotensive during pregnancy are not consistent. However, most studies determining biochemical markers of bone turnover suggest increased bone turnover in PE, probably predisposing to enhanced bone loss and osteoporosis development later in life. Further studies are needed to clarify the actual impact of gestational hypertensive disorders on maternal bone health both in the short and long term. Biochemical markers of bone turnover seem to be useful tools in evaluating the state of the skeleton during the sensitive period of pregnancy.

Summary Points

- The skeletal system undergoes a continuous process of remodeling throughout life, which involves a delicate balance between bone resorption due to osteoclastic activation and new bone formation due to osteoblastic activity.
- Biochemical markers of bone turnover are reliable indices for measuring changes of bone formation and resorption, reflecting the dynamics of bone metabolism at the cellular level.
- During normal pregnancy, major changes occur in maternal calcium homeostasis and bone metabolism, in order to fulfill the calcium demand of the fetus for skeletal growth and mineralization.
- The diphasic changes in maternal bone histology (temporary loss of cancellous bone in early pregnancy restored by term gestation) are consistent with corresponding blood biochemistry changes: increased bone resorption markers in the first trimester, followed by elevated bone formation markers at term.
- Maternal bone turnover during pregnancy is reportedly enhanced in hypertensive disorders of pregnancy, including preeclampsia and pregnancy-induced hypertension.
- Biochemical bone markers provide evidence for increased maternal bone turnover in preeclampsia, probably leading to a further reduction in maternal bone mineral density.
- In pregnancy-induced hypertension, data are scarce and do not support considerable changes in maternal bone metabolism.

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Biomarkers in Osteoarthritis: Value of Proteomics

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Abstract

Osteoarthritis (OA) is the most common rheumatic pathology accounting for much of worldwide disability. OA is related to aging and may affect the structure of any joint tissue. The early stage accurate diagnosis of OA and the ability to monitor the efficacy of putative disease-modifying drugs remain among the essential unmet medical needs for this disease. Despite the prevalence of OA, the diagnostic methods currently available are limited and lack sensitivity. Furthermore, there is currently no effective therapy capable of slowing or reversing the pathological changes that occur in the joint during the disease process. Therefore, the discovery and application of novel, noninvasive, specific biochemical markers remain a priority. This chapter will focus on the current OA protein markers and the value of proteomics for the discovery and validation of useful candidates for early diagnosis and drug discovery.

Keywords

Biomarkers • Osteoarthritis • Proteomics • Cartilage • Synovium • Synovial fluid • Mass spectrometry

List of Abbreviations

2-DE	Two-dimensional gel electrophoresis
COL2	Type II collagen
COMP	Cartilage oligomeric matrix protein
CS	Chondroitin sulfate
CTX-II	C-telopeptide region of COL2
DIGE	Differential in-gel electrophoresis
HA	Hyaluronic acid
HC	Healthy controls
iTRAQ	Isobaric tag for relative quantitation
K/L	Kellgren and Lawrence scale
KS	Keratan sulfate
LC	Liquid chromatography
MALDI-TOF/TOF	Matrix-assisted laser desorption/ionization-time of flight
MB	Magnetic beads
MMP	Matrix metalloproteinases
MS	Mass spectrometry
NAPPA	Nucleic acid programmable protein array
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PAGE	Polyacrylamide gel electrophoresis
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
SELDI	Surface-enhanced laser desorption/ionization
SF	Synovial fluid
SRM	Selected reaction monitoring

TIMP	Tissue inhibitor of matrix metalloproteinase
UF	Ultrafiltration
YKL-40	Cartilage glycoprotein 39

Key Facts About Osteoarthritis (OA)

- OA is a slowly progressive disease that can affect the structure of all joint tissues.
- OA is a major cause of pain and chronic disability in the elderly.
- To date, OA lacks effective therapies. Current therapeutic strategies are limited to symptomatic relief, but are not able to slow or reverse joint alterations.
- The diagnosis of OA relies on the description of symptoms, such as pain or stiffness, and radiography, which is not sensitive enough to detect small changes.
- The complex pathogenesis and the heterogeneity of the clinical manifestations and progression of OA have hindered development of specific and sensitive tools for its management and therapy.
- Novel specific and sensitive biomarkers are required to prevail over the medical needs of OA. Although several molecules have been assayed, to date, none is approved for use in clinical routines.

Key Facts About Proteomics

- Proteomics is a science focusing on the large-scale analysis of proteins, their abundance, interactions, and modifications.
- The set of proteins produced by a living organism is termed its proteome.
- Proteomes differ between organisms, tissues, and cell types and change continuously with time, environmental conditions, pathologies, or drug treatment.
- The use of proteomics is a valuable technology for the discovery of protein markers of disease.
- Techniques employed in proteomics approaches include gel electrophoresis, liquid chromatography, mass spectrometry, protein microarrays, and bioinformatics.

Key Facts About Mass Spectrometry (MS)

- MS is an analytical technique capable of characterizing a large variety of chemical species in pure and complex mixtures through measurement of their mass.
- MS is the most commonly employed technology for protein characterization in proteomics.
- In MS for proteomics, protein fragments are ionized and sorted to obtain their mass-to-charge (m/z) ratios.

- The ionization modes most commonly employed in proteomics are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI).
- The mass analyzers most commonly employed in proteomics are time of flight (TOF), quadrupole, ion traps, and Orbitrap or Fourier transform (FT).

Definition of Words and Terms

Articular cartilage	Hyaline tissue found on joint surfaces, covering the articular end of the bones. It is a highly structured avascular and aneural tissue, composed of an extracellular matrix in which the cells (chondrotytes) are embedded.
Biomarker	Substance or feature used as an indicator of a biological state.
Mass spectrometry	Analytical technology capable of identifying and quantifying molecules (peptides, lipids, metabolites and other small molecules) in simple and complex mixtures by measuring the mass-to-charge ratio of their ions.
Osteoarthritis	A disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including proinflammatory pathways of innate immunity.
Proteomics	An area of biology focused on the large-scale analysis of proteins (Proteome), their abundance, modifications, and interactions.
Synovial fluid	Viscous fluid found in the cavities of synovial joints, responsible of lubricating these joints and reducing friction between the cartilages that cover the articular ends of the bones.
Synovium	A specialized connective tissue localized at the surface of capsules of synovial joints. It is composed of two layers (intima and subintima).

Osteoarthritis

Osteoarthritis (OA), the most frequently diagnosed arthropathy, is a common, slowly progressive condition that may affect the structure of any joint and is a major cause of pain and chronic disability in the elderly. A definition for OA recently proposed by OARSI (Osteoarthritis Research Society International) is “a disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including proinflammatory pathways of innate immunity” (Kraus et al. 2015). Prevalence studies show that OA usually develops from the age of 45 and increases with

age. It affects more than 10% of the population, and most people over 65 exhibit OA pathology. OA is the leading cause of permanent work incapacity and one of the most common reasons for visiting a primary care physician.

OA is a very complex disease that has a multifactorial etiology. Some clearly identified risk factors include aging, obesity, genetic factors (Valdes et al. 2006), and mechanical injuries (Lohmander et al. 2004; Roos 2005). The complexity of processes underlying OA pathogenesis and the diversity of its clinical presentation hamper the development of tools sensitive and specific enough for precise diagnosis and monitoring. In most individuals, OA is characterized by an initial clinically silent phase, followed by radiographically detectable extensive deterioration of cartilage and structural joint changes. To date, the diagnosis of OA relies on the patient's subjective description of pain or stiffness symptoms and on radiographic criteria, such as joint space width. Unfortunately, these diagnostic tools lack sufficient sensitivity for detection of small changes and do not provide accurate information about disease progression. The lack of accurate and sensitive monitoring methods is especially critical in the case of OA because the development of new drugs or therapeutic strategies is hindered. Existing therapies for OA are limited to pain alleviation and have no effect in slowing or halting disease progression. Therefore, there is great interest in the discovery and validation of novel OA disease markers, both to enable early diagnosis and monitoring of joint destruction and to facilitate the development of OA-modifying therapies.

The Challenge of Finding Biomarkers for OA

Biological markers are needed to understand and characterize disease types, status, progression, and response to therapy; they must possess proven validity, reproducibility, and predictive value. By the time that OA patients manifest symptoms of the disease, cartilage degradation and other joint alterations have progressed. Therefore, markers specific for pathological joint turnover that can be screened for in advance of symptom development would be most useful. This is a difficult mission because cartilage degradation is not consistent during OA disease evolution, being characterized by intermittent periods of progressive cartilage destruction and remission. Moreover, highly sensitive methods are required to identify biological markers because the release of specific proteins or the appearance of neoepitopes during periods of cartilage degradation is slow, and because of their dilution in biological fluids.

Strong candidate biomarkers for OA should be relevant to processes occurring in the joint and to clinical endpoints, including structural damage, pain, dysfunction, or joint replacement. These biomarkers would permit screening for early diagnosis, thus enabling the selection of procedures designed to slow disease progression (Ruiz-Romero et al. 2015) before there are clinical symptoms or imaging evidence of the development of the disease (Fig. 1). This early diagnosis would facilitate the discovery of new drugs and efficacy monitoring, thus providing information concerning their success in pharmacological trials. Although both images (x-rays or MRI) and biochemical molecules can be considered to be markers of OA, this

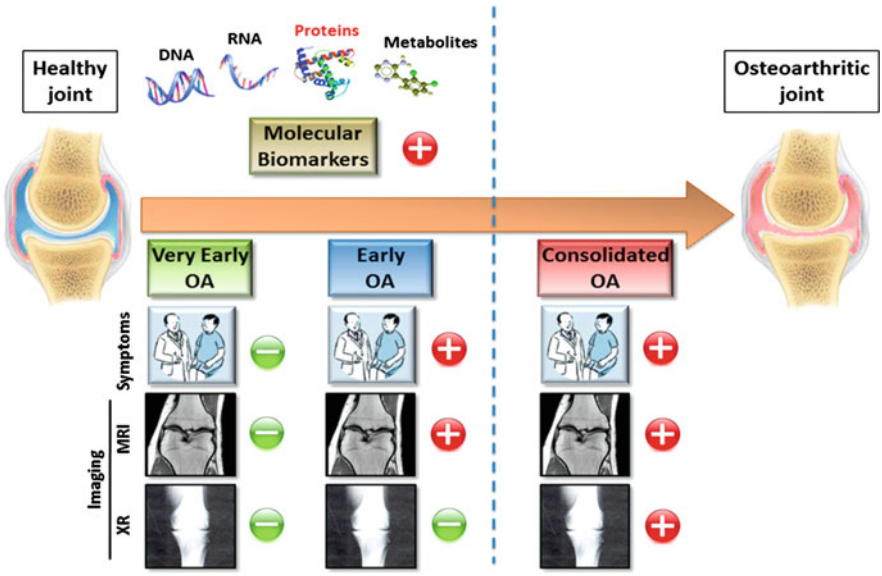

Fig. 1 The need for molecular biomarkers to improve the diagnosis of OA. Molecular biomarkers need to facilitate disease diagnosis at early stages of the process, in which there are no clinical symptoms or imaging features (Adapted from Ruiz-Romero et al. 2015)

chapter will focus on protein biomarkers and the utility that proteomic technologies have for generating novel biomarker candidates that may prove useful for early diagnosis, prognosis, and drug efficacy studies.

"Classical" Protein Biomarkers of OA

Optimal OA biomarker candidates are molecules or molecular fragments present in the cartilage, bone, or synovium of the joint. To date, several proteins directly or indirectly involved in cartilage degradation, or proteins synthesized in an attempt to repair cartilage, have been tested in clinical trials for their use as putative biomarkers of OA (Table 1). Many of these proteins shown in Table 1 are associated with the metabolism of type II collagen in cartilage or type I collagen in subchondral bone, or the metabolism of aggrecan in cartilage.

Because type II collagen (COL2) is relatively specific to and highly abundant in the articular cartilage extracellular matrix, several studies have examined this protein and its fragments (Olsen et al. 2007). One widely used biomarker for COL2 degradation is a fragment from the C-telopeptide region (CTX-II), which can be measured in serum or urine; urinary levels of this fragment are strongly associated with radiographic subtypes of OA (Valdes et al. 2014; van Spil et al. 2013). A sequence of the triple helix of COL2 and its nitrosylated form (Coll2-1 and

Table 1 Candidate biological markers for bone, cartilage, and synovial turnover that have been examined for use in osteoarthritis (OA) monitoring

Tissue	Protein	Investigated markers	
		Synthesis	Degradation
Bone	Type I collagen	N- and C-propeptides (PICP, PINP)	Pyridinoline (PYD) Deoxypyridinoline (DPD) C- and N-telopeptides (CTX-I, NTX-I, ICTP) Helical peptide
	Noncollagenous proteins	Osteocalcin Bone alkaline phosphatase	Bone sialoprotein (BSP) Tartrate resistant acid phosphatase (TRAP, 5b isoenzyme) Cathepsin K
Cartilage	Type II collagen	N- and C-propeptides (PIICP, PIIANP and PIIBNP)	PYD Type II collagen C-telopeptide (CTX-II) Type II collagen collagenase neoepitopes (C2C, C12C, TIINE) Type II collagen helical fragments (Helix-II and Coll 2-1)
	Aggrecan	Chondroitin sulfate (epitopes 846, 3B3, 7D4)	Core protein MMPs and aggrecanase neoepitopes Keratan sulfate (epitopes 5D4, ANP9)
	Nonaggrecan and noncollagenous proteins	Glycoprotein 39 (YKL-40) Cartilage-derived retinoic acid sensitive protein (CD-RAP)	COMP
Synovium	Type III collagen	Type III N-propeptide (PIIINP)	PYD CTX-I, NTX-I Glucosyl-galactosyl-pyridinoline (Glc-Gal-PYD)
	Noncollagenous proteins	Hyaluronan YKL-40 COMP MMP-1, 2, 3, 9	

Coll2-1NO2) has been recognized by two recently developed different assays and suggested for use as markers for response to viscosupplementation (McAlindon et al. 2014). COL2 degradation is also indicated by other molecules, including Helix-II, C2C, and urinary TIINE (type II collagen neoepitope). Finally, measuring COL2 synthesis has proven indicative of OA. Determination of COL2 propeptides, such as PIIANP showed increased levels in incipient knee OA compared to healthy controls, while patients in later stages of OA had lowered values (Rousseau et al. 2004). This finding suggests that there is a cartilage repair mechanism in the early development of OA that is not detectable in advanced stages.

Other noncollagenous proteins have been examined as possible markers for cartilage turnover which are as follows: cartilage oligomeric matrix protein (COMP) (Hoch et al. 2011); cartilage glycoprotein 39 (YKL-40) (Huang and Wu 2009); proteoglycans, such as keratan sulfate (KS) and chondroitin sulfate (CS); and enzymes involved in the breakdown and turnover of collagens, such as matrix metalloproteinases (MMPs), whose activity and inhibition is controlled by a variety of tissue inhibitors (TIMPs), proinflammatory cytokines, and growth factors (Raynauld et al. 2011).

Traditional OA biomarker studies have been impacted by problems created by the use of small sample sizes and case–control designs using cases recruited from secondary care facilities. In recent years, several studies have addressed these limitations by using larger cohorts. These studies have included the analysis of urinary CTX-II, serum COMP, serum MMP-degraded type II collagen (sC2M), and serum hyaluronic acid (sHA), among others (Aslam et al. 2014; Bos et al. 2013; Valdes et al. 2014; van Spil et al. 2013). These studies reported various associations between the altered presence of these molecules in urine or serum of patients and OA and suggested their possible value as predictors or to measure disease activity.

In spite of the promising results obtained with these proteins, no single biomarker has yet been introduced clinically. Because biochemical markers of joint tissue turnover would have important roles in clinical rheumatology, a classification of biomarker utility was proposed some years ago by the Osteoarthritis Biomarkers Network. This classification, designated as BIPED, focused on the description of the potential uses of a marker to qualify it for a specific clinical use, including burden of disease, investigative, prognostic, and efficacy of intervention and diagnosis (Bauer et al. 2006) (Table 2).

Because most studies performed for biomarker qualification in OA revealed a large overlap in single marker levels between OA patients and controls, they are insufficiently sensitive to be useful as diagnostic tools when used independently (Henrotin et al. 2007). These authors pointed out the utility of analyzing entire panels

Table 2 The BIPED classification scheme for the description of the clinical use of osteoarthritis (OA) biomarker candidates

BIPED	Definition	Description/goal	Example	Ref.
B	Burden of disease	To assess the severity or extent of OA among affected individuals	sCOMP	(Fernandes et al. 2007)
I	Investigative	Insufficient information to include in other category		
P	Prognostic	To provide information about the likely clinical course of disease	uCTXII sCOMP	(Chaganti et al. 2008; Reijman et al. 2004)
E	Efficacy of intervention	To discriminate between patients with and without treatment, or before and after treatment within patients	uCTXII	(Mazieres et al. 2007)
D	Diagnostic	To identify patients suffering from OA	uCTXII	(Garnero et al. 2000)

of available biomarkers as putative diagnostic tests. Accordingly, other investigators analyzing the association between individual biochemical markers and radiographic features established that this association was improved when selected biochemical markers were combined into a single factor (Davis et al. 2007). Overall, these findings suggest, as found with diagnostic biomarkers, that combinations of biomarkers may be more sensitive than individual measurements for reflecting structural damage in OA patients. Large-scale analyses, allowing for the simultaneous analysis of multiple molecules, are valuable tools for discovery and validation of biomarkers that would facilitate more OA biomarker candidates to achieve the qualification phase (Blanco 2014).

Value of Proteomics in OA Biomarker Research

As previously mentioned, the lack of a complete understanding of the complex etiology and pathogenesis of OA has hindered the identification of molecules that might serve as disease process markers, thus contributing to the difficulties of early diagnosis and evaluation of drug efficacy. In the search for novel biomarkers of OA, large-scale "omics" analyses have become vital research tools for biomarker discovery, and several approaches using this technology have been implemented (Ruiz-Romero and Blanco 2010). In contrast to nucleic acid-based expression studies, the recently emerged proteomic approaches have the advantage of studying actual functional molecules. Because possible disconnections between genes and protein expression levels are eliminated by proteomics approaches, this technology is a powerful method for the discovery of potential novel biomarkers.

Proteomic research utilizes the isolation of proteins from biological samples and their separation, identification, and quantification, usually by mass spectrometry (MS) (Fig. 2). This profound characterization of protein mixtures facilitates the

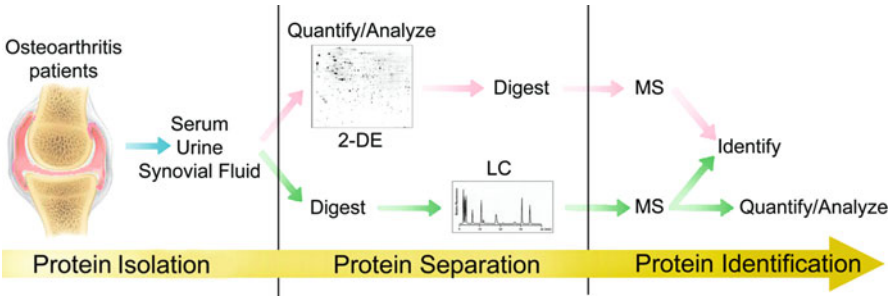

Fig. 2 Workflow of proteomic approaches for biomarker discovery in osteoarthritis. Proteomic analyses involve three independent steps: obtaining a protein extract, separation of its proteins (carried out using either gel electrophoresis- or liquid chromatography-based techniques) and identification/quantification of the proteins, generally by mass spectrometry (Ruiz-Romero and Blanco 2010). *2-DE* two-dimensional gel electrophoresis, *LC* liquid chromatography, *MS* mass spectrometry

understanding of complex biological systems and the determination of relationships among proteins, including function and protein-protein interactions. The nontargeted global approaches used in proteomics make it possible to monitor changes in abundance and structural modifications of proteins, as well as to establish putative associations of proteins with disease and treatments. During the last decade, several "shotgun" proteomics studies have been performed to increase knowledge of the pathogenesis of OA and to facilitate the search for novel protein biomarkers. Most studies on the pathogenesis of OA have used joint tissues and cells and their secreted fractions (Ruiz-Romero and Blanco 2009). On the other hand, analyses with the goal of discovering novel biomarkers have primarily made use of more accessible biological fluids and samples derived from them. A summary of the studies performed to date to search for OA biomarkers in such biological fluids as serum and synovial fluid is shown in Tables 3 and 4.

There are two general approaches to finding proteomic biomarkers: target-specific and global/nondirected. The target-specific approach uses antibodies to screen for specific proteins through enzyme-linked immunosorbent assays (ELISAs), antibody arrays, or western blot analyses. Although these techniques generally can only survey a few proteins simultaneously, and therefore are not ideal for discovering biomarkers, recent advances in the field of MS, which include selected/multiple reaction monitoring assays and protein arrays, are more suitable for detecting biomarkers. Immunoaffinity LC-MS/MS for detection of serum amyloid A-derived peptides in rheumatic SFs (Yavin et al. 2000), collagen type II neoepitope peptides in urine (Nemirovskiy et al. 2007), and endogenous aggrecan fragments in both SF and urine (Dufield et al. 2010) have been developed as targeted approaches to finding proteomic biomarkers. Additionally, a targeted nucleic acid programmable 80-protein array (NAPPA) was recently established to more completely inventory the autoantibody profile OA (Henjes et al. 2014).

In contrast, relatively unbiased, high-throughput screens using global/nondirected approaches may be better suited than target-specific approaches for biomarker discovery. Nondirected approaches encompass those that profile unidentified proteins and those that profile identified proteins (Ruiz-Romero and Blanco 2010). Protein profiling of unidentified proteins is usually accomplished through matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS), or surface-enhanced laser desorption/ionization (SELDI)-TOF-MS. These techniques have the advantage of speed, which makes them attractive tools for clinical screening. The detection of serum biomarkers associated with RA was the first peptide profiling strategy (de Seny et al. 2005, 2008). In a subsequent study specifically focused on OA, four differential MS peaks occurring among OA, RA, and control serum samples were identified by these authors (de Seny et al. 2011). Because of their high throughput, similar approaches using ion exchange chromatography magnetic beads to reduce sample complexity prior to MALDI-TOF analysis have been frequently used. This approach recently enabled the identification of potentially prognostic markers for knee OA (Takinami et al. 2013) (Table 3).

Table 3 Proteomic studies performed on human serum to search for osteoarthritis (OA) biomarkers (Adapted from Ruiz-Romero et al. 2015)

Serum/plasma	**Method**	**Mass spectrometer**	**Number of proteins identified**	**Relevant proteins**	**Ref.**
OA vs. HC	DIGE	MALDI-TOF/TOF	16	HPT	(Fernandez-Costa et al. 2012)
RA vs. OA, PsA, Asthma, Crohn's, and HC	ProteinChip array	SELDI-TOF	–	MRP-8	(de Seny et al. 2005)
OA vs. RA vs. HC	ProteinChip array	SELDI-TOF	4	V65 vitronectin fragment, C3f peptide, CTAPIII, m/z 3762	(de Seny et al. 2011)
Progressive vs. nonprogressive OA	ProteinChip array	SELDI-TOF	3	APOC-I, APOC-III, TTHY fragment	(Takinami et al. 2013)
OA K/L I/II vs. K/L IV vs. HC	iTRAQ + LC-MS/MS	MALDI-TOF/TOF	262	26 differential proteins	(Fernandez-Puente et al. 2011)
OA vs. RA vs. HC autoAb profiling	Antigen and NAPPA arrays	–	80	Anti-CHST14 autoantibodies	(Henjes et al. 2014)
Serum and synovial fluid	**Method**	**Mass spectrometer**	**Number of proteins identified**	**Relevant proteins**	**Ref.**
RA vs. OA	2-DE	MALDI-TOF	–	FIBB, SAA, MRP14 MRP-8, MRP-14	(Drynda et al. 2004; Sinz et al. 2002)

HC healthy controls, *2-DE* two-dimensional gel electrophoresis, *LC* liquid chromatography, *MALDI-TOF/TOF* Matrix-assisted laser desorption/ionization-time of flight, *MS* mass spectrometry, *RA* rheumatoid arthritis, *PsA* psoriatic arthritis, *K/L* Kellgren and Lawrence scale, *iTRAQ* isobaric tag for relative quantization, *SELDI* surface-enhanced laser desorption/ionization, *NAPPA* nucleic acid programmable protein array, *DIGE* differential in-gel electrophoresis

Table 4 Proteomic studies performed on human synovial fluid to search for osteoarthritis (OA) biomarkers (Adapted from Ruiz-Romero et al. 2015)

SF	Method	Mass spectrometer	Number of proteins identified	Relevant proteins	Ref.
Early and late OA vs. HC	1D-PAGE	Ion trap	135	18	(Gobezie et al. 2007)
Early and late OA vs. HC	2D-DIGE	MALDI-TOF/LC-Triple quadrupole	66	5 verified by MRM	(Ritter et al. 2013)
RA vs. OA	1D-PAGE	LC-MALDI-TOF/TOF	136	MMP1, BIGH3, FINC, GELS	(Mateos et al. 2012)
RA vs. OA	ProteinChip array	SELDI-TOF		3 m/z peaks. S100A12	(Han et al. 2012)
RA vs. OA	LC-MS/MS	FT-TOF/TOF Triple quadrupole	677	135 differential proteins. CAPG	(Balakrishnan et al. 2014)
RA vs. OA	LC-MS/MS	SELDI	1	MRP-8	(Uchida et al. 2002)
OA vs. HC	UF + LC-MS/MS	LTQ-Orbitrap	40	COL2, PRG4, SAA, TUB, VIME, MGP	(Kamphorst et al. 2007)
OA vs. HC	MB + MALDI-TOF	MALDI-TOF/TOF	–	Two peptide peaks	(Pan et al. 2012)
PsA vs. early OA	LC-MS/MS + SRM	LTQ-Orbitrap TSQ Vantage	137	12 proteins quantified by SRM	(Cretu et al. 2014)

MB magnetic beads, *PAGE* polyacrylamide gel electrophoresis, *SRM* selected reaction monitoring, *UF* ultrafiltration. Other abbreviations, as in Table 3

However, because peptide profiling approaches do not usually allow differential MS peak identification, validation of findings by other means is difficult and information on the biological significance of the findings is lacking. Therefore, many proteomic strategies based on protein fragmentation, identification by tandem MS, and subsequent data analysis have been developed for generating profiles of identified proteins. In OA research, quantitative proteomic profiling of sera from patients with different grades of OA and from healthy controls first removed the most abundant proteins (albumin, immunoglobulins and others) from the sample, thus enriching the levels of the less abundant proteins in the serum fraction. To identify a panel of proteins whose abundance was associated with OA, differential labeling was then performed, followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis (Fernandez-Puente et al. 2011).

Studying the synovial fluid (SF) proteome has proven highly advantageous for OA research. The progression of OA is a long process and inconsistent in development with the slow release of specific proteins or the appearance of neoepitopes. Therefore, the use of SF proteomic technologies is advantageous in the quest for biochemical markers of OA because of dilution in serum and other biological fluids (Table 4). SF is derived directly from the disease site, thus this compartment has great potential to contain OA biomarkers, which directly reflect joint cavity alterations in the composition of SF due to injury or disease and should better correlate with disease severity and progression (Hui et al. 2012). In SF samples from OA and RA patients, and in those from healthy subjects, LC-MS/MS tools have been used to identify several proteins (Gobezie et al. 2007; Kamphorst et al. 2007). A proteomic comparison of SF from OA and RA patients has identified two panels of SF proteins characteristic of each disease (Mateos et al. 2012). A more recently study identified at least 575 proteins in SF, 135 of which were differentially abundant in OA compared to RA (Balakrishnan et al. 2014). Putative SF biomarkers for psoriatic arthritis were documented using OA samples as the control group (Cretu et al. 2014). Finally, other techniques not based on LC-MS/MS have been performed on SF. Peaks associated with OA were identified in peptide profiling studies using SELDI (Han et al. 2012) and weak cation exchange magnetic beads with subsequent MALDI-MS profiling (Pan et al. 2012). Furthermore, two-dimensional electrophoresis (2-DE) strategies coupled to MS identification have also been employed to screen for differentially expressed proteins in SF. Using this approach, samples from early OA, late OA, and healthy controls were evaluated in a study using differential in-gel electrophoresis (DIGE) (Ritter et al. 2013).

Potential Applications of OA Biomarkers to Disease Prognosis and Other Diseases or Conditions

In view of the results obtained in clinical trials to date for targeted screening of proteins possibly associated with OA, C-terminal telopeptide of collagen type II (CTX-II) and serum cartilage oligomeric protein (COMP) appear to be the most promising biomarker candidates. Unfortunately, none of the candidates thus far analyzed has proven to be discriminative enough to: (i) differentiate between individual OA patients and controls (diagnostic) or between patients with different disease severities (burden of disease); (ii) predict prognosis in individuals with or without the disease (prognostic); or (iii) perform sufficiently consistently to function as a surrogate outcome in clinical trials (efficacy of intervention). In spite of the intense efforts over the last decade, to date, there is no sufficiently validated or qualified biochemical marker acceptable for systematic use in diagnostic or monitoring tests for OA (Lotz et al. 2013).

This conclusion emphasizes the need to analyze entire panels of available biomarkers as putative diagnostic tests. The limited multiplex capacity of classical

ELISA-based strategies has increased costs and hampered the simultaneous evaluation of biomarker panels in large cohorts. Therefore, the capacity of proteomics technologies to perform multiplexed analysis of proteins is very advantageous in the study of the complex disease of OA, for which no single molecule is currently the gold standard. In an effort to facilitate the movement of candidates from the discovery phase into clinical application, after 2 years of basic research, proteomics technologies have matured sufficiently that their use in clinical practice appears practical and useful (Aebersold et al. 2013). Targeted proteomics strategies, either based on MS, such as selected/multiple reaction monitoring assays (Ritter et al. 2014), or antibodies, such as multiplex bead array assays (Henjes et al. 2014), are increasingly applicable for biomarker verification in OA and other pathological conditions. However, standardization and quality control of these procedures must be established to ensure that proteomics assays are validated for use as in vitro diagnostic tests to assure the analytical validity of the test procedure and outcome.

Summary Points

- This chapter focuses on protein biomarkers for osteoarthritis (OA), the most prevalent rheumatic disease.
- To date, no single protein has been qualified to be an OA biomarker in clinical applications.
- Results obtained in clinical trials show that the power of combining marker candidates increases their association with disease.
- Proteomics is a powerful technology for large-scale monitoring of changes in protein abundance or structure and for establishing their potential association with disease or treatments.
- In the last decade, using shotgun proteomics technologies, a number of proteins have emerged as possible biomarkers for OA.
- Further efforts are required to qualify biomarker candidates for use in the management of OA.

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Adiponectin as Biomarker of Osteoporosis 38

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Abstract

Adiponectin is one of the adipose tissue hormones synthesized and released mainly by mature adipocytes of visceral white adipose tissue. So far, scientific studies have been focused on the effect of adiponectin on regulation of glucose and fatty acid metabolism and its connection to cardiovascular system diseases and diabetes mellitus as well as the occurrence of metabolic syndrome. The latest reports indicate that this hormone is expressed not only on hepatocytes, endothelial cells, skeletal muscles, and central nervous system but also on osteoblasts, as shown by the presence of its specific membrane receptors (AdipoR1 and AdipoR2). Based on many reference data, it seems that adiponectin may be a link connecting the metabolism of adipose tissue and bone tissue. Due to its connection to bone turnover markers, it is a potential marker of osteoporosis.

Keywords

Adiponectin • AdipoR1 • Adipo R2 • Osteogenesis • Osteoporosis

List of Abbreviations

5′AMP	Activated protein kinase 5′
ACC	Acetyl-CoA carboxylase
Acrp30	Adipocyte complement related protein of 30 kDa
AdipoQ	Adiponectin, C1Q and collagen domain containing
AMPK	AMP-activated(-related) protein kinase
AN	Anorexia nervosa
apM1	Adipocyte most abundant gene transcript 1
BMD	Bone mineral density
BMI	Body mass index
BN	Bulimia nervosa
DHEA-S	Dehydroepiandrosterone sulfate
ERA	Early rheumatoid arthritis
FM	Fat mass
GBP	Gastric bypass surgery
GBP28	Gelatin-binding protein of 28 kDa
GDM	Gestational diabetes mellitus
GIGT	Gestational impaired glucose tolerance
GR	Glucocorticoid receptor
HMW	High molecular weight complex
IGF-1	Insulin-like growth factor 1
IGF-2	Insulin-like growth factor 2
IL-6	Interleukin 6
LAGB	Laparoscopic adjustable gastric band
LMW	Low molecular weight trimer-dimer
MAPK	Mitogen-activated protein kinase

MCP-1	Monocyte chemotactic protein
MMP-3, MMP-9	Matrix metalloproteinase 3 and 9
MMW	Middle molecular weight
MP	Metabolic phenotype
NOS2	Nitric oxide synthase 2
NTG	Normal glucose tolerance
OA	Osteoarthritis
OGL	Oral glucose load
OPG	Osteoprotegerin
PCOS	Polycystic ovary syndrome
PPAR	Peroxisome proliferator-activated receptor
PPAR-α	Peroxisome proliferator-activated receptor alpha
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RYGB	Roux-en-Y gastric bypass
SHBG	Sex hormone binding globulin
SREBP	Sterol regulatory element-binding protein
T1DM	Diabetes mellitus type 1
T2DM	Diabetes mellitus type 2
TAL	Total adiponectin level
TNF-α	Tumor necrosis factor alpha
UA	Undifferentiated arthritis
VBG	Vertical banded gastroplasty

Key Facts of Adiponectin

Adiponectin is one of the adipose tissue hormones synthesized and released mainly by mature adipocytes of visceral white adipose tissue and to a lesser extent by adipocytes of peripheral adipose tissue and bone marrow. It circulates in three different forms: high molecular weight (18-36mer), low molecular weight (hexamer), and a trimeric form. Adiponectin level is inversely related to visceral fat along with body mass index and positively related with biochemical markers of bone loss. Concentration of adiponectin varies greatly among even in subjects with similar BMIs, and the literature shows that this hormone depends on sex and is higher in women than in men.

Specific correlations between adiponectin and other biochemical parameters during osteoporosis should give useful information and determine the role of adiponectin in progression or inhibition of osteoporotic changes. Sadly, basing on current knowledge, adiponectin cannot be used as a clear-cut predictive marker for osteoporotic fracture risk, because its concentration changes not only during osteoporosis but in different medical conditions associated with inflammation or weigh loss too.

Definition of Words and Terms

Adiponectin	A polypeptide composed of 244 amino acids with a molecular weight of approximately 30 kDa being synthesized and released mainly by mature adipocytes of visceral white adipose tissue. Adiponectin MRNA has been identified, among others, in hepatocytes, endothelial cells, skeletal muscle, central nervous system, and osteoblasts.
AdipoR	Specific membrane receptor of adiponectin occurring in two isoforms: AdipoR1 and AdipoR2.
Anorexia (AN, anorexia nervosa)	is a type of psychosomatic disorder in which a sick person subjectively assesses his/her body weight as too high, resulting in extreme cachexia through very restrictive diet.
apM1 (ACDC)	Adiponectin gene.
Bone remodeling	is an active and dynamic lifelong process where mature bone tissue is removed from the skeleton (bone resorption) and new bone tissue is formed (bone formation). The remodeling cycle consists of three consecutive phases: resorption, reversal, formation and involves the removal of mineralized bone by osteoclasts followed by the formation of bone matrix through osteoblasts.
Cell differentiation	Process by which cells become progressively more specialized to possess a more distinct form and function.
Mesenchymal stem cells (MSCs)	Multipotent stromal cells that can differentiate into a variety of cell types such as: osteoblasts, chondrocytes, myocytes, adipocytes.
Osteoporosis	Very heterogeneous disease process, dependent on many causative factors, being characterized by low bone mass and deterioration of bone tissue, leading to increased bone fragility and risk of bone fractures (mainly hip, spine, wrist, and shoulders).
Peroxisome proliferator-activated receptor gamma 2 (PPAR©2)	Group of nuclear receptor proteins that function as transcription factors regulating the expression of genes, play essential roles in the regulation of cellular differentiation, development, and metabolism.

Transforming growth factor beta (TGF-β)	Secreted protein, type of cytokine that controls proliferation, cellular differentiation, and other functions, is part of a superfamily of proteins known as the transforming growth factor beta superfamily.

Introduction

There has been increased interest in adipose tissue as an endocrine organ, and several of these secreted proteins, termed adipokines, are currently undergoing extensive study regarding roles as divergent as feeding behavior to osteoporosis protection (Pajvani et al. 2003; Kontogianni et al. 2004; Richards et al. 2007).

Adiponectin was identified and later described independently by four research teams (1995–1996) to what it owes equivalent names being in use: Acrp30 (*adipocyte complement related protein of 30 kDa*), AdipoQ (*adiponection*, *C1Q*, *and collagen domain containing*), GBP28 (gelatin-binding protein of 28 kDa), and apM1 (*adipocyte most abundant gene transcript 1*) (Scherer et al. 1995; Hu et al. 1996; Maeda et al. 1996; Nakano et al. 1996).

Adiponectin is an adipocyte-specific secretory protein produced by differentiated adipocytes and its concentration is observed in a relatively large amount in human serum. This protein plays an important role in the regulation of glucose and fatty acid metabolism in the liver and muscles, and its activity may be connected to bone structure and human osteoblastic proliferation (Hu et al. 1996; Pajvani et al. 2003). The aim of this study is to show the importance of adiponectin as a potential marker of osteoporotic lesions.

Adiponectin – Basic Information

Biosynthesis, Structure, Target Tissues

Adiponectin is synthesized and released mainly by mature adipocytes of visceral white adipose tissue, although its expression is also observed in brown adipose tissue. Recent reports have indicated that adiponectin mRNA is identified in hepatocytes, endothelial cells, skeletal muscle, central nervous system, and osteoblasts (Berner et al. 2004).

Human adiponectin is biosynthesized as a polypeptide being composed of 244 amino acids with a molecular weight of approximately 30 kDa, 17 of which are a signal sequence, the cleavage of which is followed by formation of mature protein with a molecular weight of 28 kDa (Scherer et al. 1995; Hu et al. 1996; Maeda et al. 1996). It is characterized by a complex structure which consists of an N-terminal signal sequence, a short variable section not showing homology with

any other protein, a globular subunit situated on the carboxyl terminus, and a fibrous domain located on the amine terminus (Maeda et al. 1996; Kershaw and Flier 2004). A globular domain sequence is characterized by strong similarity to one of complement proteins, i.e., C1q, and shows a certain homology to the trimeric structure of factors of the TNF family, whereas a fibrous domain resembles type VIII and type X collagen (Maeda et al. 1996; Pajvani et al. 2003). Due to its structure, adiponectin can form multimers. Globular domains assemble into homotrimers, whereas fibrous domains into higher-order structures composed of 12, 18, or more adiponectin molecules (Waki et al. 2003). Adiponectin occurs in three forms which are characterized by different degree of oligomerization: a fraction with the lowest molecular weight (low molecular weight trimer-dimer, LMW) containing adiponectin trimers, a complex with medium molecular weight (middle molecular weight hexamer, MMW), and a complex with the highest molecular weight (high molecular weight multimer, 18-36-mer HMW), which is made of multimers consisting 6 (hexamer) and 12–18 adiponectin molecules, respectively (Pajvani et al. 2003; Waki et al. 2003; Kershaw and Flier 2004). Still little is known about the regulation and significance of these adiponectin complexes in serum and about the events that lead to the generation of bioactive ligand (Pajvani et al. 2003).

A basic structural unit of adiponectin being released outside of the cell is trimers composed of three protein molecules linked by hydrogen bonds within a globular domain (Nakano et al. 1996; Pajvani et al. 2003). Further oligomerization of timers can occur in blood serum, resulting in development of more complex multimer forms. Formation of disulphide bridges within fibrous domains, being formed with the participation of cysteine in codon 36 (human adiponectin) or codon 39 (murine model), is responsible for oligomerization of trimers (hexamers and higher-order multimers, HMW) (Waki et al. 2003). Furthermore, there is also a globular adiponectin in blood serum, being a product of proteolytic degradation, with leukocyte elastase – liberated by activated monocytes and/or neutrophiles – being involved in it.

Significant differences are observed in the concentration of respective adiponectin multimeric forms and their proportion in blood serum, which probably depends on such factors as gender and obesity degree (Arita et al. 1999; Table 2). Intraindividual variation in HMW fraction concentrations is of particular interest. It is believed that the HMW isoform has a pro-inflammatory effect, whereas LMW an anti-inflammatory one. The latest studies show that HMW-form adiponectin concentration is sexually differentiated (Waki et al. 2003; Horáková et al. 2015); moreover, higher levels have been found in lean subjects, whereas such relationship has not been observed for hexamers and trimers (Horáková et al. 2015; Kobayashi et al. 2004). In patients with coronary heart disease, an increased level of trimers and no changes in hexamer concentration in blood have been shown (Kobayashi et al. 2004). Body mass reduction leads to an increase in the concentration of this particular fraction of adiponectin (Kobayashi et al. 2004).

Receptors, Transport

Adiponectin bioactivity refers mostly to liver tissue, skeletal muscle tissue, and blood vessels, but bone tissue, uterus, and the brain have been only recently taken into account, too (Kharroubi et al. 2003; Kershaw and Flier 2004; Kadowaki and Yamauchi 2005; Kim et al. 2010). Adiponectin signaling pathway is not yet fully understood but it is known that adiponectin affects the target tissues by a specific membrane receptor, being found in two isoforms: AdipoR1 and AdipoR2. The specificity of adiponectin interaction with receptors and the activation of respective signaling pathways depends on the degree of its oligomerization and posttranslation modification of adiponectin (hydroxylation and subsequent glycosylation of four lysine residues and hydroxylation of seven proline residues within a collagen domain play a key role in the formation of HMW polymers, which determines adequate adiponectin bioactivity) (Kharroubi et al. 2003; Kershaw and Flier 2004). Common features and those differentiating the above isoforms of receptors for adiponectin are presented in Table 1.

Table 1 Comparative characteristics of adiponectin receptors AdipoR1 and AdipoR2

Trait	AdipoR 1	AdipoR 2	Reference
Structure	Seven transmembrane domains		Yamauchi et al. 2003
Intracellular signaling	Kinase phosphorylation: MAPK (mitogen-activated protein kinase), AMPK (AMP-activated protein kinase) Nuclear receptor PPARa activation		Yamauchi et al. 2003
Genetic loci	ADR1 – chromosome 1 (1q32.1)	ADR2 – chromosome 12 (12p13.33)	Kharroubi et al. 2003
Tissue-specific expression	Skeletal muscle Brain Heart Kidney Liver Placenta Pancreatic β cells Macrophages Osteoblasts Chondrocytes	Liver Skeletal muscle Osteoblasts Chondrocytes	Kershaw and Flier 2004; Kim et al. 2010; Kadowaki and Yamauchi 2005; Kharroubi et al. 2003; Xibillé-Friedmann et al. 2015 Berner et al. 2004 Luo et al. 2005
Binding affinity	Adiponectin trimer	Higher-order multimers (MMW, HMW)	Yamauchi et al. 2003

Metabolism

As reported in reference data, the cDNA encoding protein Acrp30 was first described by Scherer and collaborators in 1995 (Scherer et al. 1995). Adiponectin gene is located in the region of chromosome 3 (3q27) that contains the adiponectin structural gene (apM1, ACDC) (Kissebah et al. 2000; Takahashi et al. 2000; Comuzzie et al. 2001). The apM1 spans 16 kb (kilobase pairs) and is composed of three exons, being 18, 222, and 4277 kb long, respectively (Saito et al. 1999). Exon 1 does not contain an encoding sequence which occupies only a portion of exon 2 and exon 3. The genetic location of adiponectin encoding (locus 3q27) indicates its possible connection to the occurrence of many diseases, including metabolic syndrome and type 2 diabetes mellitus phenotypes (Comuzzie et al. 2001; Al-Daghri et al. 2012). Furthermore, in the promoter region of the ACDC gene, the sequences, the so-called response elements, have been found which can indicate that ACDC gene expression may change according to body energy status and lipid store of adipose tissue. Among others, these sequences are: PPAR (peroxisome proliferator-activated receptor), SREBP (sterol regulatory element-binding protein), and GR (glucocorticoid receptor), being recognized by nuclear receptors/transcription factors (Iwaki et al. 2003; Seo et al. 2004). The apM1 gene is expressed in adipose tissue only. It is characterized by an increase with the reduction of body weight under the influence of IGF-1 but drops with the development of obesity and under the influence of glucocorticoids, TNF-α, and β-adrenergic receptor agonists (Arita et al. 1999; Fasshauer et al. 2001; Fasshauer et al. 2003).

An important factor affecting the expression of ACDC gene is insulin which may inhibit the transcription of this gene or decrease the stability of mRNA, controlling at the same time adiponectin concentration in blood or accelerating its removal from bloodstream. The adiponectin-insulin interactions are, to some extent, reciprocal because adiponectin increases tissue insulin sensitivity by decreasing the concentration of triacylglyceroles in skeletal muscles, which results in enhanced insulin signaling.

The next important factor affecting the expression of apM1 gene is peroxisome proliferator-activated receptor-alpha (PPAR-α), being a dominant PPAR isoform in adipose tissue. PPAR-α's act as ligand-activated transcription factors and stimulate the expression of genes associated with carbohydrate and fatty acid metabolism (e.g., FAT/CD36, acyl-CoA oxidase, and UCP-2) and also have an effect on the proliferation and differentiation of adipocytes (Brun and Spiegelman 1997; Yamauchi et al. 2001).

One of the main functions of adiponectin is regulation of carbohydrate and fatty acid metabolism in the liver and muscles, which is directly connected with the regulation of energy balance and the magnitude of body weight. Adiponectin bioactivity depends primarily on the degree of its oligomerization which determines the specificity of its interaction with receptors, which translates into activation of respective signaling pathways (Yamauchi et al. 2003). Adiponectin receptor AdipoR1 – which shows affinity for adiponectin trimers and, by activation of the signaling pathway with the participation of the parAMPK (5′AMP-activated protein kinase), increases the uptake and oxidation of glucose and, after inactivation of

acectyl-CoA carboxylase (ACC), the oxidation of fatty acids – prevails in muscles (Yamauchi et al. 2002, 2003). The described processes take place both with globular and full-length forms of adiponectin. Additionally, in skeletal muscles, adiponectin increases the translocation of glucose transporter GLUT-4, stimulating the uptake of glucose and the production of lactic acid and inhibiting the synthesis of glycogen by myocytes (Ceddia et al. 2005).

AdipoR2 prevails in liver tissue where the regulation of glucose and fatty acid metabolism takes place mainly under the influence of adiponectin multimers (HMW). The binding of HMW with adiponectin receptor AdipoR2 activates the signaling pathway with the participation of AMPK in hepatocytes, leading to reduced activity of acetylo-CoA carboxylase and stimulation of fatty acid oxidation, and induces suppression of the molecules being involved in the process of gluconeogenesis (e.g., glucose-6-phosphatase and phosphoenolpyruvate carboxykinase) in the liver (Yamauchi et al. 2002, 2003).

The structure of adiponectin is similar to that of tumor necrosis factor alpha (*TNF-α*) and complement system; moreover, its low concentration is associated with an increase in inflammatory markers, e.g., C-reactive proteins, which suggests its involvement in the regulation of inflammation. Adiponectin concentration shows dependence on the concentration of TNF-α and the extent of inflammation, which is observed, among others, in RA (Schaffler et al. 2003; Hamman and Twardella 2006). Furthermore, adiponectin inhibits the inflammatory response by decreasing the phagocytic activity of macrophages and the production of TNF-α and inhibits the proliferation of myelomonocytic cells (Kemp et al. 2001; Shimada et al. 2004).

Adiponectin Concentration According to Anthropometric Traits

Adiponectin concentration is about 0.01% of that of all proteins being found in blood plasma. In healthy subjects, it is about 5–30 μg/ml (Arita et al. 1999). Some reference data show a positive relationship between adiponectin concentration and gender (Cnop et al. 2003); however, these are isolated reports in relation to references not presenting such a relationship (Arita et al. 1999; Table 2).

Body composition, precisely the content of fat components, shows a strong negative correlation to adiponectin concentration in blood serum. In overweight and obese subjects, a lower expression of adiponectin is observed in adipose tissue, as well as its lower concentration in blood plasma (Arita et al. 1999; Stępień et al. 2012). Among others, a negative correlation of adiponectin concentration in blood plasma to body mass index, fat mass percentage and waist to hip ratio, fasting insulinemia, and triglyceride concentration in blood serum has been demonstrated. On the other hand, a positive correlation has been observed to HDL-fraction cholesterol (Carrasco et al. 2009; De Rosa et al. 2013). There is a profound sexual dimorphism of adiponectin levels and complex distribution in serum (Pajvani et al. 2003; Alehagen et al. 2015). Adiponectin concentration is higher in women than in men (Comuzzie et al. 2001; Alehagen et al. 2015); interestingly, the demonstrated dimorphism is maintained regardless of their body composition (Cnop et al. 2003).

Table 2 Comparison of adiponectin concentration according to gender and morbid obesity or anorexia

Respondents			Age (years)	BMI [kg/m^2]	Adiponectin (μg/ml)	Reference
Differences in gender	Women, $n = 803$		–	–	8.18 ± 4.10	Comuzzie et al. (2001)
	Men, $n = 297$		–	–	7.24 ± 3.52	
	Women, $n = 106$		–	–	7.4 ± 29	Cnop et al. (2003)
	Men, $n = 76$		–	–	5.4 ± 2.3	
	Women, $n = 80$		39 ± 12	24.3 ± 5.0	4.7 ± 1.9	Tenta et al. (2010)
	Women, $n = 234$		77.0 (3.7)	27.6 ± 5.1	7884 ± 5387 pg/mL	Alehagen et al. (2015)
	Men, $n = 242$		77.0 (3.2)	26.7 ± 3.3	4829 ± 3391 pg/mL	
Obese	Hypertensive patients with simple obesity (class I), $n = 21$		52.52 ± 14.86	32.53 ± 1.71	18.18 ± 11.93	Stępień et al. (2012)
	Hypertensive patients with severe obesity (class II and III), $n = 10$		54.30 ± 12.09	38.51 ± 2.96	20.61 ± 10.26	
	Normotensive patients with simple obesity (class I), $n = 7$		46.57 ± 13.58	32.49 ± 2.18	17.81 ± 7.20	
	Control, $N = 44$		39.3 ± 14.0	23.5 ± 3.4	TAL 28.9 ± 9.4 HMW 4.4 ± 2.2	De Rosa et al. (2013)
	Obese, $N = 25$		34.9 ± 10.5	45.6 ± 9.0	TAL 8.1 ± 3.6 HMW 5.9 ± 3.7	
Bariatric operations	Morbidly obese women before/after GBP	Baseline	37.7 ± 9.6	45.0 ± 4.3	11.4 ± 4.3	Carrasco et al. (2009)
		After 6 months		32.5 ± 3.9	15.7 ± 4.8	
		After 12 months		29.5 ± 3.9	19.8 ± 6.6	
	Laparoscopic Roux-en-Y GBP	Preoperatively, $n = 33$	–	26.71 ± 0.69	1.36 ± 0.07	Shrestha et al. (2013)
		3 months postoperatively, $n = 33$	–	24.53 ± 0.62	1.60 ± 0.09 (ng/mL)	
	Morbidly obese GBP	Control groups, $n = 18$	41 (36, 47)	22 (21, 23)	3.4 (2.0, 5.3) (ng/mL)	Quercioli et al. (2013)
		Baseline, $n = 18$	43 (36, 52)	45 (43, 49)	2.6 (2.1, 3.8) (ng/mL)	
		After 12 months, $n = 18$	44 (37, 53)	31 (28, 35)	6.0 (2.2, 10.5) (ng/mL)	

Anorexia nervosa	Comparing patients with AN and BN	Control, $n = 16$		25.7 ± 2.9	20.3 ± 1.5	18.3 ± 9.8	Tagami et al. (2004)
		Obese, $n = 9$		27.0 ± 6.8	30.3 ± 5.6	5.7 ± 2.0[4] (9)	
		AN patients, $n = 31$		25.5 ± 8.1	14.0 ± 2.5	11.0 ± 7.8[2] (31)	
		BN patients, $n = 11$		23.5 ± 3.9	20.5 ± 1.8	11.5 ± 6.2[1] (11)	
	Adolescent girls with AN and healthy adolescents (0, 30, and 60 min after ingestion of OGL)	AN	0 min	–	16.7 ± 1.3	13.3 ± 6.1	Misra et al. (2007)
			30 min			12.5 ± 8.2	
			60 min			11.2 ± 5.4	
		Healthy adolescents	0 min	–	21.8 ± 3.4	11.9 ± 7.8	
			30 min			9.8 ± 2.9	
			60 min			8.7 ± 2.8	
	Comparing patients with AN and MP	Control group, $n = 38$			22.32 ± 0.40	33.24 ± 4.41	Krízová et al. (2008)
		Anorexia nervosa, $n = 28$			15.72 ± 0.36	58.44 ± 7.17	
		Obese women, $n = 77$			43.48 ± 1.12	17.02 ± 1.19	

TAL total adiponectin level, *GBP* gastric bypass surgery, *AN* anorexia nervosa, *BN* bulimia nervosa, *OGL* oral glucose load, *MP* metabolic phenotype

Adiponectin Interaction with Other Hormones/Proteins [Hormones and Proteins/Hormone Proteins]

Adiponectin gene expression (and resulting from it adiponectin concentration) is mainly dependent on body adiposity, age, and hormonal concentrations, such as estrogen, testosterone, cortisol, and FSH levels (especially in postmenopausal women) (Pajvani et al. 2003; Wang et al. 2012). There is a large variety of pathways regulating the expression and secretion of adiponectin but most papers indicate that the activity of adiponectin gene (apM1) can be reduced by TNF-α, interleukin-6, glucocorticoids, β-adrenergic agonists, catecholamines, and high testosterone, cortisol, and estrogens levels. The stimulating effects of adiponectin concentration (with even hyperadiponectinemia) can be caused by normal or low estrogen and testosterone levels, low sex hormone-binding globulin level, and high FS level (Fig. 1; Fasshauer et al. 2002, 2003; Delporte et al. 2002; Bruun et al. 2003; Lubkowska et al. 2014). Very important but still not fully clear hormone regulation of adiponectin secretion is insulin dependent. There is a variety of reports demonstrating the stimulating effects of insulin on ACRP30 gene expression or secretion (Bogan and Lodish 1999; Halleux et al. 2001). On the other hand, there is a variety of papers showing that insulin concentration can be negatively correlated to ACRP30 gene expression and resultant low adiponectin concentration (Fasshauer et al. 2002).

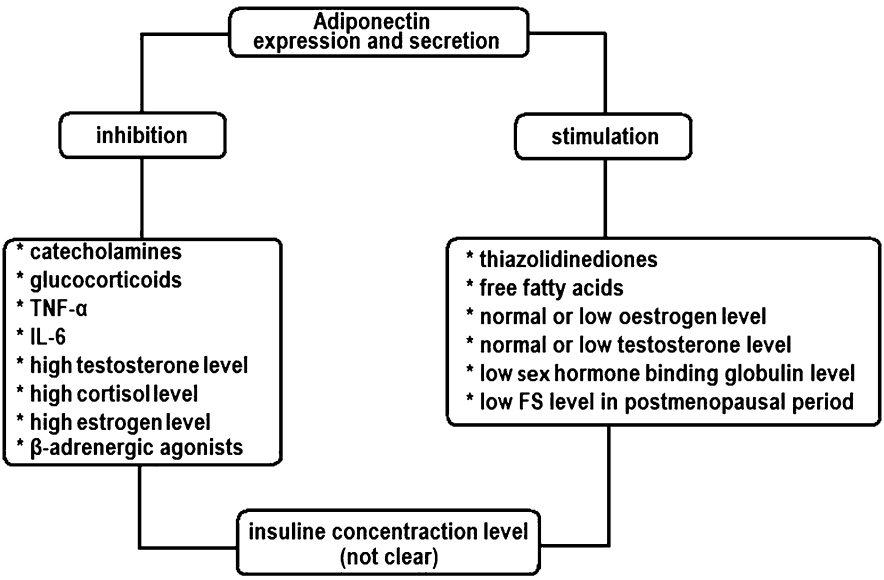

Fig. 1 Interaction of adiponectin concentration

Adiponectin Effects in Osteoporosis

Basic Information About Osteoporosis (Types, Causes, Formation Process, Consequences)

Osteoblasts and marrow adipocytes originate from a common mesenchymal progenitor. Research has shown that differentiations of bone marrow stem cells into fatty cell lines or bone cell lines are not mutually exclusive (Rosen and Klibanski 2009). Bone structure is directly dependent on the bone remodeling, an active process throughout the skeleton, being essential for calcium homeostasis and preserving the integrity of the skeleton, through the coupled activity of osteoclasts and osteoblasts (Fig. 2).

In the situation when the process of bone resorption and bone formation is dysregulated, osteoporosis is being observed with the occurrence of increased bone resorption. It is a systemic disease of the skeletal system affecting different patients at different age. It is characterized by a significantly increased likelihood of fractures due to decreased bone mineral density (BMD) and abnormal bone microarchitecture (Cummings and Black 1995).

The rate of bone resorption is greater than the rate of new bone formation; that is why a significant reduction in the weight of normal bone mass is being observed. Osteoporosis takes its greatest toll in the female population where a significant increase in incidence is observed after 50 years of age and is primarily connected with the menopause (Melton et al. 1989). Today, osteoporosis is a major public health problem, and it becomes even more serious due to the fact that the elderly population is still increasing (Van Geel et al. 2007; Kanis et al. 2007). There are many risk factors which can lead to full-blown osteoporosis but many of them are heterogeneous and not fully specific (Fig. 3).

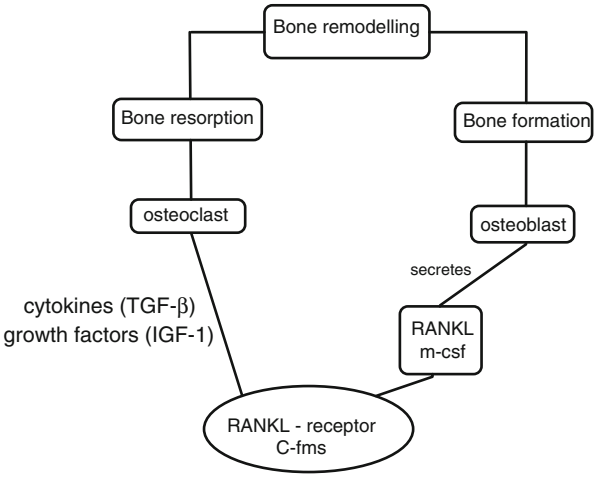

Fig. 2 Bone remodeling

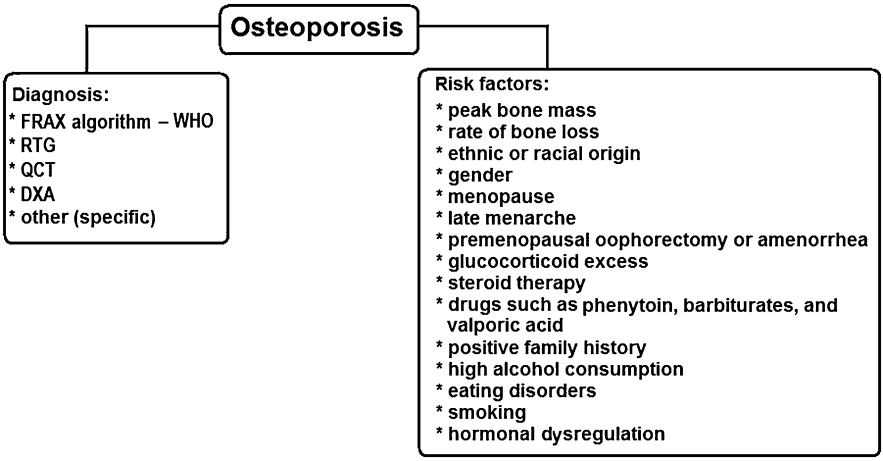

Fig. 3 Osteoporosis diagnosis and risk factors

Despite the fact that numerous papers examine this skeletal disease, some of its interactions and specific markers are still unclear. Osteoporosis is a very heterogeneous pathogenic process which depends on many causative factors. Most classifications describe osteoporosis either as primary or secondary. Primary osteoporosis is a more common form and is due to typical age-related bone loss from the skeleton. It is classified as type 1 and type 2 osteoporosis. Secondary osteoporosis results from the presence of other diseases or conditions that predispose to bone loss and is classified as type 3 osteoporosis (Ott 1998). Type 1 osteoporosis is a classic form of postmenopausal osteoporosis. Type 2 osteoporosis is being called age-related osteoporosis and affects men and women, usually after the age of 70. Secondary osteoporosis (called type 3 osteoporosis) may occur at any age; is not gender dependent; and can be caused by drugs, immobilization, or any disease (Fig. 4).

Differentiation of Osteoblasts and Adipocytes – Regulatory Factors Allowing for Adiponectin

Osteoblast Cell Differentiation

Osteoblasts are basic single nuclei bone-forming cells, differentiated from multipotent mesenchymal stem cells (Pittenger et al. 1999; Blair et al. 2008). Osteoblasts are responsible for the synthesis of cross-linked collagen and specific proteins (e.g., osteocalcin and osteopontin) which are responsible for bone matrix formation. Furthermore, osteoblasts produce a calcium and phosphate-based mineral, hydroxyapatite, that can be deposited into the organic matrix forming a specific and mineralized bone tissue (mineralized matrix) (Blair et al. 2011). These specific features of osteoblast-lineage cells make them occupy a central position in bone metabolism and structure. Of course, the formation of a structurally sound skeleton,

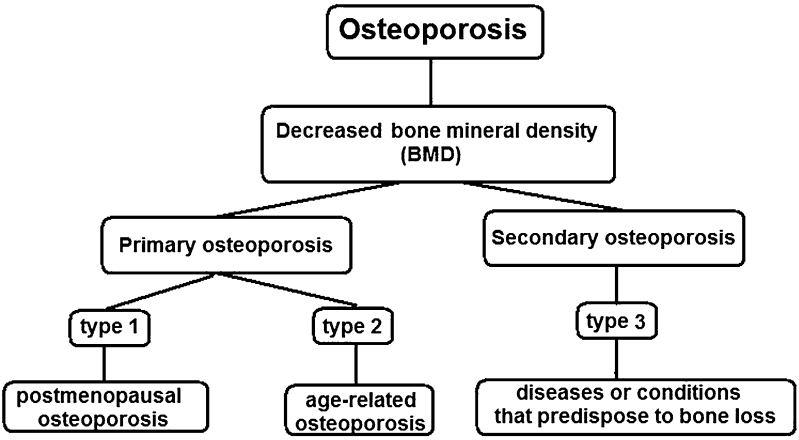

Fig. 4 Primary and secondary osteoporosis

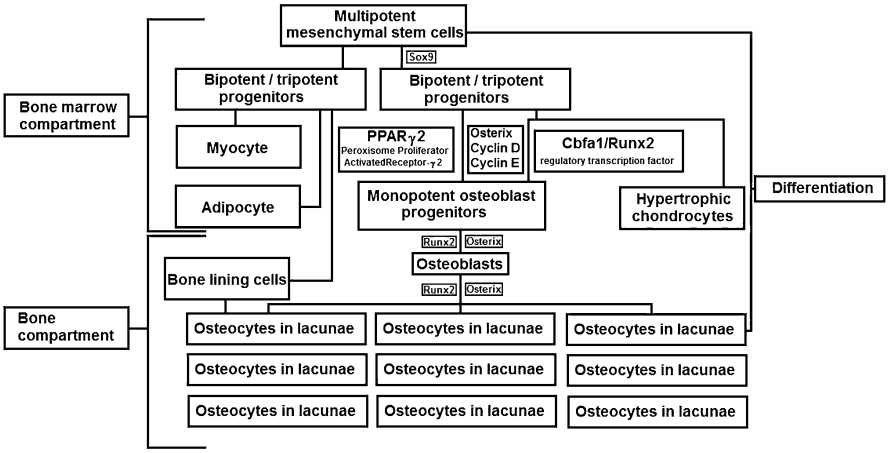

Fig. 5 Cell differentiation in the mesenchymal system

with its strength and integrity conserved by constant remodeling, and the formation as well as activation of the major bone-resorbing cell, the osteoclast, are the result of direct and indirect influences of osteoblasts. Osteocytes derive from osteoblasts and are formed by the incorporation of osteoblasts into the bone matrix (Fig. 5). Osteocytes remain in contact with each other and with cells on the bone surface via gap junction coupling of cells passing through the matrix via small channels, the canaliculi, that connect the cell body – containing lacunae – with each other and with the outside world (Aarden et al. 1994).

The membrane that covers the outer surface of all bones, except at the joints of long bones, called periosteum, contains a large number of multipotent mesenchymal stem cells. During cell differentiation, they give rise to osteoblasts (similar pathway

is mesenchymal steam cells in bone matrix). This process is being controlled under the expression of regulatory transcription factor Cbfa1/Runx2, the activity of which can also be found in hypertrophic chondrocytes. Furthermore, osteoblast differentiation is under control of osterix (Karsenty 2008; Zhu et al. 2012). Osterix regulates the expression of a set of ECM proteins which are involved in terminal osteoblast differentiation and is associated with bone mineral density.

The most important group of growth factors responsible for the skeletal differentiation and bone formation is a group of bone morphogenetic proteins (BMPs), also known as cytokines, metabologens or cartilage-derived morphogenetic proteins (CDMPs), or growth differentiation factors (GDFs).

A large group of BMPs family belongs to the transforming growth factor beta (TGF-β) superfamily of proteins, whereas the rest is being classified as a metalloproteinase. The total number of BMPs is 20, but in last few years this number has changed. Their mechanism is based on specific interaction with bone morphogenetic protein receptors (BMPRs). Activation of the signaling pathways of BMPRs results in members of the SMAD protein family reaction (Bleuming et al. 2007). The most important for osteoblast differentiation and bone formation are the BMP2 (most important), BMP3, BMP4, BMP7, and BMP8a genes. Any mutations that may occur in the BMPs genes may lead to human disorders which affect the skeleton. The SMAD intracellular protein family is proteins that are responsible for the transduction of extracellular signals into the nucleus where they activate downstream gene transcription (Park and Morasso 2002).

Other growth factors being relatively important in the skeletal differentiation and bone formation is the transforming growth factor beta (TGF-β) family which belongs to the same transforming growth factor beta superfamily as BMPs and possess similar signaling elements in the TGF-beta signaling pathway. Furthermore, the multifunctional fibroblast growth factor family (FGFs), which is formed by 22 growth factors and has a great variety of effects, is essential for the bone formation and regulation. Mostly, the family of fibroblast growth factors (FGFs) determines where skeletal elements occur in relation to the skin (Olsen et al. 2003; Moore et al. 2005).

Multipotent mesenchymal stem cells are the site of origin not only for chondrocytes and osteoblasts but also for myocytes and marrow adipocytes (Rosen and Klibanski 2009). The phenotype of cells depends on diverse ligands of PPARγ2 (peroxisome proliferator-activated receptor-γ2). The activation of PPARγ2 is responsible for regulation of different pathways which may lead to full or partial expression of the adipocyte phenotype cell, suppression of osteoblast differentiation, or both. This correlation is very important for the correct understanding of skeletal system metabolism, as there is evidence that marrow fat increases with age in humans in which osteoblast production is observed (Rosen and Klibanski 2009). Normally, bone remodeling is being observed during the lifetime but bone loss increases with aging, both in males and females. This process occurs due to a reciprocal increase in adipocyte development and a decrease in osteoblast differentiation. Adiponectin is being produced by differentiated adipocytes and changes in its concentration have been observed in many different phases of life. It seems

essential for osteoblastogenesis that adiponectin and its receptors (AdipoR1 and AdipoR2) are present in bone-forming cells, and their origin is the same – multipotent mesenchymal stem cells (Berner et al. 2004; Shinoda et al. 2006). Reports indicate a potential effect of adiponectin on bone tissue remodeling, due to induction of osteoblasts proliferation and differentiation. Human osteoblasts show the expression of both adiponectin receptors and adiponectin. Adiponectin stimulates human osteoblast proliferation and differentiation (proliferation activity via the AdipoR/JNK pathway, differentiation activity via the AdipoR/p38 MAPK pathway), as it increases the expression of alkaline phosphatase (due to the adiponectin receptor subtype AdipoR1 activity), osteocalcin, whereas type I collagen is correlated with bone density mineralization (Fig. 6; Kanazawa et al. 2007; Mitsui et al. 2011).

This conclusion fully indicates that osteoblastic proliferation and differentiation activity takes place through AdipoR1, and high adiponectin levels enhance bone mineral density and osteoblast differentiation (Luo et al. 2005).

Influence of Adiponectin on Chondrogenesis and Osteblastogenesis

The RANK-RANKL (*receptor activator of nuclear factor kappa-B* and *receptor activator of nuclear factor kappa-B ligand*) system is responsible for normal bone tissue homeostasis. RANKL has an activating effect on osteoclasts, stimulating bone resorption (osteoclastogenesis), whereas osteoprotegerin (OPG protein) neutralizes the effect of RANKL, inhibiting this process (Inage et al. 2015). Some studies suggest the further data analysis adjusting for potential confounders to reveal that the OPG/RANKL ratio is positively associated with adiponectin (Tenta et al. 2010). Excessive activation of RANKL may lead to osteoporosis, e.g., periarticular osteoporosis, as is the case of RA (rheumatoid arthritis) (Inage et al. 2015). Little is known about the influence of adiponectin on chondrogenesis processes. It has been observed that in diseases with disturbed homeostasis of this process an increased adiponectin concentration is seen; moreover, chondrocytes show the expression of both AdipoR1 and AdipoR2 (Xibillé-Friedmann et al. 2015). Some references have proven a pro-inflammatory effect of adiponectin on chondrocytes which, by inducing the expression of nitric oxide synthase 2 (NOS2), stimulates the release of interleukin 6 (IL-6), matrix metalloproteinases (MMP-3, MMP-9), and chemokine MCP-1 (*monocyte chemotactic protein*) (Lago et al. 2008; Sun et al. 2015). This is confirmed by studies on an animal model which demonstrated that adiponectin exacerbates collagen-induced arthritis via enhancing Th17 response and prompting RANKL expression. Adiponectin injection resulted in an earlier onset of arthritis, an aggravated arthritic progression, more severe synovial hyperplasia, bone erosion, and osteoporosis in CIA mice (Sun et al. 2015). There have been also reports demonstrating the stimulating effect of adiponectin on chondrocyte differentiation and proliferation (Frommer et al. 2010). Furthermore, some references indicate the anti-inflammatory antiadhesive effect of adiponectin (Challa et al. 2010).

As regards osteogenesis, many studies suggest a functional role of adiponectin in bone homeostasis. The effect of adiponectin on bone tissue may be direct, by

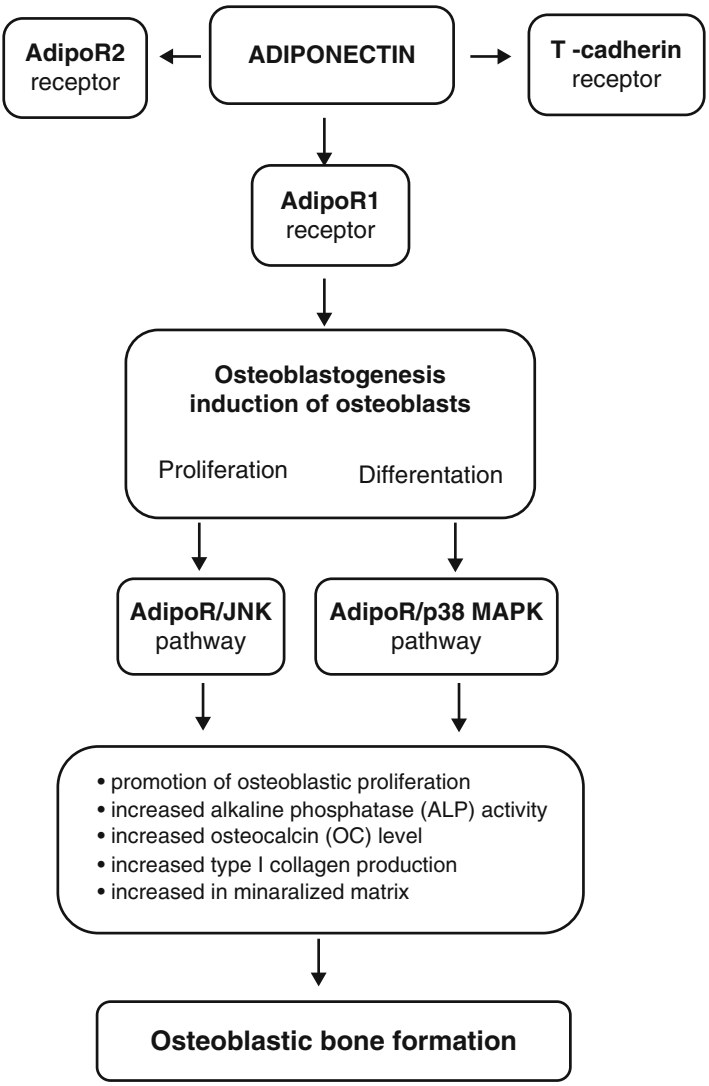

Fig. 6 Adiponectin correlation with bone mineral density

influencing osteoblasts, and indirect, by affecting osteoclasts. Adiponectin can increase bone mass by increasing the expression of alkaline phosphatase, osteocalcin, and type 1 collagen, stimulating human osteoblast proliferation and differentiation (Luo et al. 2005). Additionally, the inhibitory effect of adiponectin on differentiation of osteoclasts from CD14+ monocytes and inhibition of osteoclast resorption activity also contributes to increased bone mass (Oshima et al. 2005). Both in human and animal models, the mitogenic effect of adiponectin on osteoblasts and the inhibitory one on osteoclast proliferation have been demonstrated

independently of the RANK/RANKL/OPG system (Williams et al. 2009). More importantly, adiponectin influence can be observed at the level of mesenchymal cells, stimulating their differentiation towards osteoblasts, increasing the expression of osteoblastogenesis markers (Runx2, BMP-2). In addition, the protective effect of adiponectin on bone tissue may also result from its anti-inflammatory action, which is induced by inhibition of TNF-α-mediated NFkB activation, thus reducing the activity of osteoclasts via the RANK/RANKL/OPG pathway (Khosla 2001; Lee et al. 2009).

It would seem that the above data clearly indicate a positive effect of adiponectin on bone remodeling, but there are also references in literature showing the stimulating effect of adiponectin on osteoclastogenesis by enhancing the RANKL expression and down-regulating the expression of osteoprotegerin (OPG) (Luo et al. 2006). Consistent with this finding, culture of osteoblasts with adipocyte-conditioned media was reported to decrease the osteoblastogenic transcription factor Runx2 expression, an effect that was abrogated by knockdown of AdipoR1 (Liu et al. 2010). The index of bone mineral density (BMD) reflects to some extent the direction of bone turnover. The data referring to the effect of adiponectin on BMD are largely contradictory. There is no sufficient evidence to clearly conclude negative correlation between adiponectin concentration in blood serum and BMD but most references, nevertheless, report such a relationship (Krízová et al. 2008; Singhal et al. 2014).

Factors Affecting Bone Mass and Regulating Bone Remodeling Allowing for Adiponectin; Adiponectin Receptors Associated with Bone Metabolism

The bone remodeling cycle maintains skeletal integrity through balanced activities of its constituent cell types. It is an active and dynamic lifelong process where mature bone tissue is removed from the skeleton (bone resorption) and new bone tissue is formed (bone formation). In bone remodeling, three different cell types are involved: osteoblasts, osteocytes, and osteoclasts.

Bone-forming osteoblasts are mainly engaged in bone formation. They are specific single nuclei bone-forming cells, differentiated from multipotent mesenchymal stem cells. Their main role is to produce organic bone matrix and aid its mineralization (Pittenger et al. 1999; Blair et al. 2008; Karsenty et al. 2009).

Very important in the regulation of bone mass are osteoclasts. It is one of the types of bone cells responsible for resorption of bone tissue. This is an essential process in the maintenance, repair, and remodeling of bones of the vertebral skeleton. Osteoclasts dismount mineral bone structure in the process of bone resorption at a molecular level by acidic and enzymatic degradation of extracellular matrix (ECM) proteins through collagenase secretion (using hydrolytic enzymes, such as members of the cathepsin and matrix metalloproteinase (MMP) groups). Significant for the activity of osteoclasts being expressed by them is one of the collagenolytic, papain-like, cysteine proteases called cathepsin K. It is synthesized as a proenzyme

and activated by autocatalytic cleavage to its mature active form that is being secreted into the resorptive pit and is involved in the degradation of type I collagen and other noncollagenous proteins (Yasuda et al. 1998; Teitelbaum 2000, 2007; Teitelbaum and Ross 2003; Fuller et al. 2006).

Equally important for the enzymatic activity of osteoclasts are matrix metalloproteinases (MMPs), especially MMP-9, MMP-10, MMP-12, and MMP-14. The activity of only one of these metalloproteinases has been identified. Except MMP-9, little is known about their relevance to osteoclasts but summing up the activity of MMP-9 it can be easily noticed that it is associated with the bone microenvironment and is known to be required for osteoclast migration and as powerful gelatinase (Teitelbaum 2000, 2007).

Simultaneous and proportional activity of osteoclasts and osteoblasts is essential in bone tissue regulation and function (Pittenger et al. 1999; Teitelbaum 2007; Blair et al. 2011).

Equally important is the third group of bone cells derived from osteoprogenitors called osteocytes. They are very common cells in mature bone, reside inside lacunae and canaliculi, and, comparing to all other bone cells, their life span is very long.

During the growth of osteoblasts, they may be trapped inside the matrix that they secrete and, after transformation, they become osteocytes. Osteocytes are connected to each other through long cytoplasmic extensions. Comparing to osteoclasts and osteoblast, they are capable of molecular synthesis and modification, as well as transmission of signals.

Osteocytes are capable of producing nerve growth factors after bone fracture (due to glutamate transporters). Most papers indicate that osteocytes are thought to be mechanosensory cells that control the activity of osteoblasts and osteoclasts. Furthermore, they produce osteocyte specific proteins such as sclerostin (regulates mineral metabolism), PHEX, DMP-1, MEPE, and FGF-23 (regulates phosphate and biomineralization) (Bonewald 2011).

Potential Applications to Prognosis, Other Diseases or Conditions

Osteoporosis in Different Conditions Associated with Decreased or Increased Adiponectin Levels

One of the potential markers of **perimenopausal osteoporosis** can be adiponectin. It is believed that the main role in the mechanism of bone metabolism in the perimenopausal period is played by estrogens and androgens, especially DHEA (dehydroepiandrosteron) (Rosen and Bouxsein 2006; Ağbaht et al. 2009). However, from among the hormones being secreted by adipose tissue, particular importance is attached to the role of leptin and adiponectin as significant protective and preventive, respectively, mediators of osteoporosis (Rosen and Bouxsein 2006; Jürimäe and Jürimäe 2007; Ağbaht et al. 2009; Zillikens et al. 2010). It has been shown that adiponectin levels are considerably higher in postmenopausal women compared to premenopausal ones (Table 3). It should be noted that adiponectin receptors

Table 3 Comparison of adiponectin concentration in perimenopausal women

Respondents		Age (years)	BMI [kg/m^2]	Adiponectin (μg/ml)	Reference
Total (pre- and postmenopausal)	Adult women, $n = 1467$	47.7 ± 14.2	26.4 ± 4.7	12.3 ± 5.8	Zillikens et al. (2010)
	Adult men, $n = 1164$	48.6 ± 14.0	27.1 ± 3.9	8.0 ± 4.1	
	Adult, $n = 153$	57.8 ± 13.7	27.6 ± 2.4	12.2 ± 6.3	Jürimäe (2007) [158]
	Adult, $n = 1735$	50.0 ± 13.0	25.5 ± 4.7	8.3 (3.9)	Richards et al. (2007)
Premenopausal	Adult, $n = 98$	45.2 ± 4.3	29.9 ± 6.2	12.0 ± 4.7	Jürimäe and Jürimäe (2007)
	Middle-aged, $n = 42$	40.8 ± 5.7	25.9 ± 2.8	8.4 ± 3.2	
	Adult, $n = 25$	47.80 ± 3.14	30.01 ± 5.22	7.9 ± 5.81	Kontogianni et al. (2004)
	Adolescents, $n = 105$	15.4 ± 1.9	23.1 ± 4.0	30.79 ± 14.48	Huang et al. (2004)
Postmenopausal nondiabetic (with hip fracture), $n = 105$	Nonosteoporosis	58.4 ± 8.2	31.2 ± 5.9	6.33 ± 0.51	Özkurt et al. (2009)
	Osteoporosis	68.4 ± 8.0	25.5 ± 9.9	6.99 ± 0.5	
	Total	63.4 ± 8.1	28.5 ± 7.9	6.66 ± 0.45	
Postmenopausal	$n = 55$	54.47 ± 5.36	28.89 ± 4.19	11.94 ± 7.00	Kontogianni et al. (2004)
	$n = 84$	52.5	29.4	13.25	Ağbaht et al. (2009)
	Women, $n = 447$	76.0 ± 8.4	24.4 ± 3.8	16.28 ± 7.1	Araneta et al. (2009)
	Men, $n = 484$	74.8 ± 8.3	25.8 ± 3.2	11.1 ± 5.8	
	Control, $n = 16$	70.2 ± 1.0	27.4 ± 0.8	Peripheral plasma 12549 ± 1530 ng/m Bone marrow plasma 8939 ± 1484 ng/m	Mödder et al. (2011)
	Estrogen treated, $n = 16$	72.9 ± 1.7	28.6 ± 1.4	Peripheral plasma 12919 ± 1544 ng/m Bone marrow plasma 9615 ± 1268 ng/m	

AdipoR1 and AdipoR2 have been found in uterus, which may suggest the effect of adiponectin on the endometrium, and is involved in regulation of gonadotropin secretion (Palin et al. 2012). The role of adipose tissue in female reproductive system homeostasis is additionally emphasized by the fact that obese women go through puberty earlier and are predisposed to polycystic ovary syndrome (PCOS), whereas underweight in women is associated with later sexual maturation and a risk of premature delivery (Jürimäe and Jürimäe 2007). It is suggested that adiponectin negatively correlates with the levels of free testosterone, DHEA-S (dehydroepiandrosterone sulfate), and estradiol and positively with SHBG (*sex hormone binding globulin*) in postmenopausal women (Siemińska et al. 2006; Matsui et al. 2012). Some studies suggest that possible regulation of human osteoprogenitor cells by estrogen indicate – which is in line with previous murine studies – that estrogen suppresses the proliferation of human bone marrow lin-/Stro1+ cells, which likely represent early osteoprogenitor cells (Mödder et al. 2011). In the light of the latest data, adiponectin effects may be accomplished by modification of OPG and/or RANKL expression in osteoblasts and bone marrow stromal cells (Rosen and Bouxsein 2006). In human osteoblasts, the effect of 17β-estradiol (E2) on adiponectin and regulation of OPG and RANKL expression has been observed. Through blocking the activation of adiponectin-induced p38 MAPK, E2 suppressed adiponectin-regulated OPG/RANKL expression and then inhibited osteoclastogenesis (Wang et al. 2012). As regards the above described findings, it seems that new hormonal markers, including adiponectin, may be useful in the prediction of bone loss and risk of fractures in osteoporosis in postmenopausal women (Özkurt et al. 2009; Araneta et al. 2009).

In rheumatoid arthritis (RA), osteoporosis – localized or generalized – is secondary as a consequence of inflammatory lesions. In patients with RA, significantly higher adiponectin concentration in blood serum was observed compared to healthy subjects (Lago et al. 2006). Moreover, adiponectin concentration in synovial membrane was higher in RA patients than in those with OA (osteoarthritis) (Otero et al. 2006; Schaffler et al. 2003; Table 4). In view of the adiponectin effects in chondrogenesis and osteogenesis being described above, it can be a potential marker of osteoporosis in inflammatory diseases, such as RA or osteoarthritis. As mentioned before, TNF-α correlates with adiponectin concentration and inflammatory response; moreover, in vitro studies revealed that adiponectin may also have a pro-inflammatory effect which is associated with TNF-α activity (Schaffler et al. 2003). It is therefore considered that pro-inflammatory effects in synovial membrane, being induced by adiponectin, are probably mediated by TNF-α (Herfaarth et al. 2006). It could be suggested that serum adiponectin level is a simple useful biomarker associated with early radiographic disease progression in RA, independent of RA-confounding factors and metabolic status (Otero et al. 2006; Giles et al. 2011; Meyer et al. 2013).

Obesity is characterized by increased body weight and excess adipose tissue. Reference studies have shown that indices of body adiposity, e.g., BMI (*body mass index*) and FM (*fat mass*), negatively correlate with adiponectin concentration and positively with bone mineral density (BMD) (Arita et al. 1999; Stefan et al. 2002; Misra et al. 2007; Carrasco et al. 2009). As is well known, bone loss can lead to

Table 4 Comparison of adiponectin concentration in patients with rheumatoid arthritis

Respondents		Age (years)	BMI [kg/m^2]	Adiponectin (μg/ml)	Reference
Controls	Women, $n = 124$	57.5 ± 16.6	52.8 ± 7.0	3.6	Lago et al. (2006)
	Men, $n = 22$	45.6 ± 13.8	22.3 ± 2.8	2.3	
RA	Women, $n = 110$	59 ± 14	22.2 ± 3.8	10.1	
	Men, $n = 31$	61.0 ± 12.7	23.2 ± 3.2	2.6	
Controls, $n = 18$	Women, $n = 10$ Men, $n = 8$	48.3 ± 16.1	24.36 ± 0.83	7.6 ± 0.7 μg/mL	Otero et al. (2006)
RA, $n = 31$	Women, $n = 22$ Men, $n = 9$	46.1 ± 14.1	25.88 ± 0.63	13.56 ± 2.1 μg/ml	
RA	$n = 152$	59 ± 8	28.1 ± 5.0	32 (20–43) mg/L	Giles et al. (2011)
UA	$n = 159$	47.2 ± 13.8	24.7 ± 4.6	4.9 ± 3.4 (μg/ml)	Meyer et al. (2013)
ERA	$n = 632$	48.5 ± 12.2	25.2 ± 4.6	5.0 ± 3.7 (μg/ml)	

RA rheumatoid arthritis, *UA* undifferentiated arthritis, *ERA* early rheumatoid arthritis

osteopenia or osteoporosis and therefore it is quite popularly believed that high BMI protects from osteoporosis. Bariatric surgery is an option for morbid obesity treatment but has a negative effect on bone tissue metabolism. Regardless of the type of surgical intervention (VBG – vertical banded gastroplasty, LAGB – laparoscopic adjustable gastric band, RYGB – Roux-en-Y gastric bypass), they reduce the volume of orally ingested food. It should be noted that, apart from intended reduction of fat mass, they induce at the same time a loss in bone mass, increasing the risk of osteoporotic fractures. The reason for secondary osteoporosis may be, quite typical after bariatric surgeries, the occurrence of malabsorption syndrome, particularly of vitamins D and K as well as vitamin B12, Ca ions, and folic acid (Decker et al. 2007; Mahdy et al. 2008; Carrasco et al. 2014). Furthermore, a decrease in leptin concentration and increase in adiponectin concentration in blood serum are observed in these patients, probably as a consequence of weight loss, which can induce the activation of response pathway towards bone loss by affecting the RANK/RANKL/OPG pathway (Carrasco et al. 2009; Shrestha et al. 2013; Quercioli et al. 2013). As regards postbariatric patients, it seems that it is not the specific adiponectin level but a sudden increase of its concentration that may be a signal activating the changes towards bone loss (Table 2).

Anorexia (anorexia nervosa, AN) is a type of psychosomatic disorder which leads to lipoatrophy and weight loss and deterioration of bone tissue quality, the consequence of which is osteoporosis and increased risk of low-energy bone

fractures (Ohwada et al. 2007). The background of secondary osteoporosis in AN is hormonal disorders, including hypoestrogenism, hypoandrogenism, and hypercortisolemia (Ohwada et al. 2007). Hormonal disorders also refer to decreased IGF-1 concentration and increased growth hormone, ghrelin, and peptide Y concentrations. A consequence of the above disorders is a decreased value of peak bone mass which, as is well known, is essential for attenuation of bone loss progressing with age, especially following the menopause. In the formation of osteoporotic lesions, the lack of many vitamins and mineral compounds being normally contained in food (e.g., vitamin D, calcium, phosphorus) is also of importance. Furthermore, it is believed that increased bone resorption being induced by a decrease in the concentration of 17-beta-estradiol in blood serum of patients, which in turn induces reduced osteoprotegerin and increased osteoclast activation, is crucial for the development of osteoporosis in AN (Ostrowska et al. 2010). As regards AN patients, most references report high adiponectin values (Krízová et al. 2008; Misra et al. 2007) but not all results are conclusive (Tagami et al. 2004). Nevertheless, the levels of adiponectin concentration in AN subjects are always higher than in obese ones, which is associated with the inverse relationship of insulin levels, which significantly decrease in anorexia and increase in obesity (Tagami et al. 2004; Krízová et al. 2008; Shrestha et al. 2013; Quercioli et al. 2013). The reason for increased adiponectin concentration with weight loss is not known; nevertheless, it can be associated with the compensation mechanism of glucose metabolism reduction (Pannacciulli et al. 2003). Additionally, adiponectin as a potential marker of osteoporosis is also negatively correlated with BMD which is significantly reduced in patients with anorexia (Misra et al. 2007; Krízová et al. 2008; Singhal et al. 2014), which suggests that the increased bone resorption in AN mentioned before can be activated by an increase in adiponectin concentration being induced by reduced amount of adipose tissue which, as a further consequence, interferes with the RANK/RANKL/OPG system and shifts bone metabolism towards excessive activation of osteoclasts (Misra et al. 2007). It should be noted, however, that OPG and expression of RANKL are regulated by many factors, among others by estrogens, while hypoestrogenism induces a decrease in OPG and an increase in RANKL (Khosla et al. 2002). AN is associated with hypogonadism, therefore decreased OPG values could be expected (Khosla et al. 2002). It turns out, however, that OPG concentration in these subjects is increased, which can be associated with the hypothesis of compensation mechanism, being activated in response to low BMD which, for reasons that are not fully known, does not increase bone mass (Misra et al. 2003; Table 2).

In patients with **type 1 diabetes**, an increased adiponectin concentration is observed, while in those with type 2 diabetes, a decreased one, compared to healthy subjects (Retnakaran et al. 2010; Pala et al. 2015; Ljubic et al. 2015; Horáková et al. 2015; Table 5). Increased adiponectin concentration in patients with type 1 diabetes may be associated with reduced bone mineral density and induce diabetic osteopenia. Other causes of diabetic osteopenia are probably: insulin deficiency being characterized, among others, by anabolic effect on bone tissue (Hofbauer et al. 2007; Vestergaard 2007), accumulation of nonenzymatic protein glycosylation

Table 5 Comparison of adiponectin concentration in diabetes mellitus

Respondents			Age (years)	BMI [kg/m^2]	Adiponectin (μg/ml)	Reference
Comparing women with NTG, GIGT and defined by exceeding 2 or more NDDG glycemic thresholds (GDM)	NGT, $n = 259$	In pregnancy	33.9 ± 4.3	23.1 [21.3–26.9] +11.4 [8.6–14.5] kg	8.0 [6.2–10.0]	Retnakaran et al. (2009)
		At 3 months postpartum			8.6 [6.6–10.6]	
	GIGT, $n = 91$	In pregnancy	34.2 ± 4.2	23.5 [21.8–277] + 10.0 [7.3–145] kg	7.0 [5.2–8.7]	
		At 3 months postpartum			7.6 [5.4–9.9]	
	GDM, $n = 137$	In pregnancy	34.5 ± 4.3	25.0 [22.0–30.1] + 9.1 [5.9–12.7] kg	7.0 [5.3–8.5	
		At 3 months postpartum			8.2 [6.1–10.4]	
Comparing women with GDM and without glucose intolerance	Women with GDM, $n = 40$		–	–	At delivery 3.92 ± 4.65	Pala et al. (2015)
			–	–	In umbilical cord 20.77 ± 12.04	
			–	–	Postpartum 11.81 ± 5.81	
	Control, $n = 40$		–	–	At delivery 6.7 ± 6.49	
			–	–	In umbilical cord 27.78 ± 9.29	
			–	–	Postpartum 7.8 ± 5.97	

(*continued*)

Table 5 (continued)

Respondents			Age (years)	BMI [kg/m^2]	Adiponectin (μg/ml)	Reference
T1DM **Most (97%) were white and half were male**	1-year examination, $n = 184$		–	19.5 (3.5)	11.9	Le Caire and Palta (2015)
	4-year examination, $n = 231$		–	21.3 (4.2)	11.4	
	7-year examination, $n = 137$		–	22.8 (4.3)	11.3	
	9-year examination, $n = 187$		–	25.2 (5.0)	10.2	
	20-year examination, $n = 304$		–	28.3 (5.9)	10.2	
Diabetic nephropathy	T1DM, $n = 87$		–	–	15.37	Ljubic et al. (2015)
	T2DM, $n = 132$		–	–	8.07	
Comparing patients with and without T2DM	Control groups, $n = 269$	Women, $n = 143$	56.8	25.3 ± 1.4	TAL = 10.34; HMW = 4.71	Horáková et al. (2015)
		Men, $n = 126$	55.8	26.7 ± 3.5	TAL = 8.04; HMW = 4.46	
	T2DM, $n = 282$	Women, $n = 164$	62.1 ± 9.2	32.03 ± 5.9	TAL = 5.32; HMW = 2.92	
		Men, $n = 118$	63.9 ± 8.7	31.84 ± 5.2	TAL = 5.12; HMW = 3.03	

T1DM type 1 diabetes mellitus, *T2DM* type 2 diabetes mellitus, *GDM* gestational diabetes mellitus, *NTG* normal glucose tolerance, *GIGT* gestational impaired glucose tolerance, *TAL* total adiponectin level

(glycation) end-products in bone matrix (Vestergaard 2007), and deficiency of insulin-like growth factors (IGF-1, IGF-2) (Vestergaard 2007). So far, the pathogenesis of diabetic osteopenia has not been explained, or whether it represents a late complication of type 1 diabetes or a comorbid condition. Nevertheless, it is characterized by a higher rate of bone fractures than type 2 diabetes or hyperadiponectinemia (Hofbauer et al. 2007; Ljubic et al. 2015).

It was believed in the past that **type 2 diabetes** (T2DM, diabetes mellitus type 2) does not predispose to osteoporosis, which results from the fact that BMD in these subjects is mostly normal or even raised (Siddapur et al. 2015). This is probably a result of overweight and obesity which is often associated with type 2 diabetes and determines greater skeletal loading (Hofbauer et al. 2007). Furthermore, hyperinsulinemia, occurring in prediabetes and early DM2, reduces the production of sex hormone binding globulin (SHBG) and, consequently, increases free estradiol level in blood serum, which seems to be important in postmenopausal women (Siddapur et al. 2015). Reduced adiponectin concentration being observed in type 2 diabetes can also be of significant antiosteoporotic importance, as evidenced by the effect of adiponectin on the bone remodeling mentioned before (Ouchi et al. 2000; Williams et al. 2009; Ljubic et al. 2015; Horáková et al. 2015). Nevertheless, despite high densitometric values in patients with type diabetes, there is a high risk of fractures, which is evidenced by population studies (Janghorbani et al. 2007). This inconsistency results from the fact that bone densitometry is not able to provide complete information about the quality of bone, which consists of: its microarchitecture, rate of bone remodeling, accumulation of bone microdamages leading to microfractures, and degree of matrix mineralization. Unfortunately, modern medicine – despite its great development – does not yet have a tool which would be able to determine these traits intravitally. A probable cause of the increased risk of fractures in type 2 diabetes is the reduced number of osteoblasts and delayed formation of osteoid and its mineralization, but determination of its causes still remains an open question (Clowes et al. 2002). Moreover, a reduced concentration of adiponectin has been found in pregnant women, in whom hypoadiponectinemia in pregnancy predicts postpartum insulin resistance, beta-cell dysfunction, and fasting glycemia (Retnakaran et al. 2010; Pala et al. 2015). Researchers found that adiponectin concentrations in the circulation of GDM (gestational diabetes mellitus) patients are regulated by changes in glucose and insulin metabolism (Pala et al. 2015). Therefore, they suggest that adiponectin concentration may be relevant to the pathophysiology relating GDM with type 2 diabetes (Retnakaran et al. 2010).

Summary Points

1. Adiponectin is one of the hormones of adipose tissue which seems to link its metabolism with the metabolism of bone tissue, which is confirmed to some extent by the presence of adiponectin receptors AdipoR1 and AdipoR2 in human osteoblasts.

2. Despite still much controversy, it seems that changes in the adiponectin signaling can be associated with diseases of cartilaginous and bone tissues.
3. A number of clinical studies have shown a negative correlation of adiponectin with BMD and a positive one with biochemical markers of bone loss; moreover, in majority of in vitro studies, the stimulating effect of adiponectin on osteoblast differentiation and mineralization, as well as on osteocalcin expression, has been found.
4. It is postulated that adequate increase in adiponectin concentration affects bone loss, which may be associated with the modulation of inflammatory condition and RANK/RANKL/OPG signaling pathway.
5. Adiponectin is a noteworthy hormone of adipose tissue of potential importance as a marker of osteoporosis, both perimenopausal osteoporosis and that secondary appearing in different medical conditions associated with inflammation or weight loss.

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Use of Alkaline Phosphatase (ALP) Activity and Disease Severity to Determine Secular Changes in Bone Disease as Applied to Paget's Disease of the Bone

39

Luis Corral-Gudino

Contents

Abstract

A decrease in the prevalence of Paget's disease of the bone during recent decades has been reported for different research groups. In addition to this change in prevalence, some authors have suggested a reduction in the severity of the disease. This secular change has been documented by a reduction in the extension of skeletal involvement and the finding of lower levels of ALP at diagnosis. The reasons for the change in prevalence and severity are poorly understood. Genetic factors which confer susceptibility for Paget's disease, as SQSTM1 gene mutations, and their role on the severity of the disease have been suggested as possible explanation. Other factors, as the demographical or sociological changes in the susceptible populations, have been postulated as alternative hypothesis.

Keywords

Alkaline phosphatase • Paget's disease • Prevalence • Secular trends • SQSTM1 protein

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List of Abbreviations

ALP	Alkaline phosphatase
CSF1	Colony stimulating factor 1
NUP205	Nucleoporin 205-KD
OPTN	Optineurin
PINP	Procollagen type I N-terminal propeptide
PML	Promyelocytic leukemia
RIN3	Ras and Rab interactor 3
SQSTM1	Sequestosome 1 human protein
TM7SF4	Transmembrane 7 superfamily, member 4
TNFRS	Tumor necrosis factor receptor superfamily
UK	United Kingdom

Definitions of Words and Terms

- **Bone formation**: It is the process to form new bone by osteoblast.
- **Bone resorption**: It is the process to remove old bone tissue by osteoclast. This function is critical in the maintenance and remodeling of bones.
- **Bone turnover**: Bone is constantly remodeled through the process of active formation and resorption. Both processes occur in close temporal sequences. A balance is established between them. In bone diseases as osteoporosis or Paget's disease, this balance is lost and one of the two processes prevails.
- **Bone turnover biochemical markers**: A blood or urine test to identify changes in bone metabolism. These markers are substances produced or released during bone remodeling. The different test available could reflect bone formation or bone resorption.
- **Bone extension index**: An index designed for assessing the extent of the Paget's disease. The index is calculated according to the percentage of affected skeleton showed on X-ray or bone scintigraphy. Every bone is given a percentage value according to its volume. The index is the sum of all the values of the affected bones.
- **Monckeberg vascular calcification**: X-ray calcifications found in the muscular middle layer of the walls of arteries. The Monckeberg medial calcification is a ring-like calcification of the vascular media without associated intimal thickening. It is a form of arteriosclerosis or vessel hardening.
- **Osteoblast**: Cells specialized in bone formation. Osteoblast and osteoclast are of paramount importance in controlling the amount of bone tissue.
- **Osteoclast**: Cells specialized in bone resorption.
- **Osteolysis**: Loss of bone.
- **Paget's disease of bone**: Paget's disease of bone is a chronic bone disorder. Typically it is localized, affecting just one or a few bones. Paget's disease of bone disrupts your body's normal bone recycling process to replace the old bone tissue for new one. The disease cause excessive local breakdown and formation of bone,

followed by disorganized bone remodeling that results in enlarged and misshapen bones and cause bone to weaken, resulting in pain, fractures, and arthritis in the joints near the affected bones.

- **Secular change**: A variation appearing slowly over a sequence of data point of successive measurements made over a long period of time.
- **Serum alkaline phosphatase activity**: Alkaline phosphatase is an enzyme. It is the best characterized of the bone formation markers for assessing Paget's disease. ALP is elevated when there is active bone formation occurring. However, ALP it is not only elevated in bone diseases. High ALP levels can also occur in hepatobiliary diseases and related conditions.

Introduction

Paget's disease of the bone (Table 1) is the second metabolic bone disease in frequency after osteoporosis (Hood et al. 2001). Paget's disease has two peculiar characteristic (Bolland and Cundy 2013; Ralston 2013). On one hand, although it is a metabolic bone disease, Paget's disease has a focal nature affecting one or several bones, as opposed to other bone diseases as osteoporosis which affects the total skeleton. On the other hand, Paget's disease epidemiology is puzzling and fascinating, with large differences in prevalence between different geographical locations, and even large differences between neighboring regions in the same country. Of note, some studies show an increased observed prevalence in rural than in urban areas (Corral-Gudino et al. 2013b; Merlotti et al. 2005). This prevalence has changed over last years, as several studies have suggested that the prevalence of Paget's disease has fallen in recent years (Fig. 1) (Corral-Gudino et al. 2013a).

The factors which cause the random skeleton localization and the difference in prevalence are poorly understood. Changes in ethnic makeup of the population due

Table 1 Key facts of Paget's disease of bone. This table lists the key facts of Paget's disease including the basic concept of the role of biomarkers for the diagnosis of the disease Unpublished

Paget's disease is the second most common chronic metabolic bone disorder affecting adults after osteoporosis
Typically, it has a focal nature as it is localized, affecting just one or a few bones in a random fashion
The disease results in enlarged and misshapen bones and causes bone to weaken, resulting in pain, fractures, and arthritis
There are large differences in the prevalence of Paget's disease between different geographical locations and even within neighboring regions in the same country
The prevalence of Paget's disease is highest among the elderly
Paget's disease incidence has decreased in most regions over recent years
Paget's disease disrupts normal bone recycling process to replace the old bone tissue for new one and led to an increased bone turnover
Because Paget's disease is characterized by high rates of bone remodeling, biochemical evidence of increased bone turnover has been identified

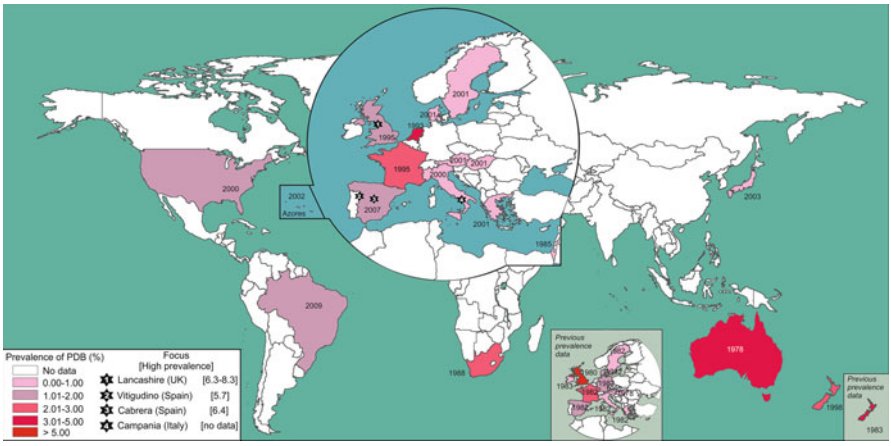

Fig. 1 Paget's disease world prevalence atlas. The figure shows Paget's disease world prevalence, including changes in prevalence in last decades (Reprinted from Corral-Gudino et al. (2013a), Copyright (2013), with permission from "Elsevier")

to immigration, or the reduction of some environmental factors which could be triggers of the disease as exposure to zoonoses or infections, have been suggested as possible causes of these changes.

In addition to the changes in prevalence, many studies have reported that the severity of Paget's disease has declined over recent years (Corral-Gudino et al. 2013b; Cundy et al. 1997, 2004, 2006; Guanabens et al. 2012; Melton et al. 2000; Morales-Piga et al. 2002). Severity in Paget's disease is classically defined as the skeleton extension of the disease and the level of increase of biomarkers of bone turnover. Alkaline phosphatase (ALP) activity, a serum marker of bone formation, has been classically used as the reference to measure Paget's disease activity.

In the present chapter, we will summarize the evidence regarding the use of ALP activity and skeleton bone lesion extension to determine secular changes in bone disease on Paget's disease. We also assess the role of genetics on severity in a disease as Paget's disease which has a strong genetic component.

Paget's Disease Severity and Biochemical Markers of Bone Turnover

There is not a universal definition for the severity of Paget's disease. Most of clinicians refer to skeletal bone extension and biochemical markers rise to define Paget's disease severity. In addition, both criteria are associated between them. Patients with large skeletal lesions have higher bone turnover markers levels and vice versa. Also, subjects with a polyostotic disease have higher bone turnover markers than monostotic patients (Alvarez et al. 1997).

Skeletal bone extension is determined by counting the number of affected bones or by using an index to estimate the percentage of bone tissue affected in patients diagnosed as Paget's disease. At least three indexes have been proposed (Coutris et al. 1975; Howarths 1953; Renier et al. 1995). The three indexes estimate a coefficient for every affected bone to calculate the percentage of affected skeleton (Table 2).

Table 2 Percentage of bone tissue affected according Howarth's, Coutris', and Renier's index. The table shows the three principal indexes proposed to estimate the percentage of skeleton affected in patients with Paget's disease. Every index estimates a coefficient for every affected bone. Unpublished

Coefficients	Howarth's	Coutris'	Renier's (♂)	Renier's (♀)
Head				
Skull and face	11	12	17	21
Mandible	–	-	2	
Spine				
Cervical spine	–	–	1.5 *(0.2% for every cervical vertebra except 0.3 for C2)*	
Thorax spine	–	–	3.7 *(0.25–0.5% for every thoracic vertebrae according to the level)*	
Lumbar spine	–	–	3.3 *(0.55–0.75% for every lumbar vertebrae according to the level)*	
Entire spine	11	17	8.5	8
Thorax				
Sternum	1	1	0.5	
Ribs	9	12	5	
Shoulder girdle				
Scapula	1.5	0.5	2	
Clavicle	0.5	0.5	0.5	
Arm				
Humerus	3.5	3	3.5	
Ulna	1	2	1	
Radius	1	2	1	
Hand-wrist	1.5	1	2	
Pelvic girdle				
Sacrum	3	5	2	
Coxal bone	5	5	4.5	
Leg				
Femur	8	5	9.5	9
Patella	0.5	0.5	0.5	
Tibia	5	4	5	
Fibula	1	2	1	
Foot	4	1	4	

To create the index, Howarth and Renier used data from skeleton bones by weighting them or by combining weights and dual X-ray absorptiometry measurement, respectively. Coutris used data from panoramic roentgenograms of the skeleton. To calculate the total percentage of affected skeleton, every coefficient is usually adjusted according to the X-ray or scintigraphic involvement; whole, three-fourths, one-half, or one-fourth; or whole, two-thirds, or one-thirds. Although Coutris' index is the more frequently used in clinical trials, Renier's index used a most robust methodology to determine skeletal involvement. In addition to the classical indexes, some authors proposed a correction of the total percentage by using a scintigraphic uptake ratio for each affected bone according to a 6-point scale (Vellenga et al. 1984). Normal bone is used as a comparator to the affected bones. The skeletal extent index is multiplied by this uptake ratio to obtain an index of activity (Alvarez et al. 1997). Lastly, apart from the extension index, a Renier's activity index has been proposed (Renier et al. 1984). The authors try to calculate pagetic bone activity by differentiating the value of ALP from the normal bone, of the ALP from the pagetic bone. The authors proposed the following formulae:

$$\text{Paget bone activity over normal ALP} = \frac{(100 \times \text{Patient's ALP}) - ((100 - \text{Coutris' index}) \times \text{ALP Normal value})}{(\text{Coutris' index} \times \text{ALP normal value})}$$

ALP activity is the best characterized of the bone turnover markers and reflect both the extent and activity over the bones of Paget's disease. Other bone markers, as bone ALP and procollagen type I N-terminal propeptide (PINP), seem to be more sensitive markers for monitoring Paget's disease (Al Nofal et al. 2015). ALP activity is a marker of bone formation. In patients diagnosed with Paget's disease, total plasma ALP activity can be viewed as being made up of three elements: the liver isoenzyme and the bone isoenzyme with its two components, the contribution from nonpagetic bone and the contribution from pagetic bone. Bone-specific ALP is one of the isoforms of ALP. Membrane-bound ALP I (bone-specific ALP) may play a role in osteoid mineralization, although we do not know its exact role (Harris 1990). The choice of ALP activity as a marker for the diagnosis of bone turnover in Paget's disease is influenced by a number of factors. Its selection is due to either by its ability to identify abnormality for the initial diagnosis or to monitor the response to treatment and by its cost and availability for a routine use in clinical practice. ALP is the biomarker of choice to monitor the response to treatment as it shows a substantial decrease with treatment. The within-subject variation in active disease is paramount to assess disease activity in order to indicate whether two sequential measurements reflect a true biological difference (Shankar and Hosking 2006). In high bone turnover patients, we need a change in total ALP of $>35\%$ to exceed the critical difference, whereas a change of only 15% is required in normal subject (Alvarez et al. 1997). For patients without high bone turnover, as monostotic patients, ALP could be an insensitive marker, and bone ALP seems to be the

most sensitive marker and was increased in 60% of patients with limited disease activity or extension even though ALP was normal (Alvarez et al. 1995). The low sensitivity of ALP is illustrated in a population based biochemical survey designed to analyze the prevalence of Paget's disease in the Netherlands (Eekhoff et al. 2004). The authors designed a nested case-control from a huge population-based cohort study. The authors compared subjects with raised serum ALP and normal liver function with controls with normal serum ALP. They showed a prevalence of 2.7% in subject >55 years of age for Paget's disease in the group of patients with normal ALP. With these data, the authors estimated that the large majority of Paget's patients have normal serum ALP as only about 14% have raised serum ALP. This finding contrasts with the percentage of subjects with raised ALP within the patients diagnosed as Paget's disease referred to bone clinics, where the prevalence of subjects with raised ALP is nearly the opposite (about 78.4%) (Tan and Ralston 2014).

In addition to diagnosis, the relation between Paget's disease and ALP has been a successful example of the use of bone markers to assist in determining the disease response to treatment. This is the reason why bone markers have played a major role during drug development. Paget's disease has been described as a model to evaluate the use of these markers because of the marked increase of bone turnover and the dramatic reduction in ALP after treatment (96% of patients had a therapeutic response at 6 months, and ALP activity was normalized in 88.6% of patients after a single infusion of zolendronic acid) (Reid et al. 2005). The percentage of reduction in ALP activity has been the main outcome for most clinical trials on Paget's disease, and there is not a single trial focused on biochemically inactive patients. There are several reasons for the pragmatic preponderance of biochemical markers over clinical complications as the primary outcomes in clinical trials. On one hand, the response of pain to treatment has a great individual variation and it is difficult to standardize, and the prevention of fractures or bone deformity have been not properly addressed on clinical trials due to the need of long follow-up periods. On the other hand, biochemical measurements of bone turnover provide an objective assessment, which allow the researchers to plan management in order to achieve optimal control of bone turnover and estimate the duration of remission according to bone turnover variations. This approach to Paget's disease management focused on achieved biochemical inactivity has been challenged in recent years (Langston et al. 2010; Tan et al. 2015), and new trials focused on clinical symptoms as the primary endpoints instead of surrogate markers as biochemical markers are needed.

Recently, the Edinburgh group has designed a novel composite index to assess Paget's disease severity (Visconti et al. 2010). This index gathers information from Paget's extension with age at diagnosis, bisphosphonates treatment data, and principal disease's complications. The index takes into account the incidence of bone fractures, previous orthopaedic surgery for Paget's disease, history of osteosarcoma, bone deformity, or the use of a hearing aid in Paget's patients who has skull involvement (Table 3). Data on biochemical markers are not included in the index.

Table 3 Composite severity score (Albagha et al. 2013; Visconti et al. 2010). Novel index designed to assess Paget's disease severity. This index gathers information from disease extension and disease's complications but doesn't include biochemical data

Item		Score
Number of bones affected (*ranges 1–26*)		1 point for each bone affected
Bone deformity (*ranges 0–14*)	Mild or moderate	1 point for each mild or moderate misshapen bone
	Severe	2 points for each severe misshapen bone
Bone pain though to be due to Paget's disease		1 point
A previous fractures through a pagetic bone		1 point
Previous orthopedic surgery for Paget's disease		1 point
History of osteosarcoma		1 point
Use of a hearing aid in patient with pagetic skull bones involvement (excluding mandible and maxilla)		1 point
Bisphosphonate treatment >12 months ago		1 point
Age at diagnosis		1 point (≥70 years) 2 point (60–69 years) 3 point (40–59 years) 4 point (<40 years)

Secular Changes of Alkaline Phosphatase (ALP) Levels and Disease Extension on Patients Diagnosed as Paget's Disease

In addition to the use of ALP for the initial diagnosis of Paget's disease or to assess the response to treatment, in recent years, the changes in ALP have been used to demonstrate a secular change in Paget's disease due to a reduction in disease severity.

To the best of our knowledge, seven groups have published data of Paget's disease severity trends. The principal characteristics of the studies' design are detailed in Table 4.

Cundy et al. (1997, 2004, 2006) published his experiences in a Paget's disease clinic in Auckland (New Zealand). Data from all the patients referred from 1973 onwards were gathered. They showed data on disease extension (including the number of bones involved and scintigraphic measures of disease extension by using Coutris index) and total ALP at diagnosis. The three indexes were significantly negatively correlated with the date of birth and the year of diagnosis. In New Zealand cohort, more recently born subjects and more recently diagnosed patients had substantially less severe bone disease than was seen previously. The fall in mean ALP over time was due to both a greater number of patients with normal ALP at diagnosis and a reduction in the number of patients with extremely high ALP levels at presentation. In addition to biochemical trends, skeletal involvement declined significantly. The proportion of patients with monostotic disease increased from 24% in the 1985–1993 period to 36% in the 1994–2002 period, and the median skeletal

Table 4 Studies characteristics. This table describes the principal characteristics of the principal published studies that show data on Paget's disease severity trends Unpublished

Reference	Location	Setting	Period	N	Method	Assessment of severity	Comparison	Comments
Corral-Gudino et al. (2013b)	Spain	Metabolic bone disease outpatient clinic	1986–2009	280	Register of all the patients referred to the clinic	ALP Disease extent (measured by radionuclide bone scan) Percentage of the skeleton affected by Paget's disease (quantitated according to the Renier Anatomic Index)	Year of diagnosis Date of birth Age at diagnosis	A comparison was made between patients from a high prevalence focus and the rest of patients.
Cundy et al. (1997, 2004, 2006)	New Zealand	Metabolic bone disease outpatient clinic	1973–2002	832	Register of all the patients referred to the clinic	ALP Disease extent (measured by radionuclide bone scan) Percentage of the skeleton affected by Paget's disease (quantitated according to the Coutris Index)	Year of diagnosis Date of birth Age at diagnosis	None
Guañabens et al. (2012)	Spain	25 centers in different areas of Spain	2006–2007[a]	208	National register	ALP Disease extent (measured by radionuclide bone scan) Percentage of the skeleton affected by Paget's disease (quantitated according to the Coutris Index) Disease activity	Year of diagnosis	None

(*continued*)

Table 4 (continued)

Reference	Location	Setting	Period	N	Method	Assessment of severity	Comparison	Comments
						(quantitated according to Renier Activity Index)		
Merlotti et al. (2005)	Italy	Different centers in Italy	2002–2005[a]	147	National register	Disease extent (measured by radionuclide bone scan)	Date of birth (before or after 1930)	None
Morales-Piga et al. (2002)	Spain	Metabolic bone disease outpatient clinic	1980–1992	233	Register of all the patients referred to the clinic	ALP Disease extent (measured by radionuclide bone scan) Percentage of the skeleton affected by Paget's disease (quantitated according to the Coutris Index)	Date of birth (before or after 1926) Age at diagnosis	A comparison was made according to patient's occupation.
Seton et al. (2003)	USA	Different centers in New England	2001–2003[a]	202	Regional Register	Disease extent (measured by number of bone involved)	Date of birth	None
Tiegs et al. (2000)	USA	Different centers in Olmsted County, Minnesota	1950–1994	236	Regional Resgister	Fracture	Year of diagnosis	None

[a]Period when national register was open to include patients

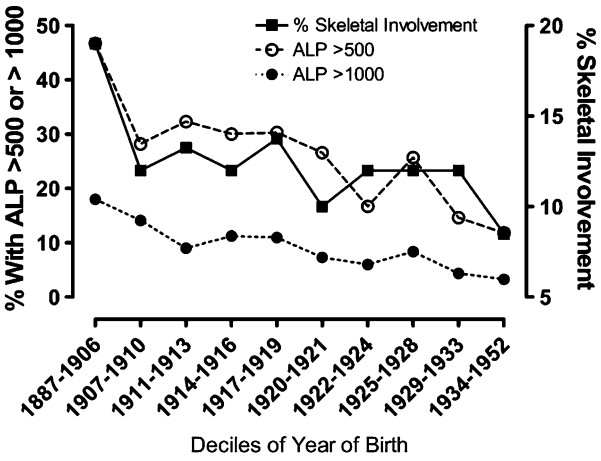

Fig. 2 Decline in Paget's disease severity in Auckland, New Zealand (1973–2002). The figure shows the proportion of patients with pretreatment total ALP activity of >500 or >1000 u/L at presentation and the mean skeletal involvement according to deciles of year of birth (Reprinted from Cundy et al. (2004), Copyright (2004), with permission from "Springer Science and Business Media")

involvement declined from 14.25% to 5.75%. Figure 2 shows the strong negative relationship between the year of birth and the mean percentage of skeletal involvement and the pretreatment level of ALP activity.

With a similar methodology, our group published our experiences in a metabolic bone clinic in Salamanca, Spain (Corral-Gudino et al. 2013b). Data from all the patients referred from 1986 to 2009 were gathered including data on disease extension (by using total number of bones affected and Renier anatomic index) and total ALP at diagnosis. We analyzed separately the data from a focus with a high prevalence of Paget's disease (Vitigudino area) from the rest of the area covered by the metabolic bone clinic (an area with a non-high prevalence of Paget's disease). Paget's disease was more severe in the high prevalence focus, with higher values of ALP, number of bones involved, and Renier extension index. Indexes of severity decreased during the study period in both regions (Fig. 3). Total ALP was negatively associated with year of diagnosis and year of birth in the non-high prevalence area but only with the year of diagnosis in the high prevalence area. Disease extension was associated with both, year of diagnosis and year of birth in the non-high prevalence area but with neither of them in the focus of high prevalence. Age at diagnosis was not associated with severity parameters.

Other two Spanish groups have tested the hypothesis that clinical expressiveness of Paget's disease has declined over the time. In a hospital-based study carried out in Madrid (Spain) (Morales-Piga et al. 2002) two groups of patients divided by their year of birth (categorized as before or after 1926) were compared. The percentage of monostotic patients was higher in the subjects who were born later. The mean percentage of affected bone differed significantly between groups and showed a weak but significant negative correlation with year of birth (Fig. 4). On the contrary, no significant differences were observed in the mean serum ALP, other biochemical markers, or different clinical characteristics (bone pain, any pagetic symptom, bone deformity, localized skin hyperthermia, fracture, cranial symptoms). The subjects

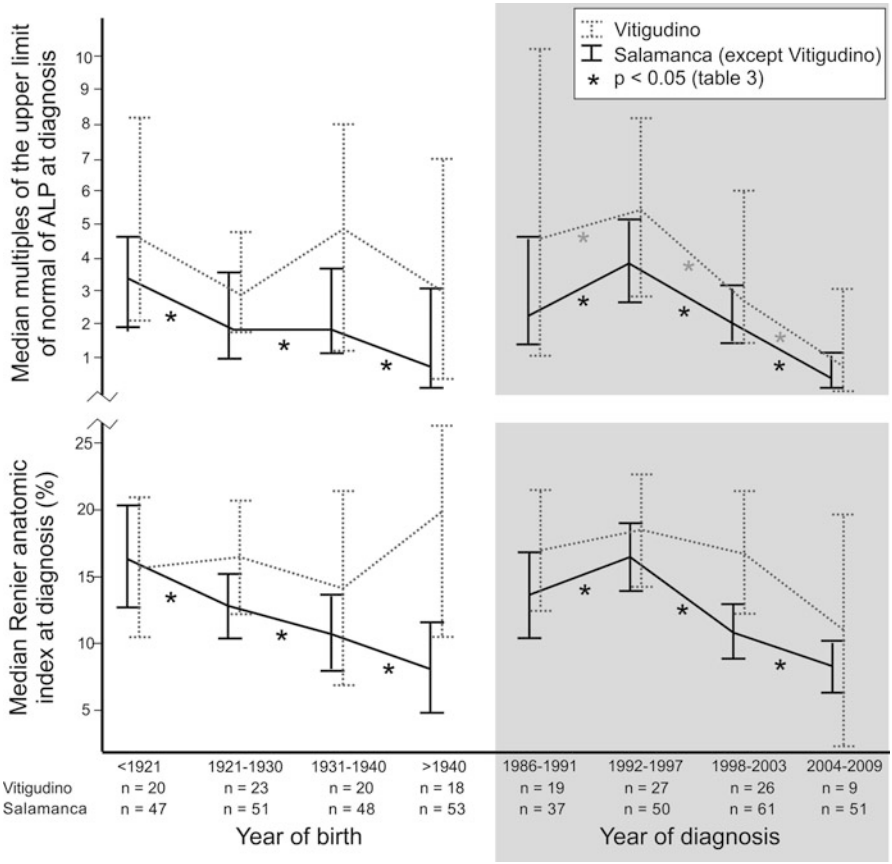

Fig. 3 Decline in Paget's disease severity in Salamanca, Spain (1986–2009). The figures show the evolution of indices of disease severity (mean percentage of skeleton affected and ALP values at diagnosis) according to year of birth or year of diagnosis in a non–high prevalence for Paget's disease area (Salamanca) and in a high prevalence for Paget's disease focus (Vitigudino) (Reprinted from Corral-Gudino et al. (2013b), Copyright (2013), with permission from "Springer Science and Business Media")

who were born earlier had more than twice the prevalence of Monckeberg-type vascular calcifications than the other patients.

Data from a national Spanish Paget's disease register were published (Guanabens et al. 2012). The median Coutris index decreased over time, with lower values in the most recently diagnosed patients. In addition, this decline in the Coutris index was associated with a lower number of involved bones throughout the study. There was also a trend toward lower values of serum ALP activity over time. However, there were no significant differences throughout the year of diagnosis in activity measured by Renier activity index. Figure 5 shows the evolution of both indexes over time. As in the previous studies, the mean age at diagnosis increased during the study.

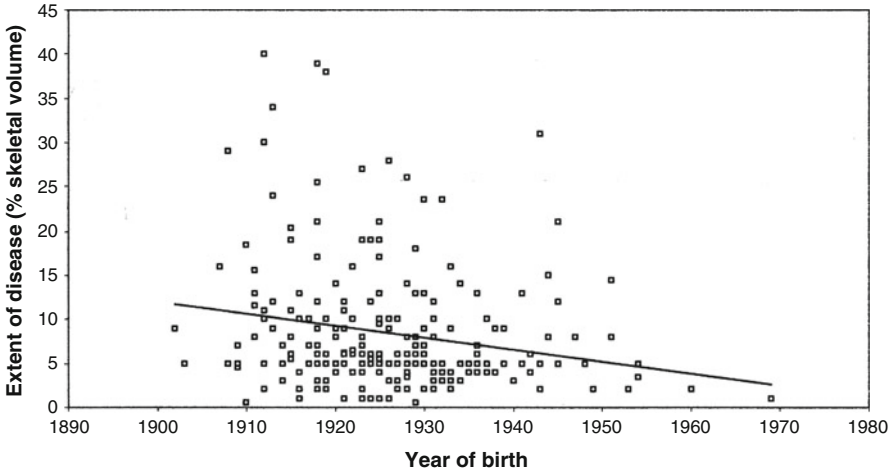

Fig. 4 Decline in Paget's disease severity in Madrid, Spain (1980–1999). The figure shows the relationship between percent of bone involvement and the date of birth (Reprinted from Morales-Piga et al. (2002), Copyright (2002), with permission from "Elsevier")

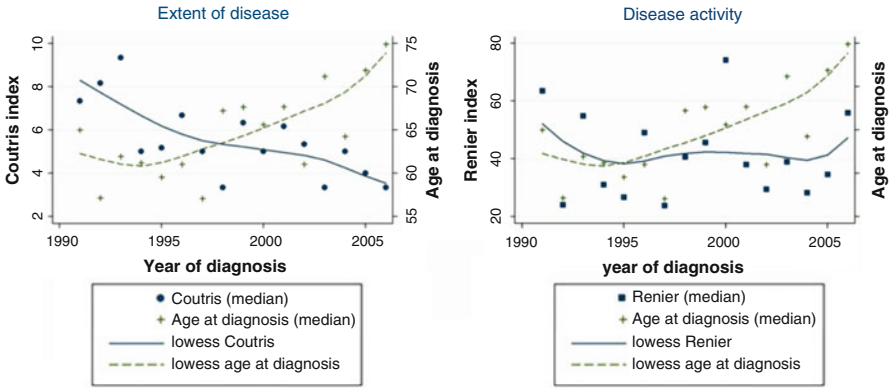

Fig. 5 Decline in Paget's disease severity in Spain-Rheumatology national Paget's disease register (1990–2005). The figure shows the trend for a decline in Coutris extension index according to the year of diagnosis. There was not a trend when Paget's disease activity was measured using the Renier activity index (Reprinted from Guañabens et al. (2012), Copyright (2012), with permission from "Springer Science and Business Media")

An Italian series has reported data on severity secular changes (Merlotti et al. 2005). There was no significant correlation between year of birth and bone involvement extension. To further explore this subject, the authors compared patients who were born before 1930 with patients who were born after 1930. There were no differences in the number of affected bones, although more subjects were monostotic in the later group. Age of diagnosis was higher in subjects who were born before 1930. To justify the lack of a secular trend in Paget's disease in

Italy, the authors hypothesize about an earlier decrease in Paget's disease severity in this country (Gennari et al. 2006). This hypothesis is supported by two facts. Firstly, the less severe disease in Italian contemporary patients when compared with studies from other countries. Secondly, a clear decrease in the number of affected bones was showed when the current series from Siena was compared with an historical series (1950–1956) from the same hospital. Lastly, as in our study, the authors showed a large skeleton involvement on patients from a high prevalence area when compared with patients from non–high prevalence areas.

Data from a regional register in New England, USA (Seton et al. 2003), did not show a significant correlation between birth year and extent of bone involvement, although there were no patients with more than five bone sites affected in the last decades in comparison with first decades.

Lastly, the Rochester epidemiology project provides data about the population in Olmsted County, USA (Melton et al. 2000; Tiegs et al. 2000). The authors describe an increase in age at diagnosis. The year of diagnosis was an independent predictor of vertebral fracture risk.

To sum up the seven studies, all but one (Merlotti et al. 2005) showed a decline on Paget's disease bone extension at diagnosis over the last decades by the finding of lower extension index, lower number of bone affected sites, or higher percentage of monostotic patients. The Italian series is the only one which does not show this difference.

Regarding ALP activity, three of the groups (New Zealand, Salamanca, and Spanish National Register) show a trend toward lower values of this bone marker. The other Spanish group (Madrid) showed a trend on serum ALP activity correlation with year of birth, although this was not statistically significant ($p = 0.10$). ALP trends were not studied in the Italian or American papers. Noteworthy, the secular trends on prevalence were different in high prevalence focus (decreasing prevalence), when compared with non–high prevalence focus (constant prevalence) in one study, but the trends to the reduction on bone extension and ALP levels were similar in both regions.

Lastly, for all the studies, the more recently the diagnosis of Paget's disease was done, the older was the patient.

The reasons for this decline in ALP levels and disease bone extension have not completely been understood. In addition, the evidence supporting the severity decline should be subjected to several limitations. As authors stated, readers have to be cautious when interpreting data from referrals to a metabolic bone disease clinic. Firstly, this research design is subject to selection bias, as only clinically recognized patients are referred to the clinics. Secondly, the changes in the patterns of referral, practice, or demography can affect which subjects are referred and give the false impression of a genuine change in the disease characteristics. To test the hypothesis that the decline in ALP could be due to depletion of the community pool of prevalent severe cases during the first years of the establishment of their specialist clinic, the New Zealand group made analysis excluding the first 6 years of their clinic's operations. The decline in the number of patients with extremely high ALP remained significant in this subanalysis. Thirdly, other possibility is that due to the

greater familiarity of the physicians with Paget's disease because of the metabolic bone disease clinic, the diagnosis was made earlier, at a time when disease is less advanced and severe. A marked trend of increased age at presentation argues strongly against this.

Paget's epidemiology, with a high rate of familiar cases and the highest prevalence rates in British people from United Kingdom or from former British colonies, as New Zealand or Australia, suggests the presence of an inherited predisposition for the disease. Indeed, mutations in SQSTM1 have been identified in familiar cases from different parts of the world. The mechanisms by which SQSTM1 mutations cause osteoclast activation are poorly understood.

The recent changes in Paget's epidemiology, with a decrease in prevalence and a less severe presentation, suggested the importance of factors, other than genetics, playing a role in the pathogenesis of the disease. One example to highlight the importance of these environmental factors is the differences in prevalence and in secular trends between neighboring areas. The causes of these differences between high prevalence rural areas and non–high prevalence urban areas are poorly understood. A genetic component due to familial aggregation could be supposed as one of the major causes of the regional clustering. However, none of the two groups that compared the clinical characteristic of rural versus urban cases show a higher proportion of familial cases in the rural high prevalence areas (16 vs. 13%, and 18 vs. 21% in Vitigudino vs. Salamanca and Campania vs. Siena, respectively) (Corral-Gudino et al. 2013b; Rendina et al. 2006). This finding is in keeping with the role of environmental factor in the pathogenesis of Paget's disease. These factors should facilitate the expression of Paget's disease in genetically susceptible subjects. The most commonly highlighted environmental factors have been the infective agents. Paramyxoviruses as canine distemper virus (Selby et al. 2006) or measles virus (Friedrichs et al. 2002a) have been involved in Paget's pathogenesis, although their role has not been confirmed by different research groups (Helfrich et al. 2000; Matthews et al. 2008) and have generated intense debates (Friedrichs et al. 2002b; Mee 1999; Ralston and Helfrich 1999; Rima et al. 2002). Previous or current contact with animals, specially cattle or unvaccinated dogs, (Khan et al. 1996; Lopez-Abente et al. 1997; Merlotti et al. 2005) or the consumption of untreated water (Miron-Canelo et al. 1997) have been signaled as possible predisposing factors for the disease. The improvements in animal health (development of veterinary pharmaceutical industry or vaccination programs) and water treatment, with subsequent reduction in the prevalence of infections, could be suggested as possible explanations for the decrease in Paget's disease prevalence and severity. A decline in the exposure of the population to other unidentified environmental determinants as childhood vitamin D deficiency (Barker and Gardner 1974), physical activity (Solomon 1979), or occupational exposures (Lever 2002) could also be suggested. As an alternative explanation, changes in the ethnic makeup of the population due to the influx of immigration could explain the changes in prevalence and severity. This hypothesis was questioned by the New Zealand group when analyzing their data. The people with a British descent remained stable in Auckland during last decades while the prevalence and severity of Paget's disease were decreasing continuously on

Dunedin, where the study was limited to people of European background (Cundy et al. 2006).

Environmental and genetic hypothesis are compatible, and both factors seem to be acting jointly in Paget's pathogenesis. Future data on the follow-up of subjects with known inherited mutations, the study of the development of new cases of Paget's disease in these subjects, and the analysis of the severity of the new cases will help to clarify this issue.

Genetic Mutations and Paget's Disease Severity

Biochemical markers and skeletal extension have been the classical approach to assess the severity of Paget's disease. In recent years, genetic susceptibility has been proposed as a new marker for the severity of the disease.

Paget's disease has a strong genetic component. The role of genetic factors in regulating susceptibility to the disease is well established. About 15% of patients with Paget's disease have a positive family history. In these families, the disease is inherited in an autosomal dominant manner, with incomplete penetrance. A mutation in SQSTM1 is carried for between 40% and 50% of patients with a family history and about 5–10% of patients with sporadic disease. SQSTM1 encodes p62, a protein that plays a key role in regulating osteoclast function (Ralston and Layfield 2012). Recent genome wide association studies have identified additional susceptibility loci for Paget's disease near the CSF1 gene on chromosome 1p13, the NUP205 gene on chromosome 7q33, the TM7SF4 gene on chromosome 8q22, the OPTN gene on chromosome 10p13, the RIN3 gene on chromosome 14q32, the PML gene on chromosome 15q24, and near the TNFRSF11A gene on chromosome 18q21 (Albagha et al. 2010).

The role of genetic factors in regulating severity of Paget's disease is less known, however. Different research groups have reported that SQSTM1 mutations influence the severity of Paget's disease (Collet et al. 2007; Hocking et al. 2004; Visconti et al. 2010). The patients with SQSTM1 mutations are younger at diagnosis, they have a greater number of affected bones, the disease is more extended, and they have a higher mean maximal level of ALP, have more bone fractures or fissures over pagetic bones, and have required orthopaedic surgery and courses of bisphosphonates more frequently than those patients without mutation.

In addition, a recent study (Albagha et al. 2013) analyzed the relationship between the severity of Paget's disease and susceptibility alleles of Paget's disease in negative SQSTM1 subjects. The study analyzed a subgroup of patients who participated in a UK-based clinical trial (Langston et al. 2010) and replicated these data on four clinic-based cohorts of Paget's disease: 2 from Italy, 1 from Spain, and 1 for Australia. In all cohorts, Paget's disease extent was assessed on the basis of radionuclide bone scan by counting the number of affected sites with evidence of Paget's disease. A composite index was used to assess disease severity (Table 1). The allele association was measured using a risk allele score based on whether the subjects carried the wild type allele or were heterozygous or homozygous for alleles

associated with Paget's disease on seven locus, with every loci adjusted depending on the strength of association with the disease. The trial showed a significant association between risk allele score and the number of affected bones, with evidence of an allele dose effect. Also a trend was observed for the composed diseases severity index, but the differences were not significant. Data were analyzed to discriminate the role of the aforementioned locus as predictors of disease extent and severity either alone or in combination with SQSTM1 mutations. According to the authors, both genetic markers were independent predictors of disease extent and severity, although the effect size of SQSTM1 mutations on disease extension and severity was about three times larger than the risk allele score. The authors combined information from both genetic markers and created a genetic risk score. This combined genetic score had 70% specificity and 55% sensitivity for predicting severe disease. The combined genetic score was not significantly associated with the response to bisphosphonate treatment in terms of change in ALP activity. Further research is required to define the role of the genetic testing for SQSTM1 mutations and other susceptibility alleles in clinical practice to define subgroups of patients with different levels of severity of the disease.

Whether changes in ethnic makeup of populations classically affected by Paget's disease, especially high prevalence areas as Lancashire (UK) or Vitigudino (Spain), could have had a role in the severity decline of Paget's disease in last decades by reducing the prevalence of SQSTM1 mutations or other susceptibility alleles is only a hypothesis which has not been tested.

Potential Applications to Prognosis, Other Diseases, or Conditions

Despite their common use, both skeletal bone extension and ALP activity are inefficient as prognostic indicators for Paget's disease. Neither the skeletal extension nor the biochemical markers have been associated with the main clinical consequences for patients diagnosed as Paget's disease such as pain, fractures, or arthritis in the joints near the enlarged and misshapen Paget's bones. Notwithstanding the aforementioned limitations of ALP activity in Paget's disease, the use of ALP activity as an approach to the evaluation of the response to treatment and as a tool to adopt treatment decisions is still a common use. We think that most clinicians will keep using it in the next few years.

The uses of ALP activity in other metabolic bone diseases, such as osteoporosis or bone cancer metastases, which are characterized by more subtle alterations of bone remodeling, are poorly defined. Clinical reports do not show a relationship between ALP or its isoform, bone-specific ALP, with the risk of fracture or the antifracture efficacy of treatment in postmenopausal osteoporosis (Cremers and Garnero 2006).

Regarding patients with bone cancer metastasis, serum ALP levels have been likely associated with a higher rate of skeletal complications on patients with bone metastatic prostate cancer (Berruti et al. 2000), but there is no other published

evidence in literature about the existence of a relationship between ALP activity and skeletal related events in patients with bone metastasis regardless of whether they received or not bisphosphonates. For its part, bone-specific ALP has been related with hormone refractory prostate cancer, non-small cell lung cancer, myeloma multiple, breast cancer, and other solid tumors (Cremers and Garnero 2006). ALP activityis not a standardized measure for the initial diagnosis or follow-up assessment in any of these diseases.

Due to its poor role in diagnosis and severity assessment in bone cancer metastasis or postmenopausal osteoporosis, we could hypothesize that ALP activity will not become a useful tool for the determination of secular changes for any of these conditions.

Summary Points

- This chapter focuses on ALP activity and extent of skeletal lesions as tools to measure changes over time in Paget's disease characteristics.
- Generally, the degree of elevation of serum AP activity reflects the extent and severity of the pagetic process.
- The degree of reduction in the levels of serum AP activity is the most commonly used surrogate outcome in clinical trials.
- Decreases in disease activity (assessed by biochemical markers) and in disease skeletal extension in recent decades have been reported for different research groups.
- The reduction in Paget's disease severity is even more marked in non–high prevalence focus.
- These changes support the hypothesis that clinical severity of Paget's disease is decreasing.
- The reasons for the change in severity in recent years are poorly understood.
- Changes in sociological (sedentary lifestyle, kind of work) or demographic (ethnic makeup, migration from rural to urban areas) population characteristics have been postulated as possible explanations.
- Genetic mutations which confer susceptibility for Paget's disease have been proposed as new biomarkers of disease severity.
- SQSTM1 mutations are associated with Paget's disease severity in several studies.

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Bone Turnover Markers and Glucocorticoid Treatments

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Abstract

Glucocorticoids (GCs) remain as the cornerstone of therapy in most inflammatory diseases, even if newly developed biological molecules became available. GCs are potent, possess a fast action, and are cheap and relatively easy to prescribe. However, their beneficial therapeutic activity has a nasty counterpart: quite a lot of complications, notably secondary osteoporosis, aseptic bone osteonecrosis, and fractures. The skeleton is continuously remodeling, old bone being resorbed and replaced by new young bone. GCs interfere with the bone turnover and provoke a disequilibrium in favor of bone loss and fragility. The mechanisms of bone fragility consist of a decreased activity and in apoptosis of osteoblasts, as well as an increase in bone resorption. These changes have already been observed histomorphometrically a long time ago in transiliac bone biopsies. Biological parameters of bone turnover, chiefly degradation products of type I collagen, can help to assess atraumatically the bone metabolism. If, in idiopathic osteoporosis, they can have a predictive value of bone loss, they cannot be considered as surrogates for bone mineral density measurements. In GC-OP, the concentrations of the bone turnover markers (BTMs) of bone formation dramatically and rapidly decrease, whereas the BTMs of bone resorption slightly increase. During GC therapy, they cannot be used as predictive tools of bone fragility on an individual basis. Other markers such as RANKL/RANK/osteoprotegerin seem to be promising in this aim, but this still awaits confirmation.

Keywords

Glucocorticoid • Bone turnover • Biomarkers • Osteoporosis • Bone mineral density • Bone remodeling • Collagen • Telopeptide

List of Abbreviations

ALN	Alendronate
BDP	Beclomethasone dipropionate
BMD	Bone mineral density
BSAP	Bone-specific alkaline phosphatase
BTMs	Bone turnover markers
BUD	Budenoside
CD	Crohn's disease
COMP	Cartilage oligomeric matrix protein
COPD	Chronic obstructive pulmonary disease
CTX	Carboxy-terminal cross-linking telopeptide of type I collagen
DAS	Disease activity score
Dkk-1	Dickkopf-1
FN	Femoral neck
GC	Glucocorticoid
ICTP	Carboxy-terminal telopeptide of type I collagen
Il-6	Interleukin-6

JIA	Juvenile idiopathic arthritis
MMP	Metalloproteinase
MP	Methylprednisolone
NTX	Amino-terminal cross-linking telopeptide of type I collagen
OBS	Osteoblasts
OC	Osteocalcin
OCS	Osteoclasts
OP	Osteoporosis
OPG	Osteoprotegerin
PICP	Procollagen type I C-terminal propeptide
PINP	Procollagen type I N-terminal propeptide
PTH	Parathyroid hormone
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor NF-kB
RANK-L	Receptor activator of nuclear factor NF-kB-ligand
rh	Recombinant human
RIS	Risedronate
Scl	Sclerostin
SLE	Systemic lupus erythematosus
TPTD	Teriparatide
TRAP	Tartrate-resistant acid phosphatase
UC	Ulcerative colitis
uDPD	Urinary deoxypyridinoline
uPYD	Urinary pyridinoline
VF	Vertebral fracture

Introduction

Owing to their immunomodulatory, immunosuppressive, and anti-inflammatory properties, glucocorticoids (GCs) are frequently prescribed in many conditions as various as intestinal, locomotor, skin, vascular inflammatory, or allergic diseases, as well in organ transplantation. Besides their favorable actions, GCs can beget a lot of complications such as bruising and skin atrophy, truncal obesity, cataracts, hypertension, salt and fluid retention, and disorders in the glucose and lipids metabolism. One of the most devastating complications consists of the development of osteoporosis and bone fragility leading to an increased incidence of fractures, which can occur soon after the onset of GC therapy (Van Staa et al. 2002; Kanis et al. 2004).

In a retrospective study, the risk of fracture was shown to be commensurate to the daily dose. Compared to controls, the adjusted relative rate for a daily dose of $\leq$ 2.5 mg equivalent predniso(lo)ne increased from 1.17, 1.10, 0.99, and 1.55 for nonvertebral, forearm, hip, and vertebral fractures, respectively, to 1.64, 1.19, 2.27, and 5.18 for a dose of $\geq$ 7.5 mg/day (Van Staa et al. 2000a, b).

Even since the availability of potent biologic agents such as human tumor necrosis factor alpha antibodies, anti-cytotoxic T-lymphocyte antigen 4-immunoglobulin antibody, and recombinant humanized antihuman interleukin 6 receptor monoclonal antibody, GCs are still frequently prescribed, because they are cheap and demonstrate a rapid and potent therapeutic response, all characteristics of particular importance in chronic conditions with severe flares. It is therefore mandatory to systematically consider preventive measures as soon as GC therapy is started. The chapter addresses the role of bone turnover markers (BTMs) in the development of bone loss and bone fragility and in deciding a preventative therapy.

Available Glucocorticoids in Daily Clinical Practice

In Cushing's syndrome, cortisol (or hydrocortisone) is secreted in excess by the adrenal glands. It has been recognized for years that this condition is complicated by osteoporosis. Hydrocortisone is chiefly utilized for hormone replacement in states of adrenal insufficiency. Derivatives were synthesized in the aim of augmenting the therapeutic potency of GCs without significantly increasing the side effects. Various preparations of GCs are available for a daily clinical use. They are shown in Table 1, with their respective potencies.

The Routes of Administration of Glucocorticoids

According to the severity of the condition, various routes of administration and dose regimens have been proposed for therapy, such as for systemic administration (daily oral, constant, intermittent, or alternate-day doses; step-up, step-down, intravenous

Table 1 Equivalence of the more frequently used preparations of glucocorticoids

	Approximative equivalence in mg	Relative anti-inflammatory potency
With a short biologic half-life (8–12 h)		
Hydrocortisone	20	1
Cortisone	25	0.8
With a long biologic half-life (36–72 h)		
Betamethasone	0.75	25
Dexamethasone	0.75	30
With an intermediate biologic half-life (12–36 h)		
Prednisone	5	4
Prednisolone	5	4
Oxazolone derivative of prednisone (Deflazacort)	6	4
Methylprednisolone	4	5
Triamcinolone	4	5

Modified after Haynes (1990), p. 1447

pulse therapy) or for local administration (inhaled, skin topical, or intra-articular). It became rapidly evident that in most conditions, every route of GC administration could provoke detrimental effects (Nagant de Deuxchaisnes et al. 1984; Emkey et al. 1996; Richy et al. 2003; Dovio et al. 2004; Dhar et al. 2014) on bone mineral density (BMD) and on biochemical markers of bone metabolism.

The Mechanisms of Action of Glucocorticoids

The potent immunosuppressive and anti-inflammatory actions of GCs are expressed through GC receptors, which are considered to have positive genomic effects on anti-inflammatory proteins (an action so-called transactivation) or negative effects on the production of pro-inflammatory proteins (so-called transrepression). Transactivation is considered accountable for side effects of GCs (notably on the skeleton). Transrepression, on the contrary, is seen as favorable (Stahn and Buttgereit 2008). However, GCs can also exert positive effects through an acute genomic-independent activity (Jiang et al. 2015). This particularity could help to the development of new GCs with nongenomic mechanisms and provoking less adverse effects (Jiang et al. 2015). These promising drugs are not yet clinically available. In the bone tissue, osteoblasts (OBS) seem to be the main target of GCs. This has been histomorphometrically demonstrated already a long time ago (Bressot et al. 1979; Aaron et al. 1989). The number and the longevity of active OBS and the wall thickness of the trabecular plates are dramatically decreased. Furthermore, GCs promote apoptosis of OBS and osteocytes (Weinstein et al. 1998). Moreover, GCs promote osteoclast (OCS) survival (Weinstein et al. 2002). The apoptosis of osteocytes could also favor the malfunction of the mechanostat, potentially leading to GC-induced osteonecrosis (Weinstein et al. 2000).

The discovery of the receptor activator of nuclear factor NF-kB ligand (RANK-L), its receptor RANK, and the decoy receptor of RANK-L, osteoprotegerin (OPG), has further improved our understanding of the bone remodeling in physiologic and pathologic conditions (Manolagas 2000). Various theoretical mechanisms of GC-induced OP and their actions on biomarkers of bone remodeling are summarized in Fig. 1.

The Bone Turnover Markers

Bone is a tissue which is physiologically and relentlessly remodeled, in order to eliminate the weakened old bone and replace it with new more solid bone. This mechanism in equilibrium in normal conditions allows the maintenance of bone mass and of its mechanical resistance in healthy young males and premenopausal women. During growth, the bone modeling is accompanied by spontaneous changes in the BTMs. This has been studied for some BTMs (Pereira et al. 1999). In healthy adolescent girls (aged 13–18 years) and boys (aged 15–18), a progressive decrease in the BTMs concentration toward adult levels was observed comparatively with

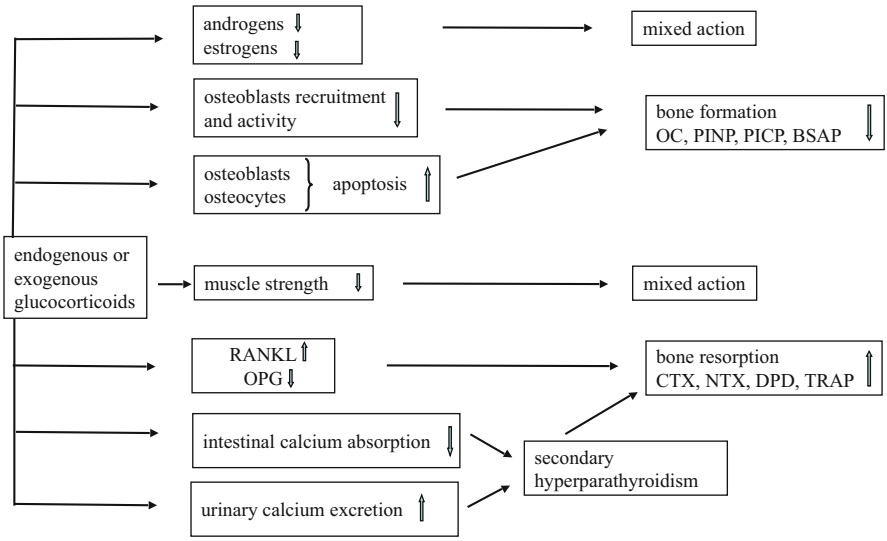

Fig. 1 Various mechanisms potentially involved in the development of glucocorticoid-induced osteoporosis and action on bone turnover markers. *DPD* deoxypyridinoline, *OPG* osteoprotegerin, *TRAP* tartrate-resistant acid phosphatase

values of children aged 5–12 years (girls) or 5–14 years (boys). A further decrease in BTMs was observed in girls suffering from juvenile idiopathic arthritis (JIA) treated by GCs (Pereira et al. 1999). Beyond menopause in women as well as in elderly in men, there is an acceleration of the bone remodeling, with some preponderance of the markers of resorption over the markers of bone formation (Devogelaer et al. 2011). Pathologic conditions, notably inflammatory diseases and mainly endogenous or exogenous GC excess, modify the bone remodeling. The biomarkers which can be measured in GC-OP are summarized in Tables 2, 3, and 4. These tables include the methods of dosage, the precautions to be respected for sampling, and the possible interferences. First of all, it should be remembered that most BTMs have a circadian rhythm, being high during the night and in the morning and low in the afternoon. It is therefore important to take the blood or urine samples in the early morning and preferably for most of them in a fasting condition or, at the worst, always at the same time of the day in a very same patient (Devogelaer et al. 2011). Moreover, some BTMs demonstrate a seasonal variation, with a zenith in February and a nadir in August (Rapuri et al. 2002). These changes parallel the changes in the level of 25OH vitamin D3 (Rapuri et al. 2002). Furthermore, BTM levels increase after a fracture – a frequent event during GC therapy – and can remain elevated for about 1 year (Ivaska et al. 2007). The practitioner who cares for the patients should have in mind these characteristics when interpreting the clinical significance of BTMs.

Table 2 The OPG/RANKL/RANK system

Biomarker	Available assay format	Sampling	Pre-analytical variability	Analytical variability	Biological variability
Receptor activator of nuclear factor kappa B ligand (RANKL)	EIA	Serum	Poorly documented	Intra-laboratory within run CV: below 15% Intra-laboratory between run CV: below 20% Interlaboratory/method CV: ND	Limited circadian rhythmic variation Affected by thyroid function Intra-individual variability: ND
Osteoprotegerin (OPG)	EIA	Serum Plasma (OPG concentrations are lower in serum than in plasma)	Poorly documented	Intra-laboratory within run CV: below 10% Intra-laboratory between run CV: about 15% Interlaboratory/method CV: ND OPG concentrations are falsely diminished with hemoglobin interference	Limited circadian rhythmic variation Affected by thyroid function Intra-individual variability: ND

Table 3 Biomarkers related to bone resorption: available assays, sampling precautions, pre-analytical factors, analytical and biological variability

Biomarker	Available assay format	Sampling	Pre-analytical variability	Analytical variability	Biological variability
Amino-terminal cross-linking telopeptide of type I collagen **(NTX)**	EIA ECi	Serum Plasma Urine	Morning collection Urine: second morning void	Intra-laboratory within run CV: about 10% Intra-laboratory between run CV: about 10% Interlaboratory/ method CV: 40% (urine)	Circadian rhythm Food intake Age Gender Kidney and liver functions Fractures Pregnancy If urinary measurement, must be corrected with urinary creatinine levels Intraindividual variability: about 10%
Carboxy-terminal cross-linking telopeptide of type I collagen (CTX)	AI ECLIA EIA	Serum Plasma Urine	Morning collection after overnight fast Urine: second morning void	Intra-laboratory within run CV: below 5% Intra-laboratory between run CV: below 8% Interlaboratory/ method: 10–30%	Important circadian rhythm Food intake Age Gender Fractures Pregnancy Kidney function If urinary measurement, must be corrected with urinary creatinine levels Intraindividual variability: about 10%
Carboxy-terminal telopeptide of type I collagen (ICTP)	EIA	Serum	Morning collection after overnight fast	Intra-laboratory within run CV: about 10% Intra-laboratory between run CV: about 10% Interlaboratory/ method CV: 25%	Important circadian rhythm Food intake Age Gender Fractures Pregnancy Kidney function Intraindividual variability: ND

Deoxypyridinoline (DPD)	EIA AI RIA	Urine	Morning collection Avoid exposure to ultraviolet light Second morning void	Intra-laboratory within run CV: below 8% Intra-laboratory between run CV: below 10% Interlaboratory/ method CV: about 25%	Circadian rhythm Age Gender Fractures Pregnancy Measurement must be corrected with urinary creatinine levels Intra-individual variability: about 20%
Dickkopf-related protein-1 (DKK1)	EIA	Serum	Poorly documented	Intra-laboratory within run CV: about 15% Intra-laboratory between run CV: about 15% Interlaboratory/ method CV: ND	Poorly documented
Hydroxyproline	HPLC method	Urine	Second morning void Keep samples refrigerated or frozen until analysis	Intra-laboratory within run CV: about 15% Intra-laboratory between run CV: about 15% Interlaboratory/ method CV: ND	Nonspecific for the bone Affected by food intake Measurement must be corrected with urinary creatinine levels Intra-individual variability: ND
Osteopontin (OPN)	EIA	Serum Plasma (Plasma showed higher concentrations than serum)	Poorly documented	Intra-laboratory within run CV: below 10% Intra-laboratory between run CV: about 15% Interlaboratory/ method CV: ND	Potential circadian rhythmic variation Intra-individual variability: ND

(*continued*)

Table 3 (continued)

Biomarker	Available assay format	Sampling	Pre-analytical variability	Analytical variability	Biological variability
Pyridinoline (PYD)	EIA AI RIA	Urine	Morning collection Avoid exposure to ultraviolet light Second morning void	Intra-laboratory within run CV: about 15% Intra-laboratory between run CV: about 15% Interlaboratory/ method CV: about 25%	Circadian rhythm Age Gender Fractures Pregnancy Liver function Active arthritis Measurement must be corrected with urinary creatinine levels Intraindividual variability: about 20%
Sclerostin (SCL)	EIA	Serum Plasma Levels reported to be about 30% higher in plasma than in serum	Poorly documented	Intra-laboratory within run CV: below 10% Intra-laboratory between run CV: about 15% Interlaboratory/ method CV: 10–20%	Circadian rhythm Associations reported with age, height, dialysis vintage, troponin, homocysteine, phosphate, PTH Increased in patients with type 1 and type 2 diabetes Higher concentrations observed in hemodialyzed patients

Secreted frizzled-related protein 1 (sFRP-1)	EIA	Serum	Poorly documented	Intra-laboratory within run CV: below 15% Intra-laboratory between run CV: about 20% Interlaboratory/ method CV: ND	Poorly documented
Tartrate-resistant acid phosphatase (TRAP)	EIA AI	Serum	Avoid hemolysis Keep samples frozen until analysis to avoid degradation	Intra-laboratory within run CV: about 10% Intra-laboratory between run CV: about 10% Interlaboratory/ method CV: ND	Circadian rhythm Age Gender Fractures Intraindividual variability: about 25%

AI automated immunoassay, *ECLIA* electrochemiluminescent immunoassay, *EIA* enzyme immunoassay, *ECi* enhanced chemiluminescent assay, *RIA* radioimmunoassay, *HPLC* high-performance liquid chromatography, *CV* coefficient of variation, *ND* not documented

Table 4 Biomarkers related to bone formation: available assays, sampling precautions, pre-analytical, analytical, and biological variability

Biomarker	Available assays	Sampling	Pre-analytical variability	Analytical variability	Biological variability
Bone-specific alkaline phosphatase (BSAP)	CLIA EIA AI	Serum	Keep samples refrigerated or frozen until analysis Avoid hemolysis	Intra-laboratory within run CV: below 8% Intra-laboratory between run CV: below 10% Interlaboratory/method CV: 30%	Circadian rhythm (low) Age Gender Fractures Pregnancy Intraindividual variability: about 8%
Osteocalcin (OC)	CLIA EIA ECLIA AI	Serum Urine	Keep samples frozen until analysis to avoid degradation Avoid hemolysis Urine: second morning void	Intra-laboratory within run CV: below 8% Intra-laboratory between run CV: below 10% Interlaboratory/method CV: 10–30%	Circadian rhythm Kidney function Age Gender Fractures Pregnancy If urinary measurement, must be corrected with urinary creatinine levels Intraindividual variability: about 20%
Procollagen type I N-terminal propeptide (PINP)	ECLIA EIA AI RIA	Serum	Relatively stable biomarker No specific need of fasting	Intra-laboratory within run CV: below 8% Intra-laboratory between run CV: below 10% Interlaboratory/method CV: ND	Circadian rhythm (low) Age Gender Fractures Pregnancy Intraindividual variability: about 12%
Procollagen type I C-terminal propeptide (PICP)	EIA RIA	Serum	Poorly documented	Intra-laboratory within run CV: about 10% Intra-laboratory between run CV: about 10% Interlaboratory CV: ND	Circadian rhythm (low) Age Gender Fractures Pregnancy Intraindividual variability: ND

AI automated immunoassay, *ECLIA* electrochemiluminescent immunoassay, *CLIA* chemiluminescent immunoassay, *EIA* enzyme immunoassay, *RIA* radioimmunoassay, *CV* coefficient of variation, *ND* not documented

Bone Markers and Inflammatory Diseases Likely to be Treated by Glucocorticoids

In active rheumatoid arthritis (RA), not having started GCs yet, most biomarkers of bone remodeling have been frequently found elevated. Procollagen type I N-terminal propeptide (PINP) and procollagen type I C-terminal propeptide (PICP), both markers of bone formation and CTX a marker of bone resorption, were significantly higher than in controls (Cortet et al. 1998). In this study OC, PINP, and PICP levels were correlated with femoral neck (FN)-BMD. In another study, serum OC was significantly lower in patients with active RA than in normal controls and inactive RA patients (Al-Awadhi et al. 1999). Serum OC was confirmed to be lower than in normal controls in both severe erosive RA and less destructive RA, whereas CTX was more elevated in active RA. Based on the low levels of OC and high CTX levels, the authors concluded to an uncoupling in bone turnover in RA patients, even not taking GCs (Garnero et al. 1999). The predictive value of increased serum levels of CTX, even if associated with radiological progression of the disease, was lower than classical markers of RA such as erythrocyte sedimentation rate, C-reactive protein, and DAS28 score of disease activity (Jansen et al. 2004). The predictive value for an annual radiological progression over 11 years was improved when the baseline values of the RANKL/OPG ratio were added to the classical markers (van Tuyl et al. 2010). However, these markers cannot be used as surrogate markers for radiological endpoints in the follow-up of RA (Syversen et al. 2009).

In 30 female patients with systemic lupus erythematosus (SLE), sOC was found significantly lower than in controls, contrary to the values of BSAP, PICP, ICTP, and uDPD which were not significantly different from controls, whether they had a normal, osteopenic, or osteoporosis BMD (Redlich et al. 2000). OC levels were also found low in another study with 20 SLE females at the time of diagnosis. In this study, however, urinary cross-link excretion was increased (Teichmann et al. 1999). In another study, uPYD and uDPD were significantly higher in postmenopausal women than in premenopausal women with SLE, which was not unexpected (Kipen et al. 1998). The interference of the urinary concentration of creatinine as denominator in the elevation of the urinary marker should be taken into account in such systemic conditions.

In inflammatory bowel diseases (IBD), whether Crohn's disease (CD) or ulcerative colitis (UC), sOC has been found to be low and ICTP to be high (Bischoff et al. 1997), while sOC and PICP, both markers of bone formation, and ICTP, a marker of bone resorption, were not different from controls in another study. However, urinary PYR and uDPD were elevated (Bjarnason et al. 1997). Serum RANKL and OPG levels were found to be increased in CD, with RANK expressed in the mucosa of the colon at a higher level than in normal colon (Franchimont et al. 2004).

It is important to assess the levels of BTMs in the disease states before initiation of GC therapy, in order to be able to appreciate the changes due to the treatment versus the disease activity itself.

Hyperparathyroidism Caused by Glucocorticoids, Yes or Not?

A theoretical mechanism for the development of GC-OP was attributed a long time ago to a secondary hyperparathyroidism which could occur as GCs provoke a decrease in intestinal calcium absorption and an increase in urinary calcium excretion (Morris et al. 1990; Suzuki et al. 1983). This assumption did not resist to the current availability of modern assays of parathyroid hormone (Paz-Pacheco et al. 1995).

Effects of Glucocorticoid Therapy on Bone Turnover Markers in Volunteers

The effect on OC of 60 mg of prednisone administered orally at 8 a.m. over 5 consecutive days was studied by Godschalk and Downs (1988) in young volunteer males. They observed a rapid fall in sOC of 32% from baseline already evident 24 h after the first dose. Forty-eight to 96 h after the first dose, serum OC had fallen 63% from baseline values. Twenty-four hours after the last day of dosing, OC remained significantly low (−49%) but reached basal values back 48 h after weaning from GCs. In two other studies, Lems et al. (1995, 1998) treated male volunteers with 10 mg oral prednisone/day for 7 days. Serum OC decreased of – 20% to – 33% from the second to the fourth days of GC therapy, and of – 25% to – 27% the seventh and eighth days, but returned to badeline values 4 days after weaning from GC. A study of dose-ranging effect of GCs on sOC is illustrated in Table 5. In this Table, the study by Godschalk and Down (1988) is compared with studies by Nielsen et al. (1988a) and Kotowicz et al. (1990). Similar changes in OC levels were evidenced after equivalent GC doses in these studies. Kotowicz et al. observed a negative correlation between the prednisone dosage and sOC ($r = -0.7, p < 0.001$) in 50 patients with various rheumatoid disorders receiving long-term prednisone therapy. By using a multiple regression analysis, they demonstrated a significant relationship between sOC and prednisone dosage ($R^2 = 0.72$; $p < 0.001$). In a preliminary study, an inverse correlation between serum undercarboxylated osteocalcin level and oral glucocorticoid dose was also observed in patients suffering from rheumatoid arthritis (Mokuda et al. 2012). As

Table 5 Effects of various doses of prednisone on serum osteocalcin estimated from three studies

Daily dose of prednisone	Serum osteocalcin decrease in %		
	By Godschalk and Downs (1988)	By Nielsen et al. (1988a)	By Kotowicz et al. (1990)
5 mg	NS	(–)	−2%
10 mg	−17%	(–)	−19%
15 mg	−22%	(–)	−32%
20 mg	−26%	(–)	−43%
40 mg	(–)	−74%	(–)
60 mg	−63%	(–)	(–)

undercarboxylated osteocalcin could influence muscle function in humans, this drop might favor falls and increase the fracture risk by affecting the lower limb muscle strength (Levinger et al. 2014).

The comparison of the slopes of the decreases in sOC levels in volunteers naive to GCs and in patients on long-term GC therapy demonstrates that the reduction in sOC concentration does not continuously decrease and levels off. The OBS remain at the ready to secrete OC again as soon as GC therapy is discontinued. By giving 40 mg prednisone daily to 18 volunteers, Nielsen et al. observed a 74% decrease in sOC the day after the last dose of GC (Nielsen et al. 1988a). The comparative series of numbers mentioned in Table 5 are rather similar.

In another study, Nielsen et al. (1988b) administered 2.5 mg or 10 mg prednisone orally at 08:00 H p.m. or placebo. In the placebo group, the circadian rhythm of serum OC was maintained (rise from 11.30 p.m. and peak at 02:30 a.m., decrease after that time until a nadir around 03:30 p.m). The two doses of prednisone similarly inhibited the circadian rhythm of sOC (lack of increase, even a decrease in the expected nocturnal rise in sOC), with a similar maximal decrease. What was different was the duration of the inhibiting effect of the 10 mg versus the 2.5 mg prednisone dose, which was twice longer for the high dose (12 h versus 6 h). Therefore, even a very low dose of prednisone can inhibit and revert the circadian rhythm of sOC. Administering GCs at night could at long term become more deleterious for the bone than if they had been administered early in the morning. However, it should be recalled that the administration of small doses of predniso(lo)ne (5–7.5 mg) daily at bedtime had been many years ago recommended in rheumatoid arthritis with the aim of reducing the morning stiffness, with apparently clinical benefits and not more side effects (de Andrade et al. 1964).

Intra-articular injection of 40 mg triamcinolone acetonide provoked a decrease of 50% in the OC concentration within 24 h after injection, with a reincrease after 7 days and normalization after 14 days (Emkey et al. 1996). Intra-articular injections of triamcinolone hexacetonide in the knee provoked a similar decrease in sOC within 24 h in 20 patients suffering from RA. If the rheumatoid patients were submitted to a bed rest of 24 h, a slightly larger decrease of serum cartilage oligomeric matrix protein (COMP) was observed, suggesting a better cartilage protective effect (Weitoft et al. 2005). The bone resorption marker uDPD was not affected. Such a short bed rest time of 1 day did not apparently influence the bone remodeling.

Intravenous pulse therapies of large GC doses provoke also a rapid drop in sOC values, as well as also in PICP (Lems et al. 1993), but unexpectedly in ICTP too (Lems et al. 1993, 1996), with a normalization in a few weeks. In our experience and that of others, this kind of GC administration is generally devoid of deleterious effect on BMD, provided of course if the pulse injections are not too numerous (Devogelaer 2006; Frediani et al. 2004). Compared with oral methylprednisolone (MP) 16 mg/day for 1 month, followed by a slow tapering down to 4 mg/day, three intravenous pulses of 1000 mg on alternate days did not provoke any significant bone loss after 1 year versus a significant loss amounting to – 9.3%, – 7.8%, and – 10.0%, respectively, at the lumbar spine, whole body, and femoral neck BMD for the oral doses. Bone-specific alkaline phosphatase (BSAP) showed a significant

decrease in the oral group only (−56%, $p < 0.01$) after 1 year (Frediani et al. 2004).

Inhaled glucocorticoids (InGCs) are more and more frequently used in the treatment of asthma and chronic obstructive pulmonary diseases (COPD). A preliminary remark is that the crude effects of InGCs on bone metabolism were difficult to determine, because, as in daily clinical practice, a lot of patients were either simultaneously on oral GCs, had received GCs for a while just before initiation of InGCs, or received oral GCs for treating flares during InGC therapy. In the majority of studies with InGCs, a significant decrease in sOC was observed (Hanania et al. 1995; Wisniewski et al. 1997; Jones et al. 2002; Richy et al. 2003), within some studies, a larger decrease in sOC and a larger increase in uDPD and uPYR provoked by beclomethasone dipropionate (BDP) compared with budesonide (BUD) administered at equivalent doses (Struis and Mulder 1997; Tattersfield et al. 2001). There was no correlation between the changes in BTMs and the changes in BMD, but a negative correlation between the cumulative doses of InGCs and BMD was observed in some studies (Hanania et al. 1995; Wisniewski et al. 1997).

Other Biomarkers of Bone

Other biomarkers of bone formation such as BSAP, PINP, and PICP have been much less frequently studied. In general, they have demonstrated directionally the same behavior as sOC, but with a much lower magnitude of changes. Such a comparative example is shown in healthy postmenopausal women on 5 mg prednisone per day for 6 weeks (Table 6) (Ton et al. 2005).

As a rapid bone loss occurs soon after initiation of GCs therapy, particularly in the first year of therapy, an elevation of the biomarkers of bone resorption is expected. Urinary and serum amino telopeptide of type I collagen (uNTX), urinary and serum carboxy-terminal telopeptide of type I collagen (CTX), serum cross-linked telopeptide domain of type I collagen (ICTP), serum tartrate-resistant acid

Table 6 Effects of low dose of prednisone (5 mg/day for 6 weeks) on bone markers in healthy postmenopausal volunteers (percentage from baseline before therapy)

Markers	Week 2	Week 4	Week 6	Week 8	p from baseline
OC	−19%*	−23%*	−26%*	+ 1%*	<0.01
BSAP	−40%	−12.6%	−16%	−4%	=0.06
PICP	−15%*	−19%*	−8%**	+7%*	<0.01
PINP	−7%*	−16%*	−11%	+4%	<0.01
sNTX	+5%	+1%	−2%	−11%	=NS
uNTX/cr	+5%	−2%	−6%	−2%	=NS
uDPD/cr	−10%	−12.6%	−14%	−4%	=NS

OC osteocalcin, *BSAP* bone-specific alkaline phosphatase, *PICP* type I carboxyl-terminal propeptide, *PINP* type I amino-terminal propeptide, *sNTX* serum type I collagen N-telopeptide, *uNTX* urinary type I collagen N-telopeptide, *Cr* creatinine, *uDPD* urinary free deoxypyridinoline. Values estimated from graphs of Ton 2005

*$p < 0.01$ from placebo

phosphatase (TRAP), urinary pyridinoline (uPYD), and deoxypyridinoline (DPD) were the most frequently measured. Discordant results were observed according to the time period of the studies.

ICTP levels decreased on GC in the studies of Lems et al. 1993, 1996, 1998, as well as uPYD (Lems et al. 1996) and uNTX (Lems et al. 2006). Serum CTX increased significantly of 149–248% in the studies of Paglia et al. 2001 and Dovio et al. 2004. Such a discrepancy in the response to GCs in sCTX and sICTP has been observed also in another study in patients suffering from rheumatoid arthritis and treated with oral prednisolone (7.5 mg per day) (Engvall et al. 2013). The increase in CTX and the decrease in ICTP releases are due to the fact that the liberation of these collagen fragments is generated by different proteinases (Garnero et al. 2003). CTX is released by cathepsin K and ICTP by matrix metalloproteinases (MMPs) MMP-2, MMP-9, MMP-13, or MMP-14. This partly explains that small doses of prednisolone can retard the progression of inflammatory erosions in rheumatoid hands and feet (van Everdingen et al. 2002) but are still deleterious for bone mass (Devogelaer 2006).

Serum NTX did not change (Fujii et al. 2007). Urinary NTX, however, increased significantly in another study (+60%) simultaneously with a decrease in sOC (−40%) (Kaji et al. 2010a). No correlation between the change in the levels of biomarkers and BMD changes was observed. These authors found in another study that uDPD levels were significantly higher in women with vertebral fractures (VF) than in women without VF. Furthermore, uDPD was a factor linked to prevalent VF in postmenopausal women only (Kaji et al. 2010b). Such a cross-sectional study does not allow to conclude to the utility of the measurement of uDPD in the aim at measuring the risk of fracture in patients treated by GCs. It should be recalled that biological parameters of bone turnover may remain elevated at long term after a fracture.

Urinary PYD did not change in other studies (Hanania et al. 1995; Siomou et al. 2003). Urinary DPD did not change significantly either (Hanania et al. 1995; Bornefalk et al. 1998). The last authors also observed a significant decrease in iCTP (−19%) in patients older than 65 years and suffering from asthma treated with oral GCs, but no change was seen in younger patients. The unexpected results of uNTX, uPYD, and uDPD could simply be explained by the correction for urinary creatinine. However, it was suggested that this was potentially attributable to a decrease of 62% in the level of Il-6 (Bornefalk et al. 1998). This hypothesis needs confirmation. Further prospective studies with fracture and biomarkers as endpoints should therefore be implemented in order to be able to confer a prognostic value on changes in parameters of bone remodeling induced by GCs.

RANK, RANKL, and OPG System

In several studies with patients suffering from pathologic conditions necessitating GC therapy (e.g., renal diseases (Sasaki et al. 2001, 2002), Crohn's disease (von Tirpitz et al. 2003), cardiac transplantation (Fahrleitner et al. 2003), and various

rheumatic conditions (Brabnikova Maresova et al. 2013)), OPG significantly decreased after the initiation of the GC treatment. The decrease in OPG confirms in vivo what was observed in preclinical studies (Hofbauer et al. 1999). Rather unexpectedly, patients suffering from Cushing's syndrome, if they have low sOC values, were shown to have significantly higher values of OPG (Ueland et al. 2001), even persisting for 6–18 months after the cure of the condition. Contrary to OPG, sOC levels were already rapidly normalized (Camozzi et al. 2010). In another study, OPG levels were shown to be the only independent predictor of BMD changes at femoral neck and lumbar spine, using multiple regressions ($r = 0.98$, $p < 0.001$) compared with changes in serum OPG, CTX and creatinine, body mass index, months since heart transplantation, cumulative dose of prednisolone, renal function, and parathyroid hormone (Fahrleitner et al. 2003). Patients with vertebral fractures had levels of OPG 2.9-fold lower than patients without fracture. OPG was shown to have a predictive value for prevalent vertebral fractures, contrary to age, body mass index, serum creatinine, femoral neck BMD, and months since heart transplantation. A decrease by 20 or 30% in OPG levels increased the risk of prevalent fractures by 7.9 or 22 times, respectively (Fahrleitner et al. 2003). These interesting predictive values for fracture await confirmative studies before to recommend OPG dosage in patients receiving GC treatment.

The explanations of the apparently inconsistent data stem from the small number of studied patients, the various doses of GC used, and our ignorance about the correlation between the dosages and the biological activity of the biomarkers in peripheral blood and their local concentration and action at the cellular level. Therefore, the results observed in Dickkopf-1 (Dkk-1) and sclerostin (Scl) levels should be interpreted with caution (Brabnikova Maresova et al. 2013). The significant decrease in Scl and the nonsignificant increase in Dkk-1 observed within 4 days after initiation of GC therapy await confirmation. Indeed, another small clinical study showed on the contrary an increase in Scl and a decrease in Dkk-1 after 12 months of GC therapy (Gifre et al. 2013). Sclerostin deficiency in humans is complicated by sclerosteosis, a condition which consists of a skeleton with a high bone mass (Balemans et al. 2001). Expression of DKK-1 in cultured human osteoblasts has been shown to be enhanced by GCs (Ohnaka et al. 2004) and to attenuate the induction of apoptosis of osteoblasts (Wang et al. 2008), an action of this cytokine of possible interest in the prevention of bone complications in GC therapy.

Treatment of Glucocorticoid-Induced Osteoporosis

Vitamin D and calcium supplementation are weak antiresorptive agents. In a study of patients suffering from various rheumatic conditions and necessitating GC treatment, we compared the effect of calcium supplementation (800 mg) alone, with intravenous disodium pamidronate (90 mg at once at the start of therapy) and quarterly disodium pamidronate 30 mg. The results are shown in Table 7. The depressive effect of pamidronate was far more marked than that of calcium on sOC and BSAP, on top of the known depressive effect of GCs on bone formation.

Table 7 Preventive therapy in patients starting glucocorticoids. Percentage change from the start

		Calcium alone 800 mg/day	Cumulative GC dose mg equivalent prednisone	Pamidronate 90 mg at the start only	Cumulative GC dose mg equivalent prednisone	Pamidronate 90 mg at the start + 30 mg/ 3 months	Cumulative GC dose mg equivalent prednisone
Initial GC dose		19 (16)		28 (25)		25 (23)	
Parameters							
BSAP µg/L	Baseline	9.4 (8.1)		6.5 (1.6)		8.6 (6.2)	
	+3 months	−22%	1185 (311)	−45%	1976 (1736)	−45%	1548 (652)
	+6 months	−47%	2060 (657)	−49%	2954 (2064)	−57%	3004 (1794)
	+9 months	−40%	2734 (1081)	−46%	3603 (2430)	−47%	3959 (2488)
	+12 months	−42%	3233 (1401)	−1.5%	3993 (2787)	−51%	4962 (3300)
OC ng/mL	Baseline	9.3 (3.3)		10.5 (7.4)		12.1 (8.3)	
	+3 months	−23%	1185 (311)	−70%	1976 (1736)	−70%	1548 (652)
	+6 months	−33%	2060 (657)	−54%	2954 (2064)	−67%	3004 (1794)
	+9 months	−33%	2734 (1081)	−39%	3603 (2430)	−63%	3959 (2488)
	+12 months	−2%	3233 (1401	−20%	3993 (2787)	−73%	4962 (3300)
CTX pM/L	Baseline	2648 (1115)		2702 (1279)		2828 (2488)	
	+3 months	−25%	1185 (311)	−56%	1976 (1736)	−56%	1548 (652)
	+6 months	−45%	2060 (657)	−53%	2954 (2064)	−65%	3004 (1794)
	+9 months	−62%	2734 (1081)	−21%	3603 (2430)	−57%	3959 (2488)
	+12 months	−41%	3233 (1401	−10%	3993 (2787)	−71%	4962 (3300)

Modified after Boutsen et al. (2001)
GC glucocorticoids, *BSAP* bone-specific alkaline phosphatase, *OC* osteocalcin, *CTX* C-telopeptide (mean +/− SD)

The antiresorptive effect of pamidronate was much more marked than that of calcium (cf CTX). Lumbar BMD increased 1.7 (2.2)% and 2.3 (3.4)% after 12 months on single pamidronate infusion and quarterly pamidronate, respectively, whereas it decreased 4.6 (2.9)% on calcium alone. Similar changes were observed at the hip regions. There was no correlation between the changes in BMD and BTMs (Boutsen et al. 2001).

Most commercially available bisphosphonates (BPs) proved to maintain and even increase BMD in patients treated by GCs, as it is the case in the treatment of postmenopausal osteoporosis. Alendronate (ALN) and risedronate (RIS) have been the most frequently studied and became the standard care of GC-OP. Moreover, they have served as comparative agents for new molecules in trials of prevention and treatment of GC-OP. For example, zoledronic acid has been compared with risedronate in preventive and curative treatment of glucocorticoid-induced OP (Devogelaer et al. 2013). Figure 2 shows the changes in the median concentrations of bone resorption and bone formation markers in treatment and prevention in males and females already treated with GCs. A more marked decrease in the levels of serum CTX, PINP, BSAP, and uNTX was observed in patients on zoledronic acid compared with risedronate, both in males and in pre- or postmenopausal females, independently of the GC dose (Devogelaer et al. 2013).

As the main effect of GCs consists of a decrease in bone formation, and secondarily an increase in bone loss, it is consistent to prescribe teriparatide (TPTD), a recombinant human parathyroid hormone (rhPTH 1–34), a bone anabolic drug (Saag et al. 2009). The study of 3 years compared subcutaneous injections of TPTD (20 μg/day) with oral ALN (10 mg/day) in patients on daily minimum 5 mg prednisone or its equivalent for at least 3 months and put on daily calcium (1000 mg) and vitamin D (800 IU). BMD increased more in the TPTD group than in the ALN group after 36 months (11.0% versus 5.3% at the lumbar spine, 5.2% versus 2.7% at the total hip, and 6.3% versus 3.4% at the femoral neck, respectively). Incident VF was observed in 1.7% of patients on TPTD versus 7.7% on ALN, most of VF occurring in the first 18 months (Saag et al. 2009). Figure 3 shows the median percent changes from baseline of biomarkers (PINP, BSAP, OC, PICP, and CTX). The time course of changes in their concentrations is interesting to scrutinize. A significant increase in all markers was observed from the first month in the TPTD group, with highest values for OC and PICP already after 1 month and for BSAP, PINP, and CTX after 6 months. PICP became not significantly different from baseline values after 6 months, 18 months, and 36 months. CTX became not different from baseline at 18 and 36 months. In the ALN group, PICP, PINP, and CTX levels decreased significantly from 1 month, with a nadir after 6 months for PINP; after 18 months for BSAP, PICP, and CTX; and after 36 months for OC. For all biomarkers, there was a significant difference between the TPTD and ALN groups at all illustrated times (Saag et al. 2009). Gains in BMD on TPTD were pursued even when a fading in the concentrations of biomarkers was observed. It should be noted, however, that the balance between markers of bone formation and of bone resorption was all along the

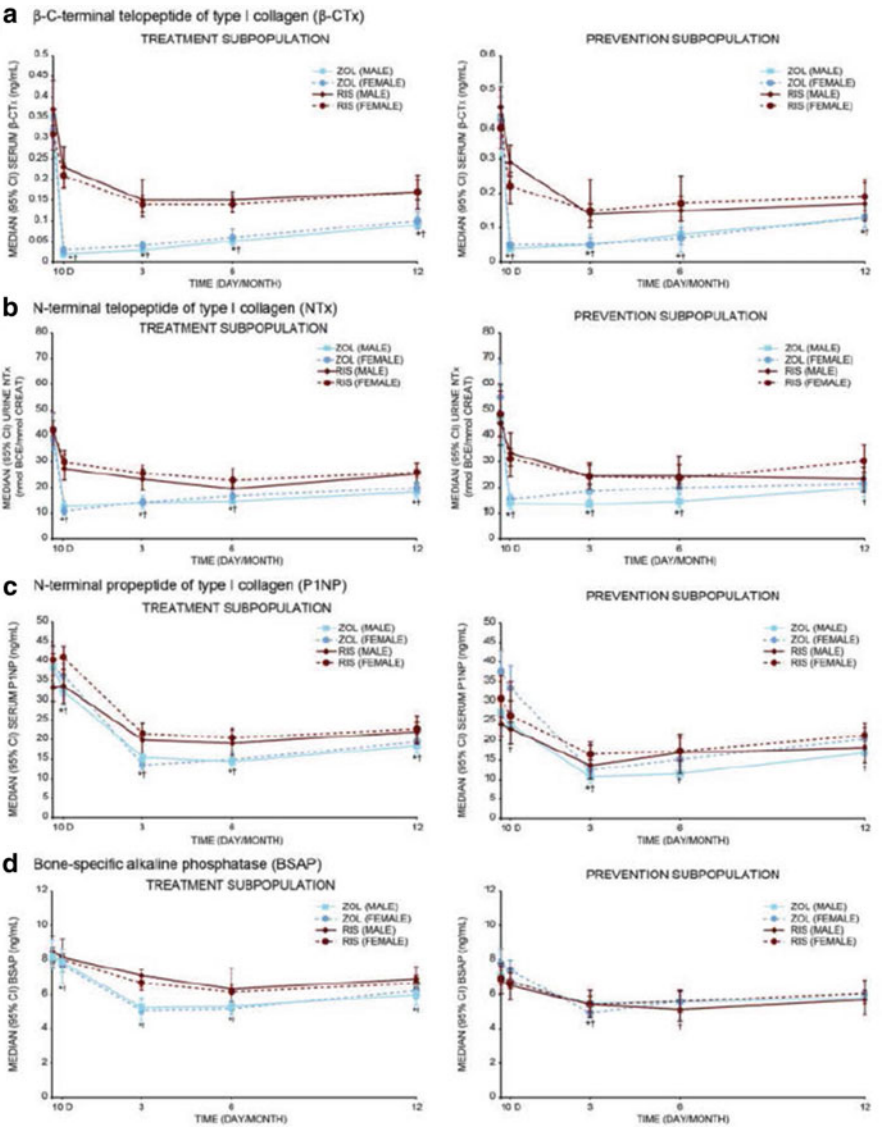

Fig. 2 Changes in the concentrations (median) of bone resorption and bone formation markers. Bone resorption markers [serum β-CTx (**A**)] and urine NTx (**B**) and bone formation markers [serum PINP (**C**) and serum BSAP (**D**)], overtime in the male and female subgroups of the treatment and prevention subpopulations. $P < 0.05$ shows statistical significance; $^{*}P < 0.05$ (male subjects), $^{\dagger}P < 0.05$ (female subjects). Error bars represent 95 % Cls. *ZOL* zoledronic acid, *RIS* risedronate (Reprint from Devogelaer JP et al (2013). Rheumatology (Oxford) 52(6), 1058-1069 with permission)

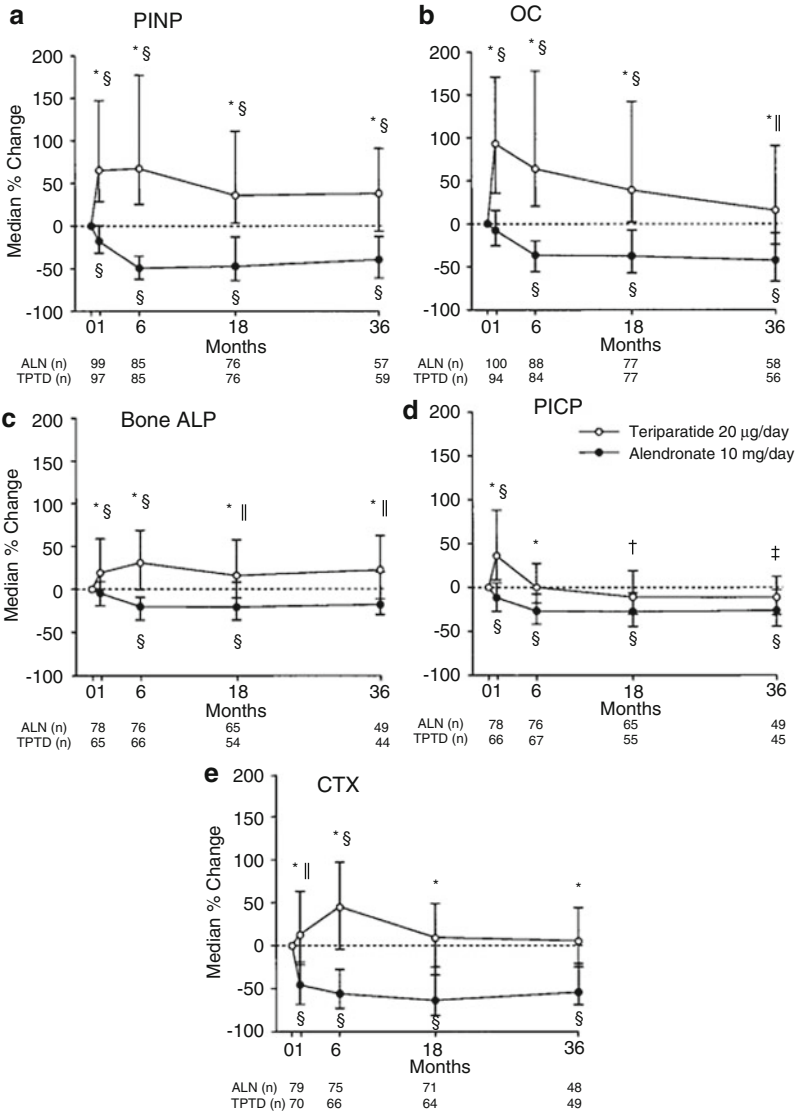

Fig. 3 Teriparatide versus alendronate in glucocorticoid-induced OP. Effect on BTMs concentrations: median changes from baseline. *PINP* N-terminal type I procollagen propeptide, *OC* osteocalcin, *bone ALP* bone alkaline phosphatase, *PICP* C-terminal type I procollagen propeptide, *CTX* C-terminal telopeptide of type I collagen (Reprint from Saag KG et al. (2009) Arthritis Rheum 60(11), 3346–3355, with permission)

study in favor of TPTD treatment, probably explaining the continuous BMD gain. On the contrary, there was some leveling off in lumbar BMD during the months 24–36 in the ALN group. It should be noted, however, that if an increase in lumbar BMD and hip BMD was observed across various baseline GC doses both in TPTD

and ALN groups, lumbar BMD increased significantly more in patients with a low-dose GC (<5 mg/day) treated with TPTD, compared with patients on medium (>5 and <15 mg/day) and high GC-dose (≥ 15 mg/day) group (Devogelaer et al. 2010). ALN therapy increased lumbar BMD the same way at any GC dose. Such a blunting of the anabolic effect of PTH (1–34) by simultaneous GC treatment had already been shown in a rat model (Oxlund et al. 2006). No weakness of bone mechanical resistance attributable to this blunting was observed, however. As the biomarkers of bone formation decrease during therapy in the ALN group, it seems unlikely that the increase in BMD should be the consequence of new bone formation (Eastell et al. 2010). The increase in BMD is probably due to an increased mineralization of bone, similarly as it is observed in the treatment of postmenopausal osteoporosis (Boivin et al. 2000). In the ALN group, contrary to the TPTD group, the increase in the femoral neck BMD after 18 months was correlated with the baseline biomarkers concentrations ($p < 0.05$). There was also a negative correlation between changes in CTX at 1 month and the increase in lumbar BMD in the ALN group (Burshell et al. 2010). In the TPTD group, the early changes in PINP were significantly correlated with later increases in BMD. PINP could be, therefore, a useful marker for monitoring the BMD response to TPTD (Burshell et al. 2010). Further prospective studies should, however, be necessary before recommending the use of biomarkers of the bone for the follow-up of patients on GC therapy.

Conclusion

Generally speaking, the biomarkers of bone remodeling are valuable scientific instruments. They have been frequently used in clinical trials. They can help to assess the compliance of the patients and could be considered in the evaluation of the response or failure to therapy. The currently available data remain, however, not sufficient to predict the increase or the reduction in fracture risk. The frequency of bone complications in GC therapy is that high that it precludes the use of such biomarkers to settle the initiation of a preventative therapy. Most of them cannot predict bone loss. When anti-osteoporosis therapy has been initiated, the changes in biomarkers are directionally similar to the changes observed in idiopathic osteoporosis, in which the utility of the use of markers has so far not yet encountered unanimity.

Potential Applications to Prognosis, Other Diseases, or Conditions

Bone complications begotten by glucocorticoid therapy are extremely frequent and may occur soon after the initiation of therapy. Bone loss is rapid particularly in the first 6–12 months of treatment. It is therefore recommended to prescribe preventative therapies (calcium, vitamin D, bisphosphonates, teriparatide) early after the GC-start, particularly if GCs are foreseen to be maintained for a long time. This is

mandatory in the daily clinical practice, without waiting for the results of bone turnover markers. They chiefly serve eventually to follow the compliance of the patients to the antiresorptive or bone anabolic agents. As the concentrations of BTMs are different in the various conditions justifying glucocorticoid treatments, it is commonsense to measure some of them before initiating a preventative therapy, if one desires to assess the compliance of the patient, in order to be able to demonstrate significant changes in their values. They cannot so far constitute surrogates of BMD measurements to evaluate the fracture risk. In pivotal studies of medicines for prevention and/or therapy of glucocorticoid-induced osteoporosis, they have been used to add rapid biological changes to slow BMD changes observed by dual energy X-ray absorptiometry. If the predictive value of OPG dosage for fractures is confirmed, this dosage could be used in the future for a rapid decision of preventive therapy, even in patients necessitating only low doses of GC.

Moreover, in the perspective of the clinical development of selective glucocorticoid receptor agonists (SEGRAs) with potentially less bone side effects, the BTMs could be used to rapidly demonstrate a lesser toxicity to the bone from these dissociated GCs, compared with predniso(lo)ne.

Summary Points

- Glucocorticoids remain nowadays the cornerstone of therapy for most inflammatory diseases.
- They are cheap and potent and possess a fast therapeutic action.
- Their use can provoke multiple complications notably bone fragility, fractures, and aseptic osteonecrosis.
- Glucocorticoids rapidly induce a decrease in bone formation evidenced by a drop in the concentration of osteocalcin and other biomarkers of bone formation such as PINP, PICP, and BSAP.
- Glucocorticoids also provoke an increase in bone resorption evidenced by an elevation in the concentration of serum and urine CTX, NTX, DPD, and a decrease in the levels of osteoprotegerin.
- The combination of the abovementioned actions on bone turnover markers begets a rapid bone loss leading to bone fragility.

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Effect of Statins on Bone Turnover Markers 41

Magdalena Fernández García and José L. Hernández

Contents

Abstract

The link between statins, the most prescribed drugs worldwide, and the bone remodeling process have been recently suggested. Thus, there is growing evidence about an anabolic and even anticatabolic effect of statins on bone metabolism.

Bone turnover markers (BTMs) reflect the remodeling status and provide a dynamic assessment of the skeleton which may complement the information obtained by bone mineral density (BMD) assessment. However, the effect of statins on BTMs has been reported in a few randomized controlled trials, with contradictory results, mainly based on its small sample size and shorter duration of treatment. Most of them concluded that serum BTMs are lower in patients on

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statins than in nonusers. Overall, decreases in BTMs have been more evident in subjects receiving the more lipophilic statins, especially when using for long periods of time. Simvastatin and atorvastatin have been the most studied statins regarding its effects on bone metabolism. In this chapter, the main data on the effect of all the marketed statins on BTMs will be reviewed.

Keywords

Statins • 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors • Bone remodeling • Bone turnover markers • Alkaline phosphatase • Procollagen type I N-terminal peptide • C-terminal type I collagen telopeptide • Osteocalcin • Bone morphogenetic protein

List of Abbreviations

ALP	Alkaline phosphatase
AOBs	Primary alveolar osteoblasts
AUC	Area under the curve
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMP-2	Bone morphogenetic protein-2
BP	Bisphosphonates
BSAP	Bone-specific alkaline phosphatase
BTMs	Bone turnover markers
CTX	Carboxy-terminal cross-linked telopeptide of type 1 collagen
FPP	Farnesyl pyrophosphate
GGPP	Geranylgeranyl pyrophosphate
HMG-CoA reductase	3-Hydroxy-3-methylglutaryl coenzyme A reductase
IOF	The International Osteoporosis Foundation
LDL	Low-density lipoprotein
mRNA	Messenger ribonucleic acid
NA	Not available
No	Number
NTX	Amino-terminal cross-linked telopeptide of type 1 collagen
OC	Osteocalcin
OPG	Osteoprotegerin
OVX	Ovariectomy
PINP	N-Terminal propeptide of type I procollagen
PDLs	Periodontal ligament cells
RANKL	Receptor activator of nuclear factor-kappa B ligand
T½	Terminal half life
t-AP	Total alkaline phosphatase
TGF-β	Transforming growth factor-beta

TRAP	Tartrate-resistant acid phosphatase
ucOC	Undercarboxylated osteocalcin
VLDL	Very low-density lipoprotein
Vol.	Volume

Key Facts of Bone Turnover Markers

- Bone turnover markers (BTMs) represent a dynamic way for assessing the bone remodeling process.
- They are classified as markers of bone formation or bone resorption.
- The clinical utility of BTMs in everyday's clinical practice is still limited.

Key Facts of Statins

- Statins are the most prescribed drugs worldwide. Its overall benefit appears to exceed their lipid-lowering effects (pleiotropic effects).
- Statins act in the same metabolic pathway as bisphosphonates (BP) do (mevalonate).
- The effect of statins on BTMs seems to be dual: enhancing formation and inhibiting resorption.

Definitions of Words and Terms

Alkaline phosphatase	Group of isoenzymes that catalyze the hydrolysis of organic phosphate esters in an alkaline pH.
Biochemical markers of bone metabolism	Proteins and enzymes released from bone cells during the process of bone remodeling.
Bone remodeling	Process where mature bone tissue is removed from the skeleton (bone resorption) and new bone tissue is formed (bone formation).
Carboxy-terminal cross-linked telopeptide of type 1 collagen	Carboxy-terminal product of degradation of type I collagen released from the activity of osteoclasts.
N-Terminal cross-linked telopeptide of type 1 collagen	Amino-terminal product of degradation of type I collagen released from the activity of osteoclasts.

N-Terminal propeptide of type I procollagen	Amino-terminal section of the non-helical portions of tropocollagen.
Osteocalcin	Noncollagenous small protein with calcium-binding properties, synthesized by osteoblasts.
Statins	Class of drugs used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase.

Introduction

Bone, as a metabolically active tissue, has the skill for self-regeneration. This continuous renewal process is called "bone remodeling." Osteoclasts, osteoblasts, and osteocytes are the responsible cells for this process. Usually bone formation and resorption occur closely coupled, in a coordinated manner, regulated by different cytokines, growth factors, and systemic hormones that orchestrate all the steps.

Bone Turnover Markers

Biochemical markers of bone metabolism, also known as bone turnover markers (BTMs), are proteins and enzymes released from bone cells, mainly osteoclasts and osteoblasts, during the process of bone remodeling. Thus, it is possible to distinguish between markers of bone formation and markers of bone resorption. BTMs reflect whole body rates of bone resorption and formation providing a dynamic assessment of the skeleton as a whole, which may complement the static information given by bone mineral density (BMD) measurement. In this sense, measurement of the rate of bone turnover represents a useful tool to assess patients with metabolic bone disorders (Hernández et al. 2013).

Considerations on BTMs in Clinical Practice

BTMs are not usually ordered in everyday's practice, due to they are relatively expensive and not widely available. Besides, its values may be affected by many variables such as age, gender, ethnicity, history of a previous fracture, certain diseases (thyroid and parathyroid diseases, renal impairment, Paget's disease, Cushing's disease, etc.) or drugs affecting bone metabolism (antiosteoporotic agents, antiepileptic drugs, vitamin K antagonists, corticosteroids, etc.), assay variability (equipment, poor standardization of techniques, etc.), type of BTM used, collection and storage of the samples, vitamin D deficiency, circadian variations, physical exercise, smoking, menstrual variations, use of oral contraceptives, or food intake (Puri and Meeta 2013). Table 1 summarizes the main BTMs used in clinical studies.

As noted above, the utility of BTMs in clinical settings may seem controversial. Notwithstanding, many studies have analyzed the relationship between BTM levels and the prediction of bone loss rate. Moreover, several studies have found that increases in the rates of bone resorption are associated with an increased risk of

Table 1 Characteristics of the main bone turnover markers mentioned in this chapter

Marker	Specimen	Origin	Assay	Circadian rhythm	Comments
BSAP	Serum	Bone	RIA*/ Ab	No	*Cross-reactivity with liver isoform
OC	Serum	Bone, platelets	RIA	Yes	Rapidly degraded in serum, requires collection on ice
PINP	Serum	Bone, soft tissue, skin	ELISA/ RIA	Yes	Nonskeletal tissues may contribute (a little) in levels
CTX	Serum Urine	All tissues	RIA/ ELISA/ Ab	Yes	The most commonly used. An isomerized bond between the aspartate and the glycine is specific from collagen in bone Values should be corrected by measuring the marker in the morning
NTX	Serum Urine	All tissues	RIA/ ELISA/ Ab	Yes	A collection bottle that contains an acidic preservative results in substantial artifactual elevations of apparent NTX concentrations Values should be corrected by measuring the marker in the morning

vertebral and non-vertebral fractures. Finally, BTMs may be particularly useful to monitoring the response to antiosteoporotic drugs and could play a role to identify nonresponder patients, including those not adherent to osteoporosis treatment (Hernández et al. 2013) and could also be useful to detect oversuppression of bone turnover in patients on long-term therapy.

Bone Formation Markers

Alkaline Phosphatase

Serum total alkaline phosphatase (t-AP) activity is determined by an extent group of isoenzymes that catalyze the hydrolysis of organic phosphate esters in an alkaline pH. It came from different tissues such as liver, intestine, bone, spleen, kidney, and placenta. Bone-specific alkaline phosphatase (BSAP) is anchored to the cell membranes of osteoblasts. Its exact function is unclear, but it reflects osteoblastic activity. Physiological elevations of t-AP are seen in children and adolescents, due to an increase in osteoblastic activity associated with growing and also in pregnancy. Immunoassays with monoclonal antibodies against specific AP bone fraction improve its specificity (Farley et al. 1981).

Osteocalcin

Osteocalcin (OC) or bone Gla-protein is a noncollagenous small protein with calcium-binding properties, characterized by containing three g-carboxyglutamic

acid residues (Rehder et al. 2015). It is synthesized by osteoblasts and participates in the osteoid mineralization, regulating calcium homeostasis and inhibiting calcium phosphate precipitation, in order to avoid excessive mineralization of the bone matrix. Serum osteocalcin levels are considered a sensitive and specific marker of osteoblast activity. Its values follow a circadian rhythm, with a peak in the morning, and are not influenced by food intake. Its synthesis is vitamin K dependent and regulated by vitamin D.

N-Terminal Propeptide of Type I Procollagen (PINP)

Osteoblasts synthesize type I collagen, which constitutes about 90% of the organic content of the bone. The basic unit of collagen is tropocollagen. In this process, series of three amino acids are assembled in tandem to form polypeptide chains, called α-chains, connected by intramolecular hydrogen bonds. Three of these alpha-chains are assembled to form procollagen which is secreted into the extracellular space, thus becoming tropocollagen. The non-helical portions of tropocollagen at the amino and carboxy terminal sites are known as N-telopeptide and C-telopeptide regions, respectively. Serum concentration of PINP reflects changes in synthesis of new collagen. PINP is a more sensitive marker of bone formation rate than other available markers, and it is particularly useful for monitoring antiosteoporotic therapies (Vasikaran et al. 2011).

Bone Resorption Markers

Most of the numerous markers of bone resorption are the result of the degradation of collagen type I by the osteoclasts. Crosslaps are degradation products of type I collagen released from the activity of osteoclasts and are markers of bone collagen loss. Carboxy-terminal cross-linked telopeptide of type 1 collagen (CTX) and amino-terminal cross-linked telopeptide of type 1 collagen (NTX) are synthetized in a stoichiometric ratio (1:1), and their serum concentrations correlate with the intensity of degradation of type 1 collagen. CTX is the most widely used in clinical and experimental studies. Its values decrease rapidly and markedly in response to antiresorptive therapies, particularly bisphosphonates and denosumab (Vasikaran et al. 2011) being the most accurate BTM when monitoring the effect of these agents on bone metabolism.

Statins

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also called statins, are potent inhibitors of cholesterol biosynthesis widely used for the treatment of hypercholesterolemia as first-line therapy for reducing the cardiovascular risk. They represent the most commonly prescribed drugs worldwide. The overall benefit of these agents appears to exceed their defining lipid-lowering effects, resulting in the so-called pleiotropic effects, including anti-inflammatory and

immunomodulator effects, antioxidant activity, neuroprotection, antithrombotic properties, reduction of the incidence of some types of cancer (Zhou and Liao 2010), and beneficial effects on bone metabolism (Hernández et al. 2014).

Statins are a class of drugs that act as competitive inhibitors of HMG-CoA reductase inhibiting the rate-limiting step of cholesterol biosynthesis, the conversion of HMG-CoA to L-mevalonic acid. At the same time, statins also decrease the isoprenylation of many intracellular signaling molecules and lead to an upregulation in the expression of LDL receptors, increasing the clearance of plasma LDL-cholesterol and its catabolism. Moreover, statins reduce moderately the hepatic production of VLDL-cholesterol, which contribute to reduce LDL-cholesterol levels due to the lack of precursor lipoproteins.

In addition to the cholesterol-lowering effect, the pleiotropic effects of statins are mainly related with their capacity to modulate the pathophysiology of the endothelium and their immunomodulatory properties arising from their effects both on T lymphocytes and on antigen-presenting cells (Kwak et al. 2000; Weitz-Schmidt et al. 2001). The mechanism underlying some of these pleiotropic effects is the inhibition of isoprenoid synthesis which leads to the inhibition of intracellular signaling molecules such as Rho, Rac, and Cdc42 (Zhou and Liao 2010).

A Common Metabolic Pathway for Aminobisphosphonates and Statins

Bisphosphonates are synthetic analogs of inorganic pyrophosphate. The nitrogen-containing BP interfere with the mevalonate pathway, key precursor of the cholesterol synthesis, by inhibiting farnesyl pyrophosphate synthase, enzyme that generates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). FPP and GGPP are essential for the posttranslational isoprenylation and activation of small GTPases (Rho GTPases), involved in the coordinated movement of subcellular protein, cell survival, and integrity of the cytoskeleton. Thus, inhibition of FPP synthase impairs protein prenylation which will ultimately result in the inhibition of protein prenylation because of the depletion of farnesyl diphosphate or geranyl diphosphate (Luckman et al. 1998).

Noteworthy, a link between statins and bisphosphonates is plausible, since both agents act on the same metabolic pathway. Statins, by blocking the mevalonate pathway, may interact with bone metabolism. They are competitive inhibitors of HMG-CoA reductase and thus can reduce the endogenous synthesis of cholesterol. The inhibition of mevalonate blocks the formation of GGPP. As we have commented on above, this inhibition in the synthesis of cholesterol also leads to a reduced formation of intermediates which play a role in posttranslational modification of proteins and cell signaling pathways, participating in osteogenesis, suppression of osteoblasts apoptosis, and inhibition of osteoclastogenesis (Zhang et al. 2014).

Statins and BTMs

A role for statins in bone metabolism was first proposed by Mundy et al. (1999) when they proved, in vitro and in rodents, that lovastatin and simvastatin promoted new bone formation through upregulation of bone morphogenetic protein-2 (BMP-2) expression. The mechanisms by which these drugs have been proposed to determine this increase in bone formation differ in nature. On the one hand, they could stimulate bone formation through an increase in expression and synthesis of bone morphogenetic protein-2 (BMP-2) and OC (Mundy et al. 1999; Ruiz-Gaspa et al. 2007). On the other hand, they could carry out an antiresorptive effect by interfering with the fusion process through which osteoclast precursors become multinucleated cells (Woo et al. 2000).

BMPs, Osteoblastogenesis, and Osteoclastogenesis

The role of BMPs in skeletal homeostasis is critical as suggested by its extensive conservation among species, illustrated by the characteristic seven-cysteine knot domains (Kawabata and Miyazono 2000). They are a group of over 20 proteins synthesized by skeletal cells, although their synthesis is not limited to bone. They belong to the transforming growth factor-beta (TGF-β) superfamily of polypeptides, which include TGF-βs, activins, and inhibins (Lee et al. 2000; Ozkaynak et al. 1992; Wang et al. 1990; Wozney et al. 1988). Bone morphogenetic proteins (BMPs) are brisk regulators of osteoblast differentiation. Several BMPs promote osteoprogenitors to differentiate into mature osteoblasts. This requires interactions of the BMP and decapentaplegic (Smad) 1/5 and Runx-2/Cbfa-1 (Leboy et al. 2001). Once osteoblasts undergo terminal differentiation and the cellular matrix mineralizes, they suffer apoptosis (Pereira et al. 2001). This programmed cellular death is an expected result of cell maturation, and the blocking of BMP actions not only arrests osteoblast differentiation but also prevents apoptosis. In cultures of human osteoblasts, BMP-2 induces apoptosis by protein kinase C-dependent and Smad 1-independent mechanisms (Hay et al. 2001). The direct effects of BMPs on osteoclastogenesis also include sensitization of osteoclasts to the effects of receptor activator of nuclear factor-kappa B ligand (RANKL) (Gao et al. 1998; Itoh et al. 2001; Kaneko et al. 2000).

BMPs also induce osteoprotegerin (OPG) gene transcription and, so, may modulate their effects on osteoclastogenesis (Wan et al. 2001). In fact, BMPs stimulate osteoprotegerin (OPG) transcription through two Hoxc-8 binding sites. The BMP signaling Smad 1 interacts with Hoxc-8 and dislodges Hoxc-8 from its binding element, enhancing gene expression (Wan et al. 2001). According to this positive effect on OPG, BMPs inhibit collagenase type 3 expression in osteoblasts, a matrix metalloprotease that cleaves type I and type II collagen fibrils and is also required for normal bone resorption (Varghese and Canalis 1997; Zhao et al. 1999).

Statins have been identified as enhancers of the BMP-2 gene expression and bone formation in vivo. As we discuss in this chapter, this hypothesis is supported by experimental data, so lipophilic statins increase BMP-2 mRNA and synthesis of this protein by osteoblasts (Mundy et al. 1999). On the contrary, hydrophilic statins, such as pravastatin, seem to have no effect on BMP-2 (Chuengsamarn et al. 2010). This is a worthy point, since several investigators have suggested that lipophilic statins could add further benefit to bone metabolism (Chuengsamarn et al. 2010; Stein et al. 2001).

Effects of Statins on BTMs: Clinical Evidence

The potential relationship between statins and bone metabolism has been usually focused on BMD (Hernández et al. 2014). It has been suggested that BTMs are good predictors of subsequent changes of BMD (Bauer 2003) or in the risk of fractures. Nevertheless, observational studies and randomized clinical trials devoted on BTMs, in patients on statins, are scarce and often have shown conflicting and even contradictory results (Bauer et al. 2004; Hatzigeorgiou and Jackson 2005; Uzzan et al. 2007; Yue et al. 2010). This may be the result, at least in part, from the heterogeneity of the study designs as well as the different confounders applied. Besides, available studies are difficult to compare due to several reasons, such as the type of statin, duration of use, dosage, BTM considered, age and gender of the included patients, and the small size of control groups.

Due to the hepatic metabolism (Table 2), it is important to consider that with oral administration at the dosages currently prescribed in clinical practice, less of 5% of a given dose reaches the systemic circulation and is available to exert its effects on bone tissue (Hamelin and Turgeon 1998). Furthermore, as we have commented on above concerning limitations in determining BTMs, the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) have recommended that a marker of bone formation (serum PINP) and a marker of bone resorption (serum CTX) are used as reference analytes for BTMs in clinical studies (Vasikaran et al. 2011).

For the sake of completeness, in this chapter, we will analyze the main published studies on the effect of statins on BTMs. Only data concerning to BTMs are presented. Firstly, we will comment on the main experimental studies, and secondly, we will summarize the clinical ones.

Experimental Studies Analyzing the Effect of Statins on BTMs

Several experimental studies have pointed out a role for statins in bone metabolism. Table 2 summarizes the main findings on this topic.

As we have previously commented on, Mundy (1999) found that statins, in appropriate doses, enhanced new bone formation in vitro and in rodents, effect associated with increased expression of BMP-2 gene in bone cells. They added lovastatin, simvastatin, fluvastatin, and mevastatin in cultures to Swiss white mice

Table 2 Pharmacokinetics of statins

	Simvastatin	Fluvastatin	Atorvastatin	Rosuvastatin	Pravastatin	Pitavastatin	Lovastatin
Absorption, %	60–85	98	NA[a]	NA	35	NA	30
Effect of food intake on absorption[b]	=	↓15–25	↓ 13	↓ 20	↓ 30	↓ 43	↑ 50
Bioavailability	5	20–30	12	20	18	60–80	5
Protein binding, %	>95	>98	>98	88	50	>99	>98
Lipophilicity	Yes	Yes	Yes	No	No	No	Yes
Passage of the blood–brain barrier[c]	Yes	No	NA	NA	No	NA	Yes
Cytochrome P450 metabolism	CYP3A4	CYP2C9	CYP3A4	Marginal	CYP3A4	Marginal	CYP3A4
Hepatic extraction[d], %	≥80	≥70	>70	63	40–60	80	≥70
Urinary excretion[d], %	13–60	6	<2	10	60	15	30
$t_{(1/2)}h$[e]	2–3	1,2	14	19	3	10–13	3

a) NA: Not Available; b) % Area under curve (AUC); c) BBB: Blood-Brain Barrier; d) % of absorbed dose; e) t: terminal half-life.

calvarial bones and found an increase in new bone formation by approximately two- to threefold. Then, they injected lovastatin and simvastatin into the subcutaneous tissue overlying the murine calvarian in vivo and observed an almost 50% increase in new bone formation after 5 days of treatment. Furthermore, to evaluate the systemic action of statins in vivo, they tested their effect on trabecular bone volume after oral administration. Statins lead to a 39–94% increase in trabecular bone volume and also induce a concomitant decrease in the number of osteoclasts.

Du et al. (2013) investigated the effect of simvastatin in 3-month-old female Sprague–Dawley rats, randomly divided into three groups: sham-operated group (SHAM; $n = 18$), ovariectomized group (OVX; $n = 18$), and ovariectomized treated with simvastatin group (OVX + SIM; $n = 18$). Fifty-six days after ovariectomy, screw-shaped titanium implants were inserted into the tibiae. Simvastatin (5 mg/kg each day) was orally administered after the placement of the implant in the OVX + SIM group. The animals were sacrificed at either 28 or 84 days after implantation. Serum BSAP, t-AP, and OC were measured and correlated with the histological findings. No differences were detected among the three groups, concerning serum t-AP levels. In the OVX + SIM group, serum BALP concentrations were significantly higher than both OVX and SHAM groups at day 28. In the case of OC, there were no significant differences between the three groups at day 28, but at day 84, the OVX + SIM group showed significantly higher levels than the other groups. BTMs were associated positively with the histological assessment in the OVX + SIM group, suggesting a favorable effect on osseointegration probably mediated by simvastatin. Similar results were found by Ayukawa et al. (2010) in 30-week-old female rats that received pure titanium implants in both tibiae and treated with different doses of intraperitoneal simvastatin. The more pronounced effects on bone contact ratio and bone density were observed with the higher doses of statin. No significant differences in the percentage of cortical bone were found, but simvastatin increased bone formation markers and decreased bone resorption markers. Atorvastatin was also found to have the same effects in orchidectomized rats after 12 weeks of treatment (12 mg/kg/day). Atorvastatin increased IGF-1 concentrations and reduced CTX, BMP-2, and OPG compared to sham-operated animals (Gradosova et al. 2012). The effect of lovastatin on bone turnover has been also studied in vitro and in vivo (Jadhav et al. 2006). Intravenously administered lovastatin was more effective than oral in reducing the ovariectomy-induced increase in BTMs, especially urinary cross-links.

In the same line of the abovementioned studies, Liu et al. (2012) analyzed the effect of different concentrations of simvastatin on the primary alveolar osteoblast (AOB) and periodontal ligament cell (PDL) proliferation/viability. They also measured the t-AP activity and the mRNA expression levels of OC, RANKL, and OPG. The authors concluded that simvastatin seems to slightly increase the expression of osteogenic markers in AOBs and PDLs, indicating its ability to influence alveolar bone formation and periodontal regeneration.

Finally, the effects of statins in experimental models of arthritis were investigated by Funk et al. (2008). Hydrolyzed simvastatin (20 mg/kg/day) was administered subcutaneously to female Lewis rats 4 days before or 8 days after induction of

arthritis by intraperitoneal injection of streptococcal cell wall or vehicle. Regarding BTMs, simvastatin suppressed serum pyridinoline levels in arthritic animals, while it had no effect in normal animals. Besides, serum OC levels, which were decreased in arthritic animals, were unaltered by statin treatment. Taking into account these data, the authors suggest that statins may be useful in arthritic joints, preserving periarticular bone by reducing bone resorption.

Clinical Studies Analyzing the Effect of Statins on BTMs

Simvastatin

Simvastatin has been, along with atorvastatin, the most studied statin regarding its effects on bone metabolism (Table 3). Chuengsamarn et al. (2010) conducted a

Table 3 Results of the main experimental studies analyzing the effect of statins on BTMs

Author/year	Statin	Type of study	Result
Mundy et al. (1999)	Lovastatin, simvastatin, fluvastatin, mevastatin	Cultures to Swiss white mice calvarial bones	Increase in new bone formation by approximately two- to threefold
Du et al. (2013)	Simvastatin	Evaluate the relationship between serum level of bone formation markers and the osseointegration of pure titanium implants in osteoporotic rats treated with simvastatin	Total ALP, BSAP, and OC in the OVX + SIM group was significantly higher than both OVX and SHAM groups
Ayukawa et al. (2010)	Simvastatin	Enhancement of bone formation around titanium implants in rat tibiae	Increase in OC and decrease in TRAP
Gradosova et al. (2012)	Atorvastatin	Bone protective effect in orchidectomized rats after 12 weeks of treatment	Reduced CTX, BMP-2, and OPG
Jadhav et al. (2006)	Lovastatin	Distribution of lovastatin and its active metabolites to bone, with respect to plasma and liver compartments after oral and intravenous administration in female rats	Oral and intravenous lovastatin treatment significantly reduced serum t-AP and urinary cross-links in OVX rats
Liu et al. (2012)	Simvastatin	Effects on primary alveolar osteoblasts and periodontal ligament cells in vitro	Increase in OC mRNA, RANKL expression, and OPG expression
Funk et al. (2008)	Simvastatin	Simvastatin (20 mg/kg/day) was administered subcutaneously to female Lewis rats 4 days before or 8 days after induction of arthritis by intraperitoneal injection of streptococcal cell wall or vehicle	No effect in normal animals. OC levels, which were decreased in arthritic animals, were unaltered by statin treatment

prospective randomized controlled trial enrolling 212 patients with hyperlipidemia and osteopenia. All of them were randomized to simvastatin at an initial dosage of 40 mg/day or 80 mg (if the initial dosage failed to meet accepted target lipid levels), and non-statin group, receiving either gemfibrozil ($n = 69$) or fibrates ($n = 36$) and followed-up for 18 months. Serum PINP increased significantly in the simvastatin group ($p < 0.01$). Besides, serum CTX was also reduced with statin therapy ($p = 0.02$). In summary, this study shown that an 18-month treatment with 40–80 mg of simvastatin daily significantly increased bone formation and reduced bone resorption.

Montagnani et al. (2003) published a 1-year prospective randomized controlled trial in 30 postmenopausal hypercholesterolemic women and 30 healthy age-matched postmenopausal women. Both groups received simvastatin (40 mg/day) during 12 months. In controls, BTMs of bone formation and of bone resorption did not show any significant change over the whole study. Nevertheless, t-ALP and BALP showed a moderate increase in the simvastatin group, as early as 3 months. Thus, t-ALP significantly increased by 2.2%, 8.8%, 15.8%, and 16.1% ($p < 0.05$) at 3, 6, 9, and 12 months, respectively. Regarding BALP, at 6 months, its value was significantly higher than baseline (+9.3%; $p < 0.05$) and continued to increase by 10.1% and 10.4% ($p < 0.05$), at 9 and 12 months, respectively. In the statin group, serum CTX levels showed a moderate, albeit nonsignificant, positive trend. These results seem to be, at least in part, in contrast with the data reported by Mundy et al., which demonstrated in vitro an influence of statins, also on osteoclastic activity. However, the results in this sense may have been limited by the small size of the sample and the short follow-up period of the study.

Tikiz et al. (2005) conducted a 1-year prospective study in 32 postmenopausal osteopenic women receiving simvastatin (20 mg/day) and not on osteoporosis treatment. At 3 months, serum levels of BSAP and OC were found to be significantly increased, from 120.8 ± 56.6 to 149.5 ± 57.6 IU/l ($p = 0.008$) and from 20.8 ± 12.6 to 34.7 ± 18.4 microg/l ($p = 0.015$), respectively. No significant change was observed concerning serum CTX. At 6 and 12 months, serum BSAP and OC levels decreased below the pretreatment values. While a significant reduction was found in BSAP levels (from 120.8 ± 56.6 to 55.9 ± 18.8 IU/l; $p < 0.001$), no significant change was observed in CTX levels after the 6-month treatment period. Therefore, simvastatin exhibit a short-lasting anabolic effect on bone metabolism, effect that was lost in the long run. The decrease in both BTMs at 6 and 12 months suggests that simvastatin affects bone metabolism mostly in favor of inhibition of the bone turnover in a long-term observation period although this inhibitory effect was not reflected in terms of BMD.

The same author (Tikiz et al. 2004) had previously found in a 3-month prospective study aimed to evaluate the effect of simvastatin (20 mg/day) on bone metabolism and its correlations with changes in BTMs. For this purpose, 38 postmenopausal women with hypercholesterolemia, not on osteoporosis treatment, were studied. Pre- and posttreatment samples were analyzed for serum BSAP and OC levels, as markers of bone formation, and serum CTX, as a marker of bone resorption. Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha)

cytokine levels were also measured. While no significant changes were observed in serum CTX levels, BSAP and OC levels were significantly increased from 120.8 ± 56.6 to 149.5 ± 57.6 IU/l ($p = 0.008$) and from 20.8 ± 12.6 to 34.7 ± 18.4 mcg/l ($p = 0.015$), respectively. No significant change was observed regarding IL-6 levels, but serum TNF-alpha levels significantly decreased after simvastatin treatment (from 77.9 ± 31.6 pg/ml to 23.5 ± 12.6 pg/ml; $p = 0.021$). Changes in serum TNF-alpha concentrations showed a moderate negative correlation with changes in BSAP and OC levels ($r = -0.550$; $p = 0.001$, and $r = -0.497$; $p = 0.004$, respectively). In conclusion, simvastatin at a dose of 20 mg daily for 3 months significantly increased BSAP and OC levels in hypercholesterolemic postmenopausal women without affecting bone resorption. One of the most important findings of this study was the observation of a moderately significant negative correlation between changes in TNF-α and changes in OC and BSAP concentrations after simvastatin therapy. This observation suggests that the cytokine-lowering effect of simvastatin, in addition to its lipid-lowering effect, may exert some additional benefits on bone metabolism.

Chan et al. (2001) conducted a small prospective study using BTMs, in 17 hypercholesterolemic non-osteoporotic subjects treated with simvastatin (20 mg/day) for 4 weeks. They found that serum OC levels significantly increased ($p < 0.05$) at 4 weeks, whereas serum BSAP, urine deoxypyridinoline, and urine CTX levels did not show any significant change. Nevertheless, Hsia et al. (2002) carried out a placebo-controlled dose-ranging trial in 24 women whom were randomized to receive simvastatin 20 mg/day, 40 mg/day, or placebo for 12 weeks. Age, blood pressure, heart rate, body mass index, creatinine, and T-score were similar among groups. At baseline, levels of bone ALP, NTX, and CTX were similar in the three treatment groups. At 6 and 12 weeks, serum BTM levels remained similar in the three groups. These results confirm and extend those of Chan et al. who found, as we have stated above, no effect of simvastatin 20 mg on serum BSAP or urine NTX levels. The effect of higher versus lower doses of statins on bone metabolism was analyzed by Stein et al. (2001). They conducted a 12-week, randomized, multicenter, open-label study to compare the safety and lipid-lowering efficacy of simvastatin, 40 mg/day or 80 mg/day, with that of atorvastatin, 20 mg/day or 40 mg/day, in 846 hypercholesterolemic patients. Besides, they assessed the effect of both statins on serum BSAP and CTX levels. Treatment with simvastatin 40 and 80 mg/day, but not atorvastatin 20 or 40 mg/day, led to significant ($p < 0.05$) reductions in BSAP in both men (−4.1% to 5.4%) and women (−4.2% to 7.4%). Furthermore, a dose-dependent effect, with greater reductions in BSAP with simvastatin (80 mg) was observed. Treatment with either atorvastatin 20 mg or 40 mg had no significant effect on BSAP levels in the whole group or in the gender-specific subgroups. Serum CTX showed a small, albeit not statistically significant decrease with simvastatin, again with an apparent dose-related trend.

Rosenson et al. (2005) investigate whether statin therapy acutely altered bone turnover as measured by changes in BSAP, OC, and NTX in 55 (M/F: 39/16) healthy nonsmoking adults. Subjects were randomized to four 8-week treatment regimens: placebo ($n = 14$), pravastatin 40 mg/day ($n = 12$), simvastatin 20 mg/day ($n = 14$),

and simvastatin 80 mg/day ($n = 15$). High-dose simvastatin (80 mg/daily) was related to a significant reduction in BSAP as compared with other therapies ($p = 0.009$). However, there were no changes in urinary N-telopeptide cross-links, a sensitive marker of bone resorption. Short-term use of high-dose simvastatin reduced serum BSAP levels, which suggests the possibility of reduced bone turnover. Although high-dose simvastatin (80 mg/day) also lowered serum OC by 9% as compared with simvastatin 20 mg/day, this effect was not significant. Among the high-dose simvastatin-treated subjects, changes in serum BSAP were positively associated with changes in OC levels ($r = 0.47$; $p < 0.00001$). Noteworthy, in this study, 5 of 16 postmenopausal women also received hormone replacement therapy.

Studies analyzing the long-term effect of statins on bone remodeling are scarce. Rejnmark et al. (2002) in a cross-sectional study studied BTMs in 140 postmenopausal women who had been treated with simvastatin for more than 2 years (median 4 years) and in 140 age- and gender-matched controls. Serum BTM levels were lower in subjects on statins than in controls: OC (−9%; $p = 0.03$), BSAP (−14%; $p < 0.01$), and CTX (−11%; $p < 0.01$). No association could be demonstrated between changes in BTMs and dose or duration of statin treatment.

Atorvastatin

There are several clinical studies devoted on the effect of atorvastatin on bone metabolism, most of them with conflicting results (Table 4). Thus, Chen et al. (2014) conducted a 1-year prospective randomized controlled study including 64 elderly men with osteopenia and mild dyslipidemia, receiving therapeutic doses of atorvastatin. All the parameters were recorded at baseline and at 6 and 12 months. Serum CTX levels were significantly reduced in the group on atorvastatin at 12 months, compared with baseline ($p < 0.001$). Therefore, in this study, therapeutic doses of atorvastatin were associated with positive effects on lumbar BMD, probably mediated by suppressed bone resorption. In the same line, Majima et al. (2007a) performed a prospective study including 22 Japanese male patients with untreated hypercholesterolemia. After a 3-month period on atorvastatin 10 mg/day, serum BSAP did not significantly change, but NTX decreased by nearly 20% ($p = 0.020$). These findings are in agreement with those published by Chen et al., suggesting that atorvastatin may have potential beneficial effects on bone metabolism in patients with hypercholesterolemia mostly by reducing bone resorption rather than by stimulating bone formation.

Berthold et al. (2004) suggest that short-term treatment with atorvastatin may have age-dependent effects on BTMs in postmenopausal women. They conducted an 8-week, multicenter, prospective-randomized, double-blind, placebo-controlled trial in three hospital-based outpatient metabolism clinics. Forty-nine postmenopausal women were treated with atorvastatin, 20 mg/day ($n = 24$) or matching placebo ($n = 25$). At the end of the study, serum CTX, as well as intact OC levels, were not significantly different for baseline in either group. Serum OC levels did not change in the statin group but increased by 9% ($p < 0.03$) in the placebo group. The CTX/OC ratio remained unchanged, and the same effect was observed in the case of undercarboxylated OC (ucOC). Serum BSAP remained unchanged in the statin group but

Table 4 Summary of studies analyzing the effect of simvastatin on BTMs in humans

Author (year)	Statin(s)	Sex (M/F)	Age (mean years)	No	Type of study	Result
Chuengsamarn et al. (2010)	Simvastatin	32/74	63	106	Prospective randomized controlled trial study	PINP statistically significant increase ($p < 0.01$) CTX-1 significantly reduce ($p = 0.017$)
Montagnani et al. (2003)	Simvastatin	F, postmenopausal	61	30 cases 30 controls	1-year prospective randomized controlled trial study	Total ALP and BASP showed a moderate increase after 3 months. BSAP increases in cases ($p < 0.05$) at sixth month. CTX showed a weak and nonsignificant increase In controls, BTMs did not show any significant change
Tikiz et al. (2005)	Simvastatin	F, postmenopausal with osteopenia	57	32	1-year prospective uncontrolled study	Significant reduction in BSAP levels ($p < 0.001$) No significant change in CTX-1 levels after the 6-month
Tikiz et al. (2004)	Simvastatin	F, postmenopausal	57	38	3-month prospective study	BSAP and OC levels significantly increased No significant change was observed in serum CTX

Chan et al. (2001)	Simvastatin	6/11	40–79 (range)	17	4-week prospective study	OC concentration increased significantly ($p < 0.05$) No significant effect in BSAP, urine deoxypyridinoline, and CTX
Hsia et al. (2002)	Simvastatin	F, osteopenia	56	24	Randomized controlled trial	No changes in BSAP or CTX
Stein et al. (2001)	Simvastatin/ atorvastatin	See text	See text	846	Randomized controlled trial	Simvastatin 40 and 80 mg/day led to significant ($p < 0.05$) reductions in BSAP in both men and women CTX showed a small, but not statistically significant, decrease with simvastatin
Rosenson et al. (2005)	Simvastatin/ pravastatin	39/16	50	55	Prospective, double-blind, placebo-controlled trial	Significative reduction ($p = 0.009$) in BASP (simvastatin 80 mg/daily) No changes in urinary NTX
Rejnmark et al. (2002)	Simvastatin	F, postmenopausal	62	140 cases 140 control	Cross-sectional	OC (−9%, $p = 0.03$), BSAP (−14%, $p < 0.01$), and CTX (−11%; $p < 0.01$)

increased by 7.3% in the placebo group, a small but statistically significant change. When analyzed the effect of age, atorvastatin increased serum CTX and OC concentrations in younger subjects, while it decreased them in older ones. Noteworthy, in elderly participants, atorvastatin led to a significant decrease in the CTX/OC ratio, an indicator of bone remodeling, while this ratio was increased in younger subjects, suggesting that beneficial effects of atorvastatin on bone turnover could be more pronounced in older individuals.

In this sense, Kuzuya et al. (2003) performed a prospective study in elderly subjects (mean age, 75 years) to examine the effect of atorvastatin on bone metabolism. They analyzed serum BSAP levels, as a marker of bone formation, and urinary NTX/creatinine ratio, as a marker of bone resorption. Sixteen elderly patients with hypercholesterolemia (three men, 13 women, mean age ± standard deviation = 75.3 ± 6.3), who had not received statins, and 27 age-matched control subjects without hypercholesterolemia (nine men, 28 women; mean age = 71.8 ± 6.5) were recruited from a geriatric outpatient clinic of a university hospital. Blood and urine were sampled at 3 months and 6 months after the commencement of treatment with atorvastatin (or follow-up for control subjects). The two groups were matched for age, sex, body mass index, and serum BASP. The NTX/Cr ratio in urine was lower in the control group, and the serum total cholesterol and LDL-C levels were higher in the atorvastatin group. No significant difference in BMD was observed between the control and atorvastatin groups. No statistically significant change in serum concentration of BSAP was observed during follow-up in either group, but the urine NTX/Cr ratio decreased significantly in the atorvastatin group at 3 and 6 months. This report shows that 1-year treatment with atorvastatin had no effect on BMD changes compared with that of the control group, who were not receiving atorvastatin treatment, despite substantial reduction of bone resorption, which was confirmed by a 20% decrease in bone resorption marker, NTX/Cr. The observed effect on bone resorption may not be sufficient to affect BMD for a short period of observation.

However, these results were not confirmed by other investigators. Bone et al. (2007) carried out a prospective, randomized, double-blind, placebo-controlled, dose-ranging comparative clinical trial at 62 sites in the United States. Six-hundred and twenty-six postmenopausal women with LDL-cholesterol levels of at least 130–190 mg/dl and lumbar T-score between 0.0 and −2.5 were enrolled. BTMs, specifically serum NTX, OC, BSAP, and urinary deoxypyridinoline, were measured. Participants were allocated to once-daily placebo or atorvastatin 10, 20, 40, or 80 mg/day. At 52 weeks, there were no significant differences among groups concerning BTMs. This is an interesting study, since all the therapeutic doses of atorvastatin (10–80 mg) were explored, and no effect on BMD or BTMs was detected. Similar results were published by Braatvedt et al. (2004) who examined the effect of atorvastatin (40 mg/day) on serum BTMs in 25 patients (9 men and 16 postmenopausal women; mean age, 56 years) with type 2 diabetes and hypercholesterolemia in a 12-week, double-blind, placebo-controlled study. Serum BSAP, t-AP, OC, and CTX concentrations were not different in both groups and did not change over the follow-up period. Therefore, in this study, atorvastatin did not seem to have any significant effect on BTMs.

Other Statins

A few studies have analyzed the effect of statins other than simvastatin or atorvastatin on BTMs (Table 5). Regarding fluvastatin, Bjarnason et al. (2001) investigated the metabolic effects of this statin administered along with vitamin C in 68 elderly postmenopausal women with osteoporosis and mild hypercholesterolemia. Participants were randomly assigned to 12-week open-label treatment with either fluvastatin (40 mg daily) + 500 mg vitamin C ($n = 45$) or only vitamin C ($n = 23$). No statistical power calculation was performed because the study was planned to be exploratory and the analysis of the results to be descriptive. BTMs were measured at baseline and after 4 and 12 weeks. Serum OC, t-AP, and urinary CTX levels were measured. Fluvastatin in combination with vitamin C had no effect on bone formation markers. Concerning bone resorption markers, there was a marginal but statistically significant ($p = 0.04$) decrease in urinary CTX in the group on fluvastatin + vitamin C. Serum CTX concentrations showed a slight but significant difference from baseline in both groups although no statistical differences between them were detected.

Concerning pravastatin, Mostaza et al. (2001) evaluated the factors affecting cholesterol response to pravastatin in a prospective controlled study in 36 hypercholesterolemic postmenopausal women. After a 6-week period on a 30% fat diet, participants received pravastatin (20 mg/day) during a 16-week follow-up period. Serum BSAP and CTX levels did not vary during the study period. No correlation between cholesterol reduction and changes in BSAP or CTX was observed. However, pravastatin significantly increased PINP levels (37.4 ± 16 vs. 33.6 ± 13; $p = 0.03$) without affecting bone resorption. Moreover, this increase correlated positively with the lipid-lowering response to pravastatin ($r = 0.34$; $p = 0.04$). In addition, patients with the greater cholesterol response corresponded to those with the greater increase in PINP concentrations. Serum PIIINP levels were not affected by the statin treatment.

Watanabe et al. (2001) conducted a 6-month open-label prospective study in 31 postmenopausal women randomly assigned to receive 20 mg of fluvastatin or 10 mg of pravastatin daily. Serum BSAP, OC, and urinary NTX were collected at baseline and 1 and 6 months after the start of the treatment. BMD of the lumbar spine (L2–L4) was also measured at baseline. There were no effects on bone formation markers between the two groups, but the percentage of the urinary excretion of NTX was statistically significantly lower in the fluvastatin group at 1 month respect to its baseline values. Besides, the percent change in OC levels in the fluvastatin group was higher than in the pravastatin group. Concerning BMD, in the pravastatin group, lumbar BMD decreased during the overall study period. In contrast, lumbar BMD during 1 year of treatment held steady in the fluvastatin group. These differences may be related to the lipophilicity of these two statins.

Kanazawa et al. (2009) conducted a 3-month open-label prospective study in 36 diabetic patients with hypercholesterolemia, randomized to either rosuvastatin (2.5 mg/day) or ezetimibe (10 mg/day). Serum BSAP, OC, urinary NTX, and urinary deoxypyridinoline were collected and compared at baseline and at 3 months. Changes in total cholesterol or LDL-cholesterol levels were not significantly

Table 5 Summary of studies analyzing the effect of atorvastatin on BTMs in humans

Author/year	Statin(s)	Sex (M/F)	Age (mean years)	No	Type of study	Result
Chen et al. (2014)	Atorvastatin	64/0	80	64	Prospective, randomized, controlled study	CTX was reduced in atorvastatin group ($p < 0.001$)
Majima et al. (2007a)	Atorvastatin	22/0	62	22	3 months prospective study	BSAP did not change significantly NTX significantly decreased ($p = 0.02$)
Berthold et al. (2004)	Atorvastatin	F, postmenopausal	61	24 cases 25 controls	Prospective, randomized, double-blind, placebo-controlled trial	Increased in CTX and OC in younger subjects, while it decreased in older individuals
Kuzuya et al. (2003)	Atorvastatin	3/13	75	16 cases 27 controls	1-year prospective study	No statistically significant change in serum concentration of BSAP. NTX:Cr ratio decreased in the atorvastatin group at 3 and 6 months
Bone et al. (2007)	Atorvastatin	F, postmenopausal	40–75 (range)	626	Prospective, randomized, double-blind, placebo-controlled, dose-ranging comparative clinical trial	No significant changes in BTMs
Braatvedt et al. (2004)	Atorvastatin	9/16	56	25	Prospective double-blind placebo-controlled trial	No significant effect on bone turnover in men

correlated with the changes in BTMs. In the rosuvastatin group, serum OC levels were significantly increased ($p = 0.03$), while no other BTM was changed in the ezetimibe group. The authors suggest that rosuvastatin might have a beneficial effect on bone metabolism in patients with type 2 diabetes and hypercholesterolemia. This effect seems to be mediated by stimulation of bone formation and probably is unrelated to the cholesterol-lowering effect of rosuvastatin.

Pitavastatin does not undergo first-pass metabolism and for this reason could be the most suitable statin for examining the effects of statins on bone metabolism (Fig. 1). Thus, Majima et al. (2007b) carried out a prospective, randomized, double-blind study including 101 patients with untreated hypercholesterolemia. Participants were divided into two groups (66 patients allocated to pitavastatin, group A, and 35 allocated to placebo group B). Serum BSAP and NTX levels were compared between both groups and between baseline and at 3 months. After 3 months of treatment, serum BSAP did not change in group A compared with either the baseline value or that in group B. However, in patients on pitavastatin, serum NTX significantly decreased compared with both the baseline value and that in group B. The magnitude of the percentage change of NTX in group A was also greater than that in

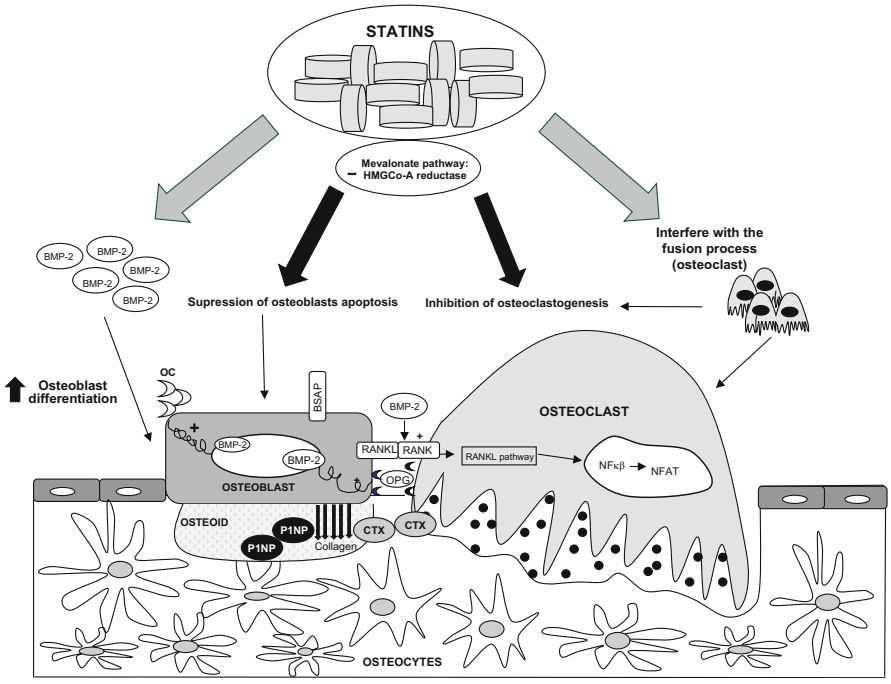

Fig. 1 Proposed effects of statins on bone cells. Statins act as competitive inhibitors of HMG-CoA reductase inhibiting the conversion of HMG-CoA to L-mevalonic acid. Statins could stimulate bone formation through an increase in expression and synthesis of bone morphogenetic protein-2 (BMP-2) and OC. Besides, they could carry out an antiresorptive effect by interfering with the fusion process through which osteoclast precursors become multinucleated cells

group B ($p = 0.008$), while serum BSAP did not. The percentage change of NTX was over the minimum significant change (14.2%) in 28 (44.4%) of the patients on pitavastatin, but none in the other group. In addition, in group A, the reduction of NTX was much more prominent in postmenopausal patients. This study suggests that pitavastatin may have positive effects on bone metabolism mainly by reducing bone resorption rather than by stimulating bone formation.

The effect of multiple statins on bone metabolism was addressed in a recent paper (Hernández et al. 2013). The authors analyzed the effects of statin use (pravastatin, lovastatin, fluvastatin, simvastatin, and atorvastatin) on BTMs, in a cross-sectional study nested in a prospective population-based study including 1401 women and 930 men. They also studied the effect of the type of statin, dose, pharmacokinetic properties, and length of treatment on BTMs. Serum PINP and CTX were lower in participants receiving statins versus nonusers, and this effect was modulated by diabetes status. Overall, this effect was more pronounced in subjects receiving the more lipophilic statins and specifically when taking for more than 3 years.

In summary, there is growing evidence about a possible beneficial effect for statins on bone mineral metabolism. Nevertheless, this potential association has been usually focused on BMD or fracture risk, and clinical studies analyzing the effect of these lipid-lowering agents on BTMs are scarce, and results have frequently been controversial. Besides, several important areas of debate are whether the dose, the lipid-lowering potency, or the length of therapy could influence biochemical parameters of bone metabolism (Table 6).

Potential Applications to Prognosis, Other Diseases, or Conditions

Biochemical markers of bone turnover (mainly serum PINP and CTX) have been recently proposed as a tool to monitoring the response and adherence to antiresorptive drugs in patients with osteoporosis or other bone metabolic diseases, such as Paget's disease, hyperparathyroidism, or even with rheumatic disorders or neoplasms affecting bone.

On the other hand, current evidence does not support the use of statins as a treatment for osteoporosis. Moreover, an effect of statins in preventing fractures is not supported by the most recent meta-analysis.

Summary Points

- A link between statins and bone remodeling process has been recently suggested.
- Measurement of the rate of bone turnover represents a useful tool to assess patients with bone metabolic diseases.
- Recent recommendations propose to determine a specific marker of bone resorption (CTX) and bone formation (PINP) in clinical studies.
- The effect of statins on bone turnover markers (BTMs) has been reported in a few randomized controlled trials, with contradictory results.

Table 6 Summary of studies analyzing the effect of other statins on BTMs in humans

Author/year	Statin(s)	Sex (M/F)	Age (mean years)	No	Type of study	Result
Bjarnason et al. (2001)	Fluvastatin + vitamin C vs. vitamin C	F, postmenopausal	71	45 cases 23 controls	Prospective randomized controlled trial	No effect on OCs and total AP Decreased in serum and urinary CTX
Mostaza et al. (2010)	Pravastatin	F, postmenopausal	52	36	Prospective controlled study	PINP levels significantly increased ($p = 0.03$) without changes in BSAP or CTX
Watanabe et al. (2001)	Fluvastatin/ pravastatin	F, postmenopausal	See text	31	Prospective controlled study	The percentage of urinary excretion of NTX was statistically significant lower in the fluvastatin group at 1 month vs. baseline The percent change in OC levels in the fluvastatin group was higher than in the pravastatin group
Kanazawa et al. (2009)	Rosuvastatin	8/10	60	18	3-month prospective study	OC levels were significantly increased ($p = 0.03$)
Majima et al. (2007b)	Pitavastatin	38/25	60	66 cases 35 controls	Prospective, randomized, double-blind	NTX was significantly decreased after 3 months of treatment with pitavastatin ($p = 0.002$) but BSAP did not

- Experimental studies have suggested that statins may be associated with an increase of bone formation but also with an antiresorptive effect.
- Overall, clinical studies have suggested a potential beneficial effect for statins on bone metabolism in patients with hypercholesterolemia, mostly by reducing bone resorption rather than by stimulating bone formation.
- Several important areas of debate are whether the dose, the lipid-lowering potency, or the length of the treatment with statins could influence biochemical parameters of bone metabolism.

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Bone Turnover and Spinal Cord Injury

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Contents

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Abstract

Spinal cord injury (SCI) causes rapid, severe osteoporosis with increased fracture risk. The pathogenesis of osteoporosis after SCI is a complex process and is usually attributed to "disuse" or "immobilization." However, the exact pathophysiology of osteoporosis after SCI is still not clear. In SCI, bone remodeling becomes uncoupled with an initial decrease in bone formation and steadily increasing bone resorption. Osteoporosis after SCI can be evaluated by measuring BMD using DEXA, pQCT, and MRI; and estimating biochemical markers of bone turnover. Bone cell activity can be evaluated indirectly with techniques, such as specific serum and urine biochemical markers of bone turnover. An improved understanding of the natural history and risk factors for chronic bone loss following SCI is essential to designing therapies to reduce the rate of bone loss, define fracture risk, and ultimately prevent osteoporotic fractures and their associated morbidity. In conclusion, we are of the opinion that prospective randomized controlled trials should be conducted to evaluate, standardize, and find bone-specific biochemical marker of bone turnover, for the better understanding of the pathophysiology of osteoporosis in SCI.

Keywords

Spinal cord injury • Bone turnover • Osteoporosis • Bone mineral density • Biochemical marker

List of Abbreviations

ALP	Alkaline phosphatase
B-ALP	Bone-specific alkaline phosphatase
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
CGRP	Calcitonin gene-related polypeptide
Cre	Creatinine
CTx	C-Telopeptide cross-link of type I collagen
DEXA	Dual energy X-ray absorptiometry
DPD	Deoxypyridinoline
HYPRO	Hydroxyproline
IL-6	Interleukin-6
LHRH	LH-releasing hormone
NDY	Neuropeptide Y
NTx	N-Telopeptide cross-link of type I collagen
OC	Osteocalcin
OPG	Osteoprotegerin
PICP	Procollagen type I C-terminal peptide
PINP	Procollagen type I N-terminal peptide
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone

PYD	Pyridinoline
RANKL	NF-κB ligand
SCI	Spinal cord injury
TSH	Thyroid-stimulating hormone
VIP	Vasoactive intestinal polypeptide

Key Facts of Spinal Cord Injury

- The term "spinal cord injury" (SCI) refers to damage to the spinal cord resulting from trauma or from disease or degeneration.
- Every year, around the world, between 250,000 and 500,000 people suffer a SCI.
- There is no reliable estimate of global prevalence, but estimated annual global incidence is 40–80 cases per million population.
- The majority of spinal cord injuries are due to preventable causes such as road traffic crashes, falls, or violence.
- People with a spinal cord injury are two to five times more likely to die prematurely than people without a spinal cord injury, with worse survival rates in low- and middle-income countries.
- Spinal cord injury is associated with lower rates of school enrollment and economic participation, and it carries substantial individual and societal costs.
- Spinal cord injury is associated with a risk of developing secondary conditions that can be debilitating and even life-threatening – e.g., deep vein thrombosis, urinary tract infections, muscle spasms, osteoporosis, pressure ulcers, chronic pain, and respiratory complications.

Definition of Words and Terms

Bone mineral density (BMD)	It is a measure of bone density, reflecting the strength of bones as represented by calcium content.
Bone turnover markers	Markers of bone turnover are biochemical products measured usually in blood or urine that reflect the metabolic activity of bone but which themselves have no function in controlling skeletal metabolism.
Osteopenia	Mild thinning of the bone mass and represents a low bone mass. Osteopenia results when formation of new bone (osteoid synthesis) is not sufficient to offset normal bone loss (osteoid lysis).
Osteoporosis	Thinning of the bones, with reduction in bone mass, due to depletion of calcium and bone

	protein. Osteoporosis predisposes a person to fractures.
Paraplegia	When the paralysis affects all or part of the trunk, legs, and pelvic organs.
Spinal cord	The spinal cord encased by vertebral column functions primarily in the transmission of neural signals between the brain and the rest of the body. It gives rise to 31 pairs of nerves.
Spinal cord injury	Damage to any part of the spinal cord or nerves in the spinal canal – often causes permanent changes in strength, sensation, and other body functions below the site of the injury.
Tetraplegia	Also known as quadriplegia, this means your arms, hands, trunk, legs, and pelvic organs are all affected by your spinal cord injury.

Introduction

Spinal cord injury (SCI) leads to sudden disruption of flow of information from the central nervous system. It has wide-ranging psychological and pathological effects those including an increase in bone resorption (Biering-Sorensen et al. 1988; Roberts et al. 1998; Maimoun et al. 2005; Singh et al. 2014a), hormonal alterations (Shetty et al. 1993; Bauman et al. 1994; Maimoun et al. 2005), and modification of body composition (Wilmet et al. 1995; Maimoun et al. 2005; Singh et al. 2014b). It is well known that SCI is accompanied by less bone mass caused by accelerated bone remodeling, with bone resorption exceeding bone formation. This situation causes a net bone loss and ultimately leads to osteoporosis (de Bruin et al. 2005). Osteoporosis in SCI occurs predominantly in the pelvis and the lower extremities (Garland et al. 1992; Demirel et al. 1998; Lazo et al. 2001; Maimoun et al. 2002; Zehnder et al. 2004). The neuronal lesion and subsequent immobilization create a unique form of early and severe bone loss in SCI persons (Chantraine et al. 1986; Garland et al. 1992; Leslie and Nance 1993; Hill et al. 1993; Uebelhart et al. 1995), characterized by a specific pattern of bone loss below the level of lesion (Finsen et al. 1992; Garland et al. 1994; Biering-Sorensen et al. 1988). Development of severe osteoporosis in the paralyzed part of the body is accompanied by the loss of biomechanical strength. In addition, the biosynthesis of structurally modified matrix is unable to sustain normal mechanical stress. Therefore, the risk of fracture is dramatically increased (Szollar et al. 1998; Dauty et al. 2000). Typically up to 33% of the bone mass is lost within first 6 months of injury, stabilizing to approximately 66% of the bone mass by 12–16 months post injury, which is considered to be close to the fracture threshold of the bone (Garland et al. 1992; Garland and Adkins 2001). The time course of bone loss may depend on the bone compartment; at sites within a high proportion of trabecular bone, bone loss followed a log curve leveling off from 1 to

3 years post injury, whereas at the tibial diaphysis, a cortical bone site, bone mass appeared to decrease progressively beyond 10 years post injury (Zehnder et al. 2004). During the first month post injury, demineralization occurs exclusively in the sublesional areas and predominantly in weight-bearing skeletal sites such as the distal femur and proximal tibia which are trabecular-rich site (Chantraine et al. 1986; Garland et al. 1992; Biering-Sorensen et al. 1998; Maimoun et al. 2005), while the diaphyseal area of the femur and the tibia, which are cortical-rich sites, are relatively spared (Dauty et al. 2000). Tibial trabecular bone losses within 2 years of SCI ranged from 0.4% to 80%, and cortical changes ranged from 1.7% increase to 32.7% decrease (de Bruin et al. 2005).This clear dissociation of the bone mineral density (BMD) loss between the trabecular and cortical compartments has been confirmed using the pQCT techniques (Frey-Rindova et al. 2000; Eser et al. 2004).

Various prospective longitudinal and cross-sectional studies using dual energy X-ray absorptiometry (DEXA) and peripheral quantitative computed tomography (pQCT) have described the magnitude of bone lost in the lower limbs (Table 1) and upper limbs (Table 2) following SCI.

Paradoxically, at the lumbar spine, which is mainly constituted of trabecular bone, no reduction in BMD usually occurs. This phenomenon is named dissociated hip and spine demineralization (Leslie and Nance 1993; Bauman et al. 1999). In the lumbar spine, the trabecular bone demineralization remains relatively low compared to the cortical bone demineralization of long bones (Dauty et al. 2000). Various studies have reported a normal (Chantraine et al., 1986; Biering-Sorensen et al., 1988) or even higher than normal (Garland et al., 1992; Kunkel et al., 1993; Ogilvie et al., 1993; Sloan et al., 1994; Singh et al., 2014a) BMD in the lumbar spine. Table 3 shows BMD of lumbar spine in SCI patients reported in various studies.

The decrease in bone mineral content (BMC) and BMD is associated with a deterioration in bone microarchitecture (Minaire et al. 1974; Modlesky et al. 2004), geometric structure, and strength (Modlesky et al. 2005; Rittweger et al. 2010), as well as an altered degree of mineralization and collagen matrix composition (Chantraine et al. 1986). Minaire et al. (1974) showed a 33% decrease in iliac crest trabecular bone volume in a cross-sectional case control study at 25 weeks post injury. Modlesky et al. (2004) reported that men with long-term (more than 2 years post injury) complete SCI ($n = 10$) had markedly deteriorated trabecular bone microarchitecture in the knee, which might contribute to the increase in fracture incidence. Slade et al. (2005) investigated the trabecular bone microarchitecture of the knee in cross-sectional study of 20 pre- and postmenopausal women with complete SCI at more than 2 years post injury and found that postmenopausal women with SCI had 34% greater trabecular spacing in the tibia than the 40-year-old premenopausal women with SCI. Modlesky et al. (2005) reported that the medullary cavity had 53% more volume and was 21–25% wider in the SCI group ($p < 0.05$) compared to the men without SCI. In contrast, the cortical wall in the SCI group had a 24% lower volume and was 27–47% thinner ($p < 0.05$). Calculated cross-sectional moment of inertia, section modulus, and polar moment of inertia were lower in the SCI group. Rittweger et al. (2010) suggested that the anatomical

Table 1 Sublesional bone mineral density of lower limbs in spinal cord injury patients

Author	Type of study	Duration after injury	Males	Females	Age	Skeletal site measured	BMD (Z-score, SD or % loss or reduction of BMD or total BMD)
Bauman et al. 1999	Prospective	3–26 years	8		25–58 years	Lower limb Pelvis	−35% −29%
Biering-Sorensen et al. 1990	Prospective	9 days–53 months	8			Femoral neck Distal femur Proximal tibia Femur diaphysis Tibia diaphysis	−30_40% −48% −45% −25% −25%
Chow et al. 1996	X-sectional	Three groups <0.25 years 0.25–1 year 1–35 years	19	12	19-58 years	Pelvis Proximal femur Leg	−2% −6% −28%
Clasey et al. 2004	X-sectional	0.6–35.3 years	21	8	23–56 years	Lower extremity	−28.20%
Dauty et al. 2000	X-sectional	>1 year	31		18–60 years	Femoral neck Femoral trochanter Distal femur Proximal tibia	−30% −39% −70% −52%
de Bruin et al. 2005	Prospective	3.5 years	9	1	19–81 years	Distal tibial Trabecular bone Distal tibial Compact bone	−40% −11%
Demirel et al. 1998	X-sectional	2–30 months	32	9	19–49 years	Lower extremity	−2.19 ± 3.5 SD
Eser et al. 2004	X-sectional	0.17–50 years	89		41.5 ± 14.2 years	Femur shaft Distal femur Tibia shaft Distal tibia	−1.4% −45% −57% −2.6%

Finsen et al. 1992	X-sectional	7 months–33 years	19		15–64 years	Tibia distal diaphysis Tibia distal metaphysis	−26% −45%
Frey-Rindova et al. 2000	Prospective	12 months	27	3	19–59 years	Tibia trabecular bone Tibia cortical bone	−15% −7%
Garland et al. 1992	Prospective	114 ± 8.6 days	12		28 ± 0.8	Proximal femur Distal tibia	−13% −13%
Garland et al. 2004	Prospective		6			Distal femur Proximal tibia Os calcis	−27% −32% −38%
Garland et al. 2001	X-sectional	20–30 years 31–50 years 50+ years		31	5.7 ± 2.3 years 16.1 ± 9.4 years 28.9 ± 11.4 years	Knee, hip	−38%, −18% −41%, −25% −47%, −25%
Garland et al. 2001	X-sectional	2–8 years 3–30 years 9–44 years	6 16 9		20–30 years 31–50 years 53–77 years	Knee Hip Knee Hip Knee Hip	−37.90% −17.50% −41.30% −25% −47% −25.50%
Jones et al. 1998	X-sectional	1–30 years	5		32.6 ± 6.3 years	Femur neck Leg BMC	−26% −30%
Jones et al. 2002	X-sectional	7–372 months	20 (total)		17–52 years	Femur Hip	−27% −37%
Kannisto et al. 1998	X-sectional	1–30 years	25	10	1.5–57 years	Proximal femur	−26%

(*continued*)

Table 1 (continued)

Author	Type of study	Duration after injury	Males	Females	Age	Skeletal site measured	BMD (Z-score, SD or % loss or reduction of BMD or total BMD)
Kiratli et al. 2000	X-sectional	0.1–51 years	239	7	27–78 years	Femoral neck Femoral midshaft Distal femur	−27% −25% −43%
Leslie and Nance 1993	X-sectional	1–17 years	14		20–41 years	Femur neck	−26%
Maimoun et al. 2005	Prospective		7		31.3 ± 9.5 years	16 weeks Total proximal femur Femoral neck Trochanter Intertrochanter 71 weeks Total proximal femur Femoral neck Trochanter Intertrochanter	1.014 (1.030 ± 0.188) 1.005 (0.992 ± 0.185) 0.718 (0.769 ± 0.167) 1.163 (1.187 ± 0.211) 0.741 (0.799 ± 0.151) 0.795 (0.780 ± 0.095) 0.570 (0.616 ± 0.133) 0.847 (0.945 ± 0.198)
Modlesky et al. 2004	X-sectional	8.7 ± 7.5 years	10		34 ± 10 years	Proximal tibia	−43%
Moynahan et al. 1996	X-sectional		30	21	3–20 years	Femur neck Ward's triangle Intertrochanter	−36% −36% −44%
Paker et al. 2007	X-sectional	24.52 ± 20.9 months	33	15	38.47 ± 15.88 years	Injury duration < one year Femur neck Total femur	0.954 ± 0.195 0.992 ± 0.182 −0.766 ± 1.46 −0.5 ± 1.45 0.785 ± 0.146

						Femur neck T score Total femur T score Injury duration > one year Femur neck Total femur Femur neck T score Total femur T score	0.762 ± 0.136 −2.088 ± 1.03 −2.258 ± 1.09
Reiter et al. 2007	Prospective						
Sabo et al. 2001	X-sectional	1–26 years	46		<50 years	Proximal femur	−24.50%
Singh et al. 2014	Prospective	0–12 months	71	24	33.3 years	Initial Hip Proximal tibia Tibial diaphysis Distal tibial epiphysis 12 months Hip Proximal tibia Tibial diaphysis Distal tibial epiphysis	0.968 1.02 1.11 0.985 0.77 0.74 1.005 0.75

(*continued*)

Table 1 (continued)

Author	Type of study	Duration after injury	Males	Females	Age	Skeletal site measured	BMD (Z-score, SD or % loss or reduction of BMD or total BMD)
Uebelhart et al. 1994	Prospective	>6 months	6			Lower extremity	−6.40%
Warden et al. 2001	Prospective	1–6 months	15		19–40 years	Calcaneus Proximal tibia	−7.5 ± 3.0% −5.3 ± 4.2%
Wilmet et al. 1995	Prospective	Within 56 days	24	7	18–66 years	Pelvis, leg	Complete −40%−45%, −25% Incomplete −30%, −10%
Zehnder et al. 2004	X-sectional	<1 year <1 year 1–9 years 1–9 years 10–19 years 10–19 years 20–29 years 20–29 years	100 16 16 38 38 31 31 13 13		18–60 years	Femoral neck Tibia epiphysis Femoral neck Tibia epiphysis Femoral neck Tibia epiphysis Femoral neck Tibia epiphysis	−0.03 ± 0.25 SD −0.34 ± 0.22 SD −1.65 ± 0.17 SD −3.81 ± 0.13 SD −1.76 ± 0.25 SD −4.00 ± 0.21 SD −1.76 ± 0.28 SD −4.12 ± 0.24 SD

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Table 2 Sublesional bone mineral density of upper limbs in spinal cord injury patients

Author	Type of study	Duration after injury	Males	Females	Age	Skeletal site measured	BMD (Z-score, SD or % loss or reduction of BMD or total BMD)
Clasey et al. 2004	X-sectional	0.6–35.3 years	21	8	23–56 years	Upper extremity	+11.10%
Dauty et al. 2000	X-sectional	>1 year	31		18–60 years	Upper extremity	+6%
de Bruin et al. 2005	Prospective	3.5 years	9	1	19–81 years	Distal radius trabecular bone	−10̃ to + 14%
Demirel et al. 1998	X-sectional	2–30 months	32	9	19–49 years	Upper extremity	+0.09 ± 0.15 SD
Finsen et al. 1992	X-sectional	7 months–33 years	19		15–64 years	Forearm distal diaphysis Forearm distal metaphysis	−5% −13%
Frey-Rindova et al. 2000	Prospective	12 months	27	3	19–59 years	Radius trabecular bone Radius cortical bone Ulna trabecular bone Ulna cortical bone	−8% 0% −4% −1%
Maimoun et al. 2005	Prospective		7		31.3 ± 9.5 years	16 weeks Distal radius 71 weeks Distal radius	0.667 (0.654 ± 0.058) 0.640 (0.633 ± 0.052)
Sabo et al. 2001	X-sectional	1–26 years	46		<50 years	Distal forearm	−6.10%

(*continued*)

Table 2 (continued)

Author	Type of study	Duration after injury	Males	Females	Age	Skeletal site measured	BMD (Z-score, SD or % loss or reduction of BMD or total BMD)
Singh et al. 2014	Prospective	0–12 months	71	24	33.3 years	Initial Distal radius 12 months Distal radius	0.61 0.57
Zehnder et al. 2004	X-sectional	<1 year <1 year 1–9 years 1–9 years 10–19 years 10–19 years 20–29 years 20–29 years	100 16 16 38 38 31 31 13 13		18–60 years	Ultra distal radius Radius shaft 1/3 Ultra distal radius Radius shaft 1/3 Ultra distal radius Radius shaft 1/3 Ultra distal radius Radius shaft 1/3	+0.02 ± 0.24 SD +0.00 ± 0.41 SD +0.01 ± 0.15 SD +0.40 ± 0.17 SD +0.52 ± 0.20 SD +0.97 ± 0.20 SD +0.44 ± 0.32 SD +0.27 ± 0.31 SD

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Table 3 Sublesional bone mineral density of lumbar spine in spinal cord injury patients

Author	Type of study	Duration after injury	Males	Females	Age	BMD (Z-score, SD or % loss or reduction of BMD or total BMD)
Clasey et al. 2004	X-sectional	0.6–35.3 years	21	8	23–56 years	2%
Dauty et al. 2000	X-sectional	>1 year	31		18–60 years	−11%
Garland et al. 2001	X-sectional	2–8 years 3–30 years 9–44 years	6 16 9		20–30 years 31–50 years 53–77 years	2% 8.10% 14.80%
Liu et al. 2000	X-sectional		64		20–98 years	−2.0 ± 1.2 SD
Maimoun et al. 2005	Prospective		7		31.3 ± 9.5 years	16 weeks 1.023 (1.017 ± 0.094) 71 weeks 1.044 (1.051 ± 0.109)
Sabo et al. 2001	X-sectional	1–26 years	46		<50 years	−3.8%
Singh et al. 2014	Prospective	0–12 months	71	24	33.3 years	1.21
Zehnder et al. 2004	X-sectional	<1 year 1–9 years 10–19 years 20–29 years	100 16 38 31 13		18–60 years	−0.43 ± 0.19 SD +0.11 ± 0.15 SD +1.09 ± 0.23 SD +1.00 ± 0.42 SD

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variation in the geometry, rather than in the bone mass, can explain differential (endocortical vs. periosteal) rate of bone of loss after SCI. Chantraine et al. (1986) reported a large increase in the proportion of little calcified bone in the cortical as well as in the cancellous bone. They also reported a decreased number in hydroxyproline (HYPRO) residue in the newly synthesized organic matrix from paraplegia bone resulting either from an alteration of the prolyl hydroxylation or from the presence of an excess of non-collagen polypeptide.

Pathophysiology of Bone Turnover After SCI

The exact pathophysiology of osteoporosis after SCI is still not clear. The pathogenesis of osteoporosis after SCI is generally considered disuse. Mechanical loading is known to be a crucial stimulus for bone formation and resorption, thereby controlling bone mass, structure, and strength. The skeleton possess an inherent biological control system that directs bone formation in response to high mechanical stresses (or strains), thus strengthening skeleton in highly stress regions. This system, sometimes called the "Mechanostat," involves the resident cells within bone tissue that detect and respond to mechanical loads (Jiang et al. 2006a). According to Frost's Mechanostat Theory, bone adapts their structure that bone strength ensures strains caused by physiological loads remain within narrow window. In particular, bone strength and mass normally adapt to the largest voluntary loads on bones, whereby under physiological conditions, the largest loads are produced by muscle forces (Frost 1987, 1997, 2003). At the other end of the spectrum, immobilization due to paralyses leads to the absence of voluntary muscle contractions and hence to the absence of muscle loads. Under this condition bone "disuse-mode" is turned on, causing a distinct bone loss (Frost 1998). The findings of Frotzler et al. (2008) study support this theory. After complete SCI, bone mass and strength are lost within first years post injury until the concerned skeletal parts have adapted to the new level of mechanical loading (Rittweger et al. 2006). In SCI subject with strong muscle spasms, bone strength at the femur has been found to be greater than in subjects with weak or no muscle spasm (Eser et al. 2004).

Disuse may play an important role in the pathogenesis of the osteoporosis, but the factors that are independent of mechanical loading of the skeleton also appear to be important (Jiang et al. 2006a). Possible nonmechanical factors may include poor nutritional status (Bauman et al. 1995, 2005), lesion-induced blood circulation abnormalities at the sublesional levels that affects bone cell differentiation (Chantraine et al. 1979), alteration in gonadal functions (Morley et al. 1979; Naftchi et al. 1980; Nance et al. 1985), hypercortisolism (either therapeutic or stress related) (Bugaresti et al. 1992), and other endocrine disorders (Bauman et al. 1994). Recent work suggests that bone remodeling is regulated by nerve-derived signals, such as vasoactive intestinal polypeptide (VIP), calcitonin gene-related polypeptide (CGRP), neuropeptide Y (NPY), and substance P, as well as classical neuromediators such as noradrenaline, serotonin, and glutamate (Elefteriou 2005). It has also been reported that there is a marked modification in the osteoprotegerin/

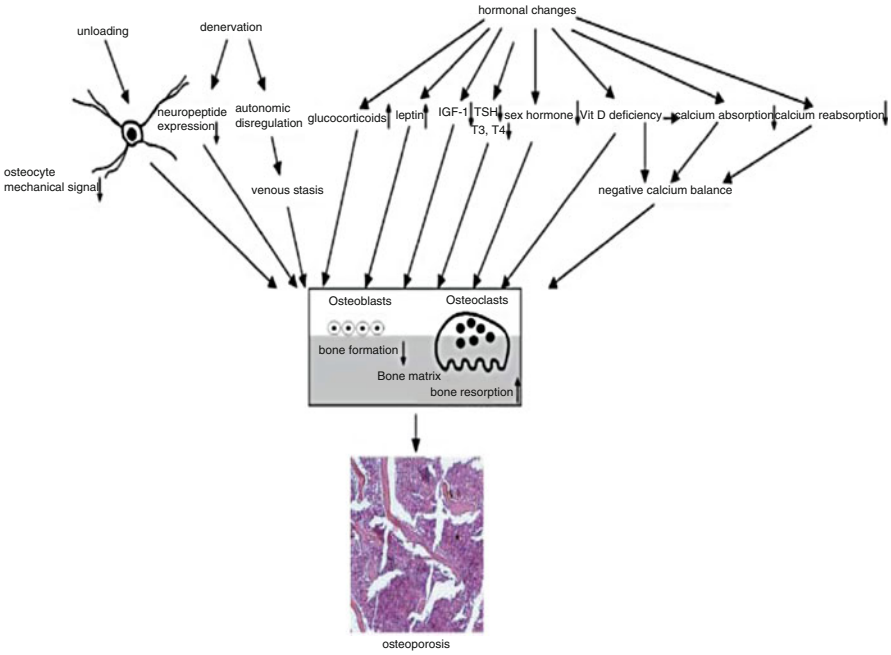

Fig. 1 A schema outlining the pathways implied in the pathogenesis of osteoporosis after SCI (Figure is from Jiang et al. (2006a), Copyright (2006), with permission from "Wiley")

RANKL system in recent SCI patients (Maimoun et al. 2005). Jiang et al. (2006a) outlined the pathways in the pathogenesis of osteoporosis after SCI (Fig. 1). The mechanism that underlies osteoporosis after SCI remains poorly elucidated and controversial (Jiang et al. 2006a).

Biochemical Markers of Bone Turnover in SCI

Bone cell activity can be evaluated indirectly with techniques, such as specific serum and urine biochemical markers of bone turnover. Biochemical markers reflect the process involved in bone turnover (Jiang et al. 2006b). Bone resorption markers reflect osteoclast activity and are the products of the protein matrix degradation of type I collagen, but noncollagenous proteins such as the enzyme of osteoclast origin tartrate-resistant acid phosphatase 5b (TRACP) and helical peptide 620–623 have also been investigated as resorption markers. At first hydroxyproline, pyridinoline (PYD), and deoxypyridinoline (DPD) adjusted to creatinine (Cre) were used; but they were relatively nonspecific and have been largely replaced by new markers such as N- and C-telopeptide cross-link of type I collagen (NTx and CTx) (Maimoun et al. 2011; Vasikaran et al. 2011). SCI is found to promote human osteoclast formation ex vivo (Demulder et al. 1998). The key molecule of osteoclast

development is the receptor activator of the NF-κB ligand (RANKL) (Lacey et al. 1998). There is evidence that bone-resorbing cytokines, such as interleukin (IL)-6, may be a potential candidate for mediating the bone loss following SCI (Demulder et al. 1998). Recent studies suggest that sclerostin is a key mediator of SCI-induced bone loss. Sclerostin, encoded by the sost gene, is produced primarily by osteocytes and is a potent inhibitor of bone formation and growth (Balemans et al. 2001; Staehling-Hampton et al. 2002). Mechanical unloading causes upregulation of sclerostin, leading to reduced Wnt/β-catenin signaling in osteoblasts. While the anti-anabolic role of sclerostin has been well characterized, recent evidence indicates that sclerostin also has catabolic activity. In fact, sclerostin causes upregulation of RANKL and downregulation of OPG expression by osteocytes, increasing osteoclast differentiation and activity, ultimately leading to bone resorption (Wijenayaka et al. 2011).

"The bone formation markers" reflect osteoblast activity and are the products of collagen synthesis (procollagen type I N-terminal peptide or C-terminal peptide [PINP & PICP], osteoblastic enzyme, i.e., bone-alkaline phosphatase [B-ALP], or matrix protein (osteocalcin [OC]) (Maimoun et al. 2011; Vasikaran et al. 2011). Type I collagen is an important component of bone matrix, and osteoblasts secrete its precursor procollagen molecule during bone formation. The extension peptides at each end of the procollagen molecule, PINP and PICP, are cleaved by enzymes during bone matrix formation and released into the circulation. Osteocalcin, one of the most abundant noncollagenous proteins in bone matrix, is also produced by osteoblasts during bone formation, and some proportion finds its way into the extracellular compartment where it can be measured. It is excreted by the kidneys and its fragments may also be measured in urine. Newly formed osteoid undergoes maturation followed by mineralization, and during this phase, alkaline phosphatase (ALP) is secreted by osteoblasts into the extracellular fluid and can be measured in serum. However, only about half of the ALP activity in blood of healthy adults derives from the bone, the other half being predominately of hepatic origin. Assays are available that detect more specifically the B-ALP (Vasikaran et al. 2011). After SCI, there is an increased turnover of bone tissue. In the meantime, bone formation and bone resorption remain uncoupling, thus leading to bone loss (Jiang et al. 2006a).

Increased bone resorption after SCI has been demonstrated clinically. After SCI, notable increase in bone resorption markers have been reported to occur as early as 2 weeks, reaching peak value 2–4 months after injury onset (Bergmann et al. 1977; Uebelhart et al. 1994; Roberts et al. 1998; Maimoun et al. 2002). However, bone formation markers remain normal or elevated (Roberts et al. 1998; Maimoun et al. 2002). Table 4 shows biochemical markers of bone turnover reported in various studies. The currently used bone markers reflect only whole-body net changes. They thus cannot determine the specific bone site affected by the intense bone demineralization or discriminate changes in a specific skeletal envelope, that is, trabecular versus cortical (Maimoun et al. 2011).

Table 4 Showing biochemical biomarkers of bone turnover reported in various studies in SCI population

	Parameters								
	Bone formation markers				Bone resorption markers				
Studies	OC	ALP	B-ALP	OPG	Serum CTx	Urinary CTx	sRANKL	HYPRO	DPD
Bagis et al. 2002 Paraplegia Tetraplegia	3.51 ± 2.07 ng/dL 3.18 ± 1.26 ng/dL	168.5 ± 44.6U/L 121.7 ± 0.1 U/L			0.49 ± 0.009 ng/mL 0.63 ± 0.12 ng/mL				
Hummel et al. 2012					263.6 ± 145.2 ng/L				
Kannisto et al. 1998 Males Females	7.2 ± 5.2 ug/L 3.8 ± 2.5 ug/dL	170 ± 86 U/L	68 ± 33 U/L		4.4 ± 2.5 ug/L	18–20 years 0.28 ± 0.2 mmol/L 21–65 years 0.12 ± 0.11 mmol/L			5.8 ± 1.8 nmol/L 8.3 ± 5.5 nmol/L
Kaya et al. 2006		100.05 ± 36.98 U/L							
Maimoun et al. 2002	24 ± 8 ng/mL		13.8 ± 5.5 ng/mL		13,340 ± 4,921 pmol/L	894 ± 371 μg. mmol/LCr			
Maimoun et al. 2005	16 weeks 25.6 (24 ± 7.9) mg/mL 71 weeks 15.3 (15.1 ± 2.44) mg/mL		16 weeks 14.5 (13.8 ± 5.5) ng/mL 71 weeks 21.7 (22 ±) mg/mL	16 weeks 7.8 (8.6 ± 1.5) U/L 71 weeks 8.4 (8.7 ± 1.5) U/L	16 weeks 13,500 (13,340 ± 4,921) pmol/L 71 weeks 3,171 (6,369 ± 5,093) pmol/L	16 weeks 849 (894 ± 371) μg. mmol/LCr 71 weeks 331 (404 ± 197) μg. mmol/LCr	16 weeks 0.13 (0.29 ± 0 30) pmol/L 71 weeks 0.25 (0.37 ± 0.33) pmol/L		
Morse et al. 2008	21.54 [17.82–28.63] ng/mL			58.39 [43.93–79.60] pg/mL	0.20 [0.15–0.31] ng/mL		0.07 [0.04–016] pg/mL		
Paker et al. 2007	23.60 ± 10.73 ng/mL	118.75 ± 84.53 U/L			1.37 ± 0.60 ng/mL				

(*continued*)

Table 4 (continued)

	Parameters								
	Bone formation markers				Bone resorption markers				
Studies	OC	ALP	B-ALP	OPG	Serum CTx	Urinary CTx	sRANKL	HYPRO	DPD
Pietschmann et al. 1992	10.5 ± 3.3 ng/mL								
Sabour et al. 2014 Male Female	17.26 ± 9.84 ng/mL 14.70 ± 6.96 ng/mL		15.0 ± 6.03 ng/mL 14.6 ± 7.72 ng/mL		0.91 ± 0.62 ng/mL 0.549 ± 0.26 ng/mL				
Singh et al. 2014 Initial 1 year		195.2 ± 47.1 U/L 231.3 ± 30.5 U/L							

Factors Affecting the Bone Turnover After SCI

Various factors may have a potential impact on bone remodeling after SCI. These include duration of injury, level of injury, physical activity, body composition, health-related complications such as diabetes mellitus, and lifestyle changes.

Duration of Injury

A positive correlation exists between the time following injury and degree of bone loss. Bone resorption decreases with post injury duration (Roberts et al. 1998; Zehnder et al. 2004; Maimoun et al. 2005; Reiter et al. 2007). However, some studies have found a negative linear relationship between markers like hydroxyproline/creatinine (Pietschmann et al. 1992) or bone alkaline phosphatase (Jones and Legge 2009). Sabour et al. (2014) and Bagis et al. (2002) found that post injury duration did not have any effect on CTx levels. Bone mineral content at the femoral shaft reaches a new steady-state at a mean 6.8 years post injury, and BMD cortical is reduced during the first 2–5 years when increased intracortical remodeling is present and recovers to normal values thereafter (Frotzler et al. 2008).

Level of Injury

SCI patients are classified into two large categories, i.e., paraplegia and quadriplegia. No significant difference in BMD is usually found between paraplegic and quadriplegic individuals (Tsuzuku et al. 1999; Demirel et al. 1998; Singh et al. 2014a). No difference in the bone resorption markers are usually observed between the two groups (Pietschmann et al. 1992; Roberts et al. 1998; Maimoun et al. 2006; Morse et al. 2008).

According to Maimoun et al. (2011), it is surprising that a clear bone remodeling profile cannot be identified according to the level of injury (paraplegia and quadriplegia) because, although the neurological level of injury does not determine the degree of bone loss, it does have an impact on the extent of alteration (Garland et al. 1992; Dauty et al. 2000). As has been noted, bone markers give a global but not site-specific evaluation of bone turnover. The lack of difference between the two groups may reflect the heterogenecity of these populations; and functionally, some patients with paraplegia and quadriplegia might not be so different, thus showing bone turnover that is quite similar (Roberts et al. 1998).

Age

In trabecular bone compartment, thinning and loss of trabeculae lead to reduced BMD with age (Inceoglu et al. 2005). This was also found in people with SCI

(Kiratli et al. 2000). Frotzler et al. (2008) reported no significant age-related changes after completion of initial bone loss and reaching of bone steady-state in the SCI population. Age-related bone adaptation may be absent when bone structure already adapted to minimal mechanical loading due to aging. They also reported no periosteal diameter at the tibial or femoral shaft to increase, indicating that there is no generalized age-related periosteal expansion in people with chronic SCI.

Age has no significance on CTx levels (Bagis et al. 2002; Savour et al. 2014). Morse et al. (2008) showed that CTx is increased by age in chronic SCI that cannot be justified with reduction of BMD loss through time. The impact of age on patients with SCI can be debated, probably injury causes a greater alteration in bone loss than age (Maimoun et al. 2011).

Complete Versus Incomplete SCI

Motor-complete (ASIA A and B) patients have significantly lower BMD than motor-incomplete (ASIA C and D) patients (Demirel et al. 1998; de Bruin et al. 2005; Singh et al. 2014). Individuals with incomplete SCI tended to lose less bone than individuals with complete SCI. The degree of mobility may be important; a cross-sectional study demonstrated that BMD in SCI patients were positively correlated with their mobility with a mobility index ranging from complete paralyses to unlimited ambulation (Saltzstein et al. 1992). However, Kaya et al. (2006) could not find significant difference in BMD values between these two groups.

Physical Activity

Physical activity in able-bodied individual is known to induce changes in bone remodeling (Maimoun and Sultan 2011), and most persons with SCI present a sedentary lifestyle (Noreau and Shephard 1995). Various studies vary in exercise modality, training duration and/or intensity, and participant level and duration of injury reveal no change (Giangregorio et al. 2005), increased (Belanger et al. 2000) or diminished BMD (Clark et al. 2007), at various sites in response to exercise training. Completion of 6 months of activity-based therapy was unable to ameliorate bone loss of the total body and hip, but reduce the expected magnitude of bone loss seen after SCI. Spine BMD was increased, and markers of bone formation and resorption were unchanged with training (Astorino et al. 2013). Jones and Legge (2009) compared two groups of male patients with SCI: an active group and a sedentary group. No difference was found for the bone resorption markers between these two groups ($p > 0.05$). Deoxypyridinoline concentration in active group (16.8 nmol/mmol creatinine $\pm$ 10.3) was higher in sedentary group (11.8 nmol/mmol creatinine $\pm$5.4). Bone alkaline phosphatase (bone formation marker) activity in sedentary SCI men was significantly higher than those of active group (sedentary, 28.0 $\pm$ 6.4 U/L; active, 14.0 $\pm$ 3.6 U/L, $p < 0.01$). Roberts et al. (1998) found no

relationship between the levels of physical activity and bone markers; and Morse et al. (2008) reported no correlation between osteocalcin and CTx concentration and ambulation mode in patients with duration of injury more than 2 years. In a cross-sectional study (Chain et al. 2012), no difference in urinary CTx was revealed between sedentary and active men with chronic SCI.

Other Factors

Individual with SCI not only lose motor and/or sensory, they experience dramatic muscle and bone changes (Giangregorio and McCartney 2006; Singh et al. 2014a). It also induces health-related complications (Bauman et al. 1994) and lifestyle changes (Noreau and Shephard 1995). Morse et al. (2008) studied 82 male patients with duration of injury more than 2 years and found no relationship between OC and CTx values and factors like body mass index (BMI), smoking history, history of heart disease, high blood pressure, and diabetes. Roberts et al. (1998) confirmed that weight and nutrition do not affect biochemical bone markers.

Effects of Hormones on Bone Turnover After SCI

Bone remodeling is a process of bone renewal accomplished by two opposing activities of bone cells: bone resorption by osteoclast and bone formation by osteoblasts. Systemic hormones such as parathyroid, vitamin D, sex steroids, thyroid hormones, and leptin may also be involved in bone loss following SCI (Jiang et al. 2006a). Table 5 shows biochemical parameters of bone turnover reported in various studies in SCI population and Table 6 various methods of assaying these parameters.

Calcium and Phosphate Homeostasis

Hypercalciuria associated with abnormally high ionized calcium (iCa) level with or without abnormal elevated total calcium was reported in patients during the acute SCI phase (Bergmann et al. 1977; Naftchi et al. 1980; Uebelhart et al. 1994; Mechanick et al. 1997; Roberts et al. 1998; Maimoun et al. 2002). The increased osteoclastic bone resorption is mainly responsible for hypercalciuria following SCI. In addition, reduced renal function has been observed in acute SCI patients (Maynard and Imai 1977), and the increased urinary elimination of calcium that occurs in response to SCI may be related to diminished renal tubular reabsorption. Urinary calcium excretion, serum phosphorous, and ionized calcium were significantly higher in acute SCI patients (Bergmann et al. 1977; Roberts et al. 1998; Maimoun et al. 2002). Serum concentration of ionized calcium in long-standing SCI population was normal (Vaziri et al. 1994). This indicates a new balance of bone formation,

Table 5 Showing biochemical parameters of bone turnover reported in various studies in SCI population

	Parameters					
	Serum					
Studies	Total calcium	Ionized calcium	Phosphorus	Proteins	Albumin	PTH
Bagis et al. 2002 Paraplegia Tetraplegia	9.21 ± 0.66 mg/dL 8.60 ± 0.58 mg/dL	1.14 ± 0.07 mmol/L 1.16 ± 0.08 mmol/L	4.06 ± 0.39 mg/dL 4.27 ± 0.26 mg/dL			41.72 ± 7.55 pmol/L 37.6 ± 8.25 pmol/L
Hummel et al. 2012		1.24 ± 0.04 mmol/L				4.4 ± 2.4 pmol/L
Kaya et al. 2006	9.60 ± 0.56 mg/dL		4.38 ± 0.70 mg/dL			2.23 ± 1.27 pmol/L
Maimoun et al. 2002	2.42 ± 0.09 mmol/L	1.27 ± 0.05 mmol/L	1.45 ± 0.24 mmol/L			5.14 ± 1.2 pmol/L
Maimoun et al. 2005	16 weeks 90.3 (90.7 ± 3.4) mg/L 71 weeks 82.8 (83 ± 3.1) mg/L				16 weeks 42 (40.1 ± 5.9) g/L 71 weeks 49 (49 ± 5) g/L	16 weeks 5 (5.1 ± 1.2) pg/mL 71 weeks 15 (16.6 ± 3.2) pg/mL
Paker et al. 2007	8.87 ± 0.57 mg/dL		4.20 ± 0.90 mg/dL			
Pietschmann et al. 1992						61.5 ± 33.0 pmol/L
Sabour et al. 2014 Male Female						3.22 ± 6.90 pg/mL 2.27 ± 1.05 pg/mL
Singh et al. 2014 Initial 1 year	8.8 ± 1.2 mg/dL 8.3 ± 0.8 mg/dL		3.8 ± 1.2 mg/dL 4.2 ± 0.8 mg/dL	6.24 ± 0.6 mg/dL 6.4 ± 1.0 mg/dL		

					Urine		
CT	T3	T4	TSH	1,25(OH) D3	24-h calcium	24-h phosphorus	Calcium/ creatinine
					288.2 ± 139.5 mg/ dL 259.5 ± 119.3 mg/ dL		0.3 ± 0.1 0.2 ± 0.001
				86.6 ± 37.8 nmol/L			
7.84 ± 3.15 mg/dL	2.47 ± 0.61 pg/mL	1.2 ± 0.23 ng/mL	1.45 ± 1.04 μIU/mL		238.81 ± 153.6 mg/ dL		0.3 ± 0.18
				13.57 ± 7.8 pg/mL		1.81 ± 1.23 mmol. mmol/Cr	0.76 ± 0.37
				16 weeks 11 (13.5 ± 7.8) pg/mL 71 weeks 24 (22.8 ± 6.6) pg/mL			16 weeks 0.72 (0.76 ± 0.37) 71 weeks 0.21 (0.23 ± 0.1)
48.3 ± 14.8 pg/mL							
	10.62 ± 9.84 pg/mL 9.64 ± 26.03 pg/mL						
					109.5 ± 24.5 mg/day 120.7 ± 39.0 mg/day	1.06 ± 0.16 mg/day 1.08 ± 0.03 mg/day	

Table 6 Showing various methods of assessment of various parameters of bone turnover

Parameter	Method of assay
Serum	
Calcium (Ca)	Colorimetric technique
Phosphorous (P)	Colorimetric technique
Ionized calcium (iCa)	Ion-specific electrode
Alkaline phosphatase (ALP)	Spectrophotometry
Bone specific alkaline phosphatase (B-ALP)	Human immunoradiometeric assay (IRMA) using two monoclonal antibodies; Scandinavian method and polyacrylamide gel electrophoresis for isoenzymes; competitive immunosorbent assay
Osteocalcin (OC)	Radioimmunoassay (RIA); human immunoradiometeric assay (IRMA)
Parathyroid hormone (PTH)	Human immunoradiometeric assay (IRMA) using two different polyclonal antibodies; solid-phase, two-site chemiluminescentimmunometeric assay; radioimmunoassay (RIA)
C-telopeptide cross-link of type I collagen (CTx)	Enzyme-linked immunosorbent assay (ELISA); radioimmunoassay (RIA); electrochemiluminescence
Procollagen type I N-terminal peptide (PINP)	Enzyme-linked immunosorbent assay (ELISA)
$1,25(OH)_2$ vitamin D_3	Radioimmunoassay (RIA) using polyclonal 1,25 vitamin D antibody; column chromatography; chemiluminescence immunoassay
RANKL: NF-κB ligand	Enzyme-linked immunosorbent assay (ELISA)
Osteoprotegerin (OPG)	Enzyme-linked immunosorbent assay (ELISA)
Urine	
Calcium (Ca)	Colorimetric technique
Creatinine (Cre)	Colorimetric technique; modified Jaffe method
Hydroxyproline (HYPRO)	Spectrophotometry
Deoxypyridinoline (DPD)	Immunoassay; high-performance liquid chromatography
C-telopeptide cross-link of type I collagen (CTx)	Radioimmunoassay (RIA); electrochemiluminescence

and resorption may be reestablished in long-standing SCI patients (Jiang et al. 2006b). Absorption of calcium from gastrointestinal tract has been found to decrease in acute phase following SCI (Zhou et al. 1993). Risk factors for hypercalcemia include children and adolescent, recently paralyzed, male gender, complete neurological injury, high cervical cord injuries, dehydration, and prolonged immobilization (Maynard 1986).

Soon after initial SCI, increased phosphorus values were reported (Bergmann et al. 1977; Naftchi et al. 1980; Roberts et al. 1998; Maimoun et al. 2002; Zehnder et al. 2004; Kaya et al. 2006). The increase could be explained by the loss of phosphorus from bone and muscle tissues (Dauty et al. 2000) and result from relative hypoparathyroidism. Secondarily, it can directly inhibit the $1,25(OH)_2$ vitamin D_3 synthesis (Mechanick et al. 1997).

Parathyroid Hormone (PTH)

In the acute phase of SCI, secretion of parathyroid hormone drops quickly (Claus-Walker et al. 1977; Chantraine et al. 1979; Pietschmann et al. 1992; Roberts et al. 1998; Maimoun et al. 2002, 2005). After acute SCI, the PTH-vitamin D axis is suppressed with depressed PTH and $1,25(OH)_2$ vitamin D_3 (Stewart et al. 1982). The initial suppression seems to be transitory because it tended to level off after 6 months (Pietschmann et al. 1992; Roberts et al. 1998; Maimoun et al. 2005); but value did not return to normal 71 weeks post injury (Maimoun et al. 2005). Hypercalcemia after injury may lead to PTH-vitamin D axis suppression in acute phase of SCI. PTH suppression in SCI patients is associated with the degree of neurological impairment. Mechanick et al. (1997) investigated serum PTH and $1,25(OH)_2$ vitamin D_3 levels in SCI patients that were tested at a mean of 76.5 days post injury in a cross-sectional retrospective study and found that patients with complete SCI, when compared to those with incomplete injury, had a greater suppression of PTH-vitamin D axis. Dysfunction of this axis soon after SCI is unlikely to be involved in the pathogenesis of bone loss after the injury (Jiang et al. 2006b).

Long-standing SCI is associated with a significant depression of $1,25(OH)_2$ vitamin D_3 and PTH concentration (Vaziri et al. 1994). Persistent inhibition of PTH in individuals with chronic SCI seems to indicate that low-grade net bone resorption continued for many years. This may be caused by persistent reduction in the mechanical stresses, the direct action of $1,25(OH)_2$ vitamin D_3 at high concentration on parathyroid tissue and changes in cytokine regulation (Jiang et al. 2006b). Approximately one-third of chronic SCI patients showed secondary hyperparathyroidism with vitamin D deficiency (Bauman et al. 1995).

Vitamin D

Patients with SCI have been reported to have higher prevalence of vitamin D deficiency than able-bodied population (Stewart et al. 1982; Vaziri et al. 1994; Bauman et al. 1995; Maimoun et al. 2002, 2005, 2006; Mechanick et al. 2006). In acute SCI, $1,25(OH)_2$ vitamin D_3 levels were suppressed compared with controls by 66% (Maimoun et al. 2002).

Findings on vitamin D metabolites in chronic SCI patients are less consistent (Jiang et al. 2006a). Vitamin D deficiency was reported in persons with chronic SCI (Vaziri et al. 1994); but in fact, it seems that only $1,25(OH)_2$ vitamin D_3 was specifically altered. Afterward, despite plasma 25-hydroxy vitamin D (25[OH]D) values that tend to decrease with duration of injury, these values remained within reference range, indicating the presence of normal vitamin D store (Stewart et al. 1982; Mechanick et al. 1997; Roberts et al. 1998). Other studies demonstrated that a proportion of subjects with 25(OH)D defect was significantly greater in individuals with chronic SCI (32%) than in able-bodied population (16%) and that 25(OH)D levels were negatively correlated with PTH levels (Bauman et al. 1995). In fact, it is likely that 25(OH)D deficiency occurs more

often in severe cases, such as patients with quadriplegia hospitalized for pressure ulcers over a long period of time (Zhou et al. 1993). Conversely, for 1,25$(OH)_2$ vitamin D_3, concentrations are generally reduced (Stewart et al. 1982; Vaziri et al. 1994; Maimoun et al. 2002, 2005, 2006).

Reduced calcium and vitamin D intake would be expected to lower the serum calcium concentrations and stimulate the release of PTH, resulting in increased bone resorption and accentuation of osteopenia. In a randomized, placebo-controlled trial of 40 chronic SCI patients, a vitamin D analogue [1-alpha D(2)] was demonstrated to increase leg BMD 24 months after treatment, and urinary N-telopeptide, a marker of bone resorption, was significantly reduced during treatment 1-alpha D(2), but not in the placebo group (Bauman et al. 2005). Bauman et al. (1995) supplemented individuals with chronic SCI with 800 IU 1,25$(OH)_2$ vitamin D_3 per day over a 12-month period and found a doubling of vitamin D levels and one-third decrease in PTH levels.

Calcitonin

The calcitonin concentration in SCI patient was higher. This may represent a compensatory response to ongoing calcium reflex from the skeleton of the paralyzed structure. Regardless of its mechanism, an elevated endogenous calcitonin may help to mitigate the rate of resorption (Vaziri et al. 1994). Pietschmann et al. (1992) reported that level of injury had no impact on calcium concentration.

Growth Hormone

Insulin resistance may be a contributing factor leading to osteoporosis following SCI. In addition, growth factors and their second messengers, such as IGF-I, have been reported to be depressed in patients with chronic SCI (Jiang et al. 2006a). Shetty et al. (1993) reported that the average plasma IGF-I level in patients with tetraplegia was depressed when compared with ambulatory controls. However, Maimoun et al. (2006) did not demonstrate a role of growth factor in accelerated bone resorption following SCI.

Gonadal Hormones

The inhibitory effect of SCI on the synthesis and secretion of sex steroids contributes to pathogenesis of SCI-induced osteoporosis (Jiang et al. 2006a). The literature provides the conflicting data: there are subsets of SCI men with relative and absolute androgen deficiency (Naftchi et al. 1980; Nance et al. 1985; Maimoun et al. 2005). Maimoun et al. (2006) reported that total testosterone and free androgen index were significantly lower in SCI patients than able-bodied controls. No significant change in serum gonadotropin concentration was observed in SCI men by Wang

et al. (1992) and Tsitouras et al. (1995), while in another cross-sectional study, it was demonstrated that there was high prevalence of low serum gonadotropins and a delayed appearance of gonadotropin peak response tKo LH-releasing hormone (LHRH) in SCI men (Naderi and Safarinejad 2003). These studies suggest that SCI may suppress the hypothalamic-pituitary-testis axis at different levels, including the hypothalamus, the anterior pituitary gland, and the gonads. The endocrine abnormalities may be the mechanism contributing to the development after SCI (Jiang et al. 2006a).

Serum estrogen levels in SCI women are also significantly lower than in controls (Rosenquist 1950). An enhanced gonadotropin response to LHRH has been reported in a group of SCI women, indicating a hypothalamic disorder within the hypothalamus-pituitary-ovary axis (Huang et al. 1996). These studies suggest that there is a high prevalence of hypothalamus-pituitary-ovary axis disorders in SCI women, and these disorders may be involved in the pathogenesis of osteoporosis after SCI (Jiang et al. 2006a).

Leptin

SCI results in progressive loss of percentage of total lean body mass and increase in percentage of fat mass (Wilmet et al. 1995; Kaya et al. 2006; Singh et al. 2014). Plasma leptin concentration is markedly elevated in SCI patients compared with able-bodied controls (Huang et al. 2000; Hjeltnes et al. 2005; Wang et al. 2005; Maimoum et al. 2004). The increased plasma concentration of leptin in SCI patients and the accompanying augmented circadian variation might distort the normal turnover of bone tissue in SCI patients, leading to osteoporosis (Hjeltnes et al. 2005).

Thyroid Hormone

Serum T3 and T4 levels remain depressed in acute SCI patients (Bugaresti et al. 1992; Claus-Walker et al. 1977). After acute stress, there may also be associated changes in thyroid hormone binding that could lower serum thyroid hormone levels (Bermudez et al. 1975). Similarly, in chronic SCI patients, serum T3 and T4 were also reduced compared to controls (Cheville and Kirshblum 1995). Patients with tetraplegia had lower serum T3 levels than did those with paraplegia (Wang et al. 1992). These data suggest a thyroid disorder within the hypothalamic-pituitary-thyroid axis (Jiang et al. 2006a).

In the literature, many studies reported normal concentration of TSH in chronic SCI patients (Prakash 1983; Huang et al. 1996). Zeitzer et al. (2000) investigated the 24 h average and circadian amplitude of TSH rhythm in the chronic SCI patients and found that they were within the low end of normal range. These data suggest that a small decline in TSH amplitude in chronic SCI patients may be a contributing factor in the pathogenesis of osteoporosis (Jiang et al. 2006b).

Glucocorticoids

Hypercortisolism found in acute SCI patients may be therapeutic or stress related (Bugaresti et al. 1992). Therefore, glucocorticoids may contribute to bone loss following SCI. However, findings on the effect of chronic SCI on serum cortisol level are less consistent. The current balance of evidence does not support the idea that the changes in serum cortisol level may be a contributing factor in the pathogenesis of osteoporosis in chronic SCI patients (Jiang et al. 2006a).

Potential Application of Bone Turnover Markers to Prognosis, Other Disease, or Conditions

Osteoporosis is a major health problem worldwide, especially in postmenopausal women. The clinical consequences of osteoporosis reside in the fracture risk. Technological developments for the measurement of bone mineral density (BMD) have led to diagnostic criteria that are widely applied (Vasikaran et al. 2011).The World Health Organization diagnostic criterion for osteoporosis is a BMD measurement equal to or more than 2.5 standard deviations (SD) below the young female (age 20–29 years) reference mean (T-score ≤ -2.5 SD) (Kanis et al. 2008). There has been interest in the clinical potential of bone turnover markers, both as tools to assess fracture risk and for monitoring treatment, to thereby aid intervention strategies (Szulc and Delmas 2008; Vasikaran 2008; Bergmann et al. 2009).

Estrogen deficiency, associated with menopause, results in a generalized increase in bone remodeling and an imbalance between bone formation and resorption (Darby and Meunier 1981; Jilka 2003). Bone turnover marker levels are also influenced by factors that are not easily modified. In postmenopausal and elderly women, the major uncontrollable factors are diseases and associated bed rest and immobility, medications, nutritional status, and recent fractures (Szulc and Delmas 2008).Increased bone turnover marker levels, especially bone resorption, are found in the institutionalized elderly and in patients with chronic diseases associated with prolonged bed rest and limited mobility such as dementia, Alzheimer's disease, stroke, hemiplegia, and Parkinson's disease(Szulc and Delmas 2008).

High levels of bone turnover markers may predict fracture risk independently from bone mineral density in postmenopausal women. They have been used for this purpose in clinical practice for many years, but there is still a need for stronger evidence on which to base practice. Bone turnover markers hold promise in fracture risk prediction and for monitoring treatment (Vasikaran et al. 2011). We need more bone turnover markers reflecting the action of enzymes involved in the catabolism of bone matrix, e.g., cathepsin K, metalloproteinases. They would be useful to assess enzymatic mechanisms of bone matrix degradation and the efficacy of drugs acting on certain metabolic pathways, e.g., cathepsin K inhibitors (Szulc and Delmas 2008).

Summary Points

- This chapter focuses on the bone turnover after spinal cord injury.
- Osteoporosis is one of the most frequent complications following SCI, resulting from uncoupling of bone remodeling in favor of bone resorption.
- This imbalance takes place right after the initial injury and persists for many years.
- Development of severe osteoporosis in the paralyzed part of the body is accompanied by the loss of biomechanical strength, and the biosynthesis of structurally modified matrix unable to sustain normal mechanical stress. Therefore, the risk of fracture is dramatically increased.
- The exact pathophysiology of osteoporosis after SCI is still not clear. The pathogenesis of osteoporosis after SCI is generally considered disuse. Mechanical loading is known to be a crucial stimulus for bone formation and resorption, thereby controlling bone mass, structure, and strength.
- The factors that are independent of mechanical loading of the skeleton also appear to be important.
- Recent works suggest that bone remodeling is regulated by nerve-derived signals, sclerostin, and that there is a marked modification in the osteoprotegerin/RANKL system in recent SCI patients.
- Biochemical bone markers of bone turnover are shown to reflect dynamic skeletal status and may help to identify increased bone loss and fracture risk.
- An improved understanding of the natural history and risk factors for chronic bone loss following SCI is essential to designing therapies to reduce the rate of bone loss, define fracture risk, and ultimately prevent osteoporotic fractures and their associated morbidity.
- We are of the opinion that prospective randomized controlled trials should be conducted to evaluate, standardize, and find bone-specific biochemical marker of bone turnover in SCI.

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Bone Biomarkers Related to Osteoarthritis 43

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Contents

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Abstract

It is evident that the bone plays a vital role in osteoarthritis (OA) disease pathogenesis, progression, and symptomatology. The close interaction of the bone and cartilage in the pathogenesis of OA and the knowledge that OA is a disease of the whole joint provide a strong rationale for investigating bone biomarker changes in OA. The evaluation of bone biomarkers is important for gaining a greater understanding of the role of bone pathology in OA and a means for developing new diagnostic and prognostic tools for therapeutic developments and early OA intervention. Although comparisons among studies are difficult because different assays and assay parameters are used and different assays reflect different outcomes, many bone-related biomarkers have shown great promise as diagnostic, prognostic, and efficacy of intervention biomarkers for OA. These include the traditional bone biomarkers, CTX-I and NTX-I and osteocalcin. The strong association of these traditional bone biomarkers with urinary C-terminal telopeptide of type II collagen (CTX-II) from the articular cartilage confirms the strong association of bone resorption with cartilage degradation. To date, results using bone biomarkers in OA trials provide examples of the modifiability of the whole joint organ by bone-acting agents. Based on recent data, tartrate-resistant acid phosphatase 5b (TRAP5b), periostin, and endothelin-1 (ET-1) show great promise and can be considered new OA-related bone biomarkers. More studies are required in the context of treatment trials to determine which bone biomarkers will be most relevant for drug development and use in the clinic.

Keywords

Osteoarthritis • Bone • Biomarkers • Resorption • Formation • Turnover • Collagen • Subchondral • Articular

List of Abbreviations

ALP	Alkaline phosphatase
BIPEDS	Burden of disease, Investigative, Prognostic, Efficacy of intervention, Diagnostic or Safety biomarkers
BMD	Bone mineral density
BML	Bone marrow lesion
BSP	Bone sialoprotein
CT	Computed tomography
CTX and CTX-I	C-terminal telopeptide of type I collagen
CTX-II	C-telopeptide of type II collagen
DKK-1	Dickkopf WNT signaling pathway inhibitor 1
DMOADs	Disease-modifying OA drugs
Dpd	Deoxypyridinoline (also called lysyl-pyridinoline or LP)
ECLIA	Electrochemiluminescence immunoassay
ELISA	Enzyme-linked immunosorbent assay
ET-1	Endothelin-1
HYL-Pyr	Hydroxylysyl-pyridinoline (also called pyridinoline or HP)

ICTP	Carboxy-terminal telopeptide of type I collagen
IL-6	Interleukin-6
JSN	Joint space narrowing
KL-score	Kellgren and Lawrence grade (of radiographic severity of OA)
KOOS	Knee injury and osteoarthritis outcome score
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
NTX and NTX-I	N-terminal telopeptide of type I collagen
OA	Osteoarthritis
OC	Osteocalcin (intact protein indicative of bone formation; fragments of OA indicative of bone resorption)
OFELY	"Os des Femmes de Lyon," a longitudinal cohort study for assessing osteoporosis and secondary OA
PICP	Procollagen type I C-terminal propeptide
PINP	Procollagen type I N-terminal propeptide
PIIANP	N-propeptide of type IIA procollagen
POSTN	Periostin
S	Serum
SF	Synovial fluid
TRACP5b	Tartrate-resistant acid phosphatase 5b
WNT	Wingless-related integration site

Key Facts of Osteoarthritis

- Osteoarthritis (OA) is the most common form of arthritis and a principal cause of disability.
- The prevalence of OA is increasing due to an aging world population (especially Japan, Europe, and North America).
- OA pathogenesis can be characterized as having disease (molecular, pre-radiographic, radiographic) and illness (symptomatic) phases.
- OA is a whole joint disease characterized by degeneration of the cartilage, menisci, and ligaments, subchondral bone changes, osteophyte formation, and synovitis with significant cross talk between the cartilage and bone.
- A disease-modifying drug treatment for OA has yet to be approved.

Introduction

Osteoarthritis (OA) is one of the most common medical conditions confronting the society today. It is estimated that 27 million people in the United States are currently diagnosed with this disease (Lawrence et al. 2008). Increased life expectancy and obesity are driving a marked increasing incidence of OA. It is, therefore, natural to expect a concomitant rise in the direct and indirect costs of managing OA.

Currently, there are no disease-modifying OA drugs (DMOADs). There are however, palliative, illness-modifying treatments such as analgesics, physical therapy, and ultimately, joint replacement. One reason for this state of affairs is that application of DMOADs requires the ability to detect early stages of OA and monitor its progression. Although an OA diagnosis typically relies on clinical and radiographic features, half of the patients with radiographic OA do not exhibit symptoms and vice versa (Bijlsma et al. 2011).

Identifying biomarkers that characterize OA at early stages, such as during the prolonged molecular, pre-radiographic, or asymptomatic phase, before the development of irreversible anatomic degradation (radiographic abnormality) or illness (pain, functional deformity, gait abnormality), has become an important goal for advancing OA research and therapeutics (see Kraus et al. for summary of disease stages (Kraus et al. 2015)). The development of clinical symptoms and/or radiological observable changes in arthritic joints is preceded by an extensive process of biochemical and mechanical disturbances of the whole joint organ. By the time that cartilage damage occurs, the joint pathology is generally considered irreversible. Early intervention requires early disease indicators. Because of the differential adaptive capacity of the bone, bone changes in OA occur rapidly and are discernible more readily than cartilage abnormalities (Goldring and Goldring 2010), the "canary in the mine" phenomenon. For this reason they present quite attractive potential biomarkers for detecting OA early. This chapter will focus on summarizing the current and potential ability of bone biomarkers to fulfill roles as OA-related *B*urden of disease, *I*nvestigative, *P*rognostic, *E*fficacy of intervention, *D*iagnostic, or *S*afety (BIPEDS) biomarkers.

Pathology

OA has long been characterized as an upregulation and imbalance of degradation (catabolism) and repair (anabolism) of cartilage components (Aigner et al. 2007). Cartilage-degrading proteinases and chondrocyte apoptosis lead to a net breakdown of the extracellular matrix. In an attempt to repair, clusters of chondrocytes form in and around damaged areas of the cartilage during early stage degeneration (Aurich et al. 2005). Both catabolic and anabolic cartilage biomarkers have been evaluated in OA, including the C-terminal telopeptide of type II collagen (CTX-II) (Valdes et al. 2014) and the N-propeptide of type IIA collagen (PIIANP) (Rousseau et al. 2004), respectively.

Role of Bone in OA

Although bone alterations on radiographs have been definitively associated with OA for over 50 years (Kellgren and Lawrence 1957), it has only recently been widely accepted that OA is a disease of the whole joint organ involving, in addition to progressive degradation of cartilage, alterations of the synovium, menisci, ligaments,

and subchondral bone (Bijlsma et al. 2011) (see Fig. 1). Bony features reflecting OA include subchondral bone sclerosis, bone marrow lesions (BMLs), bone cysts, osteophytes (Sharma et al. 2013; Hunter et al. 2014; Hunter et al. 2011), and altered bone trabecular integrity (Kraus et al. 2009; Kraus et al. 2013). Increased subchondral bone resorption occurs at an early stage in the development of OA (Funck-Brentano and Cohen-Solal 2015). A range of investigations has shown positive effects on cartilage health when bone resorption is suppressed (Karsdal et al. 2014) confirming the role of bone factors in the cross talk between the cartilage and bone and the pathogenesis of OA (Table 1).

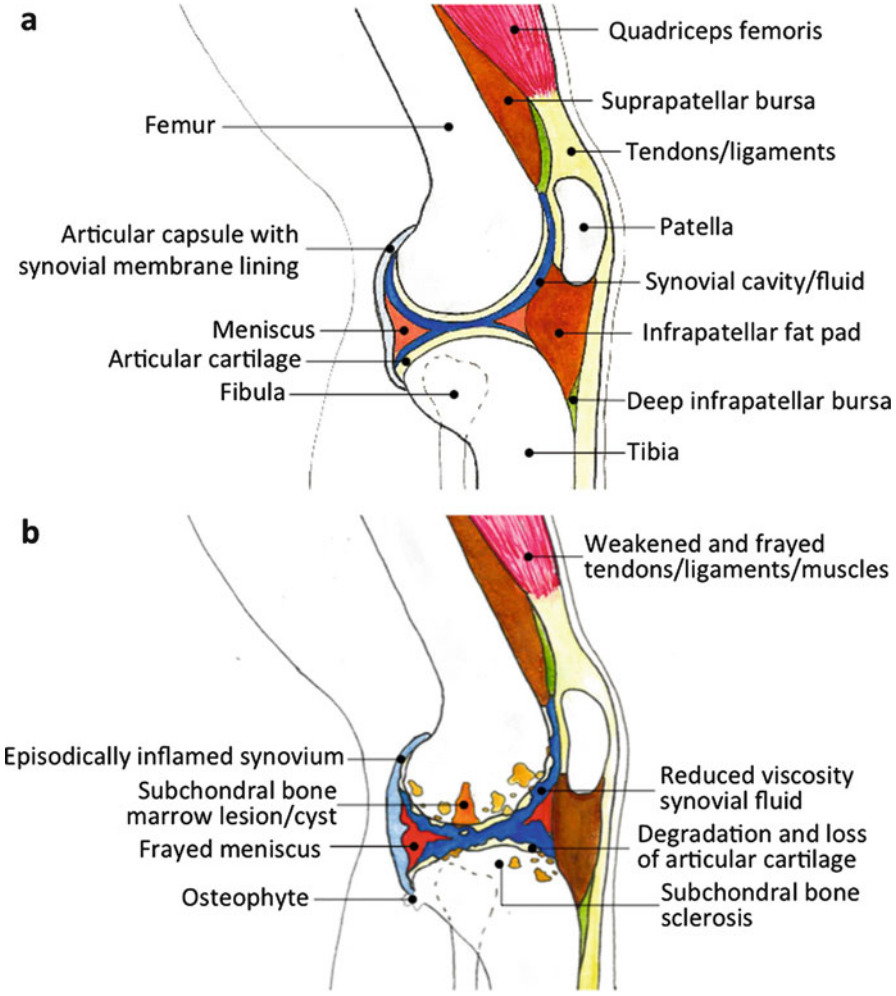

Fig. 1 Diagrammatic overview of osteoarthritis pathology. Sagittal view of the knee joint. (**a**) normal joint with joint structures labeled; (**b**) OA joint with osteoarthritis features indicated demonstrating pathology of the whole joint organ (Courtesy Hiskia Pepijn Jansen)

Table 1 Markers with a relationship to the bone and a putative role in osteoarthritis

Biomarker	Protein of origin	Relationship to the bone	Current BIPEDS[a] biomarker category for osteoarthritis
PINP	Collagen type I	Bone formation	B, P, D (↑ in OA)
PICP	Collagen type I	Bone formation	I (?↑ in early OA)
CTX-I	Collagen type I	Cathepsin K and MMP released indicator of bone resorption	P, E (↑ in OA)
ICTP	Collagen type I	MMP released indicator of bone resorption	P (↑ in hip OA)
NTX-I	Collagen type I	Bone resorption	P, E (↑ in OA)
Hyl-Pyr	Collagen of the bone, cartilage, and other tissues	Bone resorption	I (↑ in OA)
DPD	Collagen type I of the bone	Bone resorption	I (↑ in OA)
CTX-II	Collagen type II	Cartilage degradation	D, P, E (↑ in OA)
OC	Non-collagenous	Bone formation↓	B, P, E (↑ in OA)
Periostin	Non-collagenous	Non-collagenous	I (↑ in OA subchondral bone)
IL-6	Cytokine	Bone resorption	D, E (↑ in OA)
Leptin	Adipokine	Bone resorption (centrally mediated); bone formation (peripherally mediated)	D, E (↑ in OA)
ET-1	Vasoconstrictor induced by cytokines	Bone formation	I (↑ in OA)
BSP		Bone formation and mineralization	I (↑ in OA)
Dkk-1	Non-collagenous	Bone resorption by inhibiting osteoblast development	I (↓ in OA)
Trap5b	Protease	Bone-degrading enzyme	I (↑ in OA)

[a]BIPEDS = burden of disease, investigative, prognostic, efficacy of intervention, diagnostic, safety as described in Bauer et al. (2006) and Kraus et al. (2011); for names of biomarkers, see list of abbreviations

Subchondral bone marrow edema-like lesions (BMLs) and cyst-like lesions are common findings in OA. BMLs are associated with both clinical symptoms and signs, including pain and OA progression (Xu et al. 2012; Zhang et al. 2011). BMLs evolve into subchondral cyst-like lesions suggesting that BMLs could be a precystic phase or that BMLs facilitate formation of cysts (Crema et al. 2010).

Osteophyte formation is a clear indicator of radiographic OA and is a major determinant of the Kellgren and Lawrence (KL) grade, the most commonly used system for grading radiographic OA severity (Hart and Spector 1995). Even though it is still often unclear whether the bone abnormalities develop independently or secondary to articular cartilage degradation, and whether the abnormalities are

caused by an altered mechanical or biochemical environment, it is evident that the bone plays a vital role in OA disease pathogenesis, progression, and symptomatology.

Biomarkers of Collagen Type I

Collagen type I is the most abundant organic component of the bone constituting 95% of the bone collagen. Therefore, various epitopes of collagen type I are a natural choice for following changes in bone metabolism in OA. However, collagen type I is also found, but to a lesser extent, in the extracellular matrix of other tissues like the skin, dentin, cornea, vessels, and tendons (Lombardi et al. 2012), so it is important to qualify these biomarkers in the context of OA to determine their utility.

Markers of Synthesis

When a triple helical form of the collagen precursor is secreted to the extracellular matrix by osteoblasts, the procollagen protein is cleaved of its C- and N-terminal propeptides whose concentrations in the circulation reflect synthesis of collagen type I and, therefore, bone synthesis. The propeptides can be detected in serum using sandwich enzyme-linked immunosorbent assays (ELISAs) or electrochemiluminescence immunoassays (ECLIAs) (Kumm et al. 2013; He et al. 2013). Although collagen type I propeptides may also arise from other tissues (such as the skin, vessels, fibrocartilage, and tendons), most nonskeletal tissues exhibit a slower turnover than the bone and contribute very little to the circulating pool of procollagen type I N-terminal propeptide (PINP) (Research 2015). In a 6-year study of knee OA, PINP exhibited both prognostic and diagnostic potential, especially for progressive osteophytosis; OA progression was preceded by enhanced bone formation, reflected in elevated PINP (Kumm et al. 2013). Interestingly, the associations were strongest for the first 3 years of follow-up suggesting that this biomarker is most informative during the early radiographic stages of OA. Kumm et al. chose not to use KL grades but rather a system described by Nagaosa et al. (Nagaosa et al. 2000) that scores joint space separately from osteophyte; they suggest this scoring system has advantages for identifying early stages of OA. Two prior studies utilizing KL grades identified positive trends but not significant associations of PINP with an OA diagnosis (Davis et al. 2007) and paradoxically, reduced OA progression (Berry et al. 2010). Although these results would appear contradictory to Kumm et al., possibly different forms of PINP were detected in the different studies. PINP can be detected in the circulation as two different forms, the "intact" or trimeric molecule and as a monomer. In osteoporosis subjects with normal renal function, the predominant form of PINP detected in circulation is the trimeric form. However, monomeric PINP fragments may accumulate in patients with renal failure or metastatic bone disease (Research 2015; Koivula et al. 2012).

In addition, the study by Berry et al. used non-fasting samples, and serum PINP levels have been shown to vary modestly (4%) but significantly depending on fed versus fasting status (Clowes et al. 2002).

Despite the fact that the procollagen type I C-terminal propeptide (PICP) is a widely used bone turnover biomarker, much like PINP, little research has been done to evaluate PICP as an OA biomarker. A study in 2002 showed no association of synovial fluid PICP levels with grade of OA severity by the Outerbridge and Neyes classification system; however samples were obtained after a 50 ml saline lavage of the joint (Schmidt-Rohlfing et al. 2002). As a result, neither of these procollagen peptides have been researched extensively as bone synthesis biomarkers for OA. Nevertheless, PINP shows potential for use as a burden of disease and prognostic biomarker in OA. PICP and PINP behave as independent proteins with different liver and lymph clearance (Koivula et al. 2012). PINP from soft as opposed to hard (bone) tissues generally reaches the circulation indirectly via the lymph, while changes in lymph flow do not detectably affect the circulating concentration of PICP. This suggests that the contribution of soft tissues to the circulating concentration of PINP is normally quite small. Based on the fact that serum PINP has been recommended as the preferred bone synthesis reference marker for osteoporosis, further research of its particular utility as an OA biomarker would appear to be warranted.

Markers of Degradation

Collagen type I is degraded by various proteins, implying that various degradation products can be found in either serum or urine. Matrix metalloproteinases (MMPs), like MMP-8, can degrade collagen type I. Cathepsin K, involved in systemic bone resorption, can also degrade collagen type I (Fardellone et al. 2014).

The cross-linked C-telopeptide of collagen type I (CTX-I) is a preferred biomarker for monitoring bone resorption of osteoporosis (Vasikaran et al. 2011). There are a number of carboxy-terminal telopeptide epitopes of collagen type I (CTX-I) in serum and urine as well as various assays specific to each of the four possible isomers of CTX: alpha-L, beta-L, alpha-D, and beta-D, whereby alpha and beta refer to the non-isomerized and isomerized forms of aspartate, respectively, and L and D refer to non-racemized and racemized forms of aspartate, respectively (Cloos and Fledelius 2000). Baseline serum concentrations of CTX (beta isomers) have been associated with an increase in BML scores over 2 years (Pelletier et al. 2010). A recent paper by Heubner et al. showed a strong correlation between joint space narrowing (JSN) and osteophyte progression with urinary alpha CTX, the non-isomerized form of CTX indicative of turnover of new bone (Huebner et al. 2014). The same study showed a correlation between knee periarticular bone turnover, demonstrated by bone scintigraphy, and urinary alpha CTX. In a study of 600 knee OA subjects from the osteoarthritis initiative, higher baseline urinary alpha CTX-I and higher 12- and 24-month time-integrated concentrations of urinary alpha CTX-I and serum CTX-I predicted the risk of the

combination of pain and radiographic OA worsening over 4 years (Kraus et al. 2016). These results underscore a strong association of bone remodeling and OA progression.

In a 16-week study on the effect of weight loss in obese OA patients, serum (beta isomeric) CTX-I increased significantly and in proportion to the amount of weight loss, but the change in CTX-I was not associated with the change in patient-reported outcomes (quantified by four items of the knee injury and osteoarthritis outcome score (KOOS-4)) (Bartels et al. 2014).

Another carboxy-terminal cross-linked telopeptide of type I collagen includes ICTP. The generation of ICTP and CTX depends on different collagenolytic pathways; cathepsin K releases CTX but not ICTP, while matrix metalloproteinases release both carboxy-terminal epitopes (Garnero et al. 2003). There are also assays recognizing ICTP. In 2005, Berger et al. found higher levels of ICTP in the bone but not serum in association with rapidly destructive hip OA, compared to healthy controls (Berger et al. 2005). Since then, not much research has been done evaluating ICTP as a biomarker for OA.

Cross-linked N-telopeptide of collagen type I (NTX-I) is much like CTX-I and is commonly used as a biomarker of bone resorption. Urinary NTX-I may be preferred to serum or plasma NTX-I in a clinical setting because it is not as sensitive to circadian changes and is not affected by food intake (Baxter et al. 2013). Recently, urinary NTX-I concentrations did not show significant differences by differing severities of radiological knee OA by KL grade in a large cohort of 1200 subjects (Tanishi et al. 2014). However, the higher quartiles of the uCTX-II and uNTX-I levels gradually included higher numbers of grade $\geq$2 OA subjects in women over 60-year-old, suggesting a possible diagnostic value of NTX-I in women. Similarly, in another study of women, urinary NTX was not associated with any radiographic knee OA phenotypes (Blumenfeld et al. 2013).

In a UK study of 216 postmenopausal women not receiving any bone-modifying medication who had a baseline knee radiograph and a repeat radiograph 4 years later, higher baseline urinary NTX-I was associated with risk for radiographic progression of knee OA (Bettica et al. 2002). The UK study results contradict the findings of Berry et al. who reported a significant association between higher bone resorption markers, CTX-I and NTX-I, and reduced cartilage loss in a subgroup of subjects with high bone formation (as demonstrated by PINP) (Berry et al. 2010). However, the UK findings agree with results of a study of 600 knee OA subjects from the osteoarthritis initiative, wherein higher 12- and 24-month time-integrated concentrations of urinary and serum NTX-I predicted the risk of the combination of pain and radiographic knee OA worsening over 4 years with a similar trend for baseline NTX-I concentrations (Kraus et al. 2016). Baseline serum NTX has also been associated with a modest risk of incident and progressive radiographic hip OA (Kelman et al. 2006). The same group found that change in NTX-I over 6 years was not significantly correlated with concurrent radiographic hip OA progression (Chaganti et al. 2008).

As collagen is degraded by osteoclast-derived proteases, non-reducible collagen cross-links are released into the bloodstream and urine. These molecules, called

hydroxylysyl-pyridinoline (HYL-Pyr) or deoxypyridinoline (Dpd), are believed to originate from different tissues. Whereas HYL-Pyr is mainly derived from the cartilage (also bone, tendon, and vessel connective tissue), Dpd is derived almost exclusively from the bone and dentin (Lombardi et al. 2012). Overall, there have been few publications of these biochemical markers in human OA (van Spil et al. 2010). A study in the guinea pig model of knee OA, comparing a control strain (non-OA Strain 13) to an OA susceptible strain (Hartley), showed that the Hartley strain had a higher rate of bone formation (based on sOC) and bone resorption (based on uHyl-Pyr and uDPD) at a young age with persistence of a greater rate of bone formation at 12 months of age (Huebner et al. 2002). The research on the relationship between free urinary HYL-Pyr and Dpd and OA has seemingly halted over the last decade. This may be the result of a paper written in 2003 that found no significant correlations in a 3-year follow-up study of knee OA using these biomarkers (Bruyere et al. 2003).

A study in the context of acute trauma to the anterior cruciate ligament showed increased levels of collagen type I and II degradation markers (CTX, NTX, CTX-II) in synovial fluid. In turn, these levels correlated with serum levels (Catterall et al. 2010). Knee trauma in adolescence is a well-known risk factor for the later development of OA (Lohmander et al. 2007) suggesting that these changes in collagen metabolism may be early triggers of OA. Connecting collagen type I-derived biomarkers in serum or urine directly to clinical and/or radiological OA scores at the joint level however has been difficult. Thus, in general the bone collagen-related biomarkers may be useful for predicting incident OA, particularly incited by trauma, as well as progression of OA (Tables 2 and 3).

Other Collagenous Biomarkers

Collagen Type II

Collagen type II makes up 80–95% of all protein in the articular cartilage. It is therefore a logical biomarker for following cartilage metabolism. Considering the obvious role of cartilage degradation in OA pathophysiology, degradation products of collagen type II have shown great promise as OA biomarkers.

Although this might not seem directly relevant to a discussion of biomarkers of the bone, Eyre and Lohmander have suggested that uCTX-II probably originates mostly from osteoclast-degraded mineralized collagen type II. Supporting a mineralized tissue source for uCTX-II are data showing that levels are very low in individuals with pycnodysostosis compared with age-matched controls (Lohmander and Eyre 2008). Pycnodysostosis is a rare lysosomal storage disease of the bone caused by mutation of the gene encoding the enzyme cathepsin K involved in bone resorption wherein osteoclasts function normally to demineralize the bone but do not adequately degrade the organic matrix.

Support for alterations of bone collagen type I metabolism involvement in increased cartilage degradation was provided by a study of subjects with

Table 2 An overview of mentioned studies regarding type 1 collagen-derived biomarkers

Marker	Study	Assay used	Main outcome	BIPEDS category	OA site	Source
PINP	Berry et al. 2010	UniQ™ PINP RIA; Orion Diagnostica	High levels of PINP are associated with low cartilage loss (P = 0.02, n = 117)	Diagnostic	Knee	Serum
	Davis et al. 2007	Electrochemiluminescence immunoassay, Roche	No significant correlation with KL-score, sclerosis, or joint space width was found (n = 119)	–	Knee	Serum
	Kumm et al. 2013	Electrochemiluminescence immunoassay, Roche	Over the first 3 years of follow-up, significant prognostic and diagnostic value was found (n = 35, P = 0.005 and n = 33, P = 0.046); only a prognostic value for the last 3 years (n = 22, p = 0.022)	Prognostic/ diagnostic	Knee	Serum
PICP	Schmidt-Rohlfing et al. 2002	ELISA kit; Metra Biosystems, MB 004	PICP in lavage synovial fluid showed no correlation with disease severity (n = 72)	–	Knee	Synovial fluid
NTX	Bettica et al. 2002	NTx, Osteomark; Ostex	Bone resorption is increased in progressive OA and not in nonprogressive OA in postmenopausal women (n = 216, P < 0.001)	Diagnostic	Knee	Urine
	Kelman et al. 2006	Osteomark NTx	NTX associated with an increased risk of incident radiographic hip OA (adjusted OR of 1.38 per SD increase in NTX (95 % CI 1.07–1.79), n = 397)	Prognostic	Hip	Serum
	Chaganti et al. 2008	ELISA kit, Osteomark	No significant concurrent association of changes in serum NTX over 6 years and radiographic hip OA progression (n = 677)	–	Hip	Serum
	Berry et al. 2010	Serum Osteomark NTx, Inverness Medical	High levels of NTX are associated with low cartilage loss (P = 0.02, n = 117)	Diagnostic	Knee	Urine
	Blumenfeld et al. 2013	Osteomark NTx ELISA kit, Inverness Medical Innovations	NTX not significantly associated with any OA phenotypes of radiographic knee OA (n = 1007 women only)	–	Knee	Urine

(*continued*)

Table 2 (continued)

Marker	Study	Assay used	Main outcome	BIPEDS category	OA site	Source
	van Spil et al. 2013	Osteomark NTx Urine, Wampole laboratories	NTX was strongly associated with uCTX-II ($n = 1002$, $p < 0.005$)	–	Hip & knee	Urine
	Tanishi et al. 2014	Osteomark NTx ELISA kit, Inverness Medical Innovations	No significant difference between OA groups by KL-score ($n = 1200$) but highest quartile of subjects based on uNTX and uCTXXII were enriched for more severe OA (KL-score ≥ 2)	Diagnostic	Knee	Urine
	Kraus et al. 2016	Competitive inhibition ELISA, ALERE-Osteomark (Inverness Medical)	Time-integrated concentration over 12 and 24 months of both serum and urine NTX predicted combined pain and radiographic structural progression of OA over 4 years ($n = 600$)	Prognostic	Knee	Serum and urine
CTX	Pelletier et al. 2010	Serum CrossLaps ELISA, IDS Ltd	Serum values of CTX is predictive of a higher BML score ($n = 116$, $P = 0.02$)	Prognostic	Knee	Serum
	Berry et al. 2010	Serum CrossLaps® ELISA, IDS	High levels of CTX are associated with low cartilage loss ($P = 0.01$, $n = 117$)	Prognostic	Knee	Serum
	van Spil et al. 2013	Urine CrossLaps EIA, IDS	uCTX was strongly associated with uCTX-II ($n = 1002$, $p < 0.005$)	–	Hip and knee	Urine
	Bartels et al. 2014	Urine CrossLaps® EIA, IDS	CTX-I increased significantly with weight loss ($P = 0.005$, $n = 192$); CTX-I change was not associated with the changes in KOOS-4 score ($n = 192$)	–	Knee	Urine
	Huebner et al. 2014	Sandwich ELISA, Nordic Bioscience	αCTX levels were correlated with the progression of JSN and osteophytes, with the intensity of bone scintigraphic uptake ($n = 117$, $P = 0.041$)	Prognostic	Knee	Urine
	Kraus et al. 2016	Serum CrossLaps® ELISA, IDS	Time-integrated concentration over 12 and 24 months predicted combined pain and radiographic structural progression of OA over 4 years ($n = 600$)	Prognostic	Knee	Serum

	Kraus et al. 2016	Competitive inhibition ELISA, alpha and beta CrossLaps (CTX-I), IDS	Baseline CTXIα (resorption of the new bone) and time-integrated concentration over 12 and 24 months CTXIα and CTX-1β (resorption of the older bone) predicted combined pain and radiographic structural progression of OA over 4 years (n = 600)	Prognostic	Knee	Urine
ICTP	Berger et al. 2005	Radioimmunoassay, Orion Diagnostica (FIN-02101 Espoo, Finland)	Increased ICTP in OA bone but not serum of subjects with rapidly destructive hip OA compared with controls (n = 47)	Diagnostic	Knee	Serum
HYL-Pyr	Bruyere et al. 2003	Pyrilinks® competitive enzyme immunoassays (EIA), Metra Biosystems	No significant correlations found between this marker and joint space width or WOMAC score at baseline or when comparing the change after 3 years (n = 76)	–	Knee	Urine
Dpd	Bruyere et al. 2003	Pyrilinks-D™ competitive enzyme immunoassays (EIA), Metra Biosystems	No significant correlations found between this marker and joint space width or WOMAC score at baseline or when comparing the change after 3 years (n = 76)	–	Knee	Urine

Table 3 An overview of the mentioned studies regarding biomarkers not derived from type I collagen

Marker	Study	Assay used	Main outcome	BIPEDS category	OA site	Source
OC	Bruyere et al. 2003	ELSA-OSTEO radioimmunoassay (IRMA), CIS Bio International	One year changes in serum OC were correlated with 3 year progression by mean and minimal joint space width ($P = 0.04$ and $P = 0.01$ respectively, $n = 76$)	Prognostic	Knee	Serum
	Kalichman and Kobyliansky 2010	ELSA-OSTEO radioimmunoassay (IRMA), CIS Bio International	OC associated with the number of affected joints determined by KL grade, JSN, and osteophyte severity ($P = 0.015$, $P = 0.025$ and $P = 0.029$, respectively, $n = 550$)	Burden of disease	Hand	Plasma
	Kumm et al. 2013	U-MidOC assay as described by Ivaska et al. 2005 sOC electrochemiluminescence immunoassay, Roche	Increased urinary MidOC associated with an increased risk of OA progression by tibiofemoral osteophytosis ($n = 161$); to lesser extent serum OC also was also positively correlated with OA progression ($n = 161$)	Diagnostic	Knee	Urine/ serum
	Kumahashi et al. 2015	Multiplex Human Bone Panel MSD immunoassay	OC correlated with C2C during the first 33 days after knee injury ($n = 71$ injured, $n = 8$ controls)	Diagnostic	Knee	Synovial fluid
Periostin	Honsawek et al. 2015	Sandwich enzyme-linked immunosorbent assay, R&D Systems	Positive correlation of periostin in plasma and synovial fluid with radiographic severity ($P < 0.001$, $n = 110$)	Diagnostic	Knee	Plasma/ synovial fluid
	Rousseau et al. 2015	Sandwich ELISA assay, USCN	At baseline serum periostin was significantly lower in female patients with a KL-score ≥ 2 than those with a KL-score <2($P = 0.002$, $n = 83$)	Diagnostic	Knee	Serum
IL-6	Livshits et al. 2009	Ultrasensitive ELISA, BioSource, Nivelles, Belgium	IL-6 was higher in OA than control and higher concentrations predicted incident knee OA 5 years later ($n = 429$)	Prognostic	Knee	Serum

	Stannus et al. 2010a	Solid-phase, two-site chemiluminescent enzyme immunometric assay, Immulite IL-6 (EURO/DPC Llanberis, Gwynedd, UK)	IL-6 was associated with increased medial tibiofemoral joint space narrowing and predicted loss of both medial and lateral tibial cartilage volume, and change in IL-6 was associated with increased loss of medial and lateral tibial cartilage volume (n = 172)	Diagnostic, prognostic	Knee	Serum
	Stannus et al. 2010b	Solid-phase, two-site chemiluminescent enzyme immunometric assay, Immulite IL-6 (EURO/DPC Llanberis, Gwynedd, UK)	Serum IL-6 associated with hip JSN in females (N = 193)	Diagnostic	Hip	Serum
	Orita et al. 2011	IL-6 sandwich ELISA, R&D Systems	Synovial fluid IL-6 negatively correlated with KL grade and WOMAC score (n = 50)	Diagnostic	Knee	Synovial fluid
	Rubenhagen et al. 2012	Multiplex immunoassay 8-plex, R&D Systems	No correlation found with OA by KL-score (n = 82)	–	Knee	Synovial fluid
	Huebner et al. 2016	Sandwich ELISA assay, MesoScale Discovery	Weight loss was responsible for 54 % of the reduction in IL-6	Efficacy of intervention	Knee	Serum
Leptin	Stannus et al. 2010b	Radioimmunoassay LINCO Research; now part of Millipore	Leptin associated with JSN in women (P = 0.024 and P = 0.002, respectively, n = 193)	Diagnostic	Hip	Serum
DKK-1	Lane et al. 2007	Goat antihuman Dkk-1 capture ELISA, R&D Systems	Higher levels of Dkk-1 were associated with diminished risk of OA progression in elderly women (P = 0.007, n = 701)	Diagnostic, prognostic	Hip	Serum
	Honsawek et al. 2010	Sandwich ELISA, Quantikine, R&D Systems	Plasma and synovial fluid (SF) Dkk-1 both inversely correlated with radiographic severity; both correlated (SF < plasma); and plasma DKK-1 was lower than control (n = 35 OA, 15 controls)	Diagnostic	Knee	Plasma, synovial fluid

(*continued*)

Table 3 (continued)

Marker	Study	Assay used	Main outcome	BIPEDS category	OA site	Source
TRAP5b	Nwosu et al. 2016	Immunoenzymatic assay (BoneTRAP), IDS	Serum TRAP5b (detects the active isoform from osteoclasts indicating the process of bone resorption) associated with knee pain, subchondral sclerosis, and predictive of knee pain worsening	Diagnostic, prognostic	Knee	Serum
ET-1	Nahir et al. 1991	Radioimmunoassay	Synovial fluid ET-1 higher in OA than serum ET-1 in controls and similar to synovial fluid from rheumatoid arthritis (n = 7 OA, n = 11 RA)	Diagnostic	Knee	Synovial fluid
BSP	Conrozier et al. 1998	Inhibition ELISA (noncommercial)	Inverse correlation of BSP with severity of osteophyte and sclerosis (N = 48)	Diagnostic	Knee	Serum
	Otterness et al. 2000	ELISA	No difference between OA and controls (n = 40)	–	Knee	Serum

osteogenesis imperfecta (a family of collagen type I mutations giving rise to fragile bones and fractures). In this study, patients with osteogenesis imperfecta had high uCTX-II levels similar to those of subjects with knee OA. In this same study, patients with knee OA had high ratios of alpha/beta uCTX-I (a marker indicating low collagen type I maturation) that were also associated with higher uCTX-II levels (Rousseau et al. 2010). A study by van Spil et al. has also made a strong argument regarding the relationship of uCTX-II and bone resorption (van Spil et al. 2013). In a study of the CHECK cohort (1002 knee and hip patients) with early OA, uCTX-II was more strongly associated with the bone markers (uCTX-I, uNTX-I, sPINP, and sOC) than with other cartilage markers, and both uCTX-II and the bone markers showed an abrupt increase ("menopausal shift") in women aged 48–53 years. This could suggest a cross talk between the cartilage and subchondral bone, or, as van Spil et al. suggest, this biomarker could represent osteoclastic resorption of the calcified cartilage. Further investigation is needed on the exact origin of this epitope to gain a clearer appreciation and understanding of the insights it provides in OA.

Non-collagenous Biomarkers

Osteocalcin

Osteocalcin (OC), a non-collagenous and vitamin K-dependent protein, plays an active role in the organization of the extracellular matrix. Osteocalcin is synthesized by osteoblasts, odontoblasts, and hypertrophic chondrocytes (Hauschka et al. 1989). Its exact function remains unclear but it is widely regarded as a bone formation marker. In vitro, subchondral OA osteoblasts have been shown to synthesize more osteocalcin compared to normal osteoblasts (Couchourel et al. 2009).

Three-year radiological progression of knee OA could be predicted by a 1-year increase in serum OC (Bruyere et al. 2003). Plasma OC has been associated with severity of hand OA based on KL grade and osteophyte grade (Kalichman and Kobyliansky 2010). These two studies suggest that OC has prognostic and burden of disease value for knee and hand OA, respectively. One study reported no difference in serum OC comparing advanced hip OA patients sampled just prior to joint replacement and labeled "controls" in contrast to patients with osteonecrosis of the femoral head just prior to joint replacement (Floerkemeier et al. 2012). Interestingly, synovial fluid OC correlated with synovial fluid C2C, a type II collagen-specific biomarker, during the first month after severe knee injury (Kumahashi et al. 2015). This observation is indicative of a simultaneous alteration in the bone and cartilage turnover in the earliest phases after joint injury suggesting that these biomarkers may provide means of monitoring possible development of posttraumatic OA.

The main route of circulating OC catabolism is renal filtration and degradation. Fragmented but not intact forms of OC have been found in urine (Ivaska et al. 2005). Ivaska et al. developed three OC immunoassays (urinary MidOC, LongOC, and TotalOC) to detect and quantify various molecular forms of urinary OC. In contrast to intact OC, which is a bone formation marker, fragments of OC are considered bone

resorption markers. High concentrations of all three analytes were negatively correlated with bone mass, and all three correlated with bone turnover rates assessed by conventional serum markers, CTX-I, bone alkaline phosphatase (ALP), TRACP5b, and total OC. No clear evidence for qualitative differences among the analytes was observed in different clinical samples of elderly women distinguished by different bone turnover rates. Higher urinary MidOC and serum OC were both subsequently associated with risk of knee osteophyte progression (Kumm et al. 2013). Among the bone markers tested (serum PINP, CTX-I, and OC), MidOC was the strongest risk predictor of progressive osteophytosis. Taken together, these results suggest that OC and urinary MidOC show the greatest promise for use in OA.

Periostin

Periostin has only recently gained the attention of OA researchers. Like osteocalcin, periostin is also a vitamin K-dependent factor. Periostin, also called osteoblast-specific factor-2, is an integrin-binding protein, well known in the oncology field because of its involvement in cell adhesion and migration (Wong et al. 2013) and implicated in bone healing. A transcriptomic study by Chou et al. showed that the POSTN gene, which encodes periostin, was upregulated 15.65-fold in OA subchondral bone as compared to non-OA bone (Chou et al. 2013b). Another study in humans and one in rats showed similar results (Chou et al. 2013a; Zhang et al. 2012). Increased synovial and plasma periostin levels were correlated with radiographic severity of knee OA (Honsawek et al. 2015). Oddly enough, serum levels of periostin seemed to show an opposite effect. Periostin levels were lower in women with prevalent OA and in OA progressors compared to controls in the all-female, "Os des Femmes de Lyon" (OFELY) cohort (Rousseau et al. 2015). These two studies used a different sandwich ELISA and a different sample type to measure periostin levels. More research will be needed to clarify these seemingly contradictory results.

Cytokine-Related Factors

An increasing evidence is emerging with regard to the role of inflammation in the pathogenesis of OA (Orlowsky and Kraus 2015). Dysregulation of the complement system is proven to play an important role (Wang et al. 2011). Obesity and diabetes, both risk factors of OA, are often associated with systemic inflammation, especially when accompanied by metabolic syndrome. Subchondral plate bone loss is, in fact, associated with hypertension and type 2 diabetes mellitus (Wen et al. 2013). Below a role of certain cytokines that share a close relationship with the bone tissue in OA is described.

Interleukin-6 (IL-6) plays a pro-inflammatory role in OA, and in the presence of IL-1beta, TNF-alpha, and interferon gamma, expression is increased in chondrocytes (Bender et al. 1990; Guerne et al. 1990). Aside from its influence on chondrocytes and the cartilage, IL-6 induces bone resorption by acting as a stimulus to osteoclast

development (Manolagas and Jilka 1995). In OA, IL-6 levels exhibit conflicting results. In an animal (guinea pig) model of knee OA, serum IL-6 levels were higher in the OA group than controls (Huebner and Kraus 2006). In humans, synovial fluid (SF) IL-6 levels have been both negatively correlated (Orita et al. 2011) and not correlated (Rubenhagen et al. 2012) with knee OA severity based on KL grade. IL-6 was associated with increased medial tibiofemoral joint space narrowing and predicted loss of both medial and lateral tibial cartilage volume, and change in IL-6 was associated with increased loss of medial and lateral tibial cartilage volume (Stannus et al. 2010a). In women with hip OA, serum IL-6 correlated with joint space narrowing but not with the presence of osteophytes (Stannus et al. 2010b). In the Chingford female cohort, serum IL-6 was higher in OA than control and higher concentrations predicted incident knee OA 5 years later (Livshits et al. 2009). In the OA LIFE clinical trial in knee OA ($n = 129$ subjects with available longitudinal serum samples), the combination of pain coping skills training and lifestyle behavioral weight management for 6 months significantly reduced inflammatory markers including sIL-6, serum high sensitivity CRP, and leptin (Huebner et al. 2016). Reductions in leptin and IL-6 were significantly correlated with reductions in weight, BMI, and WOMAC pain; reductions in IL-6 were correlated with improvements in physical function. Weight loss was responsible for 54% of the change in IL-6. There are a number of possible explanations for mixed IL-6 results. For example, circadian rhythm could be influencing fluctuations in IL-6 levels. Modest sleep deprivation increases serum IL-6 (Mabey and Honsawek 2015). The clinical trial results with serum IL-6 suggest that the inflammatory state in OA can be monitored and successfully modified.

Leptin is an adipokine that influences bone metabolism by both a central pathway, via the hypothalamus, and a peripheral pathway, via osteoblast and bone marrow mesenchymal stem cells (Chen and Yang 2015). Leptin-deficient and leptin receptor-deficient mice have increased bone mass (Ducy et al. 2000). Central leptin seems to promote bone resorption, while peripheral leptin has a bone protective effect (Chen and Yang 2015). A role for leptin in OA was first suggested in 2003 when increased levels of leptin were observed in OA synovial fluid and cartilage (Dumond et al. 2003). In the same study, the level of cartilage destruction was also correlated with increased leptin levels. Leptin expression was also found in various OA joint tissues such as synovial membranes, osteophytes, and infrapatellar fat tissues (Gegout et al. 2008). Interestingly, in osteophyte explants, the adiponectin/leptin ratio was much higher compared to the other investigated tissues, suggesting a high level of leptin production in osteophytes. In subchondral osteoblasts of OA patients, leptin protein production was increased twofold and, gene expression, fivefold compared to non-OA osteoblasts (Mutabaruka et al. 2010). JSN grade correlated positively to serum concentration of leptin in a patient cohort of 193 individuals (Stannus et al. 2010b). In the aforementioned OA LIFE clinical trial, weight loss was responsible for all of the change in leptin (Huebner et al. 2016). These results show the promise of leptin as a biomarker of not only cartilage damage but also of subchondral bone alterations. Even though leptin seems to be involved in the pathogenesis of OA, its systemic involvement in metabolic syndrome and obesity may render it too nonspecific as a biomarker to be of clinical utility for OA.

Endothelin-1 (ET-1), a strong vasoconstrictor, induced by cytokines, has also proven to be an important factor in bone formation. Osteoblast-targeted endothelin A receptor knockout mice typically exhibit decreased bone formation rate and decreased osteoblast density (Clines et al. 2011). In RA, both synovial fluid and sET-1 are elevated compared with sET-1 of controls (Haq et al. 1999). In a small 22 patient study, ET-1 was detectable and equivalent in SF of RA, OA, and acute inflammatory arthritis; these values were twice that of plasma ET-1 from controls (Nahir et al. 1991). A recent review by Sin et al. suggests a central role of ET-1 in OA pathogenesis owing to its role in osteoblast activation and elevation of sET-1 in obesity and diabetes that are known risk factors for OA (Sin et al. 2015). Because of its involvement in bone formation, ET-1 may be a potential biomarker for early OA development and subchondral sclerosis.

Bone Sialoprotein

Bone sialoprotein (BSP) is a highly conserved glycoprotein among mammals that makes up about 10% of non-collagenous bone matrix protein. Expressed mainly by mature osteoblasts, it can activate osteoclasts, either directly or indirectly, via alpha (v) beta integrin (Nakamura et al. 2003). BSP plays a role in the nucleation of hydroxyapatite (Yang et al. 2010), the major non-organic component of the bone, thus suggesting a role in bone mineralization (Lis 2008). In fact, BSP has been identified for a long time primarily as a bone and subchondral bone formation biomarker (Wollheim 1999).

BSP has not been extensively researched as a biomarker. In a small study of 39 subjects with large joint (hip or knee) OA, no significant difference in sBSP was observed comparing OA to control individuals (Otterness et al. 2000). Similarly, an older study found that baseline levels of sBSP were unrelated to hip OA progression. However, an inverse correlation was found between sBSP level and osteophyte grade and sclerosis grade (Conrozier et al. 1998).

BSP was found to be associated with chondrocyte hypertrophy in OA chondrocytes and to promote cartilage angiogenesis. Hypertrophic differentiation of chondrocytes leads to secretion of proteases, chief among them MMP-13 intimately associated with OA development and progression. BSP-immunoreactive chondrocytes in the cartilage were associated with the severity of histological cartilage lesions, OA severity by Mankin grade and with vascular density at the osteochondral junction (Pesesse et al. 2014). These results suggest that this bone biomarker could also be a marker for cartilage alterations in the context of OA, particularly as they relate to hypertrophic-like changes in OA chondrocytes.

DKK-1

Dickkopf WNT signaling pathway inhibitor 1 (Dkk-1) is a recently discovered biomarker that acts as a negative regulator of osteoblast development and activity by

inhibiting the Wingless-related integration site (WNt) signaling pathway (Baron and Kneissel 2013). Serum Dkk-1 was measured in a cohort of 701 subjects divided into four groups: no radiographic hip OA, incident radiographic hip OA, stable radiographic OA, and progressive radiographic hip OA. Higher levels of Dkk-1 were associated with a diminished risk of OA progression (Lane et al. 2007). Honsawek et al. reported similar results in patients with knee OA. Dkk-1 levels were significantly lower in OA patients than in healthy controls, and both plasma and synovial Dkk-1 were inversely correlated with radiographic severity (KL) (Honsawek et al. 2010). These results demonstrate both OA diagnostic and prognostic capabilities for Dkk-1.

TRAP5b

Osteoclasts release the bone-degrading enzyme, tartrate-resistant acid phosphatase 5b (TRAP5b), during bone resorption. In a cohort of 129 knee OA subjects, serum TRAP5b was significantly associated with knee pain and subchondral sclerosis and baseline TRAP5b positively predicted knee pain worsening (Nwosu et al. 2016). These results suggest that TRAP5b may facilitate the selection of patients for interventions targeting the bone component of the joint.

Bone Markers in Clinical Trials

Some of the bone marker candidates mentioned above have been evaluated in the context of OA clinical trials. Oral salmon calcitonin has consistently demonstrated significant reductions in the degradation markers, urinary and serum CTX-I and CTX-II, and modest but significant reductions in the bone formation marker, sOC (Karsdal et al. 2015; Karsdal et al. 2010b; Karsdal et al. 2010a; Bagger et al. 2005). Compared to placebo, vitamin D supplementation significantly increased serum alkaline phosphatase in patients with vitamin D insufficiency with knee OA (Sanghi et al. 2013). SheaFlex70, an extract of an African shea tree, reduced serum osteocalcin and uCTX-II in a 15-week OA trial; however mean concentrations of each of these biomarkers during the first week of treatment were higher in the treatment group compared to placebo, so the subjects may not have been adequately randomized; thus results must be interpreted with caution (Cheras et al. 2010). Another trial studying the relationship between biomarker changes and radiological OA progression in patients taking risedronate showed a dose-dependent reduction of uNTX-I but no association of uNTX-I and radiological progression (Garnero et al. 2008).

Other Diseases

Although most of the abovementioned biomarkers are well known in other bone-related diseases, including osteoporosis, RA, osteosarcoma, and osteogenesis imperfecta (Lombardi et al. 2012), their use in OA appears promising for indicating

the whole joint response during disease development and treatment. They can distinguish bone from cartilage metabolism and may have a particular role in differentiating between OA patient phenotypes. They may also facilitate identification of drug targets and quantify target engagement in the case of bone-acting structural modifying OA trials.

Potential Applications to Prognosis

Up until now, none of the bone-derived biomarkers have been sufficient for the diagnosis and prognosis of OA, be it of the hand, knee, or hip. This review demonstrates the difficulties and challenges in identifying reliable biomarkers of OA (Wieland et al. 2005; Bijlsma et al. 2011; van Spil et al. 2010). There are challenges in biomarker research in general that are not unique to the OA field. First, there is the issue of identifying the relationship between the systemic compartment and the joint compartment. Many of the biomarkers are secreted in the synovial fluid, blood, and even urine. Biomarker levels in the synovial fluid can report on the local joint metabolism, while biomarkers in the blood may represent the total body burden of disease, which cannot be readily quantified by other modalities. Because whole body imaging to quantify OA burden is rarely feasible, it is particularly challenging to qualify a biomarker against a gold standard representing the context of the total body burden of disease or disease progression.

Second, due to enzymatic and nonenzymatic modifications, most biomarkers in body fluids exist in different forms. The biomarker assays used to detect a particular protein often target different forms of the protein of interest. Thus, the results of the measurements may only represent a portion of the total protein or protein fragment concentration.

Third, OA is now considered a disease with different phenotypes (pathogenic pathways). Each phenotype has its own characteristics with its own respective biomarkers or biomarker patterns. Therefore, it is ultimately important to be able to qualify biomarkers in the context of specific and clear phenotypes. This is a chicken and egg problem however, given the fact that the biomarkers themselves may be the best tools for differentiating specific phenotypes.

Fourth, the pathogenesis of OA has not been fully elucidated. Gaining more knowledge in the field will greatly aid the understanding and interpretation of the role that specific biomarkers will have in a clinical setting. Future research should attempt to solve these issues to further advance the field and, eventually, find and follow treatable targets and reduce patient burden of disease.

Although some bone-derived biomarkers have shown disappointing results for OA, others, like CTX-1, DKK-1, periostin, and osteocalcin, show great promise. The ability to overcome the challenges posed in biomarker research in general will determine the eventual role for bone-derived biomarkers in the clinic.

Summary Points

The recognition, summarized in this review, of the close interaction of the bone and cartilage in OA, provides a strong rationale for investigating bone changes in OA trials. To date, osteocalcin and CTX-I have been the most widely studied biomarkers in OA studies and clinical trials. The results of these and other bone biomarkers in OA trials provide examples of the modifiability of the whole joint organ, demonstrating changes in bone metabolism and remodeling in the context of the evaluation of cartilage preservation. Future research is encouraged that continues and expands the use of biomarkers that holistically interrogate the whole joint organ, including the bone.

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Bone-Related Proteins as Markers in Vascular Remodeling

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Alexander E. Berezin

Contents

Abstract

Recent evidences reported that bone is a metabolically active tissue that undergoes continuous remodeling that realizes through the activity of osteoclasts and osteoblasts. The family of bone-related proteins includes several active proteins, i.e., osteopontin (OPN), osteoprotegerin (OPG), osteonectin (OSN), osteocalcin (OCN), sclerostin, and RANKL/RANK system that regulate bone formation, matrix reposition, and remodeling. More evidences indicate that bone-

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related proteins are involved in extra bone mineralization, calcification at ectopic sites, and they might play a pivotal role in atherosclerosis, plaque formation, vascular remodeling and integrity, neovascularization, and malignancy. This review is dedicated to the discussion of controversial role of the bone-related proteins among patients with cardiovascular disease and a predictive value of bone-related proteins as biomarker at risk stratification.

Keywords

Bone-related proteins • Osteopontin • Osteoprotegerine • Osteonectin • Osteocalcin • Cardiovascular diseases • Age-related diseases • Metabolic comorbidities

List of Abbreviations

ACS	Acute coronary syndrome
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CHF	Chronic heart failure
CRP	C-reactive protein
MACE	Major adverse cardiac events
MI	Myocardial infarction
OCN	Osteocalcin
OPG	Osteoprotegerin
OPN	Osteopontin
OSN	Osteonectin
RANKL	Receptor activator of nuclear factor-kB ligand

Key Facts

See Tables 1, 2, and 3 and Figs. 1 and 2.

Table 1 Key Facts of the biological effects of bone-related proteins

Bone-related proteins	Main direct effects	Secondary direct effects	Indirect effects
OPN	Binding of specific apatite crystal faces thereby governing its function as a bone mineralization inhibitor	Upregulation of mineralization at sites of ectopic calcification, i.e., vascular wall, valvular leaflets, kidney, and gall	Tissue remodeling Inflammation Regulation of immunity
OPG/ RANK/ RANKL system	Inhibitor osteoclastogenesis	protection of the skeleton from excessive bone resorbtion and protection of tissue injury beyond bone	Regulation of cell metabolism and extracellular matrix modeling, mediator for innate and adaptive immunity

(*continued*)

Table 1 (continued)

Bone-related proteins	Main direct effects	Secondary direct effects	Indirect effects
OSN	Supporting of osteoblastogenesis	Regulator of fibrosis and increased extracellular matrix deposition	Regulator of cell metabolism, glucose homeostasis, myoprogenitor cell differentiation
OCN	Proosteoblastic effect	Supporting of bone-building function	Regulation of glucose homeostasis, fertile function, the fat cells and male gonad endocrine activity
Sclerostin	Negative regulator of bone growth through inhibition of osteoblastogenesis	Regulation of bone modeling, remodeling, and homeostasis	Regulator of vascular and tissue calcification

This table lists the key facts of direct and indirect effects of bone-related proteins. Abbreviations: *OPN* osteopontine, *OPG* osteoprotegerine, *RANK* receptor activator of nuclear factor-kB, *RANKL* RANK ligand, *OSN* osteonectine, *OCN* osteocalcin

Table 2 Key Facts regarding regulation of vascular calcification

Calcification promoters	Calcification inhibitors
Inflammatory interleukins	Vitamin K-dependent Gla-rich protein
TNF alpha and other inflammatory cytokines	Matrix Gla protein
Osteocalcin	Fetuin-A
Bone-morphogenic proteins	VEGF
High phosphate level	OPN
Vitamin D	OPG
Parathyroid hormone fragments	Hyperglycemia
Free reactive radicals and other components of oxidative stress	Sclerostin
Sex steroids	Dickkopf-related protein 1
Osteocyte-derived sclerostin	
Advanced glycosylation end-products	
Klotho/fibroblast growth factor-23	

This table lists the key facts of regulation of vascular calcification. Calcification promoters attenuate mineral deposition and overexpress at the site of calcification. Calcification inhibitors prevent of calcium-induced signaling pathways and directly suppress mineral binding, crystal formation, and maturation

Abbreviations: *TNF* tumor necrotic factor, *VEGF* vascular endothelial growth factor, *OPN* osteopontin, *OPG* osteoprotegerin

Table 3 Key facts of predictive value of bone-related proteins in cardiovascular diseases

Predictive value of bone-related proteins is possible	Predictive value of bone-related proteins is not clear
Acute MI, acute coronary syndrome	Systemic hypertension
Acute or acutely decompensated chronic HF	Peripheral artery disease
Acute myocarditis	Dyslipidemia
Dilated cardiomyopathy with reduced LVEF	Diabetes mellitus
Atherothrombosis	Obesity
Ischemic stroke	Resistance to insulin
Vascular calcification	Urgent or postponed PCI or CABG

This table lists the key facts of episodical assessment or serial measurements of bone-related protein level in cardiovascular diseases. Abbreviations: *MI* myocardial infarction, *ACS* acute coronary syndrome, *HF* heart failure, *PCI* percutant coronary intervention, *CABG* coronary artery bypass grafting, *LVEF* left ventricular ejection fraction

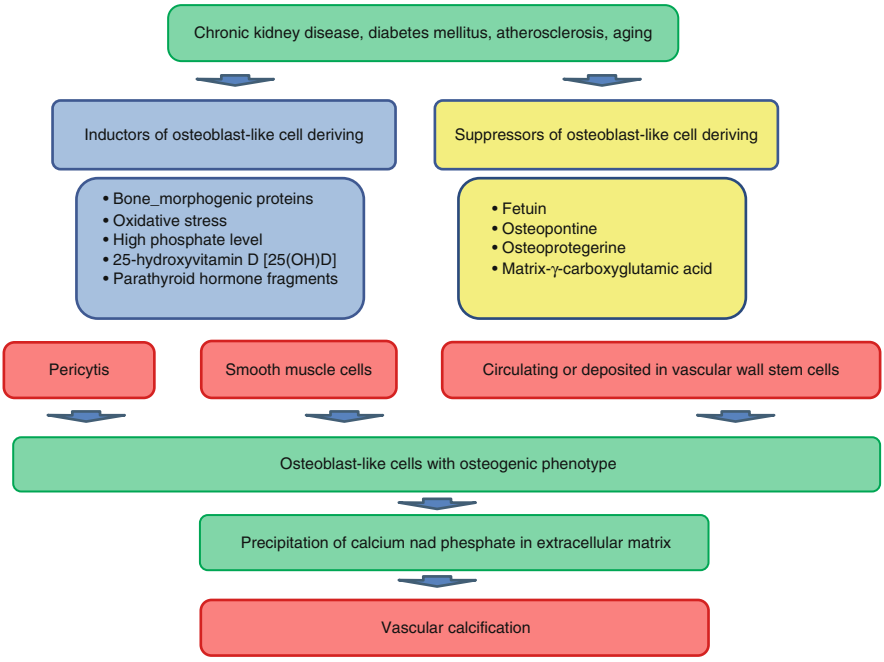

Fig. 1 The main consequent mechanisms that lead to ectopic vascular wall calcification

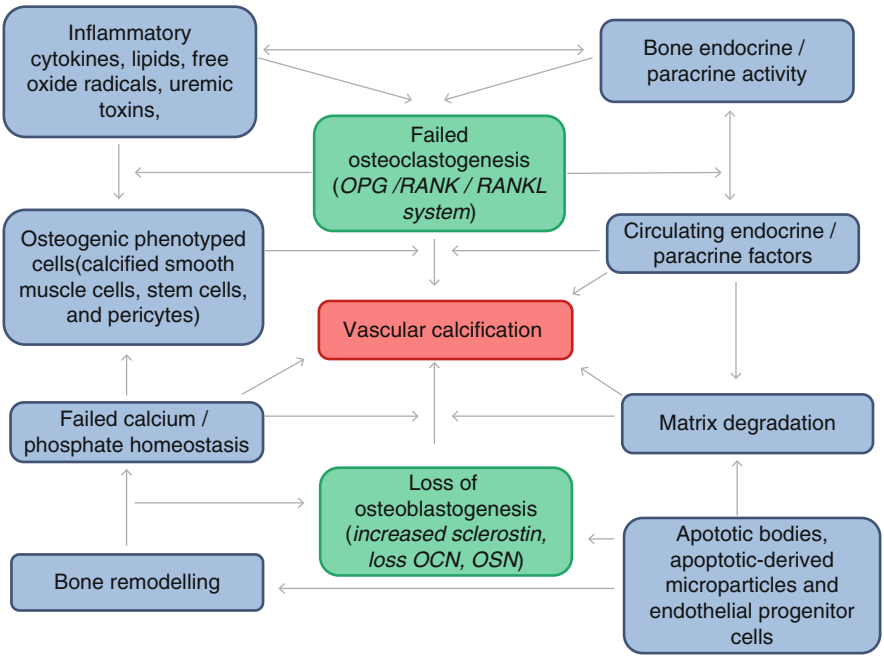

Fig. 2 Key facts of initiation of vascular calcification. Figure shows that initiation and supporting of vascular calcification are tightly regulated pathological process, which involves wide-spectrum cells, including bone environmental cells and extracellular matrix, apoptotic bodies, antigen-presenting cells, etc. Abbreviations: OPN – osteopontine; OPG – osteoprotegerine; RANK - receptor activator of nuclear factor-kB; RANKL – RANK ligand; OSN – osteonectine; OCN – osteocalcin

Definitions of Words and Terms

Biomarker	Biomarker is defined as objectively measured indicator of several faces of biological or pathological processes, pharmacologic responses, therapeutic interventions that may have diagnostic and predictive values to determine these markers as potent surrogate endpoint indicators.
Bone remodeling	Bone remodeling in normal state is defined as dynamic well-balanced process associated with bone formation and bone resorbtion that is under control of multifactorial molecular mechanisms

Bone-related proteins	Bone-related proteins are secreted proteins produced by wide spectrum of cells (dendritic cells, macrophages, osteoblasts/osteoclasts, adipocytes, etc.) and realize their direct effect toward formation, modeling, remodeling of bone extracellular matrix.
Osteoblast-like cells	Osteoblast-like cells are defined as cell with osteogenic phenotype originated from smooth muscle cells, pericytes, or stem cells.
Surrogate endpoint biomarker	Surrogate endpoint biomarker is defined as indicator of clear clinical endpoints in target patient populations only.
Vascular calcification	Vascular calcification is a consequence of closely regulated pathological processes that culminate in organized extracellular matrix deposition of calcium and phosphate produced by osteoblast-like cells.

Introduction

Bone-related proteins are referred as family members of matricellular proteins that are the main components of the extracellular matrix which are highly expressed in the bone developing, vascular remodeling, and tissue regeneration (Alford and Hankenson 2006). Members of this protein class serve as biological mediators of cell function by interacting directly with cells or by modulating the activity of growth factors, proteases, and other extracellular matrix proteins (Hruska et al. 2005; Obert et al. 2009; Wright et al. 2009). Within past decade substantial progress has been made in our understanding of the molecular mechanisms by which these proteins regulate bone mineralization, vascular integrity, and remodeling (Johnsen and Beuschlein 2010). Bone-related proteins are multifunctional growth factors that are activated in response to a hypoxic bone microenvironment stimulates the transcription of multiple genes (David et al. 2009; Kassem and Marie 2011). They contribute bone development and remodeling, as well as extra bone tissue calcification, vascular integrity and remodeling, atherosclerosis and plaque formation, angiogenesis and neovascularization (Drager et al. 2015; Hauschka et al. 1989). Moreover, bone-related proteins are intricately prone regulation by hypoxia signaling system, hormones, electrolyte and mineral changes, inflammation, and they might involve in coupling angiogenesis and osteogenesis during bone development and repair (Drager et al. 2015). In this review controversial role of the bone-related proteins among patients with cardiovascular disease and a predictive value of bone-related proteins as biomarker of vascular remodeling with possible predictive value are discussed.

Biological Role of Bone-Related Proteins

It has been previously reported that the bone-related proteins include osteopontin (OPN), osteoprotegerin (OPG), osteonectin (OSN), osteocalcin (OCN), sclerostin, and RANKL/RANK system (Hofbauer et al. 2000). The most common biological function of bone-related proteins is the control of bone mineralization processes (Okamura et al. 2011). Although the innate pathophysiological mechanisms of bone remodeling balance are not fully defined, bone-related proteins are considered turnover factors directly regulating bone formation and resorption via mediating effects of co-regulators, such as inflammatory cytokines, homocysteine, oxidized lipids, sex steroids, vitamin D, vitamin K, and others (Yasuda et al. 1998). Therefore, they are involved in multiple level controls for extra-bone mineralization at ectopic sites, i.e., vascular wall, valvular leaflets, kidney, gall, tendons, and muscles. The bone-related proteins are expressed in wide spectrum of cells (antigen presenting cells, preosteoblasts/osteoblasts, osteocytes, chondrocytes, fibroblasts, endothelial cells, smooth muscle cells, epithelial cells) as well as skeletal muscles, mammary glands, and several organs (inner ear, brain, placenta, and kidney) (Zimmermann and Ritchie 2015).

All bone-related proteins realize their direct (regulation of biological mineralization) and indirect (tissue remodeling and regulation immunity) biological effects via surface-expressed receptors that are presented as CD44 and various types of integrins (avb1, avb3, avb5, avb6, a4b1, a5b1, a8b1, a9b1) (Lund et al. 2013). Recent investigations have been shown that bone-related proteins may play a pivotal role in atherosclerosis, cardiovascular diseases, chronic rheumatic diseases, multiple sclerosis, inflammation bowel diseases, autoimmune disorders, and cancer and malignancy (Wright et al. 2009).

Osteopontin

Osteopontin (OPN, secreted phosphoprotein 1 -SPP 1, 44 kDa bone phosphoprotein, sialoprotein 1, 2ar, uropontin, and early T-lymphocyte activation-1 [Eta-1]) is a secreted low-molecular (41–75 kDa) matricellular protein. OPN is defined as integrin-binding ligand (N-linked glycoprotein) that is involved in several physiological and pathological processes. OPN belongs to SPARC (secreted protein acidic and rich in cysteine) family and demonstrates prominent roles in cell proliferation, migration, differentiation, apoptosis, adhesion, angiogenesis, tissue repair, and regulation of extracellular matrix remodeling. There are evidences regarding the pivotal role of OPN in carcinogenesis and metastasis (Nagaraju et al. 2014).

OPN is encoding by a single copy gene but exists in various isoforms (OPNa, OPNb, and OPNc) as a result of alternative splicing, alternative translation, and different posttranslational modifications (Sarosiek et al. 2015). Despite functional role of OPN isoforms in systemic inflammation is essential to understanding, overexpression of OPNa, OPNb, and OPNc isoforms was not found in similar clinical settings (Coombes and Syn 2014). In fact, presence of OPNc isoform associates well with diabetes mellitus and obesity (Sarosiek et al. 2015). The role

of OPNa, OPNb, and OPNc in vascular remodeling is under recognized. OPN interacts with several integrins via two domains: Arg159-Gly-Asp161 (RGD) sequence binding to α(v)-containing integrins, and Ser162-Val-Val-Tyr-Gly-Leu-Arg168 (SLAYGLR) sequence binding to α(4) β(1), α(4) β(7) and α(9) β(1) integrins (Ito et al. 2009). This interaction plays a pivotal role in regulating migration, survival, and accumulation of macrophage and other types of antigen presenting cells. Indeed, OPN may induce the transcription of interleukin (IL)-6 and reduced tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and IL-10 (Sarosiek et al. 2015). Therefore, after translocation into nucleus OPN may interact with p85α regulatory subunit of the signaling kinase PI(3)K and Bcl-6 that leads to protection of Bcl-6 from ubiquitin-dependent proteasome degradation and inducing apoptosis (Leavenworth et al. 2015). Overall, OPN is considered a mediator regulating the extracellular matrix modeling and interactions between cells through growth factor signaling pathway, cell adhesion, migration, and proliferation.

Osteoprotegerin

Osteoprotegerin (OPG) is a member of the tumor necrosis factor receptor superfamily and is a soluble secreted protein produced by osteoblasts, osteogenic stromal stem cells, and activated mononuclears (Simonet et al. 1997). OPG acts as a decoy receptor for RANKL and thus inhibits osteoclastogenesis. The main biological role of OPG is protection of the skeleton from excessive bone resorbtion and protection of tissue injury beyond bone (Liu and Zhang 2015). These effects realize by binding OPG with specific ligand named RANKL (receptor activator of nuclear factor-kB ligand) that leads to prevention of interacting OPG with RANK (Boyce and Xing 2007a). An imbalance in the RANKL/RANK/OPG axis, with decreased OPG and/or increased RANKL, is associated with diseases that favor bone loss, including osteoporosis (Warren et al. 2015). Therefore, recent studies showed that OPG has been identified as candidate mediators for paracrine signaling in cell metabolism and extracellular matrix regulation but have also been shown to modulate dendritic cells and activated T cells, as well as to promote B-cell maturation and antibody response, which suggests a role in both innate and adaptive immunity (Ueland et al. 2005; Yndestad et al. 2002).

Osteonectin

Osteonectin (OSN, BM-40) is secreted extracellular matrix glycoprotein that belongs to SPARC family (secreted protein acidic and rich in cysteine) and expressed in active remodeling in the skeleton and other tissues (Ribeiro et al. 2014). The main biological effect of ONC is considered a regulator of fibrosis and increased extracellular matrix deposition (Dhore et al. 2001). Overall, OSN supports osteoblastogenesis and is prone over expression on surface of osteoblasts in response of effect of proinflammatory cytokines, sex hormones, vitamin D3, and

several growth factors. Therefore, OSN together with myostatin, insulin-like growth factor I, irisin, and osteocalcin may be associated with the interactions between muscle tissues and bone metabolism through the commitment of myoprogenitor cells to the osteoblast lineage (Kawao and Kaji 2015).

Osteocalcin

Osteocalcin (OCN, bone γ-carboxyglutamic acid-containing protein, bone Gla-protein) is a small (49 amino acid residues) osteoblast-specific non-collagenous protein that is specially synthesized and secreted by osteoblast and osteocyte (Baron and Kneissel 2013). Synthesis of OCN is under control of and is regulated by 1α,25-dihydroxy-Vitamin D3 (Wei and Karsenty 2015). OCN is secreted by bone osteoblasts in response to stimulation of osteoblastic differentiation and osteocytic maturation (Fukumoto and Martin 2009). The most of OCN is found in bone matrix and only a small amount is in circulation (Garnero et al. 1992). The main biological role of OCN is proosteoblastic effect or bone-building function (Shao et al. 2015). This effect is realized through the appropriate OCN receptor (GPRC6A) (Zhang et al. 2015). Therefore, OCN regulate glucose homeostasis, fertile function, the fat cells, and male gonad endocrine activity and be regulated by insulin and the neural system (Shao et al. 2015; Wei and Karsenty 2015). There are evidences that plasma OCN is inversely related to fat mass and plasma glucose (Kindblom et al. 2009) and that leptin may effect on OCN carboxylation through the hypothalamus (Reinehr and Roth 2010). Finally, OCN is considered an effector switched dysmetabolic responces and prevented vascular calcification (Chen and Yang 2015). Notwithstanding, OCN is a well-known regulator of body energy metabolism, it still remained unclear as to how OCN might modulate extra bone mineralization and vascular function.

Sclerostin

Sclerostin (SOST) is low molecular signal secreted cystine-knot protein that is widely expressed on the surface of osteocytes and plays essential roles in bone formation, modeling, remodeling, and homeostasis (Shao et al. 2015). SOST acts a negative regulator of bone growth through inhibiting the canonical Wnt signaling cascade by binding to and blocking the Wnt co-receptor LRP5/6. Thus, in contrast of OPG, which specifically inhibits osteoclastogenesis, SOST and Dickkopf-related protein 1 (DKK1) exerting their inhibitory effects on osteoblastogenesis (Morena et al. 2015). Therefore, SOST may play a key role in vascular and tissue calcification (Morena et al. 2015; Shao et al. 2015). Moreover, recent studies have been shown that SOST is linked to bone physiology and cardiovascular disease through the Wnt/β-catenin signaling pathway (Shao et al. 2015).

RANK/RANK Ligand System

The interaction between RANKL (receptor activator of nuclear factor-kB ligand) and its receptor RANK (receptor activator of nuclear factor-kB) is essential for the differentiation and bone resorbing capacity of the osteoclastes, as well as controlling mineralization process that is suitable for several physiological and pathological states. RANKL is a type II homotrimeric transmembrane protein that is expressed as a membrane-bound and a secreted protein, which is derived from the membrane form as a result of either proteolytic cleavage or alternative splicing (Boyce and Xing 2007b). Serum RANK/RANKL have been identified as candidate mediators for paracrine signaling in cell metabolism and extracellular matrix regulation but have also been shown to modulate dendritic cells and activated T cells, as well as to promote B-cell maturation and antibody response, which suggests a role in both innate and adaptive immunity (Anderson et al. 1997; Yndestad et al. 2002; Ueland et al. 2005). There are various mutated RANKL proteins that abolish binding to OPG while preserving recognition of RANK (Warren et al. 2015). Interestingly, the physiological RANKL/RANK interaction is not optimized for maximal signaling and function, perhaps reflecting the need to maintain receptor specificity within the tumor necrosis factor (TNF) superfamily. Therefore, integrin β3, V-ATPase, CAII, CTSK, TNF receptor-associated factor (TRAP), matrix metalloproteinase (MMP)-9, parathyroid hormone, and hormonally active form of vitamin D3, 1α,25-(OH)2D3, have been identified as essential regulators of RANK/RANKL system activity (Gu et al. 2015; Silva and Bilezikian 2015).

Basic Principles of Biological Control for Mineralization Processes

According to the contemporary insight, bone mineralization is a regulated process induced by complex molecular mechanisms (Kassem and Marie 2011). In vivo bone formation requires recruitment and replication of mesenchymal precursors of osteoblasts, differentiation into preosteoblasts, osteoblasts, and mature osteoblasts ultimately result in the accumulation and mineralization of the extracellular matrix (Raouf and Seth 2000). Thus, bone development is accomplished by a cascade of biological processes that may include differentiation of pluripotential tissue, angiogenesis, osteogenesis, mineralization, and remodeling (Rachmiel and Leiser 2014). The interactions between osteoblasts and osteoclasts that is considered as pivotal mechanism of bone formation closely affects other cells present within the bone microenvironment, i.e., stem cells, endothelial progenitor, and particularly vascular endothelial cells, fibroblasts, as well as extracellular matrix. The complex communications between all components of the network in bone undergo gene regulation. There are evidences regarding bone synthesis and turnover is under control of genes regulated numerous cytokines (transforming growth factor-β, bone morphogenetic proteins, insulin-like growth factor-1, and fibroblast growth factor-2) and bone-related extracellular and matrix proteins (osteonectin, osteopontin, and osteoprotegerin).

The main inductors of mineralization are inflammation oxidative stress, sex steroids, high level of phosphate, parathyroid hormone and its fragments, and system of specific regulators, i.e., osteocalcin, bone-morphogenic proteins, and vitamin D. Vitamin K-dependent Gla-rich protein (GRP) together with fetuin-A- matrix Gla protein are a main calcification inhibitory system that prevents of calcium-induced signaling pathways and directly inhibits mineral binding, crystal formation, and resident cell maturation in vascular wall (Viegas et al. 2015). This system is able to inhibit vascular wall calcification and osteochondrogenic differentiation through α-smooth muscle actin upregulation and OPN downregulation (Hruska et al. 2005). Indeed, extracellular vesicles released from normal vascular smooth muscle cells are loaded with GRP, matrix Gla protein, and fetuin-A. In pathology state, extracellular vesicles released from vascular smooth muscle cells appear to increase calcium loading and GRP and matrix Gla protein depletion (Viegas et al. 2015). The key facts regarding bone-related proteins' effects are in the Table 1. Finally, bone mineralization is well balanced and regulated process supported by several mutual controlled molecular mechanisms. Bone-related proteins are considered an innate physiological switching involved in the positive and negative regulation of bone formation and they might mediate extra bone calcification in the pathology state.

Biology of Ectopic Vascular Calcification

On contrary to bone mineralization, vascular calcification is a pathological process, occurring in response to dysregulated/inappropriate environmental cues (Shroff and Shanahan 2007; Drüeke 2005). It results of imbalance between calcification inhibitors and promoters, which act at the systemic and the local level (Reynolds et al. 2004). Therefore, this imbalance leads probably to phenotypic change of smooth muscle cells towards osteoblast-like calcifying smooth muscle cells, which mediate organized extracellular matrix deposition in the vascular wall (Drüeke 2005; Johnson et al. 2006). Figure 1 shows consequently mechanisms that directly and indirectly lead to ectopic vascular wall calcification. Overall there is a hypothesis that ectopic vascular calcifications could be mediated by pathophysiological mechanisms underlying bone biomineralization affected residence cells allocated in vascular wall. Indeed, calcified vascular tissue expresses bone-related proteins, bone specific transcription factors, and bone morphogenetic proteins (BMPs), which contribute in osteogenesis in bone (Bostrom et al. 1993; Dhore et al. 2001). However, the origin of calcifying cells that directly promote vascular tissue mineralization is still unknown. Vascular smooth muscle cells may differentiate into calcifying osteoblast-like cells which via several molecular mechanisms regulate biomineralization in nature manner. Overexpression of extracellular matrix and biomineralization genes relevant for bone formation are sufficiently modulated by calcifying vascular smooth muscle cells (Morhayim et al. 2015; Alves et al. 2014). Moreover, these genes constitute the strongest link between residence cells and pathological vascular remodeling phenotype associated with calcification of vascular wall (Persy and D'Haese 2009). Finally, ectopic artery mineralization is frequently

accompanied by decreased bone mineral density or disturbed bone turnover (Shroff and Shanahan 2007). Interestingly, type 2 diabetes mellitus (T2DM) associates with increased fracture risk despite the fact that T2DM patients have higher bone mineral density as compared to non-diabetic individuals. Therefore, there are evidences that T2DM might contribute to decreased bone formation through interference of advanced glycosylation end-products with osteoblast development, function and attachment to collagen matrix, increased levels of osteocyte-derived sclerostin, and hyperglycemia-induced suppression of osteogenic differentiation of marrow-derived progenitor cells diverting osteoblastic precursor cells (Meier et al. 2015). Overall T2DM-dependend inflammatory process contributing to bone demineralization may lead fracture risk, but extra bone mineralization activity might increase. All these mediate augmented vascular calcification and promote atherosclerosis in T2DM patients. However, these are still unclear, whether is inversely related relationship between processes of bone and vascular calcification that appear to be inversely related (Yamamoto 2015). Further examinations are needed to improve understanding of the precise mechanism in this area.

There are evidences regarding an activation of resident pericytes in the vascular wall that may contribute vascular calcification (Yamamoto 2015). Indeed, pericytes that are discussed as mesenchymal progenitor cells have the powerful potential to develop into osteoblasts and chondrocytes in situ under influence of inflammatory cytokines, oxidated lipids, free active radicals, turbulent blood flow, high pressure, shear stress, and growth factors contributed in angiogenesis and neovascualrization (Evrard et al. 2015). Interestingly, neoangiogenesis that is suitable for atherosclerosis malignancy may facilitate migration of pericytes and thereby induce vascular wall mineralization (Stegen et al. 2015).

Bone-Related Proteins in Atherosclerosis and Vascular Remodeling

Vascular calcification frequently appears in arterial wall due to atherosclerosis, inflammation, and worsening of calcium homeostasis. Vascular calcification demonstrates increased prevalence in cardiovascular and chronic kidney disease, atherosclerosis, and dyslipidemia (Evrard et al. 2015). Recent investigations have shown that vascular calcification is a complex sophisticated pathological process affecting promoters and inhibitors of calcification, resembling skeletal metabolism, and regulated by resident cells, intermediates, hormones, cytokines, and active peptides (Viegas et al. 2015). Therefore, atherosclerosis, low-intense inflammation, stretch-induce arterial wall hypertrophy, and dyslipidemia are considered main factors that contribute in endothelial dysfunction and directly relate to clinical outcomes among subjects belong to general and selective populations. Key facts of regulation of vascular wall calcification are in Table 2.

The key point of the beginning of vascular calcification is formation of osteogenic phenotype of target cells, i.e., pericytes, vascular smooth muscle cells, and probably

stem cells. Osteogenic differentiation of target cells including vascular smooth muscle cells is characterized by the expression of bone-related molecules including bone morphogenetic protein (BMP) -2, Msx2, OPG, and OPN, which are produced by osteoblasts and chondrocytes. Osteogenic transforming target cells produce hydroxyapatite, which includes calcium deposition in extra bone sites including vascular wall, valvular leaflets, etc. Finally, calcium deposits of atherosclerotic plaque and vascular wall, which appear to be identical to fully formed lamellar bone, may worse mechanical and structural properties of vessel and lead to vascular complications. Key Facts of initiation of vascular calcification are in Fig. 2.

Recent data have linked RANKL and OPG to cardiovascular disease, including CHF, immunity, vascular calcification, osteoporosis, and bone remodeling (Loncar et al. 2010; Kearns et al. 2008; Leistner et al. 2012). Low-intense inflammation is being discussed as a powerful trigger of vascular remodeling and calcification realized through bone-related protein pathway. Recent clinical studies have shown that coronary atherosclerosis associates with a significant increase of OPG and with a trend towards a decrease of soluble RANKL and RANKL/OPG Ratio (Motovska et al. 2015). Therefore, C-reactive protein (CRP) that overexpressed in atherosclerosis, dyslipidemia, and other dysmetabolic states (diabetes mellitus, abdominal obesity, metabolic syndrome) and cardiovascular diseases (ischemic heart disease, heart failure, hypertension, etc.) may stimulate RANKL production in monocytes. The RANKL-stimulated expression of wide spectrum of transcription factors, i.e., TRAF6, p38 mitogen-activated protein kinases (MAPKs), JNK IkB-α, and NF-kB p65 DNA, triggers overproduction of bone-related proteins. Overall RANKL/RANK/OPG system and its downstream signaling pathway are closely controlled via inflammatory cytokine (TNF alpha, interleukin (IL) 1β, IL-6, IL-10, IL-21, and IL-23) productions (Doumouchtsis et al. 2007). These effects may consider an important mechanism of endogenous protection from tissue injury. Indeed, recent animal studies have shown that OPG protects large arteries from medial calcification (El Hadj et al. 2008). Overall, OPG is discussed as a vascular protector (Hofbauer and Schoppet 2004) and a surrogate marker of early coronary vascular calcification in patients with known asymptomatic coronary artery disease, dysmetabolic disease (Berezin and Kremzer 2013).

OSN has found to cause myocardial hypertrophy; increase fibrillar collagen content; stimulate cell signaling, adhesion, survival, proliferation, and migration in several cell types; mediate calcification of the vascular wall, coagulation, and endothelial dysfunction (McCurdy et al. 2010). Recent animal studies have revealed that increased circulating OSN associates with higher incidence of mortality following myocardial infarction due to increased rates of rupture and new heart failure over the first 14 days after MI that associate with left ventricular dysfunction and increased mortality in short- and long-term period (Schellings et al. 2009).

OPN is reported a surrogate marker of atherosclerotic lesions, especially in calcified plaques, and is linked to the progression of coronary artery disease. Moreover, OPN is powerful biomarker of asymptomatic coronary artery disease (Mohamadpour et al. 2015) and vascular calcification in patients with chronic kidney

disease (Gluba-Brzózka et al. 2014) with possible predictive value. OPN has a renal clearance and demonstrates a close interaction with glomerular filtration rate. Recent studies have shown that OPN and renal failure were the independent risk factors for coronary heart disease (Chen et al. 2014). There are evidences that OPN relates a systemic inflammatory activation and endothelial dysfunction that is considered a marker of negative clinical outcomes in cardiovascular disease.

Finally, circulating level of bone-related proteins associates with vascular wall calcification, target organ damage including lowered kidney function, plaque formation, and endothelial dysfunction. Key facts of dual role of bone-related proteins in cardiovascular diseases are in Table 3. However, the predictive role of these biological markers is not fully understood and requires more investigations.

The Role of Bone-Related Proteins in Age-Related Diseases

Bone has evolved to provide structural support to organisms, and therefore its mechanical properties are vital physiologically (Zimmermann and Ritchie 2015). Bone remodeling is age dependently regulated and changes dramatically during the course of development. Progressive accumulation of reactive oxygen species and hypoxia have been suspected to be the leading cause of many inflammatory and degenerative diseases, as well as an important factor underlying many effects of aging (Chen et al. 2015; Drager et al. 2015). However, the role of bone-related proteins and its co-regulators in age-related diseases is still under discussion and appears to be very controversial.

Osteopontin (OPN) and vascular endothelial growth factor (VEGF) are characterized by a convergence in function for regulating cell motility and angiogenesis, the response to hypoxia, and apoptosis (Gong et al. 2015; Ramchandani and Weber 2015). OPN and VEGF may co-express in age-related settings (Almeida 2012). In vascular diseases, these two cytokines mediate remodeling but may also perpetuate inflammation and narrowing of the arteries (Rodríguez et al. 2015). OPN and VEGF are elevated and contribute to vascularization in age-related manner (Ramchandani and Weber 2015). Indeed, cyclic stretch as a main mechanical forces influencing vascular smooth muscle cells in vasculature may initiate stimulation of NADPH oxidase isoform 1 (Nox1)-derived ROS via MEF2B, leading to endothelial dysfunction via a switch from a contractile to a synthetic phenotype (Rodríguez et al. 2015). This process is upregulated by OPN and downregulated by calponin1 and smoothelin B. Thus, OPN-dependent pathway of vascular dysfunction bases on MEF2B-Nox1-ROS upregulation under pathological stretch conditions is suitable for hypertension. Indeed, stretch-induced Nox1 activation decreases actin fiber density and augments matrix metalloproteinase-9 activity, vascular smooth muscle cells migration (Rodríguez et al. 2015). All these findings may have a pivotal role for explanation of age-related hypertension.

There are controversial data about age-related increase of OSN. However, the diagnostic and predictive role of this fact is not clear and requires more studies.

Predictive Value of Bone-Related Proteins in Patients After Stroke

The prognostic relevance of biomarkers related to atherosclerotic plaque calcification, i.e., OPN, OPG, and RANKL, was determined in several investigations. Interestingly that serum OPN may be a useful biomarker of atherosclerosis and vascular calcification. Importantly, note that there was determined a positive association between circulating OPN and the presence of CAD but not to the extent of coronary atherosclerosis (Mohamadpour et al. 2015). Therefore, serum levels of OPN, but not OPG and RANKL, peaked at day 7 after acute ischemic stroke and predicted worse neurological scores independently of age, gender, hypertension, and thrombolytic procedures (Carbone et al. 2015). Whether serial measurements of OPN, OPG, and RANKL are necessary for risk stratification of the patients after stroke is not clear.

Predictive Value of Bone-Related Proteins in Heart Failure

Notwithstanding, bone-related proteins are considered a surrogate biomarker of vascular calcification in atherosclerosis, dyslipidemia, diabetes, obesity, etc., the role of sRANKL/OPG complex in maintenance of reparative repair potency among CHF persons shows to be very intriguing while clinical data are limited. There are data OPG/RANKL/RANK system that contributes to cardiac remodeling and left ventricular dilation after acute myocardial infarction in acute phase of cardiac failure as well as in chronic phase of heart failure development, while not so profoundly (Ueland et al. 2004). It is reported that serum RANKL was a significant determinant of NT-pro-BNP independent of age, BMI, and creatinine clearance in CHF subjects (Loncar et al. 2010). There is interrelationship between OPG and serum RANKL concentrations in patients with advanced atherosclerosis in relation to medical history, risk factors, and medication intake (Giaginis et al. 2012). On the one hand, sRANKL/OPG complex contributes different stages in ischemic CHF development, whereas the clinical implication of RANKL seems uncertain (Bjerre et al. 2014). On the other hand, the independent predictive value was determined for OPG only (Montagnana et al. 2013). Finally, OPG is suggested to be a modulator rather than a marker of extracellular remodeling that may play critical role in CHF pathogenesis by neutralizing the effect of receptor activator of nuclear factor-kappa B ligand on differentiation and activation of wide spectrum cells, including circulating endothelial progenitor cells. The imbalance between free fraction of RANKL, calculated as sRANKL/OPG ratio, and circulating OPG may be responsible for the homeostatic mechanism of differentiation and apoptosis of endothelial progenitor cells (Berezin and Kremzer 2014). This effect may mediate overproduction of reactive oxygen species and oxidized lipoproteins through OPG-related activation of NOX-2 and NOX-4 and triggered phosphorylation of ERK-1/2 and p38 MAPK. All this mechanisms are suitable for ischemic CHF development. Results of our study showed that components of sRANKL/OPG complex were increased in CHF patients when compared with none-CHF persons with stable CAD as well as with healthy volunteers. Therefore, decreased circulating

EPCs -related CHF in CAD subjects were also found. However, sRANKL/OPG ratio when compared with other components of cytokines-induced bone-related proteins RANKL and OPG in ischemic CHF patients was not only significantly associated with parameters of neuroendocrine activation such as NT-pro-BNP and hs-CRP but it closely effected on EPCs with proangiogenic phenotypes. It has been suggested that sRANKL/OPG complex affected reparative face of the pathogenesis of ischemic CHF through modulating count of circulating endothelial progenitor cells. Because OPG may stimulate differentiation of the endothelial progenitor cells and positively regulate their count in circulation, it has suggested that free fraction of serum RANKL, calculated as serum RANKL/OPG ratio, consider powerful predictor for depletion of CD14 + CD309+ EPCs and CD14 + CD309 + Tie2+ endothelial progenitor cells in CHF patients. Probably, this effect may have a prognostic value for subjects with CHF. Overall the role of OPG as independent predictor of CHF development and progression requires detail investigations.

OPN has been demonstrated to be upregulated in left ventricular hypertrophy, dilated cardiomyopathy, and diabetic cardiomyopathy, and it is discussed as a possible predictor of heart failure development and heart failure-related clinical outcomes (Lok et al. 2015). Osteopontin independently predicted all-cause mortality and acutely CHF-related rehospitalization after 1 and 5 years. Compared with NT-proBNP, osteopontin was of superior prognostic value, specifically in CHF patients and for the prognostic outcome of acutely CHF-related rehospitalization (Behnes et al. 2013).

It is found that the serum OSN in patients with CHF predominantly reflected a positive pro-inflammatory response and alterations in protein metabolism that leads to biomechanical stress. However, the roles of OSN in the CHF have not been defined. Finally, further studies are needed to elucidate the potential role of bone-related proteins in the complex pathogenesis of CHF

Potential Applications to Prognosis, Other Diseases or Conditions

Although there are not sufficient evidences that the clinical correlations of circulating levels of bone-related proteins in subjects with documented cardiovascular diseases might have predictive value, it has been suggested that exaggerated level of OPG and probably OPN and OSN would confer a better prognosis in CAD patients, especially those who underwent revascularization procedures or have acute/acutely decompensated heart failure. By now there are evidences regarding an association of circulating bone-related proteins predominantly OPG and OPN with vascular calcification. Therefore, the negative effect of these proteins on progression of age-related diseases has been reported. Currently the continued monitoring for OPG/RANKL, OPN, and OSN levels is not recommended, but patient from vulnerable populations at high cardiovascular risk, probably, may have some benefit in prediction of clinical outcomes based on serial assessment of circulating bone-related proteins (Table 3).

Summary Points

- Bone-related proteins are multifunctional growth factors that are involved in extra bone mineralization, calcification at ectopic sites, and they might play a pivotal role in atherosclerosis, plaque formation, vascular remodeling and integrity, neovascularization, and malignancy.
- Bone-related proteins include osteopontin, osteoprotegerin, osteonectin, osteocalcin, sclerostin, and RANKL/RANK system.
- Bone-related proteins are intricately prone to regulation by hypoxia signaling system, hormones, electrolyte and mineral changes, inflammation, and they might involve in coupling angiogenesis and osteogenesis during bone development and repair.
- On contrary to bone mineralization, vascular calcification is a pathological process, occurring in response to dysregulated/inappropriate environmental cues as results of imbalance between calcification inhibitors and promoters.
- Ectopic vascular calcifications could be mediated by pathophysiological mechanisms underlying bone biomineralization affected residence cells allocated in vascular wall.
- The main calcifying cells that directly promote vascular tissue mineralization are vascular smooth muscle cells, stem cells, and pericytes.
- Vascular calcification demonstrates increased prevalence in cardiovascular and chronic kidney disease, atherosclerosis, and dyslipidemia.
- Elevated circulation levels of OPG, OPN, and probably OSN are considered a surrogate marker of vascular calcification, lowered kidney function, asymptomatic atherosclerosis and coronary artery disease, and severity of heart failure with possible high predictive value.

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Bone-Specific Alkaline Phosphatase and Exercise

45

Alev Alp

Contents

Abstract

It is a matter of discussion how physical exercise affects bone remodeling process and markers such as bone-specific alkaline phosphatase (BAP) which is the only marker that is not influenced by diurnal variation of bone remodeling. Dietary calcium intake and parathormone levels are strongly associated with the

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remodeling status during or after the physical training, and increase of BAP levels may not be always the indicator of bone formation process. Moreover, behavior of BAP may be different in physically active or sedentary people at different ages and sexes. Because skeletal muscle has the ability to adapt to a variety of changes in physical status, physical exercise has the ability to change turnover status in favor of formation by increasing BAP/pyridinoline ratio.

Keywords

Biomarker • Bone formation • Bone-specific alkaline phosphatase • Bone remodeling • Bone turnover • Exercise

List of Abbreviations

BAP	Bone-specific alkaline phosphatase
BMD	Bone mineral density
BMU	Basic multicellular unit
CTX	C-terminal telopeptide
DEXA	Dual-energy X-ray absorptiometry
D-PYR	Free deoxypyridinoline
HYP	Hydroxyproline
LRP5	Low-density lipoprotein receptor-related protein 5
NTX	N-terminal telopeptide
OC	Osteocalcin
PICP	C-terminal propeptide of type 1 collagen
PINP	N-terminal propeptide of type 1 procollagen
PYD	Free pyridinoline
RANKL	Receptor activator of nuclear factor kappa-B ligand
TRAP	Tartrate-resistant acid phosphatase

Key Facts of Exercise

- If a strain is detected as greater than the optimum strain, then bone formation will occur.
- Optimal physical exercise is an osteogenic stimulator, and long-term loading of the bone by means of exercise makes an anabolic effect on the bone mass and strength.
- Personal metabolic needs are the determinants of the bone turnover status in the presence of an unsteady physical loading.
- Biomarkers identified as the reflectors of bone metabolic activity enable to estimate the rate and direction of the bone turnover status.

Definition of Words and Terms

Bone remodeling (bone turnover)	Bone turnover refers to the total volume of the bone that is both resorbed and formed over a period of time which can be estimated by

	measuring bone biomarkers. Bone remodeling, on the other hand, is defined as an active process throughout the skeleton, essential for calcium homeostasis and preserving the integrity of the skeleton, through the coupled activity of osteoclasts and osteoblasts. Bone turnover occurs mainly throughout bone remodeling.
Bone-specific alkaline phosphatase	Bone alkaline phosphatase (BAP) is the bone-specific isoform of alkaline phosphatase, a bone formation marker that is found on the surface of osteoblasts.
DEXA: Dual-energy X-ray absorptiometry	It is the most widely used and most thoroughly studied bone density measurement technique to diagnose and follow osteoporosis.
Osteoblast	Osteoblasts are specialized, differentiated mesenchymal stem cells which produce a calcium- and phosphate-based mineral, deposited regularly into the organic matrix forming a very strong and dense mineralized tissue called the mineralized matrix.
Skeletal bone	Skeletal bones are made of a mix of hard stuff that gives them strength and tons of living cells which help them grow and repair themselves, and they do various jobs, such as storing body minerals like calcium.

Introduction

The skeletal bone is constantly remodeled in order to repair microfractures caused by excessive stress and biomechanical forces. The process called "remodeling" or "turnover" consists of consecutive resorption followed by formation in order not to change the bone mass. The "rate of remodeling" is an important determinant of bone physiology; therefore, measurements that correlate with this process provide useful information about bone diseases, monitoring treatment and exercise-induced changes. Yearly quantification of bone mass is done by dual-energy X-ray absorptiometry (DEXA) measurements. However, acute-subacute changes in bone mass for shorter durations (months) are inadequate to detect by means of densitometry measurements. Metabolic markers of bone turnover reflect a systemic response and are easy to detect by serum and urine samples whenever needed.

Bone formation process results in the release of collagen by-products, proteins, or bone-specific isoform of total alkaline phosphatase measured in the serum. There are also assays to measure bone resorption products released from osteoclasts reflecting

Table 1 Bone turnover markers

Formation markers	Circadian rhythm	Assay
OC	+	Serum
BAP	–	Serum
PINP/PICP	+	Serum
Resorption markers		
NTX	+	Serum, Urine
CTX	+	Serum, Urine
D-PYR	+	Urine
PYR	+	Urine
HYP	+	Urine
TRAP	+	Serum

OC osteocalcin, *BAP* bone-specific alkaline phosphatase, *PINP* N-terminal propeptide of type 1 procollagen, *PICP* C-terminal propeptide of type 1 collagen, *D-PYR* free deoxypyridinoline, *PYD* free pyridinoline, *HYP* hydroxyproline, *TRAP* tartrate-resistant acid phosphatase. Collection of serum should be performed in the morning after an overnight fast. Collection of urine should be performed in the morning. OC lacks standardization, rapidly degrades in serum, and requires collection on ice. BAP may have cross-reactivity with liver isoform (15–20%) (Seibel 2005).

the bone turnover rate (Table 1). Resorption markers fall in 2–12 weeks, while formation markers act a little bit slower. With the exception of bone-specific alkaline phosphatase, diurnal variation and complexity of these markers however complicate their availability in clinical practice (Banfi et al. 2010).

Total serum alkaline phosphatase consists of several enzymatic isoforms originating from the liver, intestine, spleen, kidney, placenta, and bone. In adults with normal liver function, about 50% of the total alkaline phosphatase activity in serum is derived half and half from the liver and the bone. The serum concentrations of bone-specific alkaline phosphatase (BAP) reflect the activity of osteoblastic cellular membrane to form new collagen and bone mineralization process. It is functionally similar to hepatic and placental forms, with some antigenic discrepancy. It is the only marker that is not influenced by diurnal variation of bone remodeling because of its unique molecular texture. Because BAP is cleared by the liver, liver diseases may raise the levels, or liver alkaline phosphatase antibodies may cause cross-reactions with BAP to reduce the levels. There are several assays developed for BAP such as electrophoresis, isoelectric focusing, lectin precipitation, and immunoassay. Immunoassay is the method of choice because of its high specificity and precision (Watts 1999).

During linear growth in the adolescent status, bone-specific isoenzyme (BAP) raises up to 90% (Banfi et al. 2010). Though serum total alkaline phosphatase is the mostly used marker of bone metabolism, BAP is preferred rather frequently in clinical practice and considered as a useful predictor of bone osteoblastic activity in all phases of bone mineralization. It is not influenced by the diurnal variation, and treatment changes become stable at 3–6 months. Nevertheless, unstable changes may occur daily. Immediate changes may be due to changes in plasma volume and renal activity which must be separated from long-term changes afterward (Banfi et al. 2010;

Table 2 Bone-specific alkaline phosphatase levels

Males	Females
<2 years: 25–221 mcg/L 2–9 years: 27–148 mcg/L 10–13 years: 35–169 mcg/L 14–17 years: 13–111 mcg/L Adults: < or =20 mcg/L	<2 years: 28–187 mcg/L 2–9 years: 31–152 mcg/L 10–13 years: 29–177 mcg/L 14–17 years: 7–41 mcg/L Adults: Premenopausal: < or =14 mcg/L Postmenopausal: < or =22 mcg/L

When used as a marker for monitoring treatment or exercise-induced changes, it is important to determine the critical difference (or least significant change). The critical difference is defined as the difference between two determinations that may be considered to have clinical significance. The critical difference for this method was calculated to be 25% with a 95% confidence level (BC 1998)

Langberg et al. 2000). Pathologic fluctuations may be the consequences of acromegaly, osteogenic sarcoma, bone metastases, leukemia, and Paget disease of the bone, which must be interpreted carefully in the first visit. Antiresorptive therapies lower BAP from baseline measurements in Paget disease, osteomalacia, and osteoporosis. Studies have shown that antiresorptive therapies for management of osteoporosis patients should result in at least a 25% decrease in BAP within 3–6 months of initiating therapy. Metabolic causes of high BAP include bone growth and fracture healing in which age (Table 2), sex, nutrition, and calcium supplementation are the other relevant factors (Kular et al. 2012; Marsell and Einhorn 2011). The essence of the skeletal response is serum calcium concentration modified by parathormone. Intense and exhausting physical stress has the ability to fluctuate parathormone with consecutive increase in serum calcium in order to be used by the muscles.

Remodeling and Mechanical Loading at Cellular Level

Bone remodeling occurs through the bone's basic multicellular unit (BMU). The BMU consists of the osteoclasts resorbing the bone, the osteoblasts replacing the bone, the osteocytes within the bone matrix, the bone lining cells covering the bone surface, and the capillary blood supply. The remodeling cycle begins with an initiation phase for the recruitment of mature osteoclasts and activation and maintenance of bone resorption. A reversal period then follows where osteoclasts undergo apoptosis and osteoblasts are recruited to differentiate and induce formation. The final stage is bone formation by osteoblasts and is termed the termination phase. This stage is the longest, as bone formation is slower than bone resorption, and involves new bone formation and mineralisation. The length of the bone remodeling process is shorter in the cortical bone than in the cancellous bone, and the average length of the remodeling phase in the cancellous bone is approximately 150 days mostly devoted to bone formation. The signaling and interaction between the cells control this coupling process and coordinate the function of the cells.

Over the past few years, much has been learned about how mechanical loading affects bone structure. The latest studies suggest that bone cells detect mechanical loads through focal integrin linkages. Loading stimulates new bone formation, due to increased signaling through the Wnt/low-density lipoprotein receptor-related protein 5 (LRP5) pathway. Conversely, physical inactivity both suppresses periosteal bone formation and increases receptor activator of nuclear factor kappa-B ligand (RANKL) signaling and bone resorption by apoptosis of osteoblasts and osteocytes, which results in rapid bone loss. However, it is unclear how mechanical signals are perceived by cells to bring about activation of diverse signaling pathways. It is likely that mechanical forces activate many receptors simultaneously to form physical link between extracellular matrix and intracellular organelles. Increasing the rate or loading frequency improves bone tissue mechanosensitivity where osteocytes serve as mechanosensors. Because bone cells quickly become insensitive to applied loads and require time to recover their mechanosensitivity, osteogenesis after mechanical loading can be improved substantially by inserting resting time between loading sessions. Bone loss following physical inactivity is greatest in weight-bearing bones, particularly the distal bones in the legs and almost not existent in bones that do not bear weight (Turner 2007).

Exercise

Aerobic exercise including brisk walking, jogging, swimming, tennis, dancing, cycling, and treadmills involves the use of large muscle groups and must be sustained for at least 20 min/session and three times a week. Muscle-strengthening exercises include weight training, weight-bearing calisthenics, or resistance training. Development of muscle strength and endurance is progressive and requires gradual increases in strengthening activities over time. It should be done a minimum of two nonconsecutive days of the week and should target eight to ten major muscle groups (abdomen, bilateral arms, legs, shoulders, and hips). Recommendations for this purpose are 10–15 repetitions of each exercise at a moderate to high level of intensity and gradually increased resistance over time.

The way in which exercise is thought to act on the skeleton is through gravitational forces or muscle pull producing strains within the skeleton which are perceived by bone cells as osteogenic and anabolic response of the skeletal bone is substantially localized to areas of strain. The skeletal muscle has the ability to adapt to a variety of changes in physical status. The type of training, endurance or resistance, affects the type of muscular adaptations. Endurance training leads to mitochondrial biogenesis, fast to slow fiber transformation, enhanced capillary circulation, and metabolic changes. Resistance training typically increases the size of muscle fibers and cross-sectional area of ligaments and tendons which leads to the ability to exert more force. In general, women and men of all ages show gain in strength although the degree of adaptation to training varies from one individual to another.

Moreover, muscle activity induces some metabolic changes which put forth the organ system-specific roles of different energy sources. A number of biochemical processes in muscle fibers are responsible for maintaining a constant supply of ATP. The main energy-producing pathways that are utilized to prevent significant decreases in ATP concentration during dynamic exercise are the phosphocreatine shuttle, oxidative phosphorylation (aerobic), and anaerobic glycolysis. The most efficient skeletal muscle ATP source is the oxidative phosphorylation of intracellular glycogen and free fatty acids (FFA) in the muscle mitochondria with a net gain of 36 ATP molecules (2 from Krebs cycle, 34 from electron transport chain). Anaerobic glycolysis is a rapid source of two molecules of ATP in which pyruvate derived from glycolysis is converted to lactate without oxygen. High-intensity strengthening exercises involve the use of anaerobic glycolysis at the first 2–5 min before activating oxidative phosphorylation (Spurway 1992).

Exercise-Induced BAP Fluctuations

In a Japanese population-based osteoporosis (JPOS) cohort study (Tamaki et al. 2013), elevated levels of resorption and formation markers were found to be associated with greater bone loss determined by BMD measurement in sedentary premenopausal women than those with lower levels, after adjustment for the effects of age, body mass index, diet, regular exercise, and smoking. Therefore, increase of BAP levels may not be always the indicator of the bone formation process.

Behavior of BAP may be different in physically active people. Male runners and gymnasts unsurprisingly have a higher BMD than male cyclists or swimmers, but markers of bone turnover do not always confirm this result (Taaffe et al. 1997). The different physical performance or different training baseline levels, sex discrepancies, and type/intensity/duration of exercise are the major contributing factors related with the changes in bone turnover status.

Physiologic Increase in BAP

Bone formation stimulation after aerobic training is reflected by elevations in BAP levels in male athletes, while no changes were observed in controls (Eliakim et al. 1997). Aerobic training causes a net increase in bone formation because of the suppression of the bone resorption while anaerobic training induces (Woitge et al. 1998). One-year BMD changes and bone turnover markers after regular running activity of premenopausal subjects and sedentary, low/moderate-intensity trained women were searched. Improvement in the bone mass and acceleration in bone turnover status showed that BAP, osteocalcin, and N-terminal telopeptide (NTX) had increased in 1-year time (Sumida et al. 2014; Shibata et al. 2003). Eliakim et al. (1997) showed an increase in bone formation markers after a 5-week

aerobic training program. On the other hand, study population consisted of adolescent males with a totally different osteogenic response than in adults. In the study of Lester et al. (2009), 8 weeks physical training consisting of aerobic and strengthening components was compared with strengthening exercise by means of osteogenic response. Bone densitometric measurements showed slight elevations in the areas of interest. But formation markers, BAP and osteocalcin, showed marked elevations regardless of the type or volume of exercise, while no concomitant alterations were observed in resorption or turnover markers. This systemic response also showed that biomarkers of bone formation such as BAP are more sensitive indicator of bone osteogenic response than resorption markers in a short-term physical training. These results are consistent with other studies about exercise and bone turnover markers that turnover status may be changed in favor of bone formation by aerobic and resistance training (Lester et al. 2009; Maïmoun et al. 2004; Lohman et al. 1995).

Physiologic Decrease in BAP

The behavior of BAP is sometimes different in discrete conditions depending on physical endurance and factors about the exercise. Marathon runners and high-impact trained male athletes are reported to have decreased BAP levels immediately after the activity, while short-distance runners' BAP levels remain unchanged (Banfi et al. 2010; Maïmoun et al. 2004; Jürimäe et al. 2006; Brahm et al. 1996). Suppression of the BAP is being associated with the increases in cortisol and parathormone due to the intensity and duration of the activity reflecting the changes in calcium homeostasis. Dietary calcium intake and PTH levels are strongly associated with the remodeling status during or after the physical training.

Short-Term Effects of Exercise

The acute exercise effects on bone markers in adults are unclear, and there isn't much data for growing children. The behavior of BAP after a single bout of exercise is controversial. Even one session of jumping exercises (144 jumps) was found to stimulate bone formation immediately after exercise in men and 10-year-old boys, reflected by the increase in BAP and osteoprotegerin. The boys' response by formation markers was greater than the men's, suggesting that growing immature skeletal bone is more sensitive to mechanical stimuli (Kish et al. 2015; Maïmoun et al. 2006). Though it is demonstrated that regular physical exercise increases bone mass by means of prolonged mechanical loading, change in bone markers may not be measurable immediately after exercise in active subjects (Maïmoun et al. 2009), untrained (Welsh et al. 1997; Whipple et al. 2004) or active/sedentary elderly subjects (Maimoun et al. 2011; Maïmoun et al. 2005) (Table 3).

Table 3 Short-term effects of physical activity on bone turnover

(a) In nontrained subjects

Study	Population	Exercise	Evaluation time	Change in bone markers
(Whipple et al. 2004)	M (n = 9; 21.9 ± 1.2 year)	45 min of resistance exercises at 75% of the 10-RM	Just before, just after, and 1, 8, 24, and 48 h of recovery	B-ALP, PICP and sNTX (=)
(Welsh et al. 1997)	M (n = 10; 25.7 years)	30 min of walking at 60% of HRmax	Before the test, just after, and after 0.5, 1, 8, 24, and 32 h of recovery	B-ALP (=) and OC (=) Pyr (+25.1%), D-Pyr (+28.9%) ↑ on day 2 of recovery
(Brahm, Piehl-Aulin, et al. 1997)	M (n = 6), W (n = 6); age, 23–36 years	30-min incremental one-leg knee extension exercise	Before the test and after 5 and 60 min of recovery	PICP and B-ALP ↑ just after exercise and (=) at 60 min of recovery

(b) In trained (active) subjects

Study	Population	Exercise	Evaluation time	Change in bone markers
(Maïmoun et al. 2009)	M and W (n = 18; 71.7 years) active M and W (n = 18; 71.9 years) less active M and W (n = 9; 25.8 years) active	Maximal exercise test (VO2max)	Just before and just after exercise	OC, B-ALP, CTX (=)
(Maïmoun et al. 2005)	M (n = 11) and W active (n = 10; 73.8 years)	Maximal exercise test (VO2max)	Just before and just after exercise	B-ALP, OC, CTX (=)
(Malm et al. 1993)	M (n = 8; 29.9 years) runners W (n = 15; 40.3 years) runners	Marathon running	Day 10, just after marathon,and 1, 3, and 5 days later	OC↓ in M and W after marathon and after 1, 3, and 5 days (only in W) B-ALP ↓ in W after marathon and until 5 days Hydroxyproline (=)
(Maïmoun et al. 2006)	M cyclists (n = 7; 24.4 years)	2 × 50 min at −15% of VT and +15% of VT	Just before, after 30 and 50 min of exercise, and after 15 min of recovery	B-ALP ↑ at 30–50 min, (=) after 15 min recovery, CTX ↑at 30–50 min, (=) after 15-min recovery

(*continued*)

Table 3 (continued)

(b) In trained (active) subjects				
Study	Population	Exercise	Evaluation time	Change in bone markers
(Wallace et al. 2000)	M athletes (n = 8, 28.3 ± 2.8 years) M athletes (n = 8, 25.5 ± 1.5) with placebo	30-min incremental cycle exercise	–60 min, −30 min, and just before exercise and at 15-min intervals during 2 h after the start of the exercise	OC (=) B-ALP (+7.4%) and PICP (+9.2%) at the end of the exercise and remain elevated thereafter (+7%)
(Guillemant et al. 2004)	M trained (n = 7; 30.7 years)	60 min at 80% VO2max on cycle ergometer	–60 min, −30 min, and just before the test 30 and 60 min during the test 30, 60, 90, and 120 min of recovery	Ca supplementation: CTX (=); B-ALP (=) No Ca supplementation: CTX ↑ until 120 min of recovery; B-ALP(=)

M men, *W* women, *HRmax* indicates maximal heart rate, *VT* ventilatory threshold, *GH* growth hormone treatment, VO2maxth, theoretical VO2max, Nontrained = <60 min/week exercise

In Active Individuals

Professional athletes, who have a higher bone turnover than sedentary individuals have shown that short exercise (20–30 min) is insufficient for modifying serum concentrations of bone metabolism markers. Marker variations are more evident after several hours or days after exercise, especially bone formation markers are more sensitive than bone resorption markers. The bone formation markers, BAP and osteocalcin (OC), change approximately after 1 month and 2 months of an exercise program, respectively. In addition, BAP is found to be sensitive to aerobic exercise, and OC is found to be sensitive to anaerobic exercise (Banfi et al. 2010).

Though it is not confirmed in 30 min (Wallace et al. 2000) and 50 min of cycling (Maïmoun et al. 2006), an activity of long duration (>60 min) and high intensity such as in marathon runners, a decline in BAP and osteocalcin may be observed without any change in resorption markers just after the exercise and recovery (Malm et al. 1993). The bone cell metabolic activity in response to cycling for 30–50 min was similarly investigated (Maimoun et al. 2011). BAP and osteocalcin levels were shown to be elevated transiently in both groups with a marked elevation in 50 min high- intensity training group which suggested the existence of a bone formation-stimulation threshold. All markers returned to initial values during the recovery. In a similar protocol, no variation was observed in BAP and CTX after 60 min of cycling and 30 min of recovery in calcium supplementation group (Guillemant et al. 2004). However the increased CTX in the group without supplementation highlights the importance of metabolic balance. BAP is reported to remain unchanged immediately after the exercise and recovery period by high-intensity exercise with cycle

ergometer (Whipple et al. 2004; Guillemant et al. 2004). Marathon runners with high-intensity and long-duration (>2 hours) physical activity may cause an immediate or longer-lasting decrease in BAP without any modification in resorption markers (Malm et al. 1993). There are several hypotheses to explain this negative metabolic modulation as a stress response such as overproduction of glucocorticoids, high lactic acid, and parathormone concentrations after the marathon. It is also shown that calcium intake can reverse this effect in favor of formation after an exhaustive exercise (Banfi et al. 2010;Guillemant et al. 2004).

In Sedentary Individuals

No immediate change in bone markers was observed in any of the studies on strength training except one, in which BAP increased and osteocalcin decreased at 60 min of recovery after 30 min of leg extension exercises (Brahm et al. 1997b).

In sedentary subjects, most of the studies report no immediate variations in BAP levels after 30-min endurance training (Welsh et al. 1997). In contravention of this conclusion, an anabolic effect was also reported associated with an increase in BAP and decrease in resorption markers with 30-min exercise of 60% of peak VO2 max. In postmenopausal women, jogging is capable of raising BAP at the end of the exercise with normalization after 20 min. However the same exercise was not sufficient to change BAP in healthy young females, highlighting the bone cell function variability at different ages. Exercise that exceeds 20–30 min seems to induce a change in bone turnover markers (Maimoun et al. 2011). It takes 60 min–24 hours of recovery for the stabilization process as observed in most of the studies. In bone formation marker, BAP appears to be a very attractive tool for investigating the immediate response of osteoblasts to exercise, particularly in choosing the type and intensity of the exercise to improve bone health.

Long-Term Effects of Exercise

Study populations have demonstrated distinctly different behaviors about remodeling and BAP. High-intensity resistance training seems to have a bigger impact on BAP/pyridinoline ratio in favor of bone formation confirmed also by bone mineral density measurements (Vincent and Braith 2002) (Table 4).

In Active Individuals

Studies that focused on both bone formation and resorption markers indicated that physically active people (athletes, swimmers, judoists) present accelerated bone turnover (Creighton et al. 2001; Hetland et al. 1993; Lima et al. 2001; Maïmoun et al. 2004, 2008; Karlsson et al. 2003) which the bone metabolic balance is mostly in favor of formation. In contrast and as an exception, endurance athletes may have

Table 4 Long-term effects of physical activity on bone turnover

(a) In nontrained subjects

Study	Population	Exercise	Evaluation time	Change in bone markers
(In children) (Eliakim et al. 1997)	M training group (n = 20; 16 year)	2 h/day, 5×/week: aerobic training (90%) and resistance training (10%) during 5 weeks	Before and after 5 weeks	In trained subjects: OC (+15%), B-ALP (+21%), PICP (+30%) UrNTx (−21%) DPD (=), urCTx (=) In control group: no variation
In adults (Woitge et al. 1998)	M aerobic training (n = 10; 25.3 ± 2.6 years) M anaerobic training (n = 10; 23.5 ± 2.9 years) M control (n = 12; 25.3 ± 2.7)	Aerobic or anaerobic training program 60 min/day; 3×/week during 8 weeks	Before and after 4 and 8 weeks	Aerobic group: B-ALP and OC 1 at week 4 and (=) at week 8. PYD and DPD 1 at week 4 and week 8 Anaerobic group : B-ALP, OC, and PYD↑ At week 8
(Fujimura et al. 1997)	M training group (n = 8; 26.4 ± 1.2 years) M control (n = 7; 24.6 ± 1.0 year)	Weight training program 45 min/day; 3×/week during 4 months	Before and after 1, 2, 3, and 4 months of training	OC (+26.3%) and B-ALP (+30%), in trained PICP (−18%) 1, in controls DPD (=)
(Menkes et al. 1993)	M training (n = 11; 59 ± 2 year) M control(n = 7;55 ± 1 year)	16 weeks strength training; 3×/ week	Before and after 12–16 weeks of training	Variation in trained subjects: OC (+19%) At 12–16 weeks, B-ALP(+26%) at 16 weeks and TRAP (=)
(Yamazaki et al. 2004)	W training (n = 27; 64.2 ± 2.9 years) W control (n = 15;65.7 ± 2.7 years)	1 year of walking; 1 h/session; 4×/week at 50% of VO2max	Just before and after 1, 3, 6, 9, and 12 months in the trained and every 6 months in the control group	In trained group: B-ALP (−20%), uNTX (−25%) OC (=) after 3–12 months In control group uNTX (=)

(Vincent and Braith 2002)	M and W HRG (n = 22; 66.6 ± 7 years) M and W LRG (n = 22;67.6 ± 6 year) M and W control (n = 16;71 ± 5 year)	6 months, 30 min/day; 3×/week. Training at 80% of their 1-RM training or at 50% of their 1-RM	Before and after 6 months	OC LRG (+25.1%) and HRG (+39%) B-ALP (+8%) and PYD (=)in HRG Ratio OC/PYD HRG > LRG > control Ratio B-ALP/PYD HRG > LRG and control
(Ryan et al. 1994) (Alp 2013)	M training (n = 21; 61 ± 1 y) Premenopausal W training (n = 50;47 ± 7 years), W control (n = 50; 49 ± 5)	16 weeks of strength training, 3×/week 8 weeks of submaximal aerobic exercise 5×40 min/week	Before training and after 16 weeks of training Just before and after 8 weeks	TRAP↑ in trained. OC, B-ALP (=) CTX ↓, BAP(+26%) in trained

(b) In trained (active) subjects

Study	Population	Exercise	Evaluation time	Change in bone markers
(Etherington et al. 1999)	M military recruits (n = 40;18.5 ± 1.6 years) 10 weeks of military training	Habitual training	Before and after 10 weeks	OC↑, B-ALP ↑ (−13.6%) TRAP (=)
(L. Maïmoun et al. 2004)	M military recruits (n = 7; 19.2 years) 15 h/week	Habitual training	Before and after 32 weeks	B-ALP (−22%), CTX (=)
(Sartorio et al. 2001)	M trained (n = 16; 72.9 ± 0.95) M control (n = 14; 73.3 ± 1.04), 45–60 min/day moderate physical activity	16 weeks high-intensity strength training, 3×/week	Before and after 16 weeks	B-ALP (+31.7%) in trained OC and PINP (=) in trained and controls

GY indicates gymnasts, *T* an increase compared with pretraining values, = no change compared with pretraining values, *j* a decrease compared with pretraining values, *HRG* high-resistance intensity group, *LRG* low-resistance intensity group

lower bone formation and resorption marker levels in comparison with less active controls (Brahm et al. 1997a). Female athletes may have normal BAP and lower resorption markers (Ryan and Elahi 1998), or amenorrheic marathon runners may have lower bone turnover with lower BAP levels and established osteopenia which is due to estrogen deficiency (Zanker and Swaine 1998; Crespo et al. 1999). In young athletes the relative stability of bone mass (decrease in BAP and no change in osteocalcin) during sport season requires new more sensitive ways of detecting minor variations in bone turnover. Anaerobic or strengthening exercises induce bone turnover or accelerate BAP with suppression or no variation of resorption (Etherington et al. 1999; Maimoun et al. 2004). The exercise intensity seems to play an important role in the bone's cellular response. A positive effect on the bone is the maintenance or gain of bone mineral density as well as decrease (Yamazaki et al. 2004) or increase (Sartorio et al. 2001) in BAP.

In Sedentary Individuals

Five to eight weeks aerobic training improves bone remodeling in favor of formation because it reduces resorption transiently (Woitge et al. 1998) or in the longer term (Eliakim et al. 1997; Woitge et al. 1998; Eliakim et al. 1996). Anaerobic and resistance training may induce bone turnover or exactly the opposite with a decrease in resorption markers and a relative increase in BAP (Woitge et al. 1998). Training intensity and its components (strength and endurance) are the contributing factors on bone remodeling. Aerobic endurance training seems to improve bone turnover in favor of formation; however, anaerobic exercise induces an overall acceleration in bone turnover.

Ten weeks of military training was reported to have a decrease in BAP (Etherington et al. 1999), while 15 weeks of training in a similar study population induced an increase in formation markers (Casez et al. 1995). Exercise intensity and duration are the key factors for this anabolic effect in young subjects. It needs a few months of training at least to stabilize the turnover for a net gain in bone mass. Eight-week training of aerobic and anaerobic exercises in young adults was investigated by Woitge et al. (1998), and it was concluded that both demonstrated different metabolic effects. While aerobic exercise led to reduction in bone formation (BAP) and resorption (pyridinoline) markers, anaerobic activity induced an accelerated bone turnover. Prolonged oxygen deficiency was charged to imbalance in bone remodeling "uncoupling." Markers of bone formation returned to baseline after the eight-week training. The influence of exercise on bone mineral density is evaluated in adolescents (Eliakim et al. 1997) and sedentary elderly subjects (Menkes et al. 1993) in which BAP is found to be elevated after an aerobic training by 21% and 19%, respectively. BAP/pyridinoline ratio was shown to be a more reliable indicator for bone formation, and it was higher in resistance training group when compared with the group of low-intensity training (Vincent and Braith 2002). Anaerobic or strengthening exercises accelerate BAP with no variation of resorption

(Fujimura et al. 1997). BAP increase accompanied by gain in bone mass may be interpreted as the positive effects of exercise (Ryan et al. 1994).

Bone turnover effects of moderate-intensity aerobic exercise in premenopausal women were investigated by Alp (2013) in comparison with a control group. Though the rise of BAP from baseline was not statistically significant at the end of the second month, marked decrease was observed in CTX levels. It seemed that submaximal aerobic exercise may be effective in preserving bone mass for this type of population, but is not enough to enhance the bone mass. In another randomized controlled study, the effects of low-intensity yoga-based exercise program twice a week were evaluated in hemodialysis patients (Yurtkuran et al. 2007). After 3 months of intervention, significant decrease in BAP (by 15%) was observed in the yoga group as the reflection of attenuated bone remodeling status when compared with the controls. These changes were determined to be related to general effects of regular exercise rather than specific effects of yoga which was accompanied by clinical improvement in pain, sleep disturbances, fatigue, and grip strength. In conclusion, if a strain is detected as greater than the standard strain for an optimum time, then bone turnover lessens and bone formation can dominate (Lanyon 1984). Studies investigating the long-term (4 months–4 years) exercise have shown a reduction (Yamazaki et al. 2004), an increase (Sartorio et al. 2001; Menkes et al. 1993; Vincent and Braith 2002), or no change (Ryan et al. 1994) in BAP in postmenopausal subjects and old men.

Potential Applications to Prognosis, Other Diseases, or Conditions

Osteoporosis is a metabolic bone disease characterized by low bone mass and abnormal bone microarchitecture with increased risk of fracture. It can result from a number of clinical conditions with high bone turnover identified by high BAP levels. These conditions may be endocrine disorders (primary and secondary hyperparathyroidism and thyrotoxicosis), osteomalacia, renal failure, gastrointestinal diseases, long-term corticosteroid therapy, multiple myeloma, and malignancy with metastasis to the bones. Paget disease is another common metabolic bone disease caused by high rates of bone remodeling resulting in local punched lesions. It is usually not recognized until the subsequent bone formation response resulting in enlarged and deformed bones. This excessive resorption and formation bring out an abnormal mosaic pattern of the lamellar bone associated with increased vascularity and fibrous tissue deposition in adjacent marrow spaces. These lesions can result in fractures or neurological involvement as the consequence of mechanic pressure. Antiresorptive therapies are used to restore the normal bone structure.

Utility of BAP as a Prognostic Factor

BAP, as a bone formation marker, is determined to be an indicator of the bone turnover status, and therefore, physical training consequences can be established and measured

by its serum levels. BAP can be used for demonstrating the existence of metabolic bone diseases or assessing the severity of Paget disease, osteomalacia, and other states of high bone turnover. Monitoring efficacy of systemic antiresorptive therapies including postmenopausal osteoporosis treatment may be another means of use.

Cautions

When used as a marker for monitoring purposes, it is important to determine the critical difference or least significant change. The critical difference is defined as the difference between two determinations that may be considered to have clinical significance. The critical difference for this method was calculated to be 25% with a 95% confidence level (Table 2).

Liver-derived alkaline phosphatase has some cross-reactivity in this assay: 100 U/L of liver ALP activity gives a result of 2.5 mcg/L to 5.8 mcg/L. Accordingly, serum specimens with significant elevations of liver ALP activity may yield elevated results.

Conclusions

The interpretation of accelerated or ameliorated bone remodeling must be done cautiously according to study populations mentioned above; thus, crucial data is still lacking on how physical exercise effects bone remodeling process and BAP. Despite the wide discrepancies among studies, some conclusions can be drawn.

The overview of the regulation in remodeling establishes that bone mass can be enhanced by both strenuous aerobic exercise and strength training (Creighton et al. 2001). Protection against osteoporosis by physical training is a will, but it somehow may not happen because of a variety of personal factors. Therefore, the need arises for a homogenous group and a crucial study design for bone metabolism investigations with serial laboratory measurements if possible. A common profile and homogenous behavior of bone turnover markers, especially BAP, must be defined entirely for sedentary subjects and professional athletes of both sexes. In conclusion, present data lacking evidence for a general use of bone formation/turnover markers to detect metabolic changes enables to recommend a certain exercise prescription for a standardized group at the moment.

Summary

- Bone mass is the net product of counteracting metabolic processes: bone formation and resorption. BAP is generally used in the prediction of high bone turnover.
- Experimental studies have revealed that long-term (6–12 months) training with high strain rates and excessive forces are more effective in the anabolic adaptation process than low-strain physical exercise of the same duration.

- Acceleration of bone remodeling may be the consequence of various metabolic conditions extremely different in athletes and sedentary healthy subjects.
- Individuals or adolescents with high-intensity training have more stable bone turnover status during and after physical activity, compared to untrained sedentary subjects. Untrained sedentary subjects may have an accelerated or suppressed bone turnover reaction to physical training, depending on personal metabolic needs as the acute or delayed effect in the recovery period.
- Female high-intensity trainers may have suppressed BAP levels immediately or at the recovery period contrary with men trainers highlighting the sex discrepancy or estrogen deficiency. It must be retained that unbearable or unusual concentrated mechanical stress especially in amenorrheic runners may be associated with a metabolic uncompensated remodeling process in favor of resorption which may lead to microdamage and stress fractures of the involved bones.
- It seems that mild general exercise such as walking is effective in preventing postmenopausal bone loss but not enhancing bone mass in younger age. Bone turnover markers associated with fracture risk reduction need to be determined in the management of osteoporosis.

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Biomarkers of Bisphosphonate Failure in Osteoporosis

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Elisa Cairoli and Iacopo Chiodini

Contents

Abstract

Bisphosphonates are the first-line agents for the management of osteoporosis. Through the suppression of bone turnover, they are able to significantly reduce fracture risk in patients with an adequate calcium and vitamin D supplementation. Bisphosphonate failure can be assumed when two or more fragility fractures occur in the course of treatment, but surrogate markers of the efficacy of bisphosphonate treatment are the variations of bone mineral density (BMD) and

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of bone turnover markers (BTM). Indeed, the demonstration of a significant decrease in BMD and the absence of a significant decrease in BTM while on therapy are considered as indicators of treatment failure. Moreover, other biochemical, clinical, and genetic parameters can be predictive of an inadequate response to bisphosphonate treatment.

Keywords

Osteoporosis • Fracture • Bisphosphonate • Treatment failure • Bone turnover • Bone mineral density

List of Abbreviations

AFF	Atypical femoral fracture
ALP	Alkaline phosphatase
BALP	Bone-specific alkaline phosphatase
BMD	Bone mineral density
BSP	Bone sialoprotein
BTM	Bone turnover marker
CTX	Carboxy-terminal cross-linking telopeptide of type I collagen
DPD	Deoxypyridinoline
FDFT1	Squalene synthase
FPPS	Farnesyl pyrophosphate synthase
GGPS	Geranylgeranyl diphosphate synthase
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IOF	International Osteoporosis Foundation
LRP5	Low-density lipoprotein receptor-related protein
LSC	Least significant change
MVK	Mevalonate kinase
NTX	Amino-terminal cross-linking telopeptide of type I collagen
OC	Osteocalcin
ONJ	Osteonecrosis of the jaw
PINP	Amino-terminal propeptide of type I procollagen
VDR	Vitamin D receptor

Key Facts of Bisphosphonates

- Osteoporosis is a skeletal disorder characterized by reduced bone mineral density and disruption of bone microarchitecture, which leads to impaired bone strength and an increased risk of fractures.
- Fragility fractures are an important cause of morbidity and mortality, and the aim of any osteoporosis treatment is the fracture risk reduction.
- Bisphosphonates are first-line drugs used for the treatment of osteoporosis since randomized clinical trials have demonstrated that they are able to reduce the risk

of fractures in association with an adequate calcium and vitamin D supplementation.

- Their action is mediated by the suppression of bone resorption which is obtained through the inhibition of farnesyl pyrophosphate synthase in the osteoclasts.
- Osteoporosis-approved bisphosphonates are alendronate, risedronate, ibandronate, and zoledronic acid. They differ from each other in terms of route of administration, dosing schedule, and antiresorptive potency.
- Potential side effects of bisphosphonates include esophageal irritation for those administered orally and acute-phase reaction for those administered intravenously.
- Rare but serious adverse effects of long-term bisphosphonate therapy are atypical femoral fractures and osteonecrosis of the jaw.

Definitions of Words and Terms

Osteoporosis	A systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue which leads to increased bone fragility and susceptibility to fracture.
Bone mineral density (BMD)	The amount of bone mass per unit volume (volumetric density) or per unit area (areal density) which can be measured in vivo by densitometric techniques.
Bone turnover	The metabolic process of bone remodeling which occurs throughout life and which consists of the dissolution of bone matrix by osteoclasts (bone resorption) followed by the deposition in the resorption cavities of new bone by osteoblasts (bone formation).
Bone turnover markers	Biochemical products of cellular and noncellular elements of the bone which are indicative of the metabolic activity of the bone. They can be usually measured in blood or urine and they are divided in markers of bone resorption and markers of bone formation, according to the phase of bone turnover they reflect.
Osteoclasts	Bone-specific multinucleated giant cells derived from the monocyte/macrophage hematopoietic lineage which have the task of bone resorption.
Fragility fracture	A fracture that occurs without any identifiable trauma or as a result of a minimal trauma that would be insufficient to fracture a normal bone (e.g., a fall from a standing height or less).

Adherence	A term including both the concepts of persistence and compliance: persistence is the duration of time from initiation to discontinuation of therapy; compliance is the degree to which a patient takes the medication as prescribed.
Least significant change (LSC)	The least variation of a specific parameter that can be considered statistically significant, that is, it represents a meaningful biological change within an individual. It depends on the analytical (CV_a) and intraindividual (CV_i) coefficients of variability. The recommended formula for calculating the LSC with a 95% level of confidence is the following: $1.96 \times \sqrt{2} \times \sqrt{(CV_a^2 + CV_i^2)}$.
Genetic polymorphism	A variation in the DNA sequence of a gene that occurs in a population with a frequency of 1% or more.
Osteonecrosis of the jaw (ONJ)	The appearance of exposed bone in the maxillofacial region that persists for at least 8 weeks in the absence of previous radiotherapy in the craniofacial region.
Atypical femoral fracture (AFF)	A subtrochanteric or femoral shaft fracture in the presence of minimal trauma, lateral cortex origin and transverse appearance, complete extension through both cortices, periosteal or endosteal cortical thickening, and minimal comminution at most.

Introduction

The aim of any osteoporosis treatment is the fracture risk reduction. However, no available treatment is able to eliminate fracture risk. According to this essential concept, the occurrence of a fragility fracture while on therapy for at least 6 months does not necessarily mean that the treatment has failed. Thus, the definition of treatment failure in osteoporosis is more complex and less obvious than expected. In 2012 a working group of the Committee of Scientific Advisors of the International Osteoporosis Foundation (IOF) recommended that a treatment failure may be postulated when two or more incident fractures have occurred during treatment (Díez-Pérez et al. 2012a). Surrogate markers of the response to the treatment are the variations in terms of bone mineral density (BMD) and bone turnover markers (BTM).

Bisphosphonates are the most frequently used agents for the management of postmenopausal, male, and glucocorticoid-induced osteoporosis. Their anti-fracture action is mediated by the suppression of bone resorption through the inhibition in the osteoclasts of farnesyl pyrophosphate synthase (FPPS), an enzyme in the

mevalonate-to-cholesterol pathway (Fig. 1), which induces the detachment of the osteoclasts from the bone surface and their apoptosis (Favus 2010).

Approved bisphosphonates for osteoporosis therapy are alendronate, ibandronate, risedronate, and zoledronic acid. They differ from each other on the basis of route of administration, dosing schedule, bone-binding affinity, and antiresorptive potency

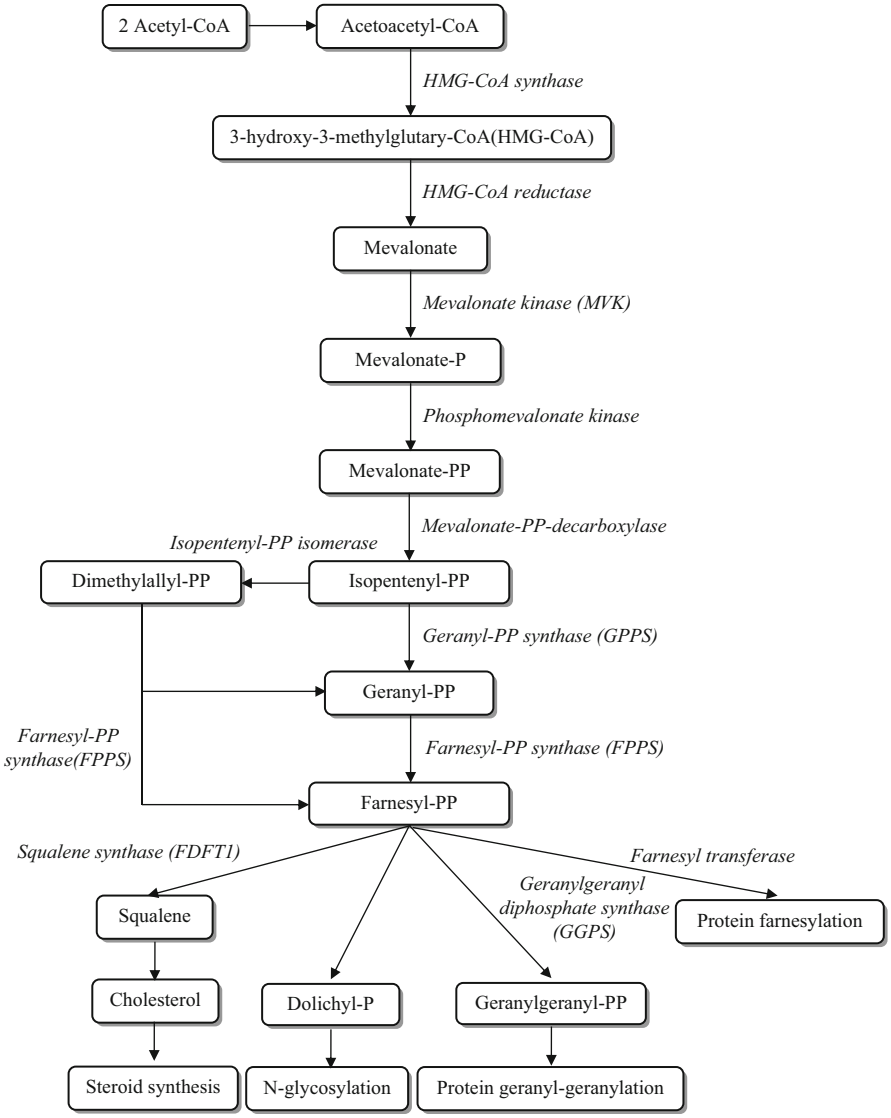

Fig. 1 The mevalonate-to-cholesterol pathway and the metabolic pathway in which bisphosphonates intervene, inhibiting the farnesyl-PP synthase (*FPPS*). *CoA* coenzyme A, *HMG* 3-hydroxy-3-methylglutaryl, *P* phosphate, *PP* pyrophosphate. *N-glycosylation* amino-glycosylation

Table 1 Bisphosphonates approved for the treatment of postmenopausal osteoporosis and their main features. Large, randomized, placebo-controlled, clinical trials demonstrated the fracture risk reduction with bisphosphonates (those administered orally in daily dose) in association with an adequate calcium and vitamin D supplementation. The comparability between daily oral doses and weekly or monthly doses has been subsequently established by assessment of comparative changes in bone mineral density and bone turnover markers

				Fracture risk reduction	
Bisphosphonates	Dosage	Dosing schedule	Route	Hip	Vertebral
Alendronate	70 mg	Weekly	Oral	x	x
Risedronate	35 mg 150 mg	Weekly Monthly	Oral Oral	x	x
Ibandronate	150 mg 3 mg	Monthly Quarterly	Oral Intravenous		x
Zoledronic acid	5 mg	Yearly	Intravenous	x	x

(Favus 2010). An overview of the features of osteoporosis-approved bisphosphonates is presented in Table 1. Randomized, placebo-controlled trials have demonstrated that these drugs are able to determine a significant reduction of the fracture risk, provided that an adequate adherence and calcium and vitamin D supplementation have been guaranteed (Harris et al. 1999; Black et al. 2000, 2007). Indeed, a poor compliance and a scarce intake of calcium and vitamin D are the most likely reasons for a suboptimal response to antiresorptive therapies (Lewiecki 2003). In addition, the presence of a secondary cause of osteoporosis can make ineffective the medical treatment (Fitzpatrick 2002). This is of utmost importance considering that a secondary cause of osteoporosis is present in up to 40% of patients with apparent primary osteoporosis (Eller-Vainicher et al. 2013). An overview of these established causes of suboptimal response to bisphosphonate treatment is presented in Table 2. However, even when these conditions are excluded, some patients do not adequately respond to bisphosphonate therapy, and two or more fragility fractures occur during treatment.

Since a fragility fracture is an important cause of disability and it is the undesirable event that the physician tries to prevent, the availability in the clinical practice of markers of bisphosphonate failure would give a considerable help to the clinician for predicting a priori which patient would have much likelihood to respond to this therapy and for understanding during treatment who should be switched to a different drug before the fracture occurs. Scientific data have demonstrated that the variations of BMD and of BTM can be used as surrogate markers of the efficacy of bisphosphonate treatment, but genetic factors and other biochemical and clinical parameters can be predictive of treatment failure.

Bone Mineral Density

Osteoporosis is, by definition, a condition characterized by bone loss (NIH Consensus Development Panel on osteoporosis prevention, diagnosis, and therapy 2001), and BMD was demonstrated to be able to predict fragility fractures (Marshall

Table 2 Established causes of suboptimal response to bisphosphonate treatment and the main causes of secondary osteoporosis

Poor adherence	
Scarce intake of calcium and vitamin D	
Secondary osteoporosis	*Endocrine diseases* Acromegaly, diabetes mellitus, growth hormone deficit, hypogonadism, hypercortisolism, hyperparathyroidism, hyperthyroidism *Gastrointestinal diseases* Celiac disease, chronic liver disease, inflammatory bowel disease, malabsorption syndromes *Hematologic diseases* Lymphoproliferative and myeloproliferative disorders, multiple myeloma, systemic mastocytosis *Renal diseases* Chronic kidney disease, idiopathic hypercalciuria, renal tubular acidosis *Rheumatologic diseases* Ankylosing spondylitis, rheumatoid arthritis, systemic lupus erythematosus *Organ transplantation* Bone marrow, heart, kidney, liver, lung *Drugs* Anticonvulsants, aromatase inhibitors, chemotherapy, glucocorticoids, gonadotropin-releasing hormone agonists, immunosuppressants, thiazolidinediones *Miscellaneous conditions* Chronic obstructive pulmonary disease, eating disorders, prolonged immobilization, severe disability

et al. 1996). Thus, it could appear presumable that a BMD increase during treatment would be a sign of the efficacy of the therapy and, conversely, a BMD reduction a sign of a useless therapy.

The working group of the Committee of Scientific Advisors of the IOF proposed that a decrease in BMD greater than the least significant change (LSC) with a 95% level of confidence is considered as an indicator of failure to respond to treatment (Díez-Pérez et al. 2012a). The LSC is a parameter which defines the change in BMD that can be confidently detected, depending on the precision error of the technique applied and on the confidence needed to assume a change.

However, a patient could benefit from a reduction in fracture risk even in the presence of a BMD decrease while on treatment, as demonstrated in the Fracture Intervention Trial (FIT), where, for similar decreases in BMD, a decrease in fracture risk in alendronate-treated patients was demonstrated compared with those receiving placebo (Chapurlat et al. 2005).

Indeed, osteoporosis is also a disease characterized by alteration of the bone quality, and bisphosphonates induce not only a BMD increase but also changes in bone microarchitecture (Díez-Pérez and González-Macías 2008). Thus, BMD variations explain only a limited part of the anti-fracture efficacy of these drugs, and treatment-induced changes in microarchitecture and in other parameters of bone quality can significantly influence the fracture risk (Seeman 2007).

Bone Turnover Markers

Markers of bone turnover are biochemical products derived from cellular and noncellular compartments of the bone which can be measured usually in blood or urine and reflect the metabolic activity of the bone. They are usually divided, according to the metabolic phase of bone turnover they reflect, in markers of bone resorption and markers of bone formation. Markers of bone resorption include degradation products of bone collagen, such as the carboxy-terminal cross-linking telopeptide of type I collagen (CTX); noncollagenous proteins, such as the bone sialoprotein (BSP); and osteoclast-derived enzymes, such as cathepsin K and L. Markers of bone formation are products of active osteoblasts expressed during different phases of osteoblast development, such as the bone-specific alkaline phosphatase (BALP), osteocalcin (OC), and amino-terminal propeptide of type I procollagen (PINP) (Seibel 2005). The most important BTM are summarized in Table 3.

The management of osteoporosis with antiresorptive treatments, like bisphosphonates, is associated with an early decrease of markers of bone resorption (already visible after 1 month on treatment with a plateau from 3 months onward) and a later decrease of markers of bone formation after a delay of about 4 weeks due to the coupling of bone resorption and formation (Fig. 2). According to this pharmacologic effect of reduction of the bone turnover, it could be conceivable

Table 3 Nomenclature of markers of bone turnover, their abbreviation, and the biological sample in which they can be measured. They are divided into markers of bone resorption, which include degradation products of bone collagen, noncollagenous proteins, and osteoclast-derived enzymes, and markers of bone formation, which are products of osteoblast activity. *MMP* matrix metalloproteinases

Markers of bone resorption	**Abbreviation**	**Sample**
Amino-terminal cross-linking telopeptide of type I collagen	NTX	Serum Urine
Carboxy-terminal cross-linking telopeptide of type I collagen	CTX	Serum Urine
Carboxy-terminal cross-linking telopeptide of type 1 collagen (generated by MMP)	ICTP or CTX-MMP	Serum
Deoxypyridinoline	DPD	Urine
Pyridinoline	PYD	Urine
Bone sialoprotein	BSP	Serum
Tartrate-resistant acid phosphatase	TRACP	Serum
Cathepsins (K, L)		Serum
Markers of bone formation	**Abbreviation**	**Sample**
Osteocalcin	OC	Serum Urine
Bone-specific alkaline phosphatase	BALP	Serum
Carboxy-terminal propeptide of type I procollagen	PICP	Serum
Amino-terminal propeptide of type I procollagen	PINP	Serum

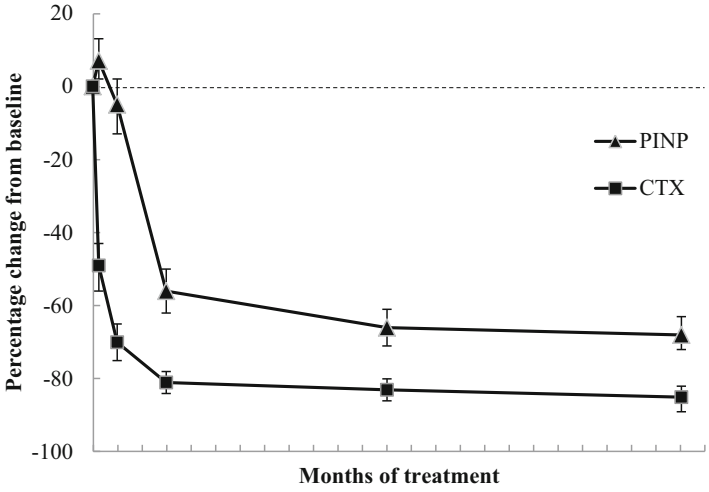

Fig. 2 The direction and magnitude of changes (expressed as mean percentage change from baseline with 95% confidence interval) for a bone formation marker (*PINP*) and a bone resorption marker (*CTX*) in response to bisphosphonate treatment (oral alendronate 70 mg once a week) over 2 years (Figure based on data from Naylor et al. 2016). *PINP* carboxy-terminal propeptide of type I procollagen, *CTX* carboxy-terminal cross-linking telopeptide of type I collagen

that the higher the baseline bone turnover, the greater the expected therapeutic response. However, the accelerated bone turnover itself may be an independent risk factor for fracture (Kanis 2002). Thus, since the available studies used different BTM and different methodologies for their assessment, it is not surprising that conflicting results about the influence of baseline bone turnover on treatment efficacy were found. One study found that risedronate treatment reduced the incident vertebral fractures in women with postmenopausal osteoporosis independent of pretreatment bone resorption as evaluated by the urinary excretion of deoxypyridinoline (DPD) (Seibel et al. 2004). In a post hoc analysis of the FIT, higher baseline levels of PINP were associated with a greater reduction in non-vertebral fracture risk in response to alendronate as compared to lower baseline levels of PINP, but the same association was not found for vertebral fracture risk (Bauer et al. 2006). Finally, another study found that, in postmenopausal women presenting baseline alkaline phosphatase (ALP) values within the upper half of the normal range for premenopausal women, the risk of an inadequate response to bisphosphonate treatment was fourfold increased as compared to women with baseline ALP values in the lower half of the normal range (Cairoli et al. 2014). Therefore, according to these not conclusive results, currently the assessment of baseline bone turnover does not seem to be crucial for treatment decision. However, the availability of baseline values of BTM could be useful in the subsequent treatment follow-up. Indeed, since many studies explored the changes of BTM in the course of therapy for monitoring the effects of treatment, a baseline assessment and another measurement at some defined point during treatment are required. The change in a BTM is then usually expressed

as a percentage of variation of the baseline values, and it is considered significant when exceeding the LSC, a parameter which takes into account both the analytical and intraindividual variability. Alternatively, if baseline levels are unknown, the variation in a BTM can be assumed significant if the value returns in the lower half of the reference interval for premenopausal women, although it is clear that this method can be unreliable since it subtends a significant reduction of BTM only if the pretreatment values were at least in the higher part of the range or abnormally high, a condition that occurs only in a limited percentage of osteoporotic subjects (Vasikaran et al. 2011).

The short-term decrease in BTM under antiresorptive therapy is strongly related to the long-term BMD increase. This association was clearly demonstrated for the hormonal replacement therapy, but a significant relationship has also been reported during bisphosphonate treatment in various studies.

In elderly osteoporotic women treated with alendronate, the changes in urinary CTX at 6 months correlated with long-term BMD changes at the hip, spine, and total body, and the patients with the greatest drop in urinary CTX ($\geq$65%) demonstrated the greatest BMD gains (Greenspan et al. 1998).

In postmenopausal women treated with alendronate, the bone-specific ALP (BALP) levels and the percent BALP change at 6 months were found to be independent predictors of long-term positive BMD response, defined as >3% increase in lumbar BMD at 2 years. Moreover, the combined use of both parameters in a logistic model allowed an accurate identification of nonresponder patients to alendronate treatment in terms of BMD gain (Garnero et al. 1999). A further study observed that in alendronate-treated women, the change from baseline at 6 months in urinary amino-terminal cross-linking telopeptide of type I collagen (NTX) and OC correlated with lumbar and femoral BMD change from baseline at 2 years, although the lack of decrease below a specific threshold in NTX or OC failed to identify women experiencing a bone loss during alendronate treatment (Ravn et al. 1999).

More recently, the TRIO study compared the effects of oral alendronate, ibandronate, and risedronate over 2 years on BMD results and BTM responses. Postmenopausal women who reached the target for response for PINP (considered as a reduction greater than the LSC) by 12 weeks of bisphosphonate treatment experienced a greater increase in lumbar spine BMD at 2 years than those that failed to reach the target for treatment (Naylor et al. 2016). A similar conclusion was obtained in another study for urinary NTX where a poor response in urinary NTX (considered as a change in urinary NTX/creatinine lower than the LSC) at 4 months was proposed to be a useful early indicator in clinical practice of a low response in lumbar BMD after 18 months of risedronate or alendronate treatment (Baxter et al. 2013). Similarly, a French study on a small sample of postmenopausal osteoporotic women demonstrated that a significant change in serum CTX after 4 months of alendronate therapy was predictive of a significant increase in lumbar BMD after 12 months of treatment (Fink et al. 2000).

Overall these studies indicate that during bisphosphonate treatment, a significant decrease of BTM is associated with a subsequent significant BMD gain and,

conversely, that small or no changes in BTM are highly suggestive of a subsequent poor BMD response to treatment.

However, an intrinsic limitation of all these studies is that their primary endpoint is to assess the ability of BTM variations under therapy to predict the BMD change, which in turn is only a surrogate marker of anti-fracture efficacy of antiresorptive treatments, as previously explained.

Post hoc analysis of randomized clinical trials on bisphosphonates assessed the correlation between the BTM changes under treatment and the fracture risk, and in general, they showed that the larger the decrease in BTM, the larger the reduction in fracture risk. In more detail, data from the FIT showed that greater reductions in one or more BTM after 1-year alendronate treatment were associated with a lower risk of vertebral, hip, and non-vertebral fractures (Bauer et al. 2004), suggesting that the measurements of bone turnover in the course of treatment may help to identify alendronate-treated women with suboptimal response. Similarly, in the Vertebral Efficacy with Risedronate Therapy (VERT) study, the reductions in urinary CTX and NTX at 3–6 months of risedronate treatment were significantly associated with the reduction in vertebral fracture risk, and the changes in these bone resorption markers accounted for a percentage of fracture risk reduction with risedronate between 49% and 77%, depending on the marker and the fracture type (vertebral or non-vertebral) (Eastell et al. 2003). Moreover, in this trial the lowest fracture risk was reached when the urinary CTX was below a level equivalent to the mean value for premenopausal women (Eastell et al. 2007). Finally, the Health Outcomes and Reduced Incidence with Zoledronic Acid Once Yearly (HORIZON) study found that lower levels of PINP 1 year after an infusion of 5 mg zoledronic acid were associated with a lower risk of clinical fractures (Delmas et al. 2009), stressing the importance of bone turnover reduction as a key mechanism to reduce fracture risk in patients treated with bisphosphonates, not only orally but also intravenously.

Further studies assessed the relationship between BTM and fracture risk reduction and similar data were reported. One of these is the Improving Measurements of Persistence on ACtonel Treatment (IMPACT) study, a multinational prospective, open-label, cluster-randomized study of postmenopausal women on oral risedronate for 52 weeks, which showed that in patients with a reduction in urinary NTX or serum CTX levels greater than 30%, the incidence of both non-vertebral and all fractures (vertebral and non-vertebral) was significantly lower compared with patients with a 30% or less reduction of BTM. This association was confirmed also in a subgroup analysis of women with good adherence (>80%) (Eastell et al. 2011).

However, beyond the results of these trials, to date it is not completely clear how the BTM should be used in the clinical practice. Indeed, although the available trials seem to suggest a clear relationship between BTM changes under antiresorptive treatment and fracture risk reduction, the use of different BTM across studies, the presence of numerous sources of pre-analytical variability of BTM (both technical and biological), and the lack of standardization of laboratory methods limit the clinical utility of BTM (Vasikaran et al. 2011).

In order to adopt international reference standards, in 2011 the IOF and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC),

on the basis of specific criteria for the selection of reference BTM standards, recommended the use of PINP to assess bone formation and of CTX to assess bone resorption (Vasikaran et al. 2011). According to this statement, the working group of the Committee of Scientific Advisors of the IOF later proposed in 2012 that a decrease in CTX and PINP less than the LSC at 95% confidence could be considered as an indicator of failure to respond to treatment with bisphosphonates (Díez-Pérez et al. 2012a).

However, specific international recommendations on the modality of assay and interpretation of BTM in clinical practice for the individual patient under treatment are lacking. Various national guidelines have recently expressed their opinion on the utility of BTM in the management of osteoporotic patients, some aiming for their routine application, while others being more cautious (Vasikaran et al. 2011). An attempt to elaborate an algorithm for the use of BTM in clinical practice was done in a Belgian consensus document which proposed to measure a BTM (serum CTX for antiresorptive therapy) at baseline and then after 3 months of therapy. If a significant decrease is not achieved, the clinician should check the treatment adherence and the method of drug administration, address potential problems detected, and then reassess the BTM after further 3 months (Fig. 3) (Bergmann et al. 2009). Conversely, if a

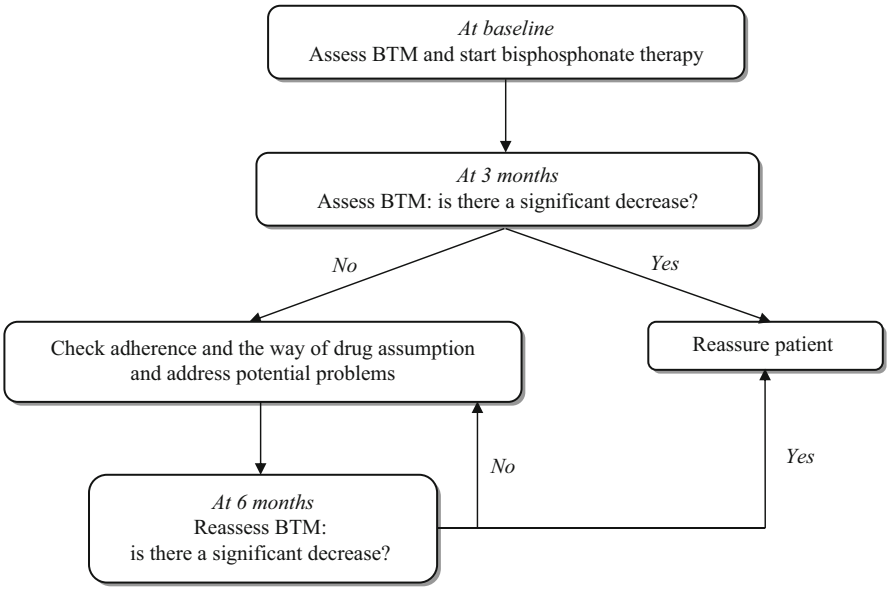

Fig. 3 An algorithm for the use of bone turnover markers (*BTM*) in clinical practice for monitoring bisphosphonate treatment efficacy for osteoporosis, suggested by the Belgian Bone Club: they proposed to measure BTM at baseline and then after 3 months of bisphosphonate therapy. If a significant decrease is not achieved, the clinician should check the treatment adherence and the method of drug administration, address potential problems detected, and then reassess the BTM after further 3 months. Conversely, if a significant change in BTM is obtained, the patient can be reassured (Bergmann et al. 2009). *BTM* bone turnover markers

significant change in BTM is obtained, the patient can be reassured and a positive feedback can be given already 3–6 months after the beginning of treatment (thus earlier than the demonstration of a BMD gain). This approach could have the advantage of maintaining or further improving adherence in the subsequent months of treatment (Delmas et al. 2007). However, other authors believe that this purpose alone does not justify the use of BTM and that the opportunity for the patient to discuss the therapy with a health-care professional is more beneficial to increase treatment adherence than the positive feedback itself given by the biochemical tests (Compston 2009).

If the changes in BTM following the initiation of osteoporosis treatment may be quite successfully used for predicting the fracture risk reduction, on the other hand, at the moment, there is no evidence to support the use of BTM to assess fracture risk after long-term bisphosphonate treatment. However, some authors consider useful the BTM for evaluating if bisphosphonates are still exerting their effects after their discontinuation, and they suggest to resume therapy when the BTM exceed the lower half of the premenopausal range (Adler et al. 2016). This approach is somewhat justified when considering that BTM in the lower range are associated with a reduced fracture risk (Vasikaran et al. 2011).

Genetic Markers

In the recent years, pharmacogenetic studies have provided several data on the genetic basis of individual response to osteoporosis treatments.

The vitamin D receptor (VDR) gene, which is located on the long arm of the chromosome 12, was one of the main genes that were investigated. VDR is a member of the steroid hormone nuclear receptor family which acts as a transcription factor after the interaction with its heterodimer partner, the retinoid X receptor. Several polymorphisms have been identified in the human VDR gene locus, of which the most studied are those identified by the restriction endonucleases ApaI, BsmI, FokI, and TaqI (Gennari et al. 2009). Among these polymorphisms, BsmI VDR genotypes were demonstrated to influence the efficacy of antiresorptive treatments in postmenopausal osteoporotic women. The bb BsmI VDR genotype (i.e., homozygous for the polymorphic allele) was associated with the highest therapeutic response to alendronate and hormone replacement therapy (Palomba et al. 2005). Another study in Southern Italy found that the FokI polymorphism of the VDR gene was associated with a better response to bisphosphonate treatment in postmenopausal osteoporosis (Conti et al. 2015).

On the basis of the mechanism of action of bisphosphonates, other pharmacogenetic studies examined the influence of genetic polymorphisms of genes encoding enzymes involved in the metabolic pathway inhibited by bisphosphonates (Fig. 1): the farnesyl pyrophosphate synthase (FPPS), the geranylgeranyl diphosphate synthase (GGPS), the mevalonate kinase (MVK), and the squalene synthase (FDFT1). In a Danish cohort of postmenopausal osteoporotic women, the homozygous CC genotype for rs2297480 FPPS single nucleotide polymorphism was associated with a decreased response of BTM to bisphosphonate

therapy when compared to the heterozygous AC and to the homozygous AA genotypes (Marini et al. 2008). Korean researchers found that the rs3840452 GGPS1 polymorphism was associated with the femoral neck BMD response rate to bisphosphonate therapy (Choi et al. 2010). Furthermore, in postmenopausal Chinese women, the rs10161126 single nucleotide polymorphism of MVK gene and the GTCCA haplotype in FDFT1 gene were associated with a better BMD response to alendronate therapy (Wang et al. 2015).

Finally, polymorphisms of the low-density lipoprotein receptor-related protein 5 (LRP5) gene, which encodes for an element of the Wnt pathway essential for the osteoblast differentiation and bone formation, were investigated. However, although the A1330V polymorphism of LRP5 gene was found to be associated with reduced BMD, available data on its influence on the response to bisphosphonates are conflicting. Whereas osteoporotic men homozygous for this polymorphism were found to respond to risedronate equally as well as the other genotype groups with respect to BMD (Kruk et al. 2009), Chinese postmenopausal women with homozygous genotype showed a higher possibility of poor BMD response at lumbar spine to alendronate treatment than those with other genotypes. Moreover, in this study, the trend of BTM reflected these different responses in BMD according to genotypes, since participants, who were homozygous for the polymorphism, had a smaller decrease in serum CTX and ALP levels than those with other genotypes after 12 months of treatment (Zhou et al. 2014).

A summary of these genetic factors found to be associated with the response to bisphosphonate treatment is presented in Table 4.

Overall, these studies provide an interesting point of view of the importance of the patient's genetic background to the response to osteoporosis therapies and of the future possibility of genotype-tailored osteoporosis therapies, in order to avoid that individuals with high probability of having an inadequate response to a treatment will take unnecessary medications.

However, further studies on larger samples are required and it must be considered that an important limitation of available pharmacogenetic studies is that the efficacy of osteoporosis treatment was not assessed as fracture risk reduction, but as BMD gain or BTM decrease which are only surrogate markers of the treatment efficacy.

Table 4 Genetic factors and polymorphisms associated with the response to bisphosphonate treatment. The sign "+" means a positive association, the sign "−" a negative association. See the text for further details. *VDR* vitamin D receptor, *FPPS* farnesyl pyrophosphate synthase, *GGPS1* geranylgeranyl diphosphate synthase, *MVK* mevalonate kinase, *FDFT1* squalene synthase, *LRP5* low-density lipoprotein receptor-related protein 5

Genetic factors	
BsmI VDR polymorphism	+
FokI VDR polymorphism	+
rs2297480 FPPS polymorphism	−
rs3840452 GGPS1 polymorphism	+
rs10161126 MVK polymorphism	+
GTCCA FDFT1 polymorphism	+
A1330V LRP5 polymorphism	−

Other Potential Markers

Several studies tried to determine other risk factors for predicting the antiresorptive treatment failure.

A multicentric, cross-sectional study of postmenopausal Spanish women on antiresorptives for osteoporosis (including not only bisphosphonates, but also raloxifene) found that the risk of inadequate response to antiresorptives (considered as the occurrence of a fragility fracture while on treatment) was significantly increased in patients with low levels of vitamin D, with low values of femoral fracture load, and with a fracture before treatment. These data suggest a worst microarchitectural deterioration could be a strong predictor of antiresorptive treatment failure (Díez-Pérez et al. 2012b). A subsequent study, based on the same cohort with the exclusion of patients on raloxifene treatment, assessed the association between the circulating levels of estradiol and sclerostin (a Wnt pathway inhibitor preferentially expressed by osteocytes) and the inadequate clinical efficacy of bisphosphonates. The authors found that increased circulating sclerostin levels and low estradiol levels were associated with the occurrence of a fragility fracture while on treatment. Moreover, serum sclerostin levels and a prior fragility fracture, adjusted by estradiol serum levels, were the only variables independently associated with the presence of an inadequate response on oral bisphosphonate treatment. However, important limitations of the present study are its retrospective design and the fact that the determinations of serum sclerostin and estradiol were performed during treatment rather than at baseline (Morales-Santana et al. 2015).

Using data from the Global Longitudinal Study of Osteoporosis in Women (GLOW), a large, prospective, observational cohort study of postmenopausal women in ten countries, the following variables were found to be independently associated with treatment failure: reduced quality of life (as measured by the SF-36 Health Survey (Brazier et al. 1992)), prior falls, and prior fracture (Díez-Pérez et al. 2014).

Finally, in a population-based cohort study in Spain and Denmark, significant predictors of multiple fragility fractures in patients with high adherence to oral bisphosphonate treatment were the older age in both populations and the history of fracture and dementia within one but not both populations (Hawley et al. 2016). At variance in an Italian study on postmenopausal women with primary osteoporosis, the current smoking was associated with an inadequate response to bisphosphonates despite a good compliance and normal vitamin D levels (Cairoli et al. 2014).

Conclusions

No osteoporosis treatments are able to eliminate fracture risk, but only to significantly reduce it. According to this assumption, an incident fragility fracture while on bisphosphonate treatment for at least 6 months cannot be considered a sign of

treatment failure, which is assumed when two or more fragility fractures occur in the course of therapy. The available evidence suggests that surrogate markers of an inadequate response to antiresorptives, such as bisphosphonates, are a decrease in BMD greater than the LSC at 95% confidence and a decrease in CTX and PINP lower than the LSC at 95% confidence (Díez-Pérez et al. 2012a). When one of these conditions occurs, the patient adherence to the drug and to the supplementation of calcium and vitamin D and the presence of a secondary cause of osteoporosis must be reviewed, since a poor compliance, an inadequate intake of calcium and vitamin D, and a secondary osteoporosis are the main reasons of a treatment failure (Lewiecki 2003; Fitzpatrick 2002).

If a good adherence is ascertained and a secondary osteoporosis is excluded, the working group of the Committee of Scientific Advisors of the IOF in 2012 recommended to consider a treatment change from bisphosphonates to a more potent drug when one of the following circumstances is fulfilled: (a) two or more fragility fractures, (b) one incident fracture and a significant decrease in BMD and/or no significant decrease in CTX or PINP, or (c) both a significant decrease in BMD and no significant decrease in CTX or PINP (Díez-Pérez et al. 2012a).

These criteria are based on the results of many studies which demonstrated that the changes in BMD and, in particular, the BTM modifications after the beginning of bisphosphonates independently correlate with the fracture risk reduction under treatment. Moreover, the BTM change, that is usually easy to measure, can be evaluated earlier than the BMD change, which requires about 18–24 months to be considered significant (Lee and Vasikaran 2012). Thus, BTM represent an attractive way to assess the treatment response. However, currently the availability of a wide number of BTM, their high pre-analytical and analytical variability, and the lack of standardization of laboratory methods limit the clinical utility of BTM (Vasikaran et al. 2011).

Further studies that apply international reference standard are needed to assess the capability for predicting fracture risk reduction under bisphosphonates of CTX and PINP, which have been defined by the IOF and the IFCC as the reference standard for bone resorption and bone formation, respectively (Vasikaran et al. 2011). The challenging goal is to establish universally accepted and reliable criteria based on the easy measurement of BTM able to early recognize the bisphosphonate failure before it becomes clinically evident.

The reasons which justify an inadequate response to antiresorptives in adherent patients with primary osteoporosis are not completely known. A hypothesis is that in these patients the bone microarchitecture is to such an extent altered that bisphosphonate therapy is not able to adequately work (Díez-Pérez et al. 2012b). Finally, other factors that may influence treatment efficacy come from genetics. The polymorphisms of the VDR gene and of genes involved in the mevalonate-to-cholesterol pathway inhibited by bisphosphonates and in the Wnt pathway were found to be associated with response to bisphosphonate. This suggests in future the interesting possibility of more personalized osteoporosis therapies (López-Delgado et al. 2016).

Potential Applications to Prognosis, Other Diseases, or Conditions

A rare but serious complication of long-term bisphosphonate treatment is the osteonecrosis of the jaw (ONJ), which is defined as the appearance of exposed bone in the maxillofacial region that persists for at least 8 weeks in the absence of previous radiotherapy in the craniofacial region. The risk of bisphosphonate-related ONJ appears to be very low in patients treated for osteoporosis (from 1/10.000 to 1/100.000 patient-treatment years), while it is significantly higher in oncologic patients treated intravenously with high doses for metastatic cancer (1–10%) (Khosla et al. 2007). Nevertheless, although the probability of this side effect is minimal in osteoporotic patients treated with bisphosphonates, the availability in clinical practice of a tool for the prediction of the individual risk of ONJ, especially if dental surgery is required, is appealing for the clinician. According to the assumption that the bisphosphonate-mediated suppression of bone turnover gives the main contribution in the pathophysiology of ONJ (Allen and Burr 2009), the markers of bone resorption have been suggested to be able to predict the ONJ risk in postmenopausal women receiving oral bisphosphonates for osteoporosis. In 2007 Marx and coauthors proposed the use of serum morning fasting CTX with this aim, considering values less than 100 pg/mL as high risk, between 100 pg/mL and 150 pg/mL as moderate risk, and greater than 150 pg/mL as minimal risk. Based on this conclusion, they suggested to defer dental surgery in the presence of CTX levels lower than 150 mg/dl, stopping temporarily bisphosphonate therapy if necessary to reach this CTX threshold (Marx et al. 2007). However, this recommendation has raised several concerns due to the lack of standardized laboratory protocols and of the estimate of the LSC of CTX levels, which takes into account both the analytical and biological variabilities. To date, the evidence does not support the use of CTX levels to predict the risk of ONJ and further studies are required on this topic (Baim and Miller 2009).

A second rare complication of long-term bisphosphonate treatment is the occurrence of an atypical femoral fracture (AFF). The diagnosis of AFF is based on subtrochanteric or femoral shaft location and the presence of $\geq$4 among minimal trauma, lateral cortex originating and transverse fracture, complete fractures extending through both cortices, periosteal or endosteal cortical thickening, and minimal comminution at most. Minor criteria are not required for the diagnosis but include increased cortical thickness of the diaphysis, bilaterality, a prodrome of thigh or groin pain, and delayed fracture healing (Dell et al. 2012). The age-adjusted AFF incidence is associated with the duration of bisphosphonate therapy, and it is estimated to rise from 1.8/100.000/year with a 2-year exposure to 113/100.000/year with 8–9.9-year exposure. Since the AFF pathogenesis seems to be related to a low bone turnover and in particular to low bone formation, the BTM have been studied for predicting the AFF occurrence. However, in the few patients studied, the correlation of bone histomorphometric parameters with BTM was poor and the urinary NTX was not consistently low (Odvina et al. 2005; Visekruna et al. 2008). Therefore, currently the BTM could not be used for predicting the AFF risk in patients treated long-term with bisphosphonates.

Summary Points

- Osteoporosis-approved therapies are able to reduce but not to eliminate fracture risk; thus the occurrence of a fragility fracture in the course of treatment does not necessarily mean a treatment failure, while the occurrence of a second fragility fracture after at least 6 months of therapy can be considered a sign that the drug has failed.
- Surrogate markers of bisphosphonate efficacy are the variations of bone mineral density (BMD) and of bone turnover markers (BTM) during therapy.
- A decrease in BMD greater than the least significant change (LSC) with a 95% level of confidence is considered as an indicator of failure to respond to bisphosphonates, although BMD variations explain only a limited part of their anti-fracture efficacy since treatment-induced changes in microarchitecture can significantly influence the fracture risk, independently of BMD.
- The short-term decrease in BTM under antiresorptive therapy is strongly related to the long-term BMD increase and to the fracture risk reduction. According to these data, a decrease in BTM less than the LSC with a 95% level of confidence is considered as an indicator of failure to respond to treatment with bisphosphonates.
- However, at the moment the clinical use of BTM is limited by their high pre-analytical and analytical variability and by the lack of standardized laboratory methods.
- Further studies based on international reference standard are needed to assess the ability of BTM to predict fracture risk reduction under bisphosphonates in order to elaborate clinical guidelines for the use of BTM to early recognize bisphosphonate failure before this condition becomes clinically evident.
- Pharmacogenetic studies have explored the genetic basis of individual response to osteoporosis treatments, and polymorphisms of the vitamin D receptor gene and of genes involved in the mevalonate-to-cholesterol pathway inhibited by bisphosphonates and in the Wnt pathway were found to be associated with the response to bisphosphonates.
- Markers of bone resorption have been suggested to predict the risk of osteonecrosis of the jaw and of atypical femoral fracture in postmenopausal women treated with bisphosphonates for osteoporosis; however, current evidence does not support their use for this aim and further studies are required on these topics.

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Serum Sclerostin as Biomarker in Osteogenesis Imperfecta

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Roland Kocijan, Sebastian Dinu, and Christian Muschitz

Contents

Abstract

Osteogenesis imperfecta (OI) is an inhomogeneous group of disorders caused by defects in collagen metabolism. OI is characterized by low bone mass, deterioration of bone structure, and high bone fragility. At present, there are no specific biomarkers available for OI. Sclerostin is a potent inhibitor of bone formation by inhibiting osteoblast activity. Sclerostin is chiefly produced by osteocytes and acts over the Wnt-pathway. Serum sclerostin levels in children with OI were

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described to be comparable to levels in healthy children. Lower sclerostin levels were found in adults with OI when compared to healthy subjects. Valuable explanations might be the altered bone microstructure and low bone mass in OI, leading to higher mechanical strain and lower sclerostin levels. Bisphosphonates, which are commonly used antiresorptive agents in OI, do not seem to affect sclerostin levels in OI. Although a direct association between fracture occurrence and sclerostin has not been proven, low sclerostin levels in OI could reflect the heightened fracture risk in OI. Moreover, sclerostin levels help to discriminate between OI patients and healthy individuals.

Keywords

Osteogenesis imperfecta • Sclerostin • Wnt-pathway • Biomarker • Bone mineral density • Fracture

List of Abbreviations

bAP	Bone-specific alkaline phosphatase
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BTM	Bone turnover marker
BV/TV	Trabecular bone volume
Col1A1	Collagen, type I, alpha 1
Col1A1	Collagen, type I, alpha 2
Co.Po	Cortical porosity
CTX	Collagen type 1 cross-linked C-telopeptide, beta-crosslaps
CV	Coefficient of variation
DAN	Differential screening-selected gene aberrative in neuroblastoma
DSSP	Dictionary of secondary structure of proteins
DXA	Dual-energy X-ray absorptiometry
ELISA	Enzyme-linked immunosorbent assay
HRPO	Horseradish peroxidase
HR-pQCT	High-resolution peripheral quantitative computed tomography
IBSP	Integrin-binding sialoprotein
LBM	Lean body mass
LOD/LLOQ	Lower limit of detection/lower limit of quantification
LRP	Low density lipoprotein receptor-related protein
OD	Optical density
OI	Osteogenesis imperfecta
PTH	Parathormone
P1NP	Procollagen type 1 amino-terminal propeptide
RCSB	Research Collaboratory for Structural Bioinformatics
ROC	Receiver operating characteristic
RUNX2	Runt-related transcription factor 2
TCF/LEF	T-cell factor/lymphoid enhancer factor

Tb.N	Trabecular number
Tb.Th	Trabecular thickness
Tb.Sp.SD	Inhomogeneity of the trabecular network
TMB	3,3′,5,5′-Tetramethylbenzidine
VIS	Visible light spectrum
WISE	Wnt-modulator in surface ectoderm
Wnt	Wingless type and integrase

Key Facts of Sclerostin

- Sclerostin is a glycoprotein which is encoded by the SOST gene (chromosome 17q12–q21).
- Sclerostin is almost exclusively produced by mature osteocytes.
- Sclerostin acts over the canonical Wnt-pathway and is a strong inhibitor of bone formation.
- Serum levels of sclerostin can quantitatively be determined by enzyme immunoassay (EIA, intra-assay coefficient of variation (CV) $<7\%$).
- Associations between sclerostin levels, microstructure, and fracture risk were shown.
- Currently two humanized monoclonal antibodies (Romosozumab, Blosozumab) targeted against sclerostin are under clinical research.
- Preliminary data show promising results regarding the osteoanabolic effects of sclerostin antibodies.

Key Facts of Osteogenesis Imperfecta (OI)

- OI is a group of genetic disorders caused by defects in collagen type 1 or by proteins interacting with collagen type 1.
- The inheritance is usually autosomal dominant. However, about 10–15% is autosomal-recessive OI.
- Clinical OI can be divided into four subtypes.
- OI type I is characterized by a quantitative reduction of structurally normal collagen.
- OI types II, III, and IV are caused by qualitative alterations of collagen.
- Genetically classified OI has more than 11 subtypes.
- OI is characterized by low bone mineral density and deterioration of bone structure.
- Bone mineralization and bone turnover are high in OI.
- Bisphosphonates are recommended as initial therapy in children and adults with OI.
- The sclerostin antibodies are promising drugs for the treatment of OI.

Definitions of Words and Terms

Bone histomorphometry	Bone histomorphometry is a histological technique that allows the measurement of bone cells, e.g., number of osteoclasts and osteoblasts, bone surface, and osteoid surface. Moreover, bone microstructure including trabecular bone volume (BV/TV), trabecular number (Tb.N), and trabecular thickness (Tb.Th) can be derived. Analysis by bone histomorphometry indicates transiliacal bone biopsy.
Bone turnover markers (BTM)	Bone turnover markers (BTM) are a group of biomarkers reflecting bone resorption and bone formation. BTM can be determined in urine or serum and are useful parameters for diagnosis and follow-up on bone diseases. In healthy bone there is a coupling of bone resorption and bone formation markers.
CTX	Collagen type 1 cross-linked C-telopeptide or beta-crosslaps are markers of bone resorption and reflect osteoclast activity. High CTX indicate high bone resorption. In case of antiresorptive treatment, CTX are suppressed.
HR-pQCT	High-resolution peripheral quantitative computed tomography is a noninvasive method for the assessment of trabecular and cortical bone microstructure including trabecular bone volume (BV/TV), trabecular number (Tb.N), and thickness (Tb.Th) as well as cortical thickness (Ct.Th) and porosity (Ct.Po). Measurements are performed at the radius and the tibia.
P1NP	Procollagen type 1 amino-terminal propeptide is a serum marker of bone formation. Thus, P1NP reflects osteoblast activity. P1NP is used for treatment monitoring. In case of antiresorptive therapy, P1NP is decreased; in case of osteoanabolic therapy, P1NP is increased.

Introduction

Sclerostin is a key regulator of bone metabolism. This protein is coded by the SOST gene and chiefly expressed by mature osteocytes. Sclerostin is a negative inhibitor of the beta-catenin-dependent canonical pathway of Wnt-signaling (wingless type and

integrase) which plays several essential roles in physiological and pathological conditions (Maeda et al. 2013).

In the canonical Wnt-pathway, the seven transmembrane receptor *Frizzled* forms a complex with the transmembrane proteins LRP5 and LRP6 (low-density lipoprotein receptor-related proteins) which act as coreceptors for *Frizzled*. Wnt binds to this receptor complex which consecutively activates the beta-catenin-mediated canonical pathway. Beta-catenin accumulates in the nucleus of the target cell and in conjunction with TCF/LEF (T cell factor/lymphoid enhancer factor) induces the expression of Wnt target genes and thereby stimulates osteoblast activity. Consequently, the Wnt-pathway has several functions in bone metabolism such as osteogenesis, proliferation from osteoprogenitor cells through preosteoblasts to osteoblasts, matrix formation, and matrix mineralization. Moreover, the beta-catenin pathway is important in bone response to loading, osteocyte apoptosis, and osteocyte communication (Bonewald 2011). The Wnt-pathway is also needed for a regular skeletogenesis (Ohyama et al. 2004).

Sclerostin is a potent endogenous inhibitor of the Wnt/beta-catenin pathway by binding at the LRP5/6 receptor and inhibiting osteoblasts and thus bone formation. Sclerostin was discovered and named over half a century ago by studying sclerosteosis among the Afrikaner population of South Africa. The research performed on this autosomal-recessive sclerosing bone dysplasia later resulted in the discovery of a nonsense mutation at the amino terminus of the peptide chain (Brunkow et al. 2001). Thereby the shortened chain can neither be folded into a functional sclerostin protein nor be secreted to the surface. Hence, significantly lower levels of sclerostin are found in subjects with this type of mutation. As a consequence, bone mass and bone mineral density are abnormally high (van Lierop et al. 2013) without adverse impact on bone quality. Both high bone mass and increased bone thickness were shown in sclerostin knockout mice models (Li et al. 2008).

Serum sclerostin levels are physiologically influenced by age, physical activity, body composition, and bone mineral density (BMD). Sclerostin correlates positively with age in both sexes. Over a lifetime, sclerostin increases by 2.4–3.7-fold in women and 4.6-fold in men (Mödder et al. 2011; Ardawi et al. 2011). This suggests that high sclerostin production may be involved in impairment of osteocytes and the expected age-related changes in bone mass. In addition, a decreased clearance of sclerostin among the elderly is under debate.

Approximately 25% higher sclerostin levels were found in healthy men than women. The reason for the higher unadjusted sclerostin levels may be the larger skeletal mass of men. As a consequence a larger skeleton may produce higher levels of sclerostin.

However, after adjustment for several factors such as bone mineral content (BMC), body mass index (BMI), physical activity, and renal function, sclerostin levels did not differ between men and women. Consequently, sclerostin levels positively correlate to BMC, BMD, and BMI (Amrein et al. 2012).

Genetics and Proteomics of Sclerostin

The coding gene for Sclerostin, SOST, is located on chromosome 17 within band range 12–21, and is comprised of two exons spanning a large intron. As previously stated, sclerostin levels may vary due to multiple reasons such as age, gender, or mechanical load (Delgado-Calle et al. 2014). On a genetic level, it has been shown that one way of sclerostin regulation is DNA methylation, which also proves to be an inheritable DNA modification mechanism (Delgado-Calle et al. 2012).

The glycoprotein's amino acid sequence totals 213 residues. According to the DSSP, hydrogen bond estimation algorithm incorporates five beta sheets. Although there are 36 models describing the regions lacking secondary structure, the most important structural discovery is the knot-like domain including five beta strands and four disulfide bonds created by cysteine residues (Fig. 1). Moreover, the cysteine-rich domain is highly conserved and described by all models (Veverka et al. 2009). This type of domain is found in several other signaling proteins such as Noggin or the differential screening-selected gene aberrative in neuroblastoma (DAN) protein family including Norrin and Wnt-modulator in surface ectoderm (WISE) (Rey and Ellies 2010;).

Therefore a technique sufficiently specific for human sclerostin detection is crucial when studying sclerostin levels in plasma or serum. The method used to determine these levels is a microtiter-plate-based enzyme-linked immunosorbent assay (Biomedica Medizinprodukte GmbH, Vienna). At the base of the microtiter strips, immobilized polyclonal goat antihuman sclerostin antibodies bind to a specific site of the protein. Subsequently, biotin-labeled monoclonal mouse antihuman sclerostin antibodies bind to different specific sites of the protein thus creating a sandwich, from this the name "sandwich ELISA" was coined. The vitamin biotin acts as a ligand for the ensuing horseradish peroxidase (HRPO)-labeled streptavidin

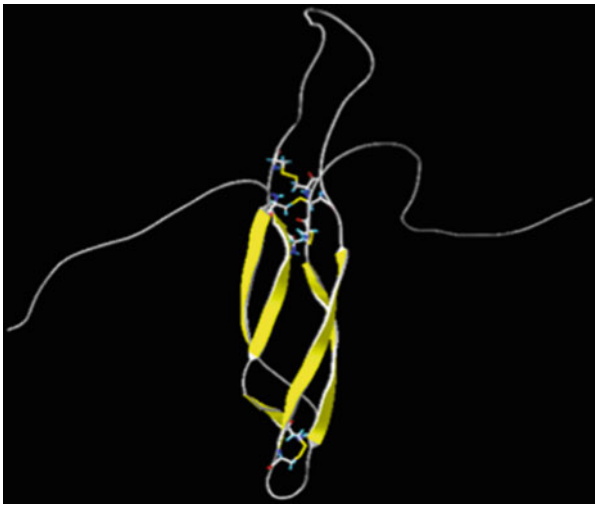

Fig. 1 Cysteine-rich knot-like domain of sclerostin. 3-D ribbon rendering of sclerostin showing amino acid range 40–170 based on information from the RCSB protein data bank. *Gray*: peptide chain showing no secondary structure. *Yellow* beta sheets including side chains: cysteine-rich knot-like domain (Performed with Swiss PDB Viewer v4.1.0)

protein. Under optimum conditions, the biotin-streptavidin bond represents one of the strongest noncovalent bonds known, hence its extensive use in life sciences. The chromogenic substrate used in this kit is 3,3,5,5-Tetramethylbenzidine (TMB), whose optical density (OD) is read on a VIS photometer at a wavelength of 450 nm corrected by 630 nm after catalytic activation via HRPO and chemical arrest by sulfuric acid. Using the four parameter logistic nonlinear regression model, a standard curve is generated and used for concentration calculation. The limit of detection (LOD) and lower limit of quantification (LLOQ) of the used kit are 3.2 pmol/L and $<$7.5 pmol/L, respectively. The intra-assay and inter-assay variation are $\leq$7% and $\leq$10%, respectively, whereas the recovery rate lies at 94%.

Despite the strong similarity of the cysteine knot-like domain of sclerostin and several other proteins, this commercially available ELISA kit does not show cross-reactivity with Noggin, WISE, or mouse sclerostin.

Since little is known about the exact interaction mechanism of sclerostin with LRP5/6, one could only hypothesize about it (Ellies et al. 2006). Underlining the fact that cysteine-rich knot-like domain containing signaling proteins such as WISE, Norrin, and sclerostin modulate the coreceptors LRP5/6 in the canonical Wnt-signaling pathway (Weivoda and Oursler 2014), a potential similar interaction mechanism can be presumed possibly including some sort of competitive inhibition. These assumptions are certainly to be further investigated in dedicated research studies.

Characteristics and Classification of Osteogenesis Imperfecta

Osteogenesis imperfecta (OI) is a group of rare genetic disorders mainly caused by defects in COL1A1 or COL1A2, the two genes encoding for collagen type 1. Over 1500 mutations have been identified to cause autosomal dominant OI (Forlino et al. 2011). However, also recessive OI caused by defects in proteins interacting with collagen type 1 is known. Recessive OI usually has null mutations, leading to lack of proteins involved in helical folding (FKBP10, SERPINH1) and collagen prolyl 3-hydroxylation (CRTAP, LEPRE1, PPIB) (Morello et al. 2006; Alanay et al. 2010).

OI is characterized by low bone mass, deterioration of bone structure, impaired bone material quality (impaired collagen bone matrix), and hence high bone fragility. As a consequence, fracture incidence is increased. Therefore OI is also called brittle bone disease. Extraskeletal manifestations such as blue or gray sclerae, dentinogenesis imperfecta, hypermobility of the joints and skin as well as hearing impairment are typical for OI.

OI can be divided into four subtypes, based on radiological and clinical factors and according to the classical Sillence classification. OI type I is dominantly inherited with a low fracture incidence and blue sclera. OI type II is lethal in the perinatal period due to multiple fractures and beaded ribs. OI type III is known as the progressively deforming OI with multiple vertebral and peripheral fractures. OI type III is the most severe form in adolescents and adults. OI type IV is the moderate type

with clinical severity between OI type I and type III with normal sclera. Recently, a clinical type V has been added. OI type V is characterized by calcification in the intraosseous membranes (Fratzl-Zelman et al. 2015b). Recently, this classification has been expanded to 11 subtypes, based on the gene defect (Forlino et al. 2011). The classical Sillence classification and its four subtypes reflecting clinical severity is still in use.

OI is caused either by a reduced synthesis of structurally normal collagen leading to clinically OI type I or by defects in type I collagen structure leading to clinical subtypes II, III, and IV.

Type I OI phenotype typically have a null COL1A1 allele, whereas OI types II–IV are caused by glycine substitutions and splice site mutations. Glycine substitution delays folding of the heterotrimer. Consequently posttranslational modification is increased. Glycine substitutions are associated to moderate and severe skeletal phenotype (Forlino et al. 2011).

Histomorphometric bone analyses of children and adults with OI proposed:

(i) High bone mineralization in OI. Although BMD is usually low in patients with OI, an abnormally high bone matrix mineralization has been described (Roschger et al. 2008; Fratzl-Zelman et al. 2015a). Moreover, the bone mineral density seems to be distributed inhomogeneously suggesting high and low mineralization in the same bone. This inconsistent distribution leads to weakness in bone strength.
(ii) Severe deteriorations of bone structure. These observations were affirmed by high-resolution peripheral quantitative computed tomography (HR-pQCT) measurements, which revealed severe alterations especially of trabecular bone in OI type I and even more in OI types III and IV (Folkestad et al. 2012; Kocijan et al. 2015).
(iii) Increased bone turnover. An increased number of osteoblasts and osteocytes (Sarathchandra et al. 2000), osteoblast and osteoclast surface and an increased formation rate per bone surface were found in bone biopsies (Rauch et al. 2000). This increase in bone turnover is only partly reflected by serum bone turnover markers.

Biomarkers in Osteogenesis Imperfecta

The diagnosis and classification of OI is made by radiological, skeletal, and extraskeletal features or by genetic testing. Since specific biochemical bone markers are not available for OI, the course of disease is usually followed by radiological techniques. Moreover, current data on serum BTM in OI are inconsistent. Most bone resorption and bone formation markers were reported to be low, normal, or even high in patients with OI.

Osteocalcin, which is secreted by osteoblasts, is a collagen-independent parameter of bone formation and has been reported to be normal or high in OI (Braga

et al. 2004; Cundy et al. 2007; Garnero et al. 2009). High osteocalcin levels reflect a high bone turnover and an increased osteoblast number (Rauch et al. 2000). Comparable osteocalcin values within the normal range were found for OI types I, III, IV, and healthy controls in adult subjects (Kocijan et al. 2014). Bone-specific alkaline phosphatase (bAP) was reported to be significantly higher in patients with OI than in respective controls, giving also evidence of the increased number of osteoblasts in OI (Braga et al. 2004; Cundy et al. 2007; Brenner et al. 1993).

Procollagen type 1 amino-terminal propeptide (P1NP) is a collagen-related marker of osteoblast activity. P1NP is a reliable and established parameter for the assessment of high and low bone turnover in osteoporosis. P1NP is furthermore a useful marker for treatment monitoring. Low P1NP values are typical for patients with antiresorptive treatment (e.g., bisphosphonates, Denosumab). In contrast, P1NP displays the *osteoanabolic window* in case of osteoanabolic treatment (Muschitz et al. 2014).

CTX, next to P1NP and bAP, is one of the most reliable biochemical bone markers in diagnosis and follow-up on osteoporosis. Low CTX values suggest low bone resorption, e.g., in case of diabetic bone or renal osteopathy or a sufficient suppression of bone resorption by antiresorptive treatments such as bisphosphonates or Denosumab. High CTX levels indicate high bone resorption, and levels above the reference range often indicate early postmenopausal stages, recent fracture, or cancer-induced osteolysis.

In contrast to osteoporosis, CTX and P1NP are unreliable parameters in OI. Despite the high bone turnover in OI, lower serum-CTX levels were reported in OI type I and OI types III-IV than in healthy subjects, and serum P1NP values were found below the reference range for all OI subtypes (Kocijan et al. 2014). However, low P1NP and serum-CTX levels might reflect the defect in collagen type I metabolism (Garnero et al. 2009). Though, most markers of bone resorption were described to be higher in OI types III and IV compared to OI type I (Braga et al. 2004), even lower P1NP levels were found for mild OI type I compared to moderate and severe OI types III and IV. A valuable explanation might be that OI type I has a 50% reduction of normal collagen type 1, whereas other OI types produce and secrete normal and abnormal type 1 procollagen molecules (Wenstrup et al. 1990). As a consequence, low levels of collagen-related parameters might be explained by the lack of pyridinoline cross-links at the C-terminal portion of collagen type 1 in OI (Braga et al. 2004).

These data indicate that the collagen-related serum parameters P1NP and CTX could give evidence of a collagen defect but do not adequately reflect the increased bone turnover in OI. It has to be taken into account that CTX and P1NP levels respond fast with a decline in case of bisphosphonate therapy. A long-term release of bisphosphonates and a persistence of many years in bisphosphonate-treated children have been reported. The prolonged recirculation of pamidronate, an often-prescribed bisphosphonate in children with OI, is up to 8 years after discontinuation (Papapoulos and Cremers 2007). A suppression of bone turnover markers up to 9 years after intravenous bisphosphonate treatment had been reported in children (Bradbury et al. 2012) and also adults with OI (Chung et al. 2012). Since

bisphosphonate therapy in moderate and severe OI starts in early childhood, low P1NP and CTX could at least partly reflect bisphosphonate therapy. Whether commercially available tests measure structurally altered collagen accurately remains unclear.

Currently no useful bone turnover markers for the classification of OI types exist. Moreover, there is no association with vertebral fractures (Aström et al. 2010). Therefore, serum sclerostin seems to be a promising biomarker for OI.

Recently, two humanized monoclonal antibodies against sclerostin (Romosozumab, Blosozumab) have been introduced (Recker et al. 2015). Preliminary data in mice models of OI suggest encouraging data regarding osteoanabolic effects caused by sclerostin inhibition. Therefore, sclerostin is not only a promising biomarker but also a possible treatment target in OI (Sinder et al. 2015).

Serum Sclerostin Levels in Children with OI

Serum sclerostin levels in healthy, untreated children and adolescents without a history of fracture were described to be higher in boys, which corresponds with the findings in adults. A nonlinear relationship between sclerostin levels and age was found with a decline of serum sclerostin levels in both sexes following the onset of puberty (Kirmani et al. 2012). In contrast, sclerostin levels in healthy children and adolescents were also described to be independent of age and gender in other observations (Fischer et al. 2012).

Sclerostin levels were assessed in 76 children with OI type I, III, and IV aged 0.9–21 years (Palomo et al. 2014) and respective controls. OI subjects were divided into patients who had previously received intravenous bisphosphonates (0.6 up to 13.6 years treatment) and treatment naive patients. Most patients had disease-causing mutations in COL1A1 and COL1A2. Sclerostin levels varied widely (5.3–77.1 pmol/L) in OI and did not differ between boys and girls or genotypic groups. No differences were found when sclerostin levels in children with OI were compared to healthy, age and gender matched controls (see Fig. 2). Moreover, similar sclerostin levels were observed in patients regardless of prior or none bisphosphonate therapy (Palomo et al. 2014).

Serum Sclerostin Levels in Adults with OI

There are only a few data available on sclerostin levels in adult OI patients. Serum sclerostin levels and BTM were assessed in Caucasian OI patients from Central Europe (Kocijan et al. 2014). Patients with OI types I, III, and IV were divided into two subgroups, mild OI type I and moderate to severe OI types III-IV for data analysis. Data were compared to healthy, age-related controls.

Three out of four OI patients had previously received bisphosphonates, and the duration of bisphosphonate therapy lasted up to 16 years in a lifetime. Patients with OI had at least sustained five nontraumatic fractures with a maximum of 80 fractures

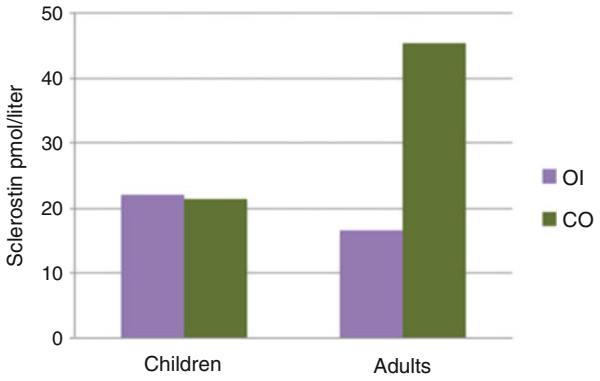

Fig. 2 Sclerostin levels in osteogenesis imperfecta. Sclerostin levels in children (*left*) and adults (*right*) with osteogenesis imperfecta (*purple*) and respective controls (*green*). No significant differences were found in children with OI when compared to healthy children. In contrast, significantly lower levels were found for adults with OI when compared to healthy subjects (Modified from Palomo et al. 2014 and Kocijan et al. 2014)

and a median of 16 fractures. Patients with OI type I and even more OI types III-IV were significantly shorter and lighter and had a reduced bone mineral content (BMC) and a lower lean body mass (LBM (kg)) compared to healthy controls.

Serum sclerostin levels were significantly decreased by 56% in OI type I and by 71% in OI types III-IV (see Fig. 2). Sclerostin levels in OI were still considerably lower when compared to control subjects after adjustment for age, sex, BMI, and BMC. Sclerostin was also substantially related to age and weight. Moreover, a positive trend for an association between sclerostin and BMC (g) as well as LBM (kg) was found. Serum sclerostin levels in adult patients with OI were neither related to the number of prevalent fractures nor body composition parameters. In the control group, sclerostin was positively associated to age, BMC, LBM, and weight (Kocijan et al. 2014).

In a ROC analysis, sclerostin and BMC were found to be significant discriminators between patients with OI and controls. A multivariable age-adjusted analysis suggested an area under the ROC of 0.996 for the combination of sclerostin and BMC (Kocijan et al. 2014).

Sclerostin, Bone Mineral Density, and Bone Microarchitecture

There seems to be a clear association between serum sclerostin levels, bone mineral density, and bone microstructure, respectively, as has been investigated in several studies.

Bone mineral density, assessed by DXA, was reported as positively related to serum sclerostin levels in elderly women and men (Mödder et al. 2011). Especially trabecular BMD was found to be significantly higher in male patients with high sclerostin levels compared to those with low sclerostin levels. A positive correlation between serum sclerostin levels and areal BMD at all skeletal sites was shown in both men and women (Szulc et al. 2013a; Sheng et al. 2012). These positive

correlations between sclerostin and BMD at the lumbar spine were also found in children with OI (Palomo et al. 2014).

Besides BMD, bone microarchitecture seems to be associated to sclerostin levels. Especially, trabecular bone parameters including trabecular bone volume (BV/TV) and trabecular number (Tb.N) were significantly related to sclerostin levels (Mödder et al. 2011). A higher trabecular number was also found in male patients with high sclerostin levels compared to those with low levels of sclerostin. Trabecular BMD, BV/TV, Tb.N, and Tb.Th were positively related to sclerostin levels in another study (Cejka et al. 2012). Moreover, an inhomogeneous trabecular network (Tb.Sp.SD) was found to be associated to low sclerostin levels (Szulc et al. 2013a). Interestingly, no consistent relationships between sclerostin levels and bone structural parameters were reported in healthy children (Kirmani et al. 2012).

Therefore, low sclerostin levels in patients with OI could be explained by low BMD and deterioration in bone microarchitecture. Despite the low bone mass, severe alterations of particularly trabecular bone have been reported. Patients with OI type I show a decreased BV/TV mainly explained by a reduced Tb.N (Folkestad et al. 2012). Patients with moderate to severe OI types III and IV have even worse bone architecture, reflected by low BV/TV, Tb.N, and Tb.Th when compared to OI type I and healthy controls, respectively (Kocijan et al. 2015). All types of OI show a heterogeneous trabecular network with a lack of cross-links (see Fig. 3).

In contrast to trabecular bone, only weak associations between cortical bone parameters and sclerostin levels have been evaluated (Szulc et al. 2013a; Mödder et al. 2011; Cejka et al. 2012). Cortical bone in OI has been described to be similar to healthy subjects with comparable cortical BMD and cortical porosity (Ct.Po) (Folkestad et al. 2012; Kocijan et al. 2015). Therefore, serum sclerostin levels are clearly positively associated to trabecular bone BMD and microstructure at different skeletal sites.

Sclerostin seems to be regulated by mechanical loading (Robling et al. 2008). A high bone mass indicates a high number of osteocytes and potentially high sclerostin levels. High bone mass leads to low mechanical strain and high sclerostin levels. In

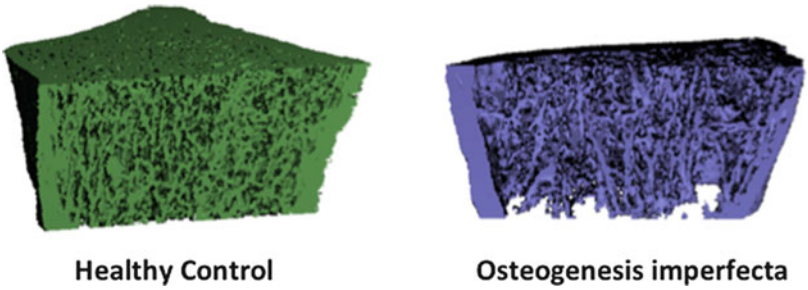

Fig. 3 Bone microstructure by high-resolution peripheral quantitative computed tomography. Bone microstructure in a healthy subject (*green*) and an adult patient with osteogenesis imperfecta type IV (*purple*). Severe deteriorations of trabecular bone are a valuable explanation for low sclerostin levels in OI

contrast, low bone mass and poor microarchitecture lead to high mechanical strain and thereby low sclerostin secretion by osteocytes (Lapauw et al. 2013). Even if the number of osteocytes is increased in OI, the severe deteriorations of bone structure seem to suppress sclerostin synthesis in adults. Comparable observations were made in male patients with idiopathic osteoporosis, low bone mass, low mineral content, and low serum sclerostin levels (Lapauw et al. 2013). Normal sclerostin levels in children with OI might be explained by low bone mass but increased osteocyte density. The sclerostin production could cancel each other out, resulting in normal sclerostin levels in children with OI (Palomo et al. 2014).

To date it remains unclear whether sclerostin levels truly reflect bone mass, bone mineral density, and microarchitecture. The power of BTM to predict bone microarchitecture is limited as has been shown in bone biopsy studies. Although, bone turnover markers P1NP and CTX were explanatory variables for BV/TV and even more for Tb.N, sclerostin was not found to be a discriminative variable for trabecular bone microarchitecture (Muschitz et al. 2015b).

Sclerostin and Bone Turnover Markers

Sclerostin is a potent inhibitor of osteoblast differentiation. Thus, an impact on markers of bone formation would be expected. In healthy people, sclerostin levels are indeed negatively associated to osteocalcin (Amrein et al. 2012; Kocijan et al. 2014), indicating low bone formation in case of high sclerostin levels. Negative correlations between sclerostin and BTM, not only of bone formation but also of bone resorption, were reported in men. Sclerostin levels were inversely related to osteocalcin, P1NP, bAP as well as CTX (Szulc et al. 2013b; Lapauw et al. 2013). In contrast to the findings in adults, sclerostin levels were positively associated to P1NP and CTX in both healthy boys and girls. A valuable explanation might be a regulating role of sclerostin in bone turnover in the growing skeleton (Kirmani et al. 2012). These findings in healthy children were also observed in children with OI. Serum sclerostin levels were positively associated with resorption and formation markers, whereas no correlations were found between sclerostin and calcium, phosphorus, or PTH (Palomo et al. 2014). Moreover, findings in adult patients with OI are similar to those in healthy adults. Serum sclerostin levels were negatively associated to osteocalcin and CTX. In adult OI patients, serum sclerostin levels were neither related to P1NP, PTH nor vitamin D (Kocijan et al. 2014).

Sclerostin Levels and Bisphosphonate Treatment

Bisphosphonates are considered initial therapy for OI. Usually treatment starts early within the first months of life in moderate and severe cases. It is well-known that bone resorption and formation markers are significantly suppressed in case of bisphosphonate therapy. Yet data regarding sclerostin levels are inconsistent. Similar sclerostin levels in postmenopausal women treated with orally administered

bisphosphonates compared to age-matched controls without therapy were reported (Chung et al 2012). In contrast, an increase of serum sclerostin levels after intravenous bisphosphonate treatment in postmenopausal women (Gatti et al. 2012) and men with idiopathic osteoporosis has been referred repeatedly (Muschitz et al. 2015a).

However, comparable sclerostin levels were reported in bisphosphonate-treated and bisphosphonate-untreated children with OI. Intravenous neridronate and pamidronate did not influence sclerostin levels in children with OI (Palomo et al. 2014).

As has also been investigated in children with OI, no differences in sclerostin levels were found between bisphosphonate-treated and bisphosphonate-untreated adult patients with OI (Kocijan et al. 2014). Taking into account that sclerostin levels occur in normal or low ranges in bisphosphonate-treated and -untreated OI patients, bisphosphonates do not seem to influence sclerostin levels significantly in OI.

Sclerostin and Fracture Risk

The association between serum sclerostin and fracture risk is complex and at present not fully understood. Similar sclerostin levels were reported in men with and without fractures (Lapauw et al. 2013). In contrast, the odds ratio for fractures decreased with increasing sclerostin levels in other observations. In this study, subjects were divided into four quartiles, based on their sclerostin levels (Szulc et al. 2013b). The prevalence of vertebral and peripheral fractures was lower in the highest quartile when compared to the three lower quartiles suggesting a higher fracture risk in patients with low sclerostin levels. Lower sclerostin levels were also found in patients with hip fractures (Dovjak et al. 2014). No significant associations between sclerostin levels and total number fractures could be shown in adult patients with OI. However, sclerostin levels were found to be lower in adult patients with moderate to severe OI types III-IV, who have sustained up to 80 fractures when compared to mild OI type I, who have only sustained a few fractures. Thus, low sclerostin levels could at least partly reflect the high fracture risk in OI. Nevertheless, sclerostin is an unreliable parameter for the prediction of low-traumatic fractures in OI (Kocijan et al. 2014).

Potential Applications to Prognosis, Other Diseases, or Conditions

Sclerostin is related to fracture risk in osteoporotic men and women and therefore a useful prognostic biomarker for low-traumatic peripheral and vertebral fractures. Whether this is also true for OI remains unclear, since data for OI are limited. However, it was shown that sclerostin levels discriminate between patients with OI and healthy subjects, especially in combination with BMC.

Sclerostin was also investigated in patients with chronic inflammatory disorders. The majority of osteocytes were sclerostin positive in healthy individuals and patients with rheumatoid arthritis. In contrast, sclerostin expression was significantly reduced in patients with osteoarthritis and was almost absent in patients with ankylosing spondylitis. Lower serum sclerostin levels were found in patients with ankylosing spondylitis than in healthy individuals, suggesting an osteocyte dysfunction. Low serum sclerostin levels were significantly associated with new bone formation. Thus, serum sclerostin is also a useful biomarker in rheumatic diseases (Appel et al. 2009).

Sclerostin also reflects changes in bone metabolism after bariatric surgery. A rapid increase of sclerostin caused an increase in bone metabolism and resulted in BMD loss in patients after Roux-en-Y gastric bypass or laparoscopic sleeve gastrectomy. The elevation of sclerostin was a significant discriminating factor for BMD loss in patients after bariatric surgery (Muschitz et al. 2014).

It has been show recently that serum sclerostin is also an independent predictor of mortality in hemodialysis patients. Higher sclerostin was found in nonsurvivals with a hazard ratio of 2.2 (Gonçalves et al. 2014).

Conclusion

Sclerostin levels are normal in OI children and decreased in adult OI patients, when compared to the respective controls. Low bone mass and severe deteriorations in bone structure are valuable explanations. Yet low values were found after adjustment for age, sex, BMC, and BMI, giving evidence for a more complex pathogenesis.

Impairments in mesenchymal stem cells and osteogenic differentiation have been proposed earlier in a knockin mouse model for OI. An incomplete differentiation from preosteoblasts to mature osteoblasts was suggested. A valuable explanation might be an upregulation of autophagy caused by collagen retention and endoplasmic reticulum stress. A decreased expression of the osteoblast-specific genes RUNX2, Sp7, Col1a1, and IBSP were found. In addition, an increase in the adipogenic lineage was reported (Gioia et al. 2012). Whether the canonical Wnt-pathway is also involved in this mechanism remains unclear. However, low sclerostin would mean a beneficial outcome regarding osteoblast differentiation and decreased adipocytogenesis. Therefore, low sclerostin levels in OI might also reflect a rescue mechanism or negative loop to restore osteoblast activity unlike the inhibiting mechanisms described above.

Summary Points

- This chapter focuses on serum sclerostin as a biomarker in patients with osteogenesis imperfecta (OI).
- At present, there are no reliable bone turnover markers for OI.
- Sclerostin levels in children with OI are comparable to those in healthy children.

- Sclerostin levels in adults with OI are significantly lower compared to respective controls.
- Bisphosphonate treatment does not seem to affect sclerostin levels in OI.
- Low sclerostin levels in OI could reflect deteriorations in microstructure - especially in trabecular bone.
- The association of sclerostin to other bone turnover markers in OI is similar to healthy subjects.
- Low sclerostin levels are associated to high vertebral and peripheral fracture risk.

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Effects of Glucose on Bone Markers: Overview of Current Knowledge with Focus on Diabetes, Glucose, and Bone Markers

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Abstract

Diabetes mellitus is associated with an increased risk of fracture. However, in patients with diabetes the bone mineral density does not explain this. Bone turnover markers give information on bone formation and bone resorption and may explain the decreased bone material competence in patients with diabetes. Diabetes mellitus is characterized by the lack of a fasting condition, which also may affect the general bone turnover and be reflected in the bone turnover markers. This chapter focuses on the relation between bone turnover markers and plasma glucose, and bone turnover markers in diabetes subjects. In clinical trials, an oral glucose tolerance test (OGTT) decreased bone resorption markers in both patients with type 2 diabetes and healthy individuals. During an OGTT, bone formation markers were decreased in healthy individuals, but the markers were not investigated in patients with diabetes. An intravenous glucose tolerance test decreases the bone resorption marker C-terminal cross-linked telopeptide of type-I collagen (CTX) but not as much as the OGTT. Therefore a gastrointestinal interaction may affect the relation between glucose and bone turnover markers. In patients with diabetes, both CTX and the bone formation marker osteocalcin were decreased compared to controls. However, heterogeneity was present in the markers, which may be due to differences in glycemic status. In vitro studies show direct effects of glucose on the bone cells: osteoblasts, osteoclasts, and osteocytes. Hyperglycemia had detrimental effects on osteoblasts and osteoclasts and increased the sclerostin production in osteocytes; thus both bone resorption and formation seemed to decrease during hyperglycemia. However, in the mild hyperglycemia with a glucose level of 11–15 mmol/l, the osteoblasts increased the mineralization. Thus, hyperglycemia may hypermineralize the bone, so the bone mineral density is increased relatively to the bone material competence due to a relative decrease in non-mineralized matrix, e.g., collagen.

Further, investigations are needed to determine if the glucose bone turnover marker interaction may be a prognostic marker of fracture in patients with diabetes.

Keywords

Glucose • Hyperglycemia • Bone turnover markers • Bone turnover • Diabetes mellitus • Osteoblasts • Osteoclasts • Osteocytes • Hypermineralization

List of Abbreviations

BAP	Bone-specific alkaline phosphatase
BMD	Bone mineral density
BSP	Bone sialoprotein
CA/P	Calcium/phosphate
CTX	C-terminal cross-linked telopeptide of type-I collagen
FGF-23	Fibroblast growth factor-23
FRAX	The fracture risk assessment tool
GIP	Gastric inhibitory peptide

GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
HbA1c	Glycated hemoglobin A1c
hMSC	Human mesenchymal stem cells
hMSC-TERT	Human mesenchymal stem cells telomerase-immortalized
IGF-1	Insulin-like growth factor-1
IVGTT	Intravenous glucose tolerance test
NTX	N-terminal cross-linked telopeptide of type-I collagen
OGTT	Oral glucose tolerance test
OPG	Osteoprotegerin
P1NP	Procollagen type 1 N-terminal propeptide
PTH	Parathyroid hormone
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor Activator of Nuclear factor Kappa beta Ligand
Runx2	Runt-related protein 2
TRAP	Tartrate resistant acid phosphatase

Key Facts

Key Facts of Diabetes Mellitus and Related Bone Disease

- Diabetes is an extremely common disease throughout the world, with an estimated 592 million cases in 2035.
- Type 1 diabetes is characterized by a near complete loss of insulin production.
- Type 2 diabetes is characterized by a decrease in insulin production relative to insulin sensitivity.
- Diagnosis of diabetes mellitus can be made by measuring fasting plasma glucose above 7 mmol/l, a 2 h oral glucose tolerance test plasma glucose value above 11 mmol/l or an HbA1c of $\geq$ 48 mmol/mol.
- Apart from the well-known microvascular and macrovascular complications, diabetes is also related to poor bone health.
- The risk of hip fracture is suggested to be sevenfold increased in patients with type 1 diabetes and twofold increased in patients with type 2 diabetes.
- Bone mineral density is increased in type 2 diabetes and slightly decreased in type 1 diabetes, but the lower bone mineral density in type 1 diabetes cannot explain the increased risk of fracture.
- Patients with diabetes display lower C-terminal cross-linked telopeptide of type-I collagen and osteocalcin levels representing lower bone resorption and bone formation.
- The mechanisms behind the increased risk of fractures in diabetes are still unclear but could be related to lack of insulin, disturbed glucose metabolism, medication use, renal impairment, falls or other factors.

Key Facts of Bone Remodeling

- Bone consists of a mineralized matrix, mainly hydroxyapatite, a non-mineralized matrix, mainly collagen, and a cellular compartment.
- The cellular component of bone consist of osteoclasts that resorb bone tissue, osteoblasts that form new bone tissue, and osteocytes that are thought to regulate the bone turnover process.
- Bone remodeling is a highly coordinated process of degradation of old bone and creation of new bone.
- Bone remodeling consists of three phases: A bone resorption phase maintained by the osteoclasts, a reversal phase where the bone is prepared for the osteoblasts, and a bone formation phase where matrix is produced by osteoblasts and subsequently matured and mineralized.
- When the bone remodeling is out of balance, typically with degradation exceeding creation of bone, osteoporosis can arise.
- Bone turnover markers are biomarkers that reflect bone remodeling.
- Bone turnover markers can easily be measured in blood and are a useful tool in assessing bone remodeling. See Table 1.

Table 1 Overview of commonly used bone turnover markers

Bone turnover marker	Secreted from	Marker of
CTX	Product of collagen degradation	Bone resorption. Is a marker of collagen degradation
NTX	Product of collagen degradation	Bone resorption. Is a marker of collagen degradation
P1NP	Product of collagen formation	Bone formation. Is cleaved from collagen and a marker of collagen production
Osteocalcin	Osteoblasts	Formation of the bone matrix
Bone-specific alkaline phosphatase	Osteoblasts	Calcification of bone mineral matrix
Tartrate resistant acid phosphatase	Osteoclasts	Reflects osteoclast number and activity
RANKL	Osteoblasts and osteocytes	Stimulates osteoclasts and bone resorption through the RANK pathway
Osteoprotegerin	Osteoblasts and osteocytes	Is the antagonist of RANKL and is thus a marker of decreased bone resorption
Sclerostin	Osteocytes	Antagonist of the Wnt pathway. Inhibits bone formation and osteblastogenesis

C-terminal cross-linked telopeptide of type-I collagen (*CTX*), N-terminal cross-linked telopeptide of type-I collagen (*NTX*), Procollagen type 1 N-terminal propeptide (*P1NP*), Receptor activator of nuclear factor kappa-B (RANK), Receptor Activator of Nuclear factor Kappa beta Ligand (*RANKL*)

Key Facts of Glucose and Bone Turnover

- C-terminal cross-linked telopeptide of type-I collagen decreases within 20 min of an oral glucose tolerance test.
- When glucose is given intravenously, a decrease in C-terminal cross-linked telopeptide of type-I collagen is seen; only it is delayed by 1 h compared to the oral glucose tolerance test.
- The decrease in C-terminal cross-linked telopeptide of type-I collagen seen in an intravenous glucose tolerance test is significantly lower than that of the oral glucose tolerance test.
- In healthy males, a hyperglycemic clamp has been shown to induce a decrease in osteoprotegerin, whereas no change was seen during euglycemia.
- Procollagen type 1 N-terminal propeptide has both been reported to be stable and to decrease during an oral glucose tolerance test.
- Hyperglycemia may decrease osteoblast differentiation and bone formation and impair bone resorption by increasing osteoprotegerin.
- Hyperglycemia decreases the number of osteoclasts, inhibits osteoclastogenesis and osteoclast differentiation, and impairs the ability of osteoclasts to resorb mineralized matrix.
- Osteocytes react to hyperglycemia by increasing sclerostin production, which in turn inhibits the Wnt pathway and thereby bone formation.

Definitions of Words and Terms

Bone formation	The process of producing new bone tissue.
Bone resorption	The process of degrading old bone tissue.
Bone turnover	The process of old and damaged bone tissue being degraded and replaced by new bone tissue.
Euglycemia	The state with a normal concentration of glucose in the blood.
Hyperglycemia	The condition of having a higher than normal concentration of glucose in the blood.
Hyperglycemic clamp	A technique used in experiments where a constant but varying amount of glucose is infused intravenously in accordance to insulin secretion and glucose metabolism to keep blood glucose at a constant high level. Same technique can be used for achieving euglycemia (euglycemic clamp).
Hypermineralization	A state where bone is over-mineralized relative to its collagenous matrix.
Mineralization	The process of impregnate mineral in the matrix of the bone. The mineral content of bone is primarily hydroxyapatite, which primarily consists of calcium and phosphate.
Osteoblasts	The cell type responsible for bone formation.

Osteoclastogenesis	The development of osteoclasts.
Osteoclasts	The cell type responsible for bone resorption.
Osteocytes	The most common cell type in bone tissue, thought to be encased osteoblasts that control the activity of osteoblasts and osteoclasts through mechanosensory mechanisms.

Introduction

Diabetes Mellitus

Diabetes Mellitus is a highly prevalent condition throughout the world with an estimate of 592 million suffering from it in 2035 (International Diabetes Federation). Diabetes is characterized by a relatively decreased insulin production, with a complete lack of insulin production in type 1 diabetes and a decreased insulin production relatively to the insulin resistance in patients with type 2 diabetes. The decreased insulin production causes unstable fasting conditions and patients with diabetes may be diagnosed by increased fasting plasma (p-) glucose of >7 mmol/l, an 2 h value of >11 mmol/l at an oral glucose tolerance test (OGTT) (American Diabetes Association 2012) or an elevated glycated hemoglobin A1c (HbA1c) level of $\geq$ 48 mmol/mol. The glycemic regulation in patients with diabetes is disturbed and fasting p-glucose levels do not follow the same pattern as in non-diabetes subjects.

Bone Remodeling

Bone remodeling is the process of degradation of old bone and creation of new bone (Hadjidakis and Androulakis 2006; Khosla and Riggs 2005). Under optimal circumstances the degradation (bone resorption) and creation (bone formation) of bone are balanced. In osteoporotic individuals, the rate of resorption is higher than the rate of formation (Khosla and Riggs 2005) which diminish bone mass and bone mineral density (BMD). Bone is constructed by a mineralized matrix consisting of mainly hydroxyapatite, a non-mineralized matrix consisting of primarily collagen, and a cellular compartment consisting of the bone cells osteoclasts, osteoblasts, and osteocytes. Mechanical resistance is provided by the hydroxyapatite crystals whereas stability and elasticity are provided by the network of type I collagen (Boskey 2013).

Osteoclasts are the bone resorping cells and osteoblasts are the bone forming cells. The osteocytes may serve as main regulators of the bone remodeling as they react to mechanical stress. The osteocytes produce and secrete sclerostin, a Wnt pathway inhibitor that decreases bone formation, and fibroblast growth factor-23 (FGF-23) that stimulate phosphate excretion in the kidneys. Furthermore, Receptor Activator of Nuclear factor Kappa beta Ligand (RANKL), a promoter of bone resorption, and its antagonist osteoprotegerin (OPG) are secreted by osteocytes.

However, the main production of OPG and RANKL are from the osteoblasts (Hadjidakis and Androulakis 2006).

The bone remodeling is divided into three phases; a bone resorption phase performed by the osteoclasts, a reversal phase where the bone is prepared for the osteoblasts, and finally the bone formation phase that consists of the production, maturation, and mineralization of the matrix. The resorption phase may take 2 weeks, whereas bone formation may continue for as long as 4 months. In healthy subjects, the production of matrix and mineralization of matrix are at the same rate (Hadjidakis and Androulakis 2006). Figure 1 depicts the systems involved in bone remodeling.

Bone Turnover Markers

Bone turnover markers are biomarkers of the bone remodeling and a specific marker represents a specific phase of the bone remodeling. Bone turnover markers are released to the blood during the bone remodeling and are thus easily measured providing information on the bone turnover. Table 1 describes the most commonly used bone turnover markers. C-terminal cross-linked telopeptide of type-I collagen (CTX) and N-terminal cross-linked telopeptide of type-I collagen (NTX) are commonly used resorption markers that reflect collagen degradation. Tartrate resistant acid phosphatase (TRAP) reflects the activity of osteoclasts and is also a resorptive

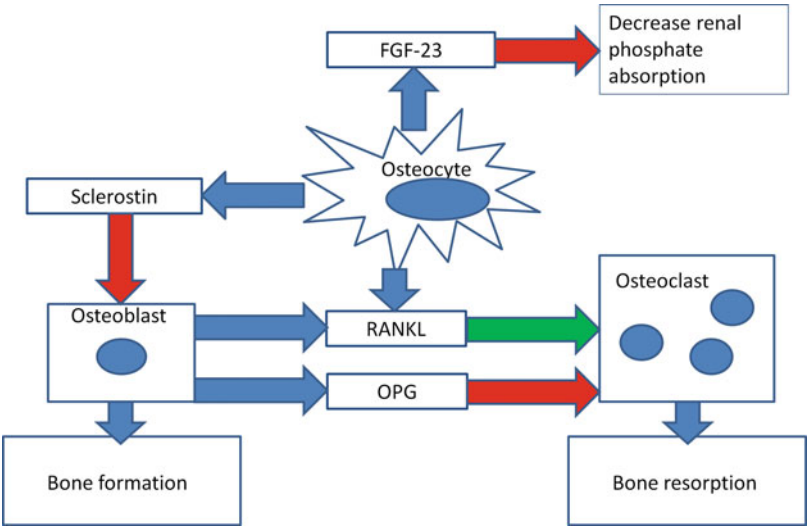

Fig. 1 ***Systems regulating bone remodeling.*** The osteocyte secretes sclerostin, which decrease bone formation and FGF-23 which increase phosphate excretion in the kidneys. The osteoblasts maintain bone formation and regulate the osteoclast through both OPG and RANKL. *Red arrows* are inhibitory actions, *green arrows* stimulatory actions, and *blue arrows* secreted products (With permission from the author (Starup-Linde 2015))

marker. Procollagen type 1 N-terminal propeptide (P1NP) is a formative marker, which is released during the cleavage of immature collagen. Osteocalcin, another formative marker, is an unmineralized matrix component and reflects the bone formation. Bone-specific alkaline phosphatase (BAP) is an enzyme produced during the mineralization phase of the bone formation (Starup-Linde 2013; Starup-Linde and Vestergaard 2015).

Analytical Factors

Bone turnover marker assays are offered by a large number of immunodiagnostic kit companies. Most are research-grade assays that are not intended for diagnostics use. Assays intended for diagnostic use are regulated by national and international bodies in terms of a range of validation parameters, particularly assay standardization (White 2011). This is in contrast to research-grade assays, which are unregulated and frequently missing assay characterization and standardization (Bowsher and Sailstad 2008). The above leads to considerable measurement differences between commercial assays and may result in conflicting research findings and slowing the implementation of bone turnover markers into routine clinical practice (Seibel et al. 2001; Whitham and Milford-Ward 2000). In this regard a recent publication stands out. The joint International Osteoporosis Foundation has recommended the use of CTX and P1NP as reference bone turnover markers in clinical trials and proposed strategies for standardization aiming for future inclusion in routine clinical practice and comparable values across assays (Vasikaran et al. 2011). Until such standardization has been attained, results and reference intervals from different assays should not be used interchangeably for clinical use, and care must be taken to address this important issue in research studies (Meier et al. 2009).

In addition to analytical issues, preanalytical factors are considered problematic with significant influence on measurements (Hannon and Eastell 2000). Preanalytical factors are factors such as sample handling, circadian, age, gender, menopausal status, and fractures. Of these, sample handling and circadian changes can be controlled by standardized sampling, sample handling, and collecting samples at the same time of day. Most other preanalytical factors cannot be controlled and their recognition is important in the interpretation of bone turnover marker results. Therefore, clinicians and researchers should be familiar with conditions where bone turnover levels are expected to be altered, for example in children, menopause, and after recent fracture (Seibel 2005). Another preanalytical factor commonly viewed as an obscuring factor but also of scientific interest and the focus of this review is the observed postprandial suppression of bone turnover levels (Clowes et al. 2003). This subject is further described in section "The Effect of Glucose Intake on Bone Turnover Markers in Humans."

Diabetes and Bone

Diabetes is related to microvascular and macrovascular complications (American Diabetes Association 2012). Until recently the increased risk of fracture was an

overseen complication. Thus, diabetes and bone may be related. The risk of hip fracture has been suggested to be sevenfold increased in patients with type 1 diabetes and twofold increased in patients with type 2 diabetes compared to non-diabetes individuals (Vestergaard 2007; Janghorbani et al. 2007). One would expect a similar lowering of BMD as it is the primary fracture predictive tool. However, BMD is increased in patients with type 2 diabetes and only slightly decreased in patients with type 1 diabetes and does not explain the increased fracture risk (Vestergaard 2007). Patients with diabetes have hypermineralized bone relative to their decreased bone material competence. When adding further fracture predictors to the model in The Fracture Risk Assessment Tool (FRAX), which also includes BMD, it underestimates both hip fracture risk and major osteoporotic fracture risk in patients with type 2 diabetes (Giangregorio et al. 2012). Furthermore, the increased fracture risk is not explained by either hypoglycemic events or the number of falls in patients with diabetes (Bonds et al. 2006; Vestergaard et al. 2005), or why the increased risk of fracture in diabetes patients seems to be bone related. The mechanism of the decreased bone quality and lack of fracture predictors is not well understood in diabetes; however, it may relate to the relative lack of insulin and disturbed glucose metabolism, but also factors as obesity, medication use, and renal impairment may affect the bone metabolism in patients with diabetes.

Glucose and Bone Turnover Markers

Clinical Studies

The Effect of Glucose Intake on Bone Turnover Markers in Humans

Patients with diabetes are at increased risk of fracture, thus their bone turnover may be altered. Clinical studies have investigated the effect of glucose on bone turnover markers. Table 2 presents the studies that have examined the effect of glucose ingestion on bone turnover markers. In men and women subjected to an OGTT, the bone resorption marker s-CTX and u-CTX decreased (Clowes et al. 2003; Henriksen et al. 2003; Bjarnason et al. 2002; Nissen et al. 2014; Chailurkit et al. 2008; Viljakainen et al. 2014; Paldanius et al. 2012; Schwetz et al. 2014; Karatzoglou et al. 2014). The decrease in s-CTX has been reported as early as 20 min after glucose ingestion (Clowes et al. 2003), whereas the decrease was delayed by one hour during the intravenous glucose tolerance test (IVGTT) (Bjarnason et al. 2002). The decrease in s-CTX was apparent in patients with type 2 diabetes but lower than in healthy controls (Chailurkit et al. 2008). Furthermore, the osteoclast specific marker TRAP decreased in both healthy obese and healthy non-obese individuals during OGTT (Viljakainen et al. 2014). A convincing effect of glucose intake on bone resorption markers was observed in these OGTT and IVGTT studies. The effect on bone formation markers was more unsettled, although both P1NP and osteocalcin have been reported to decrease. A decrease in s-osteocalcin has been shown two hours after an OGTT (Clowes et al. 2003; Viljakainen et al. 2014; Paldanius et al. 2012; Schwetz et al. 2014); however, other studies showed stable

Table 2 Studies that examine the effects of glucose ingestion and bone turnover marker response

Study and design	Population	Type of ingested glucose	Duration of the experiment	Result
Bjarnason et al. (2002) Randomized controlled cross-over	15 postmenopausal women, 12 premenopausal women, and 11 men	OGTT (75 g of glucose) and IVGTT (0.3 g glucose pr. kg)	24 h	Decrease in s-CTX after 1 h and 2 h for OGTT and IVGTT, respectively. Same pattern for u-CTX. S-osteocalcin decreased by the OGTT but not food intake
Chailurkit et al. (2008) Cross-sectional study	163 postmenopausal women (among these 54 with type 2 diabetes)	OGTT (75 g of glucose)	2 h	Decrease in s-CTX after 2 h. A significant decrease in OPG in non-diabetes women, but not in women with type 2 diabetes
Clowes et al. (2003) Randomized single blind cross-over	15 healthy subjects	OGTT (75 g of glucose) or placebo and in combinations with octreotide or saline infusion	4 h	OGTT decreased s-CTX, s-osteocalcin, and s-P1NP after 20 min and also decreased u-NTX. The effect of glucose on bone turnover markers was abolished by octreotide infusion, except for s-osteocalcin but with a delay
Clowes et al. (2002) Randomized double blind cross-over	16 healthy men	Euglycemic clamp (plasma glucose = 5 mmol/l) Hypoglycemic clamp (plasma glucose = 2.5 mmol/l). Similar hyperinsulinemia in both conditions	2 h	Euglycemic clamp did not change osteocalcin, s-CTX, or P1NP, whereas osteocalcin, s-CTX, and P1NP all decreased during the hypoglycemic clamp. PTH decreased in both groups but with partial recovery in the euglycemic group
Henriksen et al. (2003) Randomized controlled cross-over	10 healthy subjects	OGTT (75 g of glucose)	9 h	OGTT decreased s-CTX but not s-osteocalcin compared to fasting conditions

Holst et al. (2007) Cross-sectional	8 gastrectomized patients	OGTT (75 g of glucose)	3 h	OGTT decreased s-CTX and increased GLP-2. Osteocalcin remained unchanged by the OGTT
Karatzoglou et al. (2014) Cross-sectional	59 patients with Crohns disease and 45 healthy subjects	OGTT (75 g of glucose)	2 h	OGTT decreased s-CTX in both patients and healthy individuals, whereas P1NP remained unchanged
Knudsen et al. (2007) Randomized blinded cross-over study	9 healthy males	Hyperglycemic clamp (plasma glucose = 15 mmol/l) and euglycemic clamp (plasma glucose = 5 mmol/l)	4 h	OPG decreased during hyperglycemic conditions but not during euglycemia
Nissen et al. (2014) Cross-over	10 healthy males	Euglycemic (5 mmol/l) and hyperglycemic (12 mmol/l) clamps with co infusion of GIP or saline	90 min	Hyperglycemic clamp decreased CTX; however, a greater decrease was observed for the combination of GIP and hyperglycemia than for either GIP or hyperglycemia alone
Paldánius et al. (2012) Cross-sectional	23 healthy subjects	OGTT (75 g of glucose)	2 h	The OGTT decreased s-osteocalcin, s-CTX, s-TRAP, and s-P1NP
Schwetz et al. (2014) Cross-sectional	105 premenopausal women (of these 18 insulin resistant)	OGTT (75 g of glucose)	2 h	OGTT decrease CTX, P1NP, s-osteocalcin, and s-undercarboxylated osteocalcin in non-insulin resistant women, but only CTX in insulin resistant women
Viljakainen et al. (2014) Cross-sectional	34 obese individuals and 34 non-obese individuals- all non-diabetic	OGTT (75 g of glucose)	2 h	s-CTX, TRAP, BAP, osteocalcin, and P1NP all decreased during the OGTT in both groups. The osteocalcin decrease was significantly larger in the non-obese subjects compared to the obese subjects

Bone-specific alkaline phosphatase (*BAP*), C-terminal cross-linked telopeptide of type-I collagen (*CTX*), Gastric inhibitory peptide (*GIP*), Glucagon-like peptide 2 (*GLP-2*), Intravenous glucose tolerance test (*IVGTT*), N-terminal cross-linked telopeptide of type-I collagen (*NTX*), Oral glucose tolerance test (*OGTT*), Osteoprotegerin (*OPG*), Parathyroid hormone (*PTH*), Procollagen type 1 N-terminal propeptide (*P1NP*), Tartrate resistant acid phosphatase (*TRAP*)

osteocalcin levels when comparing to fasting conditions (Henriksen et al. 2003; Bjarnason et al. 2002; Holst et al. 2007). P1NP has both been reported to be stable (Karatzoglou et al. 2014) and to decrease (Clowes et al. 2003; Viljakainen et al. 2014; Paldanius et al. 2012; Schwetz et al. 2014) during an OGTT. The mineralization marker BAP decreased during OGTT in both obese and non-obese subjects (Viljakainen et al. 2014).

As the effects of OGTT and IVGTT are related to time, it was important to show whether time itself affected the bone turnover markers. Maintenance of a euglycemic p-glucose level of 5 mmol/l did not change the levels of s-CTX, osteocalcin, or P1NP (Clowes et al. 2002) when followed for two hours. Furthermore, when comparing fasting condition with OGTT, CTX decreased significantly more during the OGTT than during the fasting state (Henriksen et al. 2003). Thus, glucose intake has a time independent effect on bone turnover markers.

Glucose intake decreased the bone turnover markers, but a decrease was also observed during hypoglycemia where parathyroid hormone (PTH), P1NP, s-CTX, and osteocalcin decreased (Clowes et al. 2002). Bone turnover markers may decrease with p-glucose values both lower and higher than 5 mmol/l. Therefore the effect of glucose on bone turnover markers may be u-shaped with an optimal state in the normal healthy fasting condition. Although both IVGTT and OGTT decreased s-CTX, the decrease was significantly smaller during IVGTT (Bjarnason et al. 2002), which suggests that an additional component from the gastrointestinal tract affects bone turnover. Glucagon-like peptide-2 (GLP-2) increased while CTX decreased in gastrectomized patients (Holst et al. 2007), and this supports that the gastrointestinal absorption may affect bone turnover. Furthermore, the decrease in CTX during hyperglycemia was enhanced in combination with infusion of gastric inhibitory peptide (GIP) (Nissen et al. 2014), which suggests that the gastrointestinal hormones potentiate the effect of glucose on bone turnover. An intravenous injection of GIP and a subcutaneous injection of glucagon-like peptide-1 (GLP-1) did not affect s-CTX, whereas subcutaneous injection of GLP-2 decreased s-CTX (Henriksen et al. 2003).

The clinical studies show a strong relation between glucose intake and bone turnover, which may be either mediated or enhanced by gastrointestinal hormones. However, no direct pathway was established. The effect may be from an alteration of the OPG/RANKL pathway. During a hyperglycemic clamp OPG decreased in healthy males, while no change was observed during euglycemia (Knudsen et al. 2007). In type 2 diabetes women, OPG remained stable during an OGTT whereas it decreased in healthy women (Chailurkit et al. 2008). Thus, the OPG system may be altered in patients with diabetes compared to healthy subjects.

Bone Turnover Markers in Diabetes

Bone turnover markers in patients with diabetes have been examined in a meta-analysis (Starup-Linde et al. 2014). Both osteocalcin and CTX were decreased in patients with diabetes. compared to non-diabetes controls, whereas NTX was borderline significantly increased in diabetes patients. 25 hydroxy vitamin D levels were lower in diabetes patients, and phosphate levels were increased in patients with diabetes. PTH, calcium, and BAP were not different from controls in patients with

diabetes. When stratifying by diabetes type, patients with type 1 diabetes had lower 25 hydroxy vitamin D and osteocalcin, whereas patients with type 2 diabetes had lower phosphate levels and borderline decreased osteocalcin compared to non-diabetes subjects (Starup-Linde et al. 2014). Further studies add to a decreased bone turnover in both patients with type 1 and type 2 diabetes and report that BAP is not decreased, when other bone markers were decreased (Starup-Linde and Vestergaard 2015). Thus, bone turnover in diabetes is altered in comparison to non-diabetes individuals with lower CTX and osteocalcin levels representing lower bone resorption and bone formation. BAP, which represents mineralization, was not different, thus the bone matrix mineralization seems not to be impaired.

All bone turnover markers displayed heterogeneity between studies (Starup-Linde et al. 2014). The heterogeneity may be due to differences in patient characteristics, due to analytical and preanalytical factors, due to using different assays (no marker was evaluated with same method through all studies), or due to differences in p-glucose levels in the patients with diabetes. An in vitro study revealed that the decrease in bone turnover markers is not due to an immunochemical masking effect by bone marker glycation, as addition of glucose to serum samples with increasing dose and incubation time did not change P1NP, osteocalcin, and CTX (Starup-Linde et al. 2014). The heterogeneity among bone turnover markers also makes them unreliable fracture predictors as they may change depending on p-glucose. However, decreased osteocalcin levels and increased P1NP/CTX ratio have been associated with fractures in patients with type 2 diabetes (Starup-Linde and Vestergaard 2015).

In Vitro Studies

The Effect of Glucose on Osteoblasts

Osteoblast-like cells have been exposed to different hyperglycemic conditions, and indices of bone turnover have been assessed. Table 3 presents the studies that have evaluated the addition of glucose to osteoblast-like cells. In human osteoblast-like cells, hyperglycemia of both 12 mmol/l and 24 mmol/l for 7 and 14 days, respectively, increased the matrix calcification. The quality of the mineral was reduced with low calcium phosphate ratios (Garcia-Hernandez et al. 2012). Alkaline phosphatase activity increased at a glucose level of 12 mmol/l but decreased at a glucose level of 24 mmol/l (Garcia-Hernandez et al. 2012). Both bone formation markers osteocalcin and runt-related protein 2 (Runx2), and the bone resorptive marker RANKL increased while OPG decreased; this suggests an overall increased bone turnover (Garcia-Hernandez et al. 2012). Two other studies using a different human cell line showed decreased proliferation, alkaline phosphatase activity, and expression of OPG but with glucose concentrations from 16.7 mmol/l to 49.5 mmol/l (Terada et al. 1998; Shao et al. 2014). Continuous glucose levels of 49 mmol/l are life threatening in vivo and even sustained levels above 20 mmol/l are unphysiological and lead to ketoacidosis or hyperglycemic hyperosmolar nonketotic coma. Studies investigating murine osteoblast-like cells exposed to glucose have reported varying results. Increased proliferation and increased matrix mineralization have been

Table 3 Studies that examined the effects of in vitro added glucose on osteoblasts

Study	Cell line	Glucose dose	Duration	Results
Human cells				
Garcia-Hernandez et al. (2012)	Human alveolar bone-derived cells with osteoblastic phenotype	5.5 mmol/l, 8 mmol/l, 12 (hyperglycemia) mmol/l or 24 mmol/l (hyperglycemia)	24 h, 7 days, and 14 days	Hyperglycemia increased calcium deposits and biomineralization after 7 and 14 days; however, the quality of the mineral was decreased based on a lower Ca/P ratio. Alkaline phosphatase was increased at conditions with 12 mmol/l glucose but decreased at 24 mmol/l glucose. Hyperglycemia increased the expression of Osteocalcin, BSP, Runx2, and RANKL and decreased OPG after 7 and 14 days
Shao et al. (2014)	Human osteoblast-like cells MG63	5.5 mmol/or 16.7 mmol/l (hyperglycemia)	1, 3, 6, 7, 12, and 18 days	Hyperglycemia decreased cell proliferation, alkaline phosphatase activity, and expression of osteocalcin and OPG
Terada et al. (1998)	Human osteoblast-like cells MG-63 cells	5.5 mmol/l, 33.0 mmol/l (hyperglycemia) or 49.5 mmol/l (hyperglycemia)	7 days	Hyperglycemia decreased cell proliferation and decreased responsiveness to IGF-1
Murine cells				
Balint et al. (2001)	MC3T3-E1 mice like osteoblastic cells	5.5 mmol/l or 15 mmol/l (hyperglycemic).	30 days	More bone forming nodules were present in hyperglycemic conditions. The nodules were larger, irregular, and had a larger total calcified area in the hyperglycemic condition. However, calcium uptake was decreased, alkaline phosphatase activity increased, and osteocalcin unchanged in the hyperglycemic group
Batolomé et al. (2013)	MC3T3-E1 mice like osteoblastic cells	5.6 mmol/l or 25 mmol/l (hyperglycemic)	48 h	Hyperglycemic decreased the expression of Runx2, osterix, and osteocalcin and decreased the matrix mineralization
Botolin and McCabe (2006)	MC3T3-E1 mice like	5.5 mmol/l or 35.5 mmol/l (hyperglycemic)	29 days	Hyperglycemia increased alkaline phosphatase activity but did not change

(*continued*)

Table 3 (continued)

Study	Cell line	Glucose dose	Duration	Results
	osteoblastic cells			mineralization. Osteocalcin and Collagenase3 decreased during hyperglycemia
Cunha et al. (2014)	MC3T3-E1 mice like osteoblastic cells	5 mmol/l, 30 mmol/l (hyperglycemic), 50 nmol/of insulin or the combination of high glucose and high insulin dose	24 h	Increased organic matrix production (10x) and cell differentiation in hyperglycemic conditions. Hyperglycemia increased OPG (30x) and RANKL (2–3x) production which was attenuated by insulin, whereas alkaline phosphatase activity and mineralization decreased
López-Herradón et al. (2013)	MC3T3-E1 mice like osteoblastic cells	5.5 mmol/l or 25 mmol/l (hyperglycemia)	5 days	Hyperglycemia decreased β-catenin levels and accumulation and downregulates the Wnt pathway
Liu et al. (2015)	MC3T3-E1 mice like osteoblastic cells	5.5 mmol/l, 15.5 mmol/l, 25.5 mmol/l or 35.5 mmol/l	24 and 72 h and 7 and 14 days	Cell proliferation and Runx2 were decreased in the 25.5 and 35.5 mmol/l and increased in the 15.5 mmol/l glucose group after 72 h. Alkaline phosphatase activity was increased in the 15.5 mmol/l and decreased in the 25.5 and 35.5 mmol/l glucose groups after 7 days. Mineralization was increased at 15.5, 25.5, and 35.5 glucose levels compared to the 5.5 mmol/l group. In the group with a glucose of 15.5 mmol/l osteocalcin, OPG, osterix, Runx2, and P-AKT were increased compared to the 5.5 mmol/l
Ma et al. (2014)	Primary rat osteoblasts	5.5 mmol/l or 16.5 mmol/l (hyperglycemia)	7 days and 14 days	Hyperglycemia decreased proliferation and decrease calcium accumulation
Wu et al. (2012)	Rat osteoblasts	5.5 mmol/or 22 mmol/l (hyperglycemia)	3 days	Hyperglycemia induced the highest proliferation rate and a decrease in alkaline phosphatase. Insulin receptor and vitamin D receptor mRNA decreased in hyperglycemic

(*continued*)

Table 3 (continued)

Study	Cell line	Glucose dose	Duration	Results
				conditions. Furthermore, hyperglycemia decreased osteocalcin and undercarboxylated osteocalcin
Zayzafoon et al. (2000)	MC3T3-E1 mice like osteoblastic cells	5.5 mmol/or 16.5 mmol/l (hyperglycemia)	24 h	Hyperglycemia decreased osteocalcin mRNA expression and increased collagen I mRNA expression and c-Jun expression
Zhen et al. (2010)	Rat primary osteoblasts	5.5 mmol/, 11 mmol/l, 22 mmol/or 44 mmol/l	48 h	High glucose levels inhibited cell proliferation. Alkaline phosphatase activity was increased at glucose levels of 11 mmol/l but decreased at higher glucose levels in comparison with the level of 5.5 mmol/l. Both the number of bone forming nodules, amount of calcium deposited, and matrix mineralized were increased at the 11 mmol/l but decreased at higher glucose levels in comparison with the level of 5.5 mmol/l. IGF-1 and Runx2 expression were increased in the 11 mmol/l glucose group

Tartrate resistant acid phosphatase (*TRAP*), Calcium/phosphate (*CA/P*), Bone sialoprotein (*BSP*), Osteoprotegerin (*OPG*), Receptor activator of nuclear factor kappa-B ligand (*RANKL*), Insulin-like growth factor-1 (*IGF-1*), Runt-related protein 2 (*Runx2*)

reported (Balint et al. 2001; Liu et al. 2015; Wu et al. 2012; Zhen et al. 2010); however, both decreased matrix mineralization (Zhen et al. 2010, Bartolome et al. 2013; Cunha et al. 2014; Ma et al. 2014) and unchanged matrix mineralization (Botolin and McCabe 2006) have also been reported. Alkaline phosphatase activity reflects the mineralization process and has been reported to be both decreased (Balint et al. 2001; Liu et al. 2015; Wu et al. 2012; Zhen et al. 2010; Cunha et al. 2014) and increased (Liu et al. 2015; Zhen et al. 2010; Botolin and McCabe 2006) during hyperglycemia. Runx2 is an important transcription factor in osteoblast differentiation and osteocalcin is a marker of osteoblasts activity. RANKL and osteocalcin have been reported decreased (Wu et al. 2012; Bartolome et al. 2013; Botolin and McCabe 2006; Zayzafoon et al. 2000) and increased (Liu et al. 2015; Zhen et al. 2010) in hyperglycemic circumstances. Furthermore, the OPG/RANKL pathway may be disturbed as OPG increased 30-fold during hyperglycemia and RANKL only

increased two- to threefold, suggesting an inhibitory effect on bone resorption (Cunha et al. 2014). The Wnt pathway was also downregulated during hyperglycemia by decreasing β-catenin accumulation (Lopez-Herradon et al. 2013). Thus, hyperglycemia may decrease osteoblast differentiation and bone formation and also impair the bone resorption by increasing OPG.

Very different doses of glucose have been used in the studies to induce hyperglycemia ranging from 11 mmol/l to 49.5 mmol/l. The studies by Liu et al. and Zhen et al. (Liu et al. 2015; Zhen et al. 2010; Li et al. 2007) use different levels of hyperglycemia and present the importance of the glucose levels as both studies reported increased alkaline phosphatase activity and mineralization at the lowest level of hyperglycemia (15.5 mmol/l and 11 mmol/l) whereas higher glucose levels (22 mmol/l, 25.5 mmol/l, 35.5 mmol/l, and 44 mmol/l) decreased alkaline phosphatase activity and did not increase mineralization at the lowest levels of hyperglycemia. The glucose levels of 22 mmol/l or more are very high and is life threatening if sustained for longer periods, whereas levels of 11 mmol/l or 15 mmol/l may be tolerated for a longer period. The studies reporting decreased mineralization have used glucose levels higher than 16 mmol/l. The effect of glucose on bone markers may thus depend on the glucose levels; small increases of glucose may increase alkaline phosphatase activity and increase mineralization, whereas supraphysiological levels of glucose may decrease mineralization and alkaline phosphatase activity.

The Effect of Glucose on Osteoclasts

CTX is a marker of bone resorption and as CTX decreases during glucose intake, glucose may directly affect the osteoclasts. Table 4 presents the studies that have examined the effect of hyperglycemia on osteoclasts. Only three studies have examined this relationship and all on murine cells. Hyperglycemia was shown to have a detrimental effect when directly added to osteoclast-like cells. Hyperglycemia decreased the number of osteoclasts, TRAP expression, osteoclastogenesis, cell to cell fusion (which is an important step in creation of the multinucleated osteoclasts), and osteoclast differentiation (Wittrant et al. 2008; Xu et al. 2013, 2015). Furthermore, a decreased pit resorption area was observed during hyperglycemia. This reflects an impairment in the ability of the osteoclast to resorp mineralized matrix at elevated glucose concentrations (Xu et al. 2013). These in vitro studies clearly show a direct detrimental effect of hyperglycemia on osteoclasts. Thus, the hyperglycemia inhibits osteoclasts, this is in line with the clinical human studies where glucose ingestion decreased CTX (Bjarnason et al. 2002).

The Effect of Glucose on Osteocytes

Only a single study has assessed the effect of hyperglycemia on osteocytes. It is presented in Table 4. This study showed an increased expression of sclerostin protein, whereas RANKL was unchanged during hyperglycemia (Tanaka et al. 2015). The regulatory activity of osteocytes may thus also be affected by glucose, with an increased sclerostin production and thereby an inhibitory effect on the osteoblasts by blocking the Wnt pathway. Further research is needed to confirm the effect of glucose on osteocytes.

Table 4 Studies examining the effects of in vitro added glucose on osteoclasts and osteocytes

Study	Cell line	Glucose dose	Duration	Results
Osteoclasts				
Wittrant et al. (2008)	Murine RAW 264.7 monocytic cells	Differentiation medium using 15.5–30.5 mmol/l of D (+) glucose or L (−) glucose as control	6 days	High glucose levels inhibits RANKL induced TRAP expression, osteoclastogenesis, osteoclast differentiation, and cell migration in RAW 264.7 cells
Xu et al. (2013)	Mice osteoclasts derived from bone marrow cells	33.6 mmol/l (hyperglycemia)	4 days	Hyperglycemia decreased the number of mature osteoclasts and TRAP, RANK, and cathepsin k expression. Furthermore, hyperglycemia decreased TRAP activity, and the pit resorption area measured by absent mineral deposition
Xu et al. (2015)	Murine RAW 264.7 monocytic cells	5.6 mmol/l or 20.2 mmol/l	4–5 days	Hyperglycemia inhibited RANKL induced osteoclastogenesis, osteoclast differentiation, and decrease cell to cell fusion
Osteocytes				
Tanaka et al. (2015)	Mice osteocyte-like MLO-Y4-A2 cells	5.5 mmol/l or 22 mmol/l (hyperglycemia)	24, 48, and 72 h	Hyperglycemia increased sclerostin protein expression but did not affect RANKL

Tartrate resistant acid phosphatase (*TRAP*), Receptor activator of nuclear factor kappa-B (*RANK*), Receptor activator of nuclear factor kappa-B ligand (*RANKL*)

The Effect of Glucose on Mesenchymal Stem Cells

Both osteoblasts and osteoclasts are derived from the mesoderm, and mesenchymal stem cells may thus differentiate to both types. Only immortalized human mesenchymal stem cells proliferated during hyperglycemia. Primary human mesenchymal stem cells had lower proliferation rate but differentiated towards osteogenic cells during hyperglycemia over 4 weeks and had enhanced mineralization compared to cells exposed to lower glucose levels (Li et al. 2007). Murine mesenchymal stem cells decreased mineralization, TRAP, and alkaline phosphatase activity but increased collagen production when exposed to hyperglycemia (Dienelt and zur Nieden 2011). Glucose may thus affect the differentiation of mesenchymal stem cells to osteoblasts and osteoclasts (Table 5).

Table 5 Studies examining the effects of in vitro added glucose on mesenchymal stem cells

Study	Cell line	Glucose dose	Duration	Results
Dienelt and zur Nieden (2011)	Murine embryonic stem cells	1 g/l or 4.5 g/l (hyperglycemic)	30 days	Hyperglycemia decreased matrix calcification, TRAP, and alkaline phosphatase activity but increased collagen production
Li et al. (2007)	Human mesenchymal stem cells telomerase-immortalized (hMSC-TERT) and primary human mesenchymal stem cells (hMSC)	5.6 mmol/l, 11 mmol/l or 25 mmol/l (hyperglycemia)	4 days and 4 weeks	Hyperglycemia caused proliferation in the hMSC-TERT cell line. hMSC cells differentiated towards osteogenic cells in the hyperglycemic conditions with enhanced mineralization compared to lower glucose concentration.

Tartrate resistant acid phosphatase (*TRAP*), Human mesenchymal stem cells (*hMSC*), Human mesenchymal stem cells telomerase-immortalized (*hMSC-TERT*)

Glucose and Diabetic Bone Disease

Ingestion of glucose decreased both bone resorption markers and bone formation markers in healthy individuals and a link between glucose and bone turnover markers was established. In patients with diabetes the increased fracture risk was not explained by the apparent normal or increased BMD. This paradox suggests that BMD is not equal to bone quality in patients with diabetes. The mineralization marker BAP remained unchanged while CTX and osteocalcin were decreased in diabetes patients compared to non-diabetes subjects. Thus, a dissociative bone remodeling may be present in patients with diabetes with a decreased bone resorption and bone matrix formation whereas the mineralization is relatively increased. In this state, BMD is increased in comparison to the quality of the bone, which also is the case in some osteopetrotic patients (Starup-Linde and Vestergaard 2015). A study examining bone biopsies from patients with type 2 diabetes reports reduced bone formation rate and mineralizing surface but normal adjusted apposition rate in comparison to controls (Manavalan et al. 2012). Therefore the decreased mineralizing surface is due to a decreased bone formation. The in vitro studies on both human and murine cells report direct effects of glucose on bone cells. A physiological hyperglycemia (as in many patients with diabetes) with glucose levels of 11–15 mmol/l increased the mineralization and alkaline phosphatase activity by osteoblasts, whereas higher glucose levels decreased mineralization and alkaline phosphatase activity. Furthermore, hyperglycemia was mainly reported to decrease the Runx2 and osteocalcin expression and thus leading to decreased bone formation, although some in vitro studies also report

increased Runx2 and osteocalcin. Besides a direct effect on osteoblasts, hyperglycemia decreased osteoclastogenesis and osteoclast activity and thus impaired bone resorption and increased the osteocytes expression of sclerostin, which decrease bone formation by antagonizing the Wnt pathway.

In patients with diabetes the p-glucose level is cyclical depending on medication use and food intake. As the p-glucose level may rapidly increase, the bone formation and bone resorption may drop. However, the mineralization process seems to be spared, which may be caused by an increased mineralization in the p-glucose levels of 11–15 mmol/l. In patients with diabetes, the level of 11–15 mmol/l is a physiological level and may be sustained for longer periods. Furthermore, HbA1c was a positive effect modificator of BAP in a meta-regression analysis (Starup-Linde et al. 2014) and mineralization reflected by BAP may increase due to long-term hyperglycemia. The bone of patients with diabetes may be hypermineralized due to long-term hyperglycemia. Figure 2 presents the hypermineralization hypothesis. Hyperglycemia may decrease both bone formation and bone resorption by a direct inhibitory effect on osteoblasts and osteoclasts. During hyperglycemia, osteocytes produce more sclerostin, and osteoblasts may increase OPG production which will decrease bone formation and bone resorption, respectively. At p-glucose levels of 11–15 mmol/l, the hyperglycemia may increase the bone mineralization as reflected by both calcification and alkaline phosphatase activity but with low quality

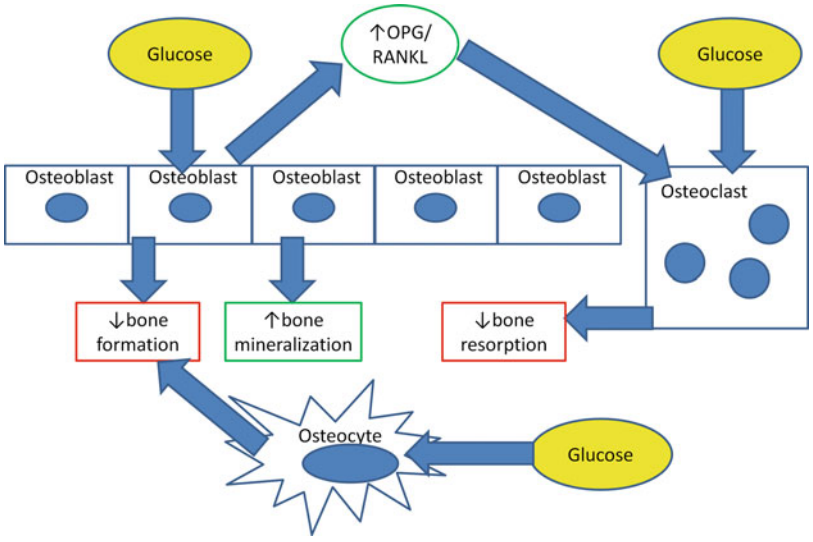

Fig. 2 ***The hypermineralization hypothesis.*** Hyperglycemia decreases the osteoclasts' ability to resorp bone directly and indirectly by increasing the OPG/RANKL ratio. Bone formation by the osteoblasts is directly inhibited by hyperglycemia reflected by decreased Runx2 and osteocalcin expression and indirectly by an increased production of sclerostin by the osteocytes. Both bone formation and resorption is decreased; however at hyperglycemic levels of 11–15 mmol/l the mineralization is increased by the osteoblast, whereas the quality of the mineralized matrix is decreased due to decreased calcium/phosphate ratio

mineralized material. Therefore, a state where the mineralization does not correspond to the bone turnover may exist in patients with diabetes, and the bone is hypermineralized relatively to the bone material strength.

Potential Applications to Prognosis, Other Diseases or Conditions

The interaction between glucose and bone turnover markers is a potential predictive and prognostic marker of fracture risk in patients with diabetes. The hyperglycemia, which characterizes patients with diabetes, may have detrimental effects on bone turnover, bone composition, and bone strength. The combination of bone turnover markers and continuous glucose monitoring may be of use to determine the bone turnover in patients with diabetes and evaluate whether this may be prognostic of fracture. No therapy of diabetic bone disease is available. Strict glucose control may be beneficial for bone health in patients with diabetes however, it is unknown whether antiresorptive therapies that decrease bone resorption are beneficial in a state already characterized by decreased bone resorption.

Future investigations may apply the advanced techniques of continuous glucose monitoring to determine the circadian rhythm of bone turnover in patients with diabetes and its relation to p-glucose levels, which may be very different from what is seen in non-diabetes subjects. Furthermore, additional studies investigating the effect of glucose on osteoblasts, osteoclasts, and osteocytes are needed. Translation of these results into animal models is also important. Randomized controlled trials are needed to determine whether a specific antidiabetic treatment is beneficial for bone health and if antiresorptive treatment may be of use.

Summary Points

- Diabetes mellitus patients have higher risk of bone fracture which cannot be explained by their bone mineral density.
- Bone turnover in patients with diabetes is altered in comparison to non-diabetes individuals as they display lower C-terminal cross-linked telopeptide of type-I collagen and osteocalcin levels representing lower bone resorption and bone formation.
- An oral glucose tolerance test induces a decrease in C-terminal cross-linked telopeptide of type-I collagen in healthy individuals, but the decrease is attenuated in patients with diabetes.
- An intravenous glucose tolerance test does not induce the same reduction in C-terminal cross-linked telopeptide of type-I collagen as an oral glucose tolerance test, indicating that gastrointestinal hormone release influence the glucose-bone interaction.
- Hyperglycemia may decrease osteoblast differentiation and bone formation and may also impair the bone resorption by increasing osteoprotegerin and inhibiting osteoclast activity directly.

- The effect of glucose on bone markers may be dependent on the glucose level, where small increases in blood glucose may increase alkaline phosphatase activity and mineralization, whereas supraphysiological levels decrease mineralization and alkaline phosphatase activity.
- The bone of patients with diabetes may be hypermineralized due to hyperglycemia that increase bone mineralization relatively to the bone turnover.

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Dietary Soy Phytoestrogens and Biomarkers of Osteoporosis

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Abstract

Osteoporosis, decreased bone strength increasing the risk of fractures, is the result of alterations in bone remodeling causing an imbalance between bone formation and resorption with a predominance of resorption. In postmenopausal women, bone loss increases due to lower levels of estrogen. One of the most common treatment strategies for osteoporosis after incidence of fractures is the use of antiresorptive agents to stimulate osteoblastic proliferation. Hormone replacement therapy (HRT) for the treatment of menopausal symptoms also reduces the risk of osteoporosis, although its adverse side effects have led researchers to investigate alternative treatments. Dietary soy phytoestrogens have gained considerable attention for exhibiting beneficial effects on bone metabolism and modulating related biomarkers of osteoporosis. Studies using cultured bone cells and postmenopausal rat models support a significant bone-sparing effect of soy phytoestrogens. These findings have initiated clinical studies for the evaluation of soy phytoestrogen effects on postmenopausal bone loss. Human clinical studies have shown both promising and conflicting results. Only few studies show that consumption of soy phytoestrogens increase bone mineral density in postmenopausal women, whereas most studies show no such effects. This short review focuses on the potential effects of soy-derived phytoestrogens on biomarkers (alkaline phosphatase, N-telopeptide of type 1 collagen) of osteoporosis by examining the evidence from in vitro cultured bone cells, in vivo animal models, and human clinical studies. These collective data suggest the bone-sparing effects of soy phytoestrogens.

Keywords

Osteoblast • Osteoclast • Ovariectomy • Orchidectomy • Genistein • Daidzein • Soy isoflavone • Bone mineral density • Ipriflavone • Postmenopause

Abbreviations

ALP	Alkaline phosphatase
AP-1	Activator protein 1
ASC	Adipose-derived stromal/stem cell
BAP	Bone-specific alkaline phosphatase
BMC	Bone marrow stromal osteoprogenitor cells
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMSC	Bone marrow-derived mesenchymal stem cell
BV/TV	Trabecular bone volume
Cbfa1	Core binding factor 1
Cd	Cadmium
$CdCl_2$	Cadmium chloride
CLO	Caged layer osteoporosis
Col I	Collagen type 1
DXA	Dual-energy X-ray absorptiometry

E2	17β-estradiol
E2B	Estradiol-3 benzoate
ER	Estrogen receptor
ER-PKCα	Estrogen receptor-protein kinase C alpha
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FDA	Food and Drug Administration
FRAX®	Fracture Risk Assessment Tool
HOB	Trabecular bone osteoblasts
IGF	Insulin-like growth factor
IP	Ipriflavone
MAPK	Mitogen-activated protein kinase
MAR	Mineral apposition rate
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTx	N-telopeptide of type 1 collagen
OCN	Osteocalcin
O-DMA	*o*-Desmethylangolensin
OPG	Osteoprotegerin
ORX	Orchidectomized
OVX	Ovariectomized
PTH	Parathyroid hormone
RANKL	Receptor activator of NF-kappa B ligand
ROI	Region of interest
SD	Standard deviation
SIE	Soy isoflavone extract
Tb.Sp	Trabecular separation
TGF-β	Tumor growth factor-beta
Th.N	Trabecular number
VDR	Vitamin D receptor
vitD3	Vitamin D3
VOI	Volume of interest
WHO	World Health Organization

Key Facts

Key Facts on In Vitro Effects of Soy Phytoestrogens on Bone Cell

- Soy phytoestrogens suppress the formation of osteoclast.
- Soy phytoestrogens promote proliferation and differentiation of osteoblast.
- Osteoblastic differentiations are induced by expressions of *BMP*, *Col I*, and *OCN* genes as well as p38 MAPK-Cbfa1 and estrogen receptor-protein kinase C alpha (ER-PKCα)-related signaling pathways.
- Osteoclastic differentiation is induced by suppression of NF-kB (RANKL).

Key Facts on In Vivo Effects of Soy Phytoestrogens in Ovariectomized (OVX) Rats

- Soy phytoestrogens prevent bone loss in OVX rats which represent the condition of postmenopausal estrogen deficiency.
- Soy phytoestrogens increase femoral mass as well as both tibia and femur BMD in OVX animals.
- The activity of soy phytoestrogen is enhanced in the presence of other supplements such as vitamin, soy extract, and soy yogurt.

Key Facts on In Vivo Effects of Soy Phytoestrogens in Intact and Orchidectomized (ORX) Rats

- Soy protein without isoflavone enhances bone quality in ORX rats.
- Soy isoflavones show effects on Tb.Sp, trabecular number, and BV/TV in ORX rats.
- Soy phytoestrogens exhibit positive effects on bone health in in utero and intact rats.
- Soy phytoestrogens show no effects on bone heath of intact rats as well as on the lactation period in female rats.

Key Facts on Effects of Soy Phytoestrogens in Postmenopausal Women

- Soy phytoestrogens exhibit conflicting results in human clinical studies.
- No effects on bone loss and bone turnover.
- Increases bone formation and reduces bone resorption in few studies.
- Shows positive effects on BMD in few studies.
- No effects on bone marker level on bone biomarkers such as bone-specific alkaline phosphatase (BAP) and N-telopeptide of type 1 collagen (NTx)/creatinine.

Key Facts on Effects of Ipriflavone, a Synthetic Isoflavone in In Vitro, In Vivo, and Human Studies

- IP is derived from soy isoflavone daidzein.
- IP increases bone formation and inhibits bone resorption in animal and human bone cells.
- IP maintains bone mineral content, restores bone mass, and increases bone or bone marrow percentage in animal models.
- IP prevents bone loss and promotes bone formation in postmenopausal women.

Introduction

Osteoporosis is defined as a condition of low mineral density resulting in fragile bones with increased risk of fracture (Bernabei et al. 2014). The World Health Organization (WHO 1994) defines osteoporosis as a bone mineral density less than 2.5 standard deviations (SD) below the standard reference for maximal bone mineral density of a young adult female. Women are more prone to develop osteoporosis as compared to men due to the decrease in estrogen level after menopause leading to the decline in bone formation and increase in bone resorption activity (Roush 2011). However, male osteoporosis is becoming an increasingly important public health problem (Gielen et al. 2011). One in three osteoporotic fractures occurs in men from age 50 onward and fracture-related morbidity and mortality are even higher than in women (Gielen et al. 2011). Hormone replacement therapy (HRT) is widely used in the prevention and treatment of osteoporosis. However, HRT has considerable side effects, such as increased risks of breast cancer, uterine cancer, and thromboembolism (Ferguson 2004). According to Women's Health Initiative studies, participants on HRT had slightly higher rates of breast cancer, ovarian cancer, heart attack, stroke, thromboembolism, and Alzheimer's disease compared to nonusers (Rossouw et al. 2002; Chlebowski et al. 2003; Shumaker et al. 2003). The problems associated with HRT lead to the development of alternative therapeutics in the management of osteoporosis incorporating phytoestrogens (Brink et al. 2008).

Phytoestrogens are polyphenolic compounds that structurally and functionally mimic the endogenous estrogen, 17β-estradiol (E2), which are broadly classified into three main groups, isoflavones, lignans, and coumestans (Dixon 2004). Soybean (*Glycine max*, Fabaceae) food contains macronutrients such as lipids, carbohydrates, and proteins and micronutrients such as isoflavones, phytate, saponins, phytosterol, vitamins, and minerals (Cederroth and Nef 2009). Soybeans are rich in isoflavones and have been widely used as a dietary source of phytoestrogens in animal and human studies (Cederroth and Nef 2009). The metabolism of isoflavones is complex. Two major isoflavones present in soybeans as β-D-glycosides, namely, genistin and daidzin (Fig. 1), are biologically inactive (Setchell 1998). Once ingested, these glycosides are hydrolyzed in the intestinal tract by bacterial β-glucosidases forming the corresponding bioactive aglycones, genistein, and daidzein, which are absorbed into the bloodstream. Daidzein can be further metabolized in the digestive tract to dihydrodaidzein, equol and *o*-desmethylangolensin (O-DMA), and genistein to *p*-ethyl phenol (Setchell 1998). Isoflavones in soybeans are tightly bound to proteins, which explains the variability of phytoestrogen contents in different soy products and therefore their availability for absorption in the digestive tract. Bhathena and Velasquez (2002) reported the soy protein contents in different soy products as follows: 0.1–5 mg isoflavones/g of soy protein in mature and roasted soybeans, 0.3 mg/g soy protein in green soybeans and tempeh, and 0.1–2 mg/g soy protein in tofu and selected soy milk preparations.

Genistein, daidzein, equol, and O-DMA are the major isoflavones detected in blood and urine of humans and animals (Setchell 1998). In rodents, equol is the

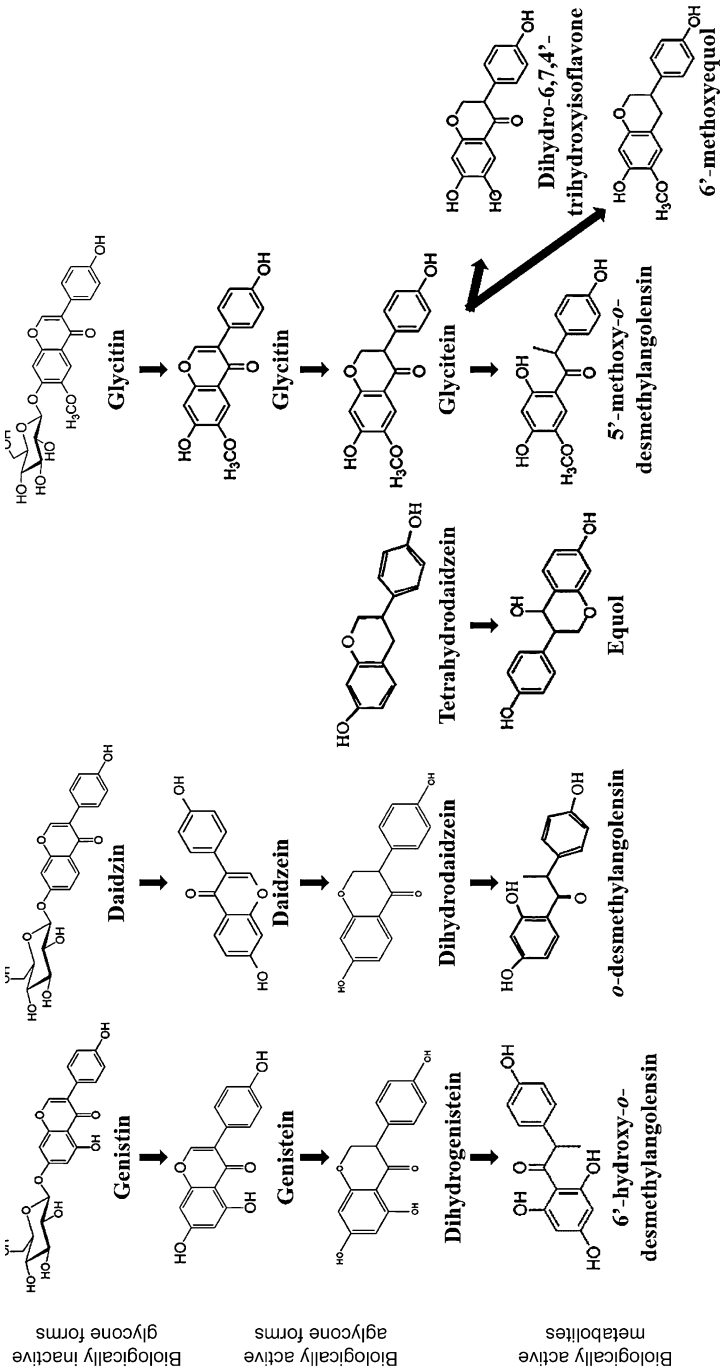

Fig. 1 Soy phytoestrogens and metabolism. The above diagram shows active forms of soy phytoestrogens and their metabolic derivatives

major circulating metabolite representing up to 70–90% of all circulating isoflavones. While all rodents are equol producers, only 30% of humans are able to metabolize daidzein into equol (Atkinson et al. 2005). Pharmacokinetic studies confirm that healthy adults absorb isoflavones rapidly and efficiently (Setchell et al. 2001). The average time for the aglycones in phytoestrogen-rich food to reach plasma concentrations after ingestion is 4–7 h. Hydrolysis of glycosidic moiety of β-glycosides in phytoestrogen-rich food is a rate-limiting step for absorption since it can delay absorption of aglycones to 8–11 h (Setchell et al. 2001).

This review aims to present a brief summary of the role of soy phytoestrogen and its synthetic derivative, ipriflavone (IP), on biomarkers of osteoporosis primarily based on studies using murine and human bone cells, experimental animal models, and human studies (Fig. 2).

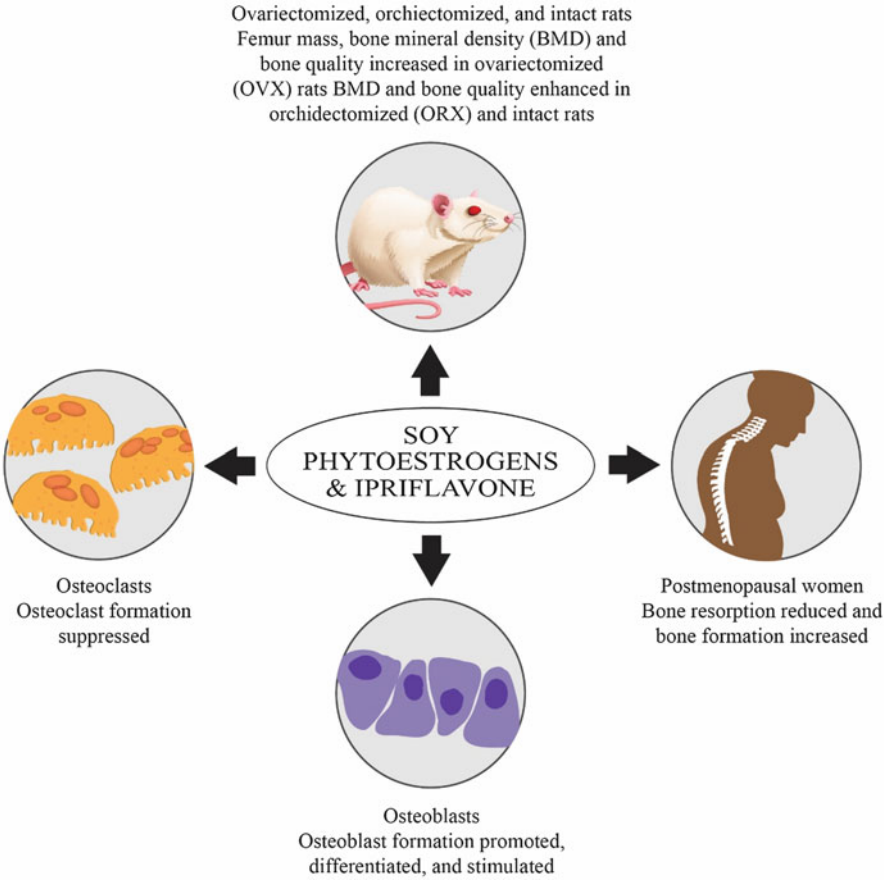

Fig. 2 Effects of soy phytoestrogens and ipriflavone on biomarkers of osteoporosis in experimental models and in humans. The above figure is a summary of the effects of soy phytoestrogens and ipriflavone on bone metabolism and biomarkers of osteoporosis using cultured murine or human bone cells, animals (intact, ovariectomized, orchidectomized), and humans (postmenopausal women)

In Vitro Effects of Soy Phytoestrogens on Bone Cell Metabolism and Biomarkers of Osteoporosis

Bone remodeling is defined as the removal of mineralized bone by osteoclasts followed by the formation of bone matrix by osteoblasts, which subsequently becomes mineralized (Hadjidakis and Androulakis 2006). The remodeling cycle consists of three consecutive phases: resorption, reversal, and formation. During resorption, partially differentiated mononuclear preosteoclasts migrate to the bone surface where they form multinucleated osteoclasts. After completion of osteoclastic resorption, in the reversal phase, mononuclear cells provide signals for osteoblast differentiation and migration and prepare the bone surface for new osteoblasts to begin bone formation. In the formation phase, osteoblasts lay down new bone completely replacing the resorbed bone (Hadjidakis and Androulakis 2006). At the end of the remodeling cycle, the bone surface is covered with flat lining cells and rests for a period of time before the next remodeling cycle (Hadjidakis and Androulakis 2006). Table 1 summarizes the effects of soy phytoestrogens on in vitro murine and human osteoblasts, osteoblast-like cells, osteoclasts, and bone marrow stromal osteoprogenitor cells (BMSCs). In general, the regulation of bone remodeling is both systemic and local. The major systemic regulators include parathyroid hormone (PTH), calcitriol, and other hormones such as growth hormone, glucocorticoids, thyroid hormones, and sex hormones. A large number of cytokines and growth factors that affect the bone cell function are attributed to the local regulation of bone remodeling (Hadjidakis and Androulakis 2006). Examples of growth actors are insulin-like growth factor (IGFs), prostaglandins, tumor growth factor-beta (TGF-β), and bone morphogenetic protein (BMP). Furthermore, through the receptor activator of NF-kappa B ligand/osteoprotegerin (RANKL/OPG) system, the processes of bone resorption and formation are tightly coupled, thus maintaining the skeletal integrity through the bone formation followed by each cycle of bone resorption (Hadjidakis and Androulakis 2006). Besides effects on systemic and local regulators, Table 1 also summarizes the effects of soy phytoestrogens through estrogen receptor (ER) and non-estrogen receptor (non-ER) pathways on bone remodeling. Studies by Chen and Wong 2006, Liao et al. 2014, Strong et al. 2014, and Wang et al. 2014 reported that osteoblastic differentiation in corresponding human and murine bone cells is mediated through the ER pathways. Human osteoblasts express both estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), although the expression of ER subtypes varies during differentiation (Onoe et al. 1997; Bodine et al. 1998). The greatly increased expression of ERβ during bone mineralization (Arts et al. 1997) is particularly pertinent to the potential hormonal effects of phytoestrogens since compounds such as genistein show a much higher affinity for ERβ than for ERα (Kuiper et al. 1998; Morito et al. 2001). Osteoblastic differentiation is also mediated through mitogen-activated protein kinase-core binding factor 1 (MAPK-Cbfa1) and other non-ER pathways (Liao et al. 2007; Strong et al. 2014). Osteoblastic differentiation and inhibition of osteoclastic formation are modulated by prevention of nuclear factor kappa B (NF-kB) translocation (Lee et al. 2014).

Table 1 In vitro effects of soy phytoestrogens on bone cell metabolism and biomarkers of osteoporosis

Author, year	Bone cells	Phytoestrogen treatment	Significant outcomes
Suh et al. (2003)	Clonal murine MC3T3-E1 osteoblastic cells	Genistein (10^{-5} M) Daidzein (10^{-5} M)	Antiresorptive activity in the presence of TNF-α in both treatments
Chen and Wong (2006)	Human osteoblastic-like SaOS-2 cells	Genistein (10^{-8} M–10^{-6} M)	Stimulation of ER-dependent ALP activities Increase of OPG protein expression Suppression of expression of NF-kB (RANKL) Modulation of osteoclastogenesis
Ge et al. (2006)	MC3T3-E1, mouse calvaria osteoblast-like cell line	Daidzein (10^{-9} M–10^{-5} M)	Differentiation and mineralization, promotion of bone formation in early and late growing osteoblasts
Liao et al. (2007)	Mouse bone marrow-derived mesenchymal stem cell (BMSC)	Genistein (0.01–1 μmol/L)	Stimulation of osteoblastic differentiation through p38 MAPK-Cbfa1 pathway
Lee et al. (2014)	Murine macrophage RAW 264.7 cells	Genistein (1–20 μM)	Inhibition of osteoclast formation of receptor activator of RANKL-induced cells by preventing the translocation of NF-kB
Liao et al. (2014)	Mouse MC3T3-E1, primary osteoblasts	Genistein (0–100 μM)	Induction of ERα gene expression via activation of MAPK/ NF-kB/activator protein 1 (AP-1) Stimulation of osteoblast differentiation and maturation through ERα-dependent expressions of the *BMP-6*, *Col I*, and *OCN* genes
Strong et al. (2014)	Human BMSCs	Daidzein analog 2c (1 μM), 2 g (1 μM), or 2 l (1 μM)	Increase in calcium deposition Activation of distinct ER and non-ER pathways resulting in differentiation of BMSCs and adipose-derived stromal/stem cells (ASCs)
Tadaishi et al. (2014)	Mouse macrophage pre-osteoclast RAW 264 cells	Genistein (10 μM) or equol (10 μM) combined with β-carotene (0.1 μM)	Enhancement of suppressive effect on osteoclast formation

(*continued*)

Table 1 (continued)

Author, year	Bone cells	Phytoestrogen treatment	Significant outcomes
Wang et al. (2014)	Rat neonatal calvaria osteoblast	Equol (0.01–1 mM)	Promotion of proliferation and differentiation of osteoblasts through activating ER-PKCα-related signaling pathway

The above table is a summary of in vitro studies examining the effects of soy phytoestrogens on cultured human or murine bone cell metabolism and biomarkers of osteoporosis

In Vivo Effects of Soy Phytoestrogens on Bone Metabolism and Biomarkers in Ovariectomized (OVX) Rats

In vitro studies provide insight into the effects of soy phytoestrogens on individual cells, whereas in vivo studies provide the advantage of using intact systems that take into account coupling effects among osteoblasts, osteoclasts, their progenitor cells, and the effects of metabolic activities that influence the efficacy of a compound (Setchell and Lydeking-Olsen 2003). Table 2 summarizes the effects of soy phytoestrogens in ovariectomized (OVX) rats. Due to acute deficiency of ovarian estrogen that leads to loss of bone mass, the OVX rat represents a good postmenopausal osteoporosis model (Setchell and Lydeking-Olsen 2003). In rats, ovariectomy leads to a selective reduction in the number of vitamin D receptors (VDR) in jejunum (Chan et al. 1984). This reduction in VDR results in lower responsiveness of intestinal cells to vitamin D signaling and lower calcium absorption by the intestine. This leads to reduction in bioavailability of dietary calcium, an essential building block for new bone formation. Arjmandji et al. (1996) reported that soy protein isolate was as effective as estradiol in retarding bone loss following OVX in rat model. Devareddy et al. (2006) observed that treatment of OVX rats with soy isoflavones did not increase the tibial bone mineral density (BMD) up to the level of sham despite a small percentage (4.5%) of increase in BMD as compared to the OVX controls. The soy isoflavone treatments also did not show any beneficial effects on lumbar microarchitectural properties in OVX rats (Devareddy et al. 2006). Om and Shim (2007) and Rachon et al. (2007) reported the positive effects of purified soy phytoestrogens daidzein and equol on OVX rats. Daidzein increased the femoral mass in cadmium-induced OVX rats (Om and Shim 2007) and equol attenuated trabecular bone loss and increased the density of lumbar spine in OVX rats (Rachon et al. 2007). Other studies (Shiguemoto et al. 2007; Jeon et al. 2009; Zhang et al. 2009; Chang et al. 2013) reported that diet supplemented with isoflavone and vitamin D resulted in high BMD and alkaline phosphatase (ALP) activity and maintained the proper bone microarchitecture indicating the bone-sparing effects of soy phytoestrogens on OVX rats.

Table 2 In vivo effects of soy phytoestrogens on bone metabolism and biomarkers in ovariectomized (OVX) rats

Author, year	Rat strain	Phytoestrogen treatment	Significant outcomes
Devareddy et al. (2006)	Female Sprague–Dawley rats Age: 9 months N = 78	Sham (N = 13) versus OVX (N = 12–13) Five treatment groups [OVX (control): E2, 10 μg/kg body wt; soy protein without added isoflavones, 0.06 mg isoflavones/g protein; soy protein with normal isoflavones, 3.55 mg isoflavones/g protein; soy protein with enriched isoflavones, 7.10 mg isoflavones/g protein]	Soy proteins unable to restore bone loss Isoflavones in higher doses reversed loss of tibial microstructural properties
Om and Shim (2007)	Female Wistar rats Age: 4 weeks N = 45	Sham (N = 9) versus OVX (N = 9) Four treatment groups (experimental diet: $CaCl_2$, 50 ppm; $CaCl_2$, 50 ppm + daidzein, 10 μg per kg of body wt.; $CaCl_2$, 50 ppm + estradiol, 10 μg per kg of body wt.)	Daidzein increased femoral mass and inhibited fast bone turnover in Cd-exposed OVX rats
Rachon et al. (2007)	Female Sprague–Dawley rats Age: 3 months N = 28	OVX (N = 4–5) Three treatment groups (control group, soy-free diet only, N = 8; E2B group, soy-free diet + E2B, N = 10; equol group, soy-free diet + equol, N = 10)	E2B lowered OVX-induced BMD loss at proximal tibia Equol showed no effect Equol and E2B attenuated trabecular bone loss and increased density of lumbar spine
Shiguemoto et al. (2007)	Female Wistar rats Age: 13 weeks N = 56	Sham (N = 7) versus OVX (N = 21) Three treatment groups for each sham and OVX [soy yogurt (aqueous soy extract + 1% lactose + 2.5% nonfat dry milk + 0.7% soy oil + 7% sucrose + 0.3% gelatin + 0.2% stabilizer/emulsifier Recodan®) + sedentary; resistive exercise; soy yogurt + resistive exercise)	Isoflavone-supplemented soy yogurt increased tibia and femur BMD and activity of serum alkaline phosphatase in all treated groups
Jeon et al. (2009)	Female Sprague–Dawley rats Age: 6 weeks N = 30	Sham versus OVX Three treatment groups (non-isoflavone-enriched milk; isoflavone-enriched milk; isoflavone- and Ca-enriched milk + vitamin D and K)	Isoflavone-enriched milk showed partial preventive effect on bone loss Addition of vitamin D and K and Ca increased bone mass

(*continued*)

Table 2 (continued)

Author, year	Rat strain	Phytoestrogen treatment	Significant outcomes
Zhang et al. (2009)	Female C57BL/6 J mice Age: 12 weeks N = 56	Sham (N = 10) versus OVX Four treatment groups [control diet, N = 10; control diet + orally administered E2, 2 mg/kg, N = 12; genistein, 500 mg/kg diet, N = 12; Novasoy (40% 1.3:1:0.3 genistein/daidzein/glycitein + 7–12% protein + 4% ash + 6% moisture + 41% natural soy phytocomponents), 2500 mg/kg diet, N = 12]	Soy extract with genistein more effective than purified genistein in improving tibial trabecular bone quality
Chang et al. (2013)	Female Sprague–Dawley rats Age: 3 months N = 48	Sham (N = 8) versus OVX (N = 40) Four treatment groups [E2; vitD3; soy isoflavone extract (SIE); SIE + vitD3]	Soy isoflavone prevented bone loss Combination of isoflavone with vitD3 increased osterix expression and preosteoblast proliferation

The above table is a summary of in vivo studies examining the effects of soy phytoestrogens on bone metabolism and biomarkers of osteoporosis in OVX rats

In Vivo Effects of Soy Phytoestrogens on Bone Metabolism in Intact and Orchidectomized (ORX) Rats

Osteoporosis poses a great challenge to the aging population in the USA, and though largely manifested in women, men also exhibit risk factors of this degenerative condition (Khosla 2010). One-third of hip fractures and one-half of symptomatic vertebral fractures are reported in men (Johnell and Kanis 2006). One of the causes for male osteoporosis is hypogonadism with aging (Becker 2008; Szulc et al. 2001; Khalil et al. 2005; Soung et al. 2006). Table 3 summarizes the effects of soy phytoestrogens on orchidectomized (ORX) and intact rat models. Few studies (Khalil et al. 2005; Soung et al. 2006; Juma et al. 2012) have focused on the effects of soy phytoestrogens on ORX rats, and even fewer studies (James et al. 2002) have concentrated on the effects on soy phytoestrogens on young and peripubertal rats.

James et al. (2002) aimed to compare calcium metabolism and bone mineralization in young female rats after feeding them casein versus isoflavone-rich diets. Results indicated that compared to soy protein, casein either alone or with the addition of isoflavones showed positive effects on growth and bone mineralization in the peripubertal period when the growth rate was at its maximum. Results also indicated that calcium metabolism was higher in casein with isoflavone-treated rats compared to soy protein. Studies on intact rats yielded mixed results detailed in Table 2. For example, a pilot study by Peterson et al. (2009) demonstrated that bone formation in female Wistar rats during lactation was not effected by soy isoflavone

Table 3 In vivo effects of soy phytoestrogens on bone metabolism in intact and orchidectomized (ORX) rats

Author, year	Rat strain	Phytoestrogen treatment	Significant outcomes
James et al. (2002)	Female Sprague–Dawley rats Age: 3 weeks N = 25	N = 3 Three treatment groups [soy protein (12 %); casein; casein + isoflavone (0.046 %)]	No difference in growth and bone mineralization between casein and casein + isoflavone groups Calcium metabolism high in casein + isoflavone group than soy protein
Khalil et al. (2005)	Male Fisher 344 rats Age: 13 months N = 72	Sham (N = 12) versus ORX (N = 12) Five treatment groups (only AIN-93 M casein-based diet; casein-based diet + 600 mg/kg of isoflavones; casein-based diet +1200 mg/kg isoflavones; soy protein-based diet + 600 mg/kg isoflavones; soy protein-based diet +1200 mg/kg isoflavones)	Casein-based diet supplemented with isoflavones Decreased loss of whole body BMD but not significantly as compared to controls Induced higher bone volume and trabecular number Decreased Tb.Sp
Nakai et al. (2005)	Sprague–Dawley female rats Age: 3 months N = 50	Control group – 200 g casein Four treatment groups (low soy, 100 g soy protein; high soy, 200 g soy protein; low isoflavone extract, 17.2 g; high isoflavone extract, 34.4 g)	Soy isoflavones and soy protein had no effects on femur and lumbar BMD
Soung et al. (2006)	Male Fisher rats Age: 13 months N = 72	Sham (N = 12) versus ORX (N = 12) Five treatment groups (AIN-93 M casein-based control diet; 600 mg/kg of isoflavones; 1200 mg/kg of isoflavones; soy + 600 mg/kg of isoflavones; soy + 1200 mg/kg of isoflavones)	Soy protein diet supplemented with isoflavones reduced ORX-induced decrease of BV/TV and Th.N and increased Tb.Sp at femoral neck site
Ward and Piekarz (2007)	Female and male CD-1 mice Age = 4 months N = 12/group/age	Control (0.4 ml corn oil) Three treatment groups [genistein (3.75 mg) in 0.4 ml corn oil; daidzein (3.75 mg) in 0.4 ml corn oil; genistein + daidzein (3.75 mg each) in 0.4 ml corn oil from day 9 to day 21 of pregnancy through subcutaneous injection]	In utero isoflavone exposure shows no effect on bone health No significant effect on femur peak load

(*continued*)

Table 3 (continued)

Author, year	Rat strain	Phytoestrogen treatment	Significant outcomes
Peterson et al. (2009)	Female Wistar rats Age: 100 day N = 48	Control group – 0 mg aglycone isoflavones/g dietary protein Three treatment groups (2 mg aglycone isoflavones/g dietary protein; 4 mg aglycone isoflavones/g dietary protein; 8 mg aglycone isoflavones/g dietary protein)	Soy isoflavones showed no effects on bone metabolism during lactation
Gautam et al. (2011)	Female Sprague–Dawley rats Age = not reported N = not reported	Two treatment groups [methoxylated daidzein (cladrin), 10 mg/kg/ day dose; formononetin, 10 mg/ kg/ day dose, oral administration for 30 consecutive days]	Cladrin showed increase in MAR and bone formation rates Formononetin showed no effect
Juma et al. (2012)	Male Sprague–Dawley rats Age: 95 days N = 40	Sham (N = 10) versus ORX (N = 10) Three treatment groups (AIN-93 M casein-based diet; soy protein + isoflavone; soy protein without isoflavone)	Regardless of isoflavone content, soy protein was unable to prevent ORX-induced femoral decrease in bone density and mineral content Isoflavone enhanced bone quality by increasing yield force

The above table is a summary of in vivo studies examining the effects of soy phytoestrogens on bone metabolism and biomarkers of osteoporosis in intact and ORX rats

consumption. Another study by Nakai et al. (2005) showed that soy protein and isoflavones have no effects on femur and lumbar BMD of intact Sprague–Dawley female rats. The study by Ward and Piekarz (2007) indicated that genistein, daidzein, or their combinations have no effect in utero and femur peak load. The positive effects of cladrin were reported on female Sprague–Dawley rats (Gautam et al. 2011). Cladrin increased the mineral apposition (MAR) and bone formation rates compared to controls, whereas formononetin showed no effect on bone formation in vivo (Gautam et al. 2011).

Effects of Soy Phytoestrogens on Biomarkers of Osteoporosis in Postmenopausal Women

Postmenopausal estrogen deficiency results in increased bone resorption, which is the major contributing factor of osteoporosis (Leboime et al. 2010). Bisphosphonates, HRT, and other antiresorptive treatments are available for the

treatment and even prevention of postmenopausal osteoporosis. However, HRT has been associated with health problems such as coronary heart disease, pulmonary embolism, and stroke (Rossouw et al. 2002), whereas the use of bisphosphonate can lead to osteonecrosis of the jaw and atypical fractures (Arrain and Masud 2008). These adverse side effects have led to the identification and use of complementary and alternative treatments, which are considered safer and effective (Barnes et al. 2008). Phytoestrogens, especially isoflavones, have been used as dietary alternatives to HRT and Food and Drug Administration (FDA)-approved drugs (alendronate, risedronate, ibandronate, zoledronic acid, raloxifene, denosumab) (Pawlowski et al. 2015). Soybean isoflavones, components of dietary supplements, are genistein, daidzein, and glycitein. Setchell et al. (2002) reported that subjects who have gut microflora that can metabolize daidzein to equol showed greater activity to isoflavones than those who do not have the proper microflora. Table 4 summarizes the effects of soy isoflavones and soy food on postmenopausal bone loss. Results of the studies mentioned in Table 4 show variability regarding the efficacy of isoflavones in preventing postmenopausal bone loss. Some studies (Morabito et al. 2002; Chen et al. 2003; Marini et al. 2007; Pawlowski et al. 2015) show positive bone-sparing effects of phytoestrogens, whereas others (Brink et al. 2008; Alekel et al. 2010; Tai et al. 2012) show no effects on reducing bone loss. These result differences could be attributed to the differences in population under study, sample size, and study duration. In addition to human clinical studies, epidemiology also revealed the protective effects of soy phytoestrogens in women against osteoporosis (Somekawa et al. 2001; Zhang et al. 2005). Thus, while some studies are promising, further research is needed on the effects of whole soy foods, soy proteins, and purified isoflavones in larger trials to support the beneficial effect of soy phytoestrogens in osteoporosis.

Effects of Ipriflavone, a Synthetic Isoflavone on Bone Biomarkers in In Vitro, In Vivo, and Human Studies

Evaluation of phytoestrogens, mainly soy isoflavones, as candidates for bone loss treatment are also supported by results on the bone-sparing effects of ipriflavone (IP) (Brandi 1993). Ipriflavone, 7-isopropoxyisoflavone, is a synthetic isoflavone derived from daidzein in the 1930s (Sziklai et al. 1992; Head 1999) showing positive effects in the treatment and prevention of osteoporosis by suppressing bone resorption, increasing bone calcium retention and enhancing the beneficial action of estrogen on bone metabolism (Reginster 1993). Ipriflavone has been used as an alternative to HRT in the prevention of acute bone loss in postmenopausal women (Reginster 1993). Arjmandi et al. (2000) reported that IP prevents bone loss in postmenopausal women and OVX rats, and Ge et al. (2010) observed the significant effect of IP in increasing BMD, osteocalcin, and hydroxyproline contents in a dose-dependent manner in OVX rats. Ipriflavone is extensively metabolized in the liver and excreted in urine. In dogs and rats, seven metabolites were identified in the plasma. However, in humans, only MI, MII (daidzein), MIII, and MV seem to

Table 4 Effects of soy phytoestrogens on biomarkers of osteoporosis in postmenopausal women

Author, year	Study design	Subject characteristics	Phytoestrogen treatment	Significant outcomes
Morabito et al. (2002)	Randomized double-blind placebo-controlled study Duration: 1 year	Age: 47–57 years N = 90 BMD: femoral neck (<0.795 g/cm^2)	Genistein (54 mg/day) tablets versus placebo	Reduction in bone resorption Increase in bone formation
Chen et al. (2003)	Randomized double-blind, placebo-controlled trial Duration: 1 year	Age: 48–62 years N = 230 BMD: spine (0.6 %) and total hip (1.53 %)	Two treatments: [medium dose (0.5 g soy extracts + 40 mg isoflavones) capsules; high dose (1.0 g soy extracts + 80 mg isoflavones) capsules] versus placebo	Attenuation of bone marrow stromal osteoprogenitor cell (BMC) loss at trochanter, intertrochanter, and total hip
Marini et al. (2007)	Randomized, double-blind, placebo-controlled trial Duration: 1 year	Age: 49–67 years N = 389 BMD: < 0.795 g/cm^2 at the femoral neck	Genistein (54 mg) tablets versus placebo	Decreased levels of bone resorption markers and increased levels of markers of new bone formation Improved BMD and markers of bone turnover
Brink et al. (2008)	Randomized, double-blind, placebo-controlled, parallel, multicenter trial Duration: 1 year	Age: 53–56 years N = 237 BMD: exclusion < −2 *z* scores	Soy isoflavone-enriched biscuits and bars containing genistein (60-75 %), daidzein (25-35 %), and glycitein (1-5 %) versus placebo	No effects on preventing bone loss and on bone turnover
Alekel et al. (2010)	Randomized, double-blind, controlled trial Duration: 3 years	Age: 45.8–65 years N = 432 BMD: lumbar spine and/or proximal femur T scores – low (1.5 SD below young adult mean) or high (1.0 SD above mean)	Two treatments [soy isoflavones (80 and 120 mg/day)] versus placebo	No bone-sparing effects of soy isoflavones Modest effects at the femoral neck

(*continued*)

Table 4 (continued)

Author, year	Study design	Subject characteristics	Phytoestrogen treatment	Significant outcomes
Tai et al. (2012)	Randomized double-blind placebo-controlled trial Duration: 2 years	Age: 45–65 years N = 431 BMD: 1 SD below the young adult female mean value (T-score < −1)	Soy isoflavones capsules (50 mg) containing genistein (57.5 %) and daidzein (42.5 %) versus placebo	No effects on BMD in lumbar spine or total femur No changes in serum BAP and urinary NTx/creatinine
Pawlowski et al. (2015)	Randomized blinded, crossover intervention trial Duration: 4 years	Age: 50–68 years N = 24; equol producers (N = 8) and no equol producers (N = 16) BMD: not reported	Five mixed isoflavone treatments with oral supplements (tablets): [genistein low dose (52.85 mg isoflavones/day); genistein high dose (113.52 mg isoflavones/day); soy low dose (105.23 mg isoflavones/day); soy high dose (219.67 mg isoflavones/day); soy + genistein (161.07 mg isoflavone/day] versus risedronate as control	Mixed isoflavones effective as bone-preserving agent as compared to genistein-enriched isoflavones Capability of converting daidzein to equol showed no effect on suppressing bone resorption

The above table is a summary of the effects of soy phytoestrogens on biomarkers of osteoporosis in postmenopausal women

predominate. Out of these metabolites, MIII is the most potent than MII and MI and MV were least potent (Head 1999). Table 5 summarizes selected in vitro, in vivo, and human studies on the effects of ipriflavone on osteoporosis. Ipriflavone stimulated osteoblast and inhibited osteoclast formation in murine and human bone cells (Giossi et al. 1996; Yao et al. 2007; Civitelli 1997). In vivo effects of IP were observed in caged hens (Yao et al. 2007; Lv et al. 2014) where IP increased egg production while maintaining the bone mineral content and alleviated caged layer osteoporosis (CLO). Ipriflavone also increased bone formation and restored bone mass in male Japanese white rabbits and Sprague–Dawley rats, respectively (Minegishi et al. 2002; Deyhim et al. 2005). According to Zhang et al. (2010), ipriflavone exhibited positive effects in postmenopausal women by inhibiting bone resorption, whereas Alexandersen et al. (2001) and Katase et al. (2001) reported no effects of IP in postmenopausal bone loss and biochemical markers of bone metabolism.

Table 5 Effects of ipriflavone, a synthetic isoflavone on bone biomarkers in in vitro, in vivo, and human studies

Author, year	Bone cells/rat strain/ human subjects	IP treatment	Significant outcomes
Giossi et al. (1996)	Fetal rat long bone cells	IP metabolites [M1, M2, M3 (10 μM), and M5]	Inhibition of parathyroid-stimulated bone resorption
Alexandersen et al. (2001)	Human prospective, randomized, double-blind, placebo-controlled Duration: 4 years Age: 45–75 years N = 474 BMD of lumbar spine (L2–L4) below 0.86 g/cm^2	IP dose: 200 mg, three times/day (N = 234) Placebo (N = 240) All received 500 mg/day calcium	No effect on bone loss No effect on biochemical markers of bone metabolism Induction of lymphocytopenia
Katase et al. (2001)	Human, randomized, placebo-controlled Duration: 2 years Age: 45–75 years N = 89 (premenopausal bilateral OVX = 37 and menopaused or OVX for >3 years before the start of study) Early stage BMD (L2–L4) 1.138 ± 0.220 g/cm^2 Late stage BMD (L2–L4) 0.929 ± 0.077 g/cm^2	IP dose: 600 mg/day for 24 months Placebo for 24 months All received 600 mg/day calcium carbonate (approximate total of 240 mg calcium/day)	Prevention of bone loss compared to placebo No effect on acute bone loss in early stage following OVX
Minegishi et al. (2002)	Male Japanese white rabbits Age: not reported N = 5	IP in a collagen gel versus collagen gel alone	Bone formation increased at an early stage
Deyhim et al. (2005)	Sprague–Dawley rats Age: 90 day N = 72	Two treatment groups OVX + IP (100 mg[sol]/kg body wt./day); OVX + E2 (10 μg[sol]/kg body wt./day) OVX control	Increase in expression of IGF-I in the femur Restoration of bone mass
Civitelli (1997)	Human BMC and trabecular bone osteoblasts (HOB)	IP and its metabolites MI (10^{-6} M), MII, MIII (10^{-5} M), and MV	Bone sialoprotein, decorin, and type I collagen expressions stimulated Bone mineralized matrix deposited

(*continued*)

Table 5 (continued)

Author, year	Bone cells/rat strain/ human subjects	IP treatment	Significant outcomes
Yao et al. (2007)	Embryonic chick calvariae osteoblasts Chick tibias and humeri osteoclasts	IP (10^{-4} M–10^{-10} M)	Stimulation of osteoblasts Inhibition of osteoclasts
Yao et al. (2007)	Caged hens Age: 58 weeks N = 500	Five treatment groups (100 hens/group): 15, 25, 50, and 100 ppm IP Control group-base layer diet	Improvement and increase in egg production while maintaining bone mineral content
Zhang et al. (2010)	Human, randomized, and double-blind Duration: 3 years Age: 45–75 years N = 60 BMD of lumbar vertebrae (L1–L4) below 1 SD in same age group	Two treatment groups [1000 mg/day compound calcium acid chelate + 1 tablet/d vit AD guttate; 1000 mg/d compound calcium acid chelate + 1 tablet/day vit AD guttate + 200 mg (3 times)/day IP] Placebo 1000 mg/d calcium acid chelate	Inhibition of bone resorption Promotion of bone formation
Lv et al. (2014)	Hy-Line Brown laying hens Age: 24 weeks N = 200	Three treatment groups [low-calcium diet CaL; low-calcium diet CaL + 8 mg/kg IP; low-calcium diet CaL + 20 mg/kg IP Control standard diet CaN	Increased in trabecular bone area and bone quality Alleviation of CLO

The above table is a summary of the effects of ipriflavone, a synthetic isoflavone on bone biomarkers in in vitro, in vivo, and human studies

Potential Applications to Prognosis, Other Diseases, or Conditions

As the human population ages, osteoporotic fractures are increasingly recognized as a common and serious health problem that significantly compromise quality of life in elderly people. Osteoporosis and its consequence of fragility fractures are characterized by highly complex phenotypes, which include BMD, bone strength, bone turnover markers, and nonskeletal traits, as reviewed earlier in this chapter. Thus, the early identification of bone biomarkers that are associated with osteoporosis phenotypes or response to therapy can eventually help individualize the prognosis, treatment, and prevention of fractures and their adverse outcomes. Bone density assessment has been identified as a clinically useful and

cost-effective tool in the prognosis and treatment of osteoporosis in older adults (Schousboe et al. 2005, 2007). Although BMD is well established as a predictor of future fracture risk and several prospective studies have demonstrated a 1.5- to 2.5-fold increased risk of fracture for every 1 SD decrease in BMD, this biomarker alone displays poor sensitivity in predicting future fractures. Thus, fracture risk assessment scores are better used in the prognosis of osteoporosis. One such example is the WHO Fracture Risk Assessment Tool (FRAX®), which combines age and sex with clinical risk factors to provide an estimate of the 5- or 10-year probability of fracture for an individual (Kanis et al. 2008). A clear advantage of fracture prediction tools is that they provide an estimate of absolute risk, in that if a 55-year-old woman has osteoporosis according to dual-energy X-ray absorptiometry (DXA), for example, she can still have a low 10-year risk of fracture that might not indicate the need for pharmacological treatment. The estrogenic effects of isoflavones have led researchers to view soy foods and isoflavone supplements as alternatives to conventional hormone therapy. However, as described earlier in this chapter, the evidence that isoflavones reduce bone loss in postmenopausal women is quite conflicting and can be largely explained by the heterogeneity in the study sample and dosing and overall limited clinical studies in this area. Based on the anabolic effects of soy phytoestrogens on bone formation in preclinical animal models of osteoporosis, the inclusion of whole soy foods and beverages may be considered a positive health choice in the older population. Further studies must identify the effects of soy phytoestrogens on novel bone biomarkers, such as those related to genomics, epigenomics, and metabolomics, as well as composite fracture risk scores in the prognosis and management of osteoporosis.

Summary Points

- Soy phytoestrogens promote osteoblastic differentiation through the expressions of biomarkers such as: *BMP* (participates in matrix differentiation and bone formation), collagen type 1(*Col I*) (stimulates osteoblast adhesion and differentiation), and osteocalcin (*OCN*) genes (control osteoblast function).
- Soy phytoestrogens suppress osteoclast formation by inhibiting translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (transcription factor and essential receptor activator for RANKL-induced osteoclast formation) to the nucleus.
- In postmenopausal (OVX) rat models, soy phytoestrogens increase femoral mass and BMD in the tibia and femur.
- In male osteoporosis represented by ORX rats, soy isoflavones show effects on trabecular separation (Tb.Sp), trabecular number, and trabecular bone volume (BV/TV).
- Soy isoflavones increase bone formation, reduce bone resorption, and exhibit positive effects in studies in postmenopausal women, whereas in few studies soy

isoflavones show no effects on bone biomarkers such as bone-specific alkaline phosphatase (BAP) and N-telopeptide of type 1 collagen (NTx)/creatinine.
- Ipriflavone, the synthetic isoflavone, shows positive effects in cultured bone cells, animals, and postmenopausal women.

Definitions of Words and Terms

Alkaline phosphatase (ALP)	An enzyme that hydrolyzes phosphate esters and liberates inorganic phosphate with an optimal pH of about 10.0 serum ALP activity increases in bone diseases such as bone cancer, hyperparathyroidism, and osteitis deformans
Bone marrow-derived mesenchymal stem cell (BMSC)	Postnatal stem/progenitor cells capable of self-renewing and differentiating into osteoblasts, chondrocytes, adipocytes, and neural cells
Bone mineral density (BMD)	Measurement of calcium in the bone which indicates the strength of bone
Bone morphogenetic protein (BMP)	30–38-kD homodimeric family of protein involved in the formation of bone and cartilage and provides morphogenetic signals guiding normal tissue architecture
Lymphocytopenia	A condition with abnormally low levels of blood lymphocytes
Orchidectomized rat	Male rats with one or both testicles removed
Ovariectomized rat	Female rats with one or both ovaries removed
Peripubertal	Early stages of puberty
Postmenopausal	Time period after which a woman undergoes a lack of menstruation for twelve consecutive months
Trabecular bone volume (BV/TV)	The fraction of a given volume of interest (VOI) occupied by mineralized bone. It is reported as a % value and is also used to evaluate a bone volume density following a given treatment
Trabecular number (Th.N)	Quantification of relative number of individual trabeculae within 3-D region of interest (ROI). It is also one of the bone microstructural indices
Trabecular separation (Tb.Sp)	Quantification of relative spacing between individual trabeculae within 3-D ROI. It is one of the bone microstructural indices

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Quality of Life as a Biomarker in Hip Fractures

50

Thoralf Randolph Liebs

Contents

Abstract

Fractures of the hip (proximal femur) are especially common in the elderly. In this age group, they represent the leading cause of injury-related mortality and pose a great economic burden on the healthcare systems.

About all of the fractures of the proximal femur require surgery. Up to this time, there is no agreement on which type of surgery should be preferred and which implants should be used. In order to develop the best treatment plan for an individual patient, treatment results have to be assessed in a comparable way. Therefore, valid and reliable biomarkers as outcome measures are needed.

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Given the high mortality after a fracture around the hip in the elderly, most commonly the mortality rates after the injury are reported. However, patients who survived the fracture commonly have significant reductions in the health-related quality of life (HRQoL). Therefore, assessing the success of the treatment solely by the survival of patients could be misleading. For this reason, it appears advantageous to report both the mortality and, if the patient has survived, the HRQoL.

In this chapter, several biomarkers as outcome measures after hip fracture are listed. In addition, efforts by several research groups are described in order to standardize the use of biomarkers after hip fractures.

Keywords
Hip fractures • Surgery • Mortality • Health-related quality of life • Registry

List of Abbreviations

ADL	Activities of daily living
CEA	Cost-effective analyses
EQ-5D	EuroQol 5 dimensions
HRQoL	Health-related quality of life
PROM	Patient-reported outcome measure
QALY	Quality-adjusted life year
SF-12	Short form 12
SF-36	Short form 36

Key Facts of Hip Fractures

- In the elderly, hip fractures are reported to be the leading cause of injury-related mortality.
- The annual incidence of hip fractures is 620,000 in the European Union, thereby posing a great economic burden on the healthcare systems.
- Most hip fractures in the elderly require surgery, but there is no agreement on which type of surgery, such as total hip arthroplasty, hemiarthroplasty, or osteosynthesis, should be preferred, which implants should be used, and when the surgery should be performed.
- Since registries have been reported to be among the most cost-effective interventions in medicine, some countries have initiated national hip fracture registries in order to improve treatment results.

Key Facts of Quality of Life as a Biomarker of Disease

- Traditionally, the main biomarkers for measuring outcomes in patients with hip fractures were mortality and the need for revision surgery.

- In the surviving patients, recovery can be measured by health-related quality of life outcome measures.
- There are several outcome measures available. These measures are, e.g., pain, physical function, mental health, physical role, general health, vitality, social function, emotional function, stiffness, depression, and self-care.
- Most of the outcomes measuring health-related quality of life can be assessed by questionnaires that are self-administered by the patient.
- Ideally, outcome measures are short, reliable, validated for the disease, and easy to administer.
- Some outcome measures allow the calculation of quality-adjusted life years.

Definitions of Words and Terms

Health-related quality of life	Traditionally health has been measured using measures of morbidity or mortality. Due to improved possibilities for the treatments of diseases and therefore a delay in mortality, it became necessary to measure health not only in terms of survival but also in terms of improving the quality of life. Quality of life is a broad multidimensional concept that includes subjective evaluations of both positive and negative aspects of life (1998). Since there are domains that could affect quality of life as well, such as housing, jobs, or schools, the concept of health-related quality of life (HRQoL) has been developed, to encompass those aspects of overall quality of life that can be clearly shown to affect health.
Hip fracture	Hip fractures (also called proximal femoral fractures) can be subdivided into intracapsular (also called femoral neck and subcapital) fractures and extracapsular (also called intertrochanteric, per-trochanteric, and subtrochanteric) fractures.
Patient-reported outcome measures	Patient-reported outcome measures (PROMs) are outcome measures, in which the responses are collected directly from the patient. The questionnaires may be disease specific (e.g., specific to osteoarthritis of the hip) or non-disease specific (e.g., covering osteoarthritis, asthma, and

	neurological diseases). The following aspects are commonly collected by PROMs: health-related quality of life (HRQoL), physical function, pain, symptoms, impairments, health status, and health perceptions. PROMs have the advantages that they are easy to administer (e.g., by mail) and that there is no observer bias.
Registry	In an implant registry, patient information is linked to information on the implant used. The registry collects, processes, and analyzes this information; calculates the revision rates; puts the calculated revision rates in a national perspective; and returns this information back to the hospital and the surgeon, who has performed the surgery. The first joint replacement registries were founded in Sweden in the 1970s. After the implementation of these registries, the probability of revision was cut in half (Herberts and Malchau 1997, 2000). Given the success of these national joint registries, several countries have set up joint replacement registries as well, and some countries have even set up registries for specific diseases, such as cancer registries or hip fracture registries.
Surgery for hip fracture	In the past, treatment of hip fracture was mainly nonoperative with several weeks of bed rest. In the last decades, the development of implants and surgical methods has allowed the surgeon to stabilize all these fractures in a way that the patients can be mobilized immediately after surgery. Surgical treatment can consist of a reduction of the fracture and osteosynthesis, which is a stabilization of the fractured bone with screws, plates, or intramedullary nails. As an alternative, a total hip arthroplasty can be performed, in which a femoral component, head component, and acetabular component are used, or a hemiarthroplasty, in which only a femoral component and a head component are used. In the latter, the artificial head articulates with the acetabulum.

Introduction

Fractures around the hip can occur at any age but are especially common in the elderly, in which these fractures are reported to be the leading cause of injury-related mortality (Panula et al. 2011). The annual incidence rate of hip fracture has been reported to be 2.24/1000 person-years in women and 1.29/1000 person-years in men (Adams et al. 2013), resulting in an annual incidence of 620,000 hip fractures in the European Union (Svedbom et al. 2013).

Fractures of the hip usually involve fractures of the femoral neck, intertrochanteric fractures, or subtrochanteric fractures. About all of these fractures require surgery (Handoll and Parker 2008; Uzoigwe et al. 2013) and pose a great economic burden on the healthcare systems. Unfortunately, there is no agreement on which type of surgery, such as total hip arthroplasty, hemiarthroplasty, or osteosynthesis, should be preferred and which implants should be used (Gjertsen et al. 2008).

In the elderly, fractures around the hip are usually associated with osteoporosis. However, in younger patients these hip fractures are associated with significant trauma, such as motor vehicle accidents, fall from height, or sport-related injuries, such as skiing.

In order to evaluate the results of the treatment chosen for an individual patient, valid and reliable biomarkers as outcome measures are needed. The evaluation of treatment results is a necessary step for the improvement of the treatment strategies.

Given the high mortality after a fracture around the hip in the elderly (Roberts and Goldacre 2003), most commonly the mortality rates after the injury are reported. However, it has been reported that in patients who survived the fracture, there are significant reductions in the health-related quality of life (HRQoL). Therefore, relying solely on the survival of patients could be misleading. For this reason, it appears advantageous to report both the mortality and, if the patient has survived, the HRQoL. This information appears crucial when evaluating different treatment strategies.

Due to the fact that the HRQoL is increasingly used to assess the outcome of surgery in several aspects of orthopedic surgery, including hip and knee arthroplasty, it is not surprising that patient-reported outcome measures (PROMs) are commonly used as an outcome measure for hip fractures as well.

The aim of the present analysis was to give an overview of outcome measures currently used for the assessment of hip fractures and to report the results of some studies that have been performed using the outcome measures.

Which Biomarkers Are Available?

The most commonly used outcome after hip fracture in the elderly is mortality, and in a recent consensus conference, it was also judged to be the most feasible outcome measure (Haywood et al. 2014).

As can be seen in Table 1, there are numerous additional outcome measures available, of which the length of stay in the hospital is most commonly used.

Table 1 Outcome measures commonly used in the assessment of hip fractures

Category	Short name	Full name	Published	Number of hits in MEDLINE	Requiring physical examination	Number of items	Further explanation
General health-related quality of life	SF-36	Short form 36	1992	104	No	36	Subscales of physical function, mental health, physical role, pain, general health, vitality, social function, and emotional function
	SF-12	Short form 12	1992	51	No	12	As the SF-36 but only 12 items
	EQ-5D	EuroQoL	1990	84	No	5 + VAS	Five items, covering activities, pain, depression, self-care, and mobility
	NHP	Nottingham Health Profile	1981	13	No	45	Six subscales of physical mobility, pain, sleep, emotional reaction, energy, and social isolation and seven questions on ADL
Activities of daily living	Barthel Index	Barthel Index	1965	169	No	10	Items on feeding, bathing, continence, mobility, and dressing
	Katz ADL	Katz Index of Independence in Activities of Daily Living assessment	1963	5	No		Functional activities (yes/no)
	FIM TM	Functional Independence Measure	1983	92	No	18	Ordinal scale covering two major dimensions of cognitive and motor function

Physical function	TUG	Timed up and go	1986	52	Yes	1	Time that is needed to rise from a chair, walk 3 m, turn around, return to chair, and sit down
	SPPB	Short Physical Performance Battery	1994	15	Yes		Sum of scores from a timed walk, balance test, and repeated rise from chair
	Timed walk	Timed walk		2	Yes		Timed walk such as timed over 10 m or distance covered in 6 min
Disease-specific outcome measures	WOMAC	Western Ontario and McMasters Universities Osteoarthritis Index	1988	82	No	24	Three subscales, with 17 questions on physical function, 5 on pain, and 2 on stiffness
	HHS	Harris Hip Score	1969	826	Yes	13	Four areas of pain, function, activity, and passive range of hip motion
	OHS	Oxford Hip Score	1996	58	No	12	Three areas of pain, range of movement, and mobility
Other	LOS	Length of stay		1160	No	1	Length of hospital stay, measured in days

The table is partially adapted from Hutchings et al. (2011). The number of hits in MEDLINE was assessed on April 30, 2016, with the search strategy: "hip and fracture and name of the outcome measure"

Of the disease-specific outcome measures, the Harris Hip Score (Harris 1969) has been used most frequently. This is easily explained by the fact that the Harris Hip Score has been published in 1969 already and could therefore be used for a much longer time than most other outcome measures that were developed later on. Furthermore, the Harris Hip Score is well known among orthopedic surgeons for the assessment of the results of total hip replacement in osteoarthritis; therefore, it is easy to understand that this score is used by orthopedic surgeons for fractures of the hip as well.

Another outcome commonly used is the EQ-5D (Brazier et al. 2004; Greiner et al. 2003, 2005), e.g., used by Desteli et al. (2015), Flodin et al. (2015), Griffin et al. (2015), Jurisson et al. (2016), Langford et al. (2015), Leonardsson et al. (2016), and Wajanavisit et al. (2015). The EQ-5D is a non-disease-specific outcome measure that has the great advantage that of all outcome measures that can be self-administered by the patient, it has the smallest number of items by far. Furthermore its data can also be used to determine a utility value that is necessary to calculate quality-adjusted life years (QALYs), which can be used for cost-effective analyses (CEA). The EQ-5D is available in a 3-level and a 5-level format. In the 3-level format, patients are offered 3 levels of severity (no problems/some or moderate problems/extreme problems), and in the 5-level format, the patients are offered a choice of 5 levels of severity (no problems/slight problems/moderate problems/severe problems/extreme problems). For the 3-level EQ-5D, it has been noted that the EQ-5D has a bimodal distribution preoperatively and a trimodal distribution postoperatively (Rolfson et al. 2011). It is thought that this distribution is not caused by a true separate grouping of the population under study but is rather caused by the design of the algorithm, since it "assigns problems in the pain dimension a relatively greater impact on the weighted index than problems in other dimensions" (Rolfson et al. 2011).

The SF-36 (Ware et al. 1995) is another frequently used outcome, for example by (de Abreu and de Oliveira 2015; Michael et al. 2016; Tseng et al. 2016), The SF-12 is a shorter version consisting of 12 instead of the original 36 items, e.g., used by Warschawski et al. (2016). As the EQ-5D, the SF-36 is also a non-disease-specific outcome measure but consists of more items compared to the EQ-5D. A utility for calculating quality-adjusted life years (QALYs) applicable for cost-effective analyses is available as well (Brazier et al. 2004).

An overview of several common outcomes used in the assessment of hip fractures is listed in Table 1.

It must be noted that there are several other outcome measures that are used less frequently, such as the Depression List (Gerritsen et al. 2007) used by Gregersen (2016); QALYs, used by Jurisson et al. (2016) and Wajanavisit et al. (2015); accelerometers, used by Fleig et al. (2016) and O'Halloran et al. (2015); the ICEpop CAPability measure for older people (Coast et al. 2008) used by Langford et al. (2015); the Hospital Anxiety and Depression Scale used by Langford et al. (2015); or the Fall Efficacy Scale-International (Kempen et al. 2008) used by Langford et al. (2015).

Why Cannot Biomarkers, e.g., from Total Hip Arthroplasty, Be Easily Used in Patients with Hip Fractures?

When performing studies in patients with osteoarthritis undergoing total hip replacement surgery, there are several aspects that are different from studies in which patients are examined that have sustained a hip fracture. First, patients with osteoarthritis have elective surgery, i.e., they have chosen and visited their doctor in advance and they have seen their anesthesiologist in advance who was able to order specific tests before surgery and who was also able to optimize underlying medical diseases of the patient before surgery. In addition, if the risk for surgery is considered to be too high, elective surgery could be delayed. Moreover, questionnaires assessing the health-related quality of life can be administered before surgery.

All this is not the case in patients with a hip fracture. The patients cannot choose their surgeons, the patients can hardly be optimized before surgery from the anaesthesiologists point of view. Therefore, high anesthesiologic risks have to the taken, since avoiding surgery in patients with a hip fracture is associated with an even higher mortality than performing surgery in the high-risk patient, who might even have limited cognitive function, several comorbidities, and a nonsupporting social situation.

Therefore, patients undergoing elective total hip arthroplasty are much more homogeneous in comparison to patients that have surgery because of a hip fracture.

Which Biomarker Should Be Used in Clinical Trials?

Given these various outcome measures mentioned above, the question arises as to which outcome should be used if a new trial is implemented. Ideally, an outcome measure should be both responsive and valid for the population under study (Liebs et al. 2013), and it should be easy to administer.

Several authors have used different approaches to select the most appropriate outcome measure for the assessment of patients with hip fractures.

Given the various outcome measures used in the literature, one author (Hutchings et al. 2011) screened abstracts of over 4000 manuscripts to identify commonly used scales in hip fractures in the five main categories of health-related quality of life measures, Activities of Daily Living scales (ADL), mobility and physical performance scales, disease-specific scales, and hip-specific scales. That author identified 14 scales. Of these there were three ADL scales and four HRQoL measures. None of the 14 identified scales were validated for the proximal femoral fracture population. The authors concluded that there is no single unifying scale in widespread use for proximal femoral fracture patients.

One researcher initiated a consensus conference that took place in the UK. The results of this conference have been published in 2014. As a result, a five-domain core outcome set, consisting of mortality, pain, activities of daily living, mobility, and health-related quality of life (HRQL), has been agreed upon. For the assessment of

mortality and mobility (indoor/outdoor walking status), single-item measures and, for the assessment of the HRQL, a generic multi-item measure – the EuroQoL EQ-5D – were recommended to be included in all hip fracture trials (Haywood et al. 2014).

Another author compared the measurement properties of PROMs among patients with a hip fracture (Parsons et al. 2014). The Oxford Hip Score (a hip-specific measure, OHS), ICEpop CAPability (a measure of capability in older people, ICECAP-O), and EuroQol EQ-5D (general health-related quality of life measure, EQ-5D) were compared. As a result, the ICECAP-O was not responsive to change. The EQ-5D was responsive to change from baseline and was considered to be almost as responsive to change as the OHS. The authors recommended using a general HRQoL tool such as the EQ-5D for the assessment of patients that suffered a hip fracture (Parsons et al. 2014).

Which Biomarkers Are Used in Registries?

As registries have been reported to be "among the most cost-effective interventions in medicine" (Capozzi and Rhodes 2010), it is not astonishing that an increasing number of hip fracture registries have been initiated. Probably Norway has introduced the very first hip fracture registry in 2005 (Gjertsen et al. 2008), followed by New Zealand and Australia (http://www.hipfracture.org.au) and the UK (http://www.nhfd.co.uk). Several publications that are based on registries have been published in the past (Hommel and Baath 2016; Leonardsson et al. 2016).

Outcomes used in registries include variables such as length of stay, return to original residence within 30 days, reoperation within 30 days, and pressure ulcers. While the National Hip Fracture Database (NHFD) of the UK has not started using PROMs yet (Griffin et al. 2015), the Norwegian Hip Fracture Registry is sending questionnaires to the patients that include the EQ-5D, visual analogue scales concerning pain and patient satisfaction, and the Charnley class for functional assessment.

In analogy to the International Society for Arthroplasty Registries, a Fragility Fracture Network has been founded in the meantime.

Which Biomarkers Are Used in National Quality Assurance Programs?

In Germany, a national quality assurance program has been started more than 10 years ago. In that program (2014), parameters such as length of stay, complications, and mortality are recorded during the initial hospital stay (2014). However, the program ends with the discharge of the patient from inpatient treatment. Therefore, the length of the survival of the patient after surgery, the number of revision surgeries that have been performed after the patient was discharged, and the patient-reported outcome measures cannot be evaluated. These limitations are known, and initiatives have been undertaken in order to address these issues.

Which Interventions Were Recently Examined in Order to Improve the Treatment Results in Patients with Hip Fractures?

Recently, several aspects were examined, such as surgical approach for hemiarthroplasty (Leonardsson et al. 2016), red blood cell transfusion (Gregersen 2016), protein-rich nutritional supplementation and bisphosphonates (Flodin et al. 2015), individualized nutrition and exercise therapy (Milte et al. 2016), or motivational interviewing (O'Halloran et al. 2015).

Leonardsson et al. (2016) has analyzed 2118 patients (mean age of 85 years) from the Swedish Hip Arthroplasty Register aged 70 years and above who underwent a hemiarthroplasty for a fracture. As a result, patients in whom a posterior approach was used reported a higher health-related quality of life, less pain, and greater satisfaction than patients in whom a direct lateral approach was chosen. However, after and adjustment for age, gender, cognitive impairment, and ASA grade was performed, no association was found between surgical approach and HRQoL, residual pain, and patient satisfaction anymore. Therefore, the authors concluded that the surgical approach for hemiarthroplasty does not seem to affect the HRQoL, pain, or patient satisfaction one year after surgery in this patient group.

Gregersen (2016) performed an RCT in which 284 elderly patients admitted to hospital for surgical hip fracture repair were randomized postoperatively to either a liberal (Hb target of 7 mmol/l (11.3 g/dl)) or a restrictive (Hb target of 6 mmol/l (9.7 g/dl)) transfusion strategy. They concluded that the group with the liberal Hb target had improved survival in the frailest elderly without impairing recovery from physical disabilities and HRQoL or increasing risk of infections compared to the restrictive transfusion group within one year after hip fracture surgery.

Fleig et al. (2016) applied an accelerometer at the waist for 7 days in 53 older Canadian adults (mean age 79.5 years) who sustained a hip fracture. That author reported that older adults have long periods of sedentary time with minimal activity after a fracture of the hip.

Griffin et al. (2015) report patient-reported outcomes of patients one year after they have sustained a hip fracture and were treated at a major trauma center in the UK. Using the EQ-5D as the outcome measure, the author concluded that hip fracture marks a "step down in the quality of life of a patient" as it accounts for approximately 0.22 disability-adjusted life years in the first year after fracture. The author noted that this value is "equivalent to serious neurological conditions for which extensive funding for research and treatment is made available."

Potential Applications to Prognosis, Other Diseases, or Conditions

Quality of life outcome measures as a biomarker in hip fractures are important, since they allow assessing the treatment results in the patients that have survived the injury. Assessing the results is crucial for the development of improvements in the treatment strategies.

The biomarkers for quality of life in hip fractures were mostly originally developed for other diseases and have not been validated for the hip fractures yet. Therefore, it is not applicable to transfer the results mentioned in the manuscript to other diseases or conditions.

Further research should include the development of outcome measures that are validated in patients that have sustained a fracture of the hip.

Summary Points

- This chapter focuses on the assessment of quality of life in patients that have sustained a hip fracture.
- Hip fractures are very common in the elderly and are associated with a high mortality and decreased health-related quality of life.
- Given the high mortality after a hip fracture, the survival of the patient is the most commonly used outcome for the assessment of the treatment result.
- For the patients that survive the fracture, there are numerous outcome measures available that are used for the assessment of the quality of life.
- None of these outcome measures have been validated for the use in patients with hip fractures up to this time.
- The non-standardized use of a large number of outcome measures limits comparability between studies.
- Therefore, several authors have employed various methods in order to develop recommendations on which outcome should be used. These methods included literature reviews, consensus conferences, and statistical analyses.
- Using these methods, some authors have concluded that five domains should be measured in every study evaluating hip fractures. These domains include mortality, pain, activities of daily living, mobility, and health-related quality of life (HRQoL).

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Part V

Resources

Recommended Resources on Biomarkers of Bone Diseases

51

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Contents

Abstract

There are a variety of conditions affecting the bone including osteoarthritis, osteoporosis, osteosarcoma, osteogenesis imperfecta, and fractures, to name just a few. In the USA, about 10% of the adult population have osteoporosis, for example. Biomarkers are of significant value in most skeletal conditions. They are used in risk stratification, diagnosis, prognostication, directing initial therapy, monitoring response to treatment, and guiding the choice of further treatments. However, biomarker discover is an ongoing scientific dialogue between the experimental scientist, clinicians, and manufacturers of equipment and diagnostic platforms. This chapter lists resources on the regulatory bodies, journals, books,

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professional bodies, and websites that are relevant to an evidence-based approach to the use of biomarkers in diseases of bone.

Keywords
Biomarkers • Cardiovascular Disease • Evidence • Resources • Books • Journals • Regulatory bodies • Professional societies

Introduction

There are a variety of bone diseases or adverse conditions affecting the skeletal system. In the USA, for example, there are 40 million adults with low bone mass and 10 million adults with osteoporosis (Wright et al. 2014). These rates are higher in those aged over 80 where the rate of osteoporosis is 26% (Wright et al. 2014). The prevalence figures in women are much higher than in men. For example, in women over 80 years of age the prevalence of osteoporosis is 35% (Wright et al. 2014). Such a high prevalence represents a considerable burden on the individual, family unit, and health providers. This is particularly so as the forward trajectories on a world-wide basis indicate an increasing proportion of elderly in the population. Of course there are many other conditions affecting this grateful system. It is therefore important that the package of care involves diagnosis, treatment, and monitoring. Biomarkers are measurable characteristics of normal biological processes, pathogenic processes, or responses to a therapeutic intervention (Atkinson et al. 2001). Biomarkers have significant scientific and clinical value in the management of bone diseases which are common causes of morbidity. Some biomarkers of diseases of the skeleton (e.g., osteoporosis and rheumatoid arthritis) may be released from bone or result from a specific systemic response to the disease (Yavropoulou et al. 2011). The understanding of biomarkers of bone turnover has increased significantly in recent years (e.g., see Starup-Linde and Vestergaard 2016). Monitoring bone metabolism requires measurement of analytes released during bone formation as well as measurement of the products of bone restoration. A variety of genetic, epigenetic, proteomic, glycomic, and imaging biomarkers can also be used for the diagnosis, prognosis, and epidemiology of diseases of the bone. Ideally, such biomarkers should be assayed noninvasively (Atkinson et a1. 2001). There is an ongoing scientific dialogue in biomarker discovery ranging from the identification of newly discovered pathways to the development of physical platforms which depend on advances in chemistry.

There are four main uses for biomarkers in diseases of bones:

1. Risk stratification
2. Diagnosis
3. Prognostication
4. Monitoring response to treatment

While challenges exist in translating research on biomarkers into clinical practice, several biomarkers are already being used routinely by rheumatologists (doctors who specialize in the management of patients with musculoskeletal disease). However, the use of emerging high-throughput technologies to integrate biomarkers into clinical practice will allow "personalization" of disease management.

It is now difficult even for experienced scientists and clinicians to remain up-to-date with the rapid pace of the developments in this field. For those new to the field, it is difficult to know which of the myriad of available sources are reliable. To assist colleagues who are interested in understanding more about biomarkers of diseases of bones, we have therefore produced tables containing reliable resources. The experts who assisted with the compilation of these tables of resources are acknowledged below.

Examples of the definitions, measurement, and applications of biomarkers can be found in this book and also via the tables below.

Tables 1–7 list information on regulatory bodies (Table 1), professional bodies (Table 2), journals on bone and biomarkers (Table 3), books on bone biomarkers (Table 4), books on bone (Table 5), Emerging techniques and platforms (Table 6) and websites (Table 7) that are relevant to an evidence-based use of biomarkers in bone disease.

Summary Points

- There are a variety of conditions that can affect the skeletal system.
- Diseases of the bone can be considered as common particularly in the elderly.
- Biomarkers are used in screening for such diseases.

Table 1 Regulatory bodies and organizations. This table lists the regulatory bodies and organizations involved with various aspects of biomarker discovery and applications

Biomarker, Imaging and Quality of Life Studies Funding Program, National Cancer Institute, USA. http://www.cancer.gov/aboutnci/organization/ccct/funding/BIQSFP
Biomarker Qualification Program US Food and Drug Administration. http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/ucm284076.htm
Centers for Disease Control and Prevention (CDC). http://www.cdc.gov/
European Medicines Agency. http://www.ema.europa.eu/ema/index.jsp?curl=pages/special_topics/general/general_content_000349.jsp
International Federation of Clinical Chemistry and laboratory Medicine (IFCC). www.ifcc.org
Medicines and Healthcare products Regulatory Agency (MHRA). http://www.mhra.gov.uk
National Institutes of Health. http://www.nih.gov/
US Food and Drug Administration (FDA). www.fda.gov

Table 2 Professional societies. This table lists the professional societies involved with biomarkers and/or bone disease

American Association Clinical Chemistry (AACC)
www.aacc.org
American Cancer Society
www.cancer.org
American Society for Bone and Mineral Research (ASBMR)
www.asbmr.org
Androgen Excess and Polycystic Ovaries Society
www.ae-society.org
Biomarkers Consortium
www.biomarkersconsortium.org/consortium.php
European Musculo-Skeletal Oncology Society (EMSOS)
www.emsos.org
Liddy Shriver Sarcoma Initiative
sarcomahelp.org/osteosarcoma.html
National Bone Health Alliance (NBHA)
www.nbha.org
Orthopedic Research Society
www.ors.org
Osteoarthritis Research Society International (OARSI)
www.oarsi.org
Sun Valley Workshop
www.ors.org/sunvalley
Osteoarthritis Imaging Workshop
www.iwoai.org
Osteoarthritis Research Society International
www.oarsi.org

Table 3 Journals publishing on biomarkers of bone disease. This table lists the top 20 journals publishing original research and review articles related to bone and biomarkers. The list was generated from SCOPUS (www.scopus.com) using general descriptors related to bone and biomarkers. The journals are listed in descending order of the total number of articles published in the past 5 years. Of course, different indexing terms or different databases will produce different lists so this is a general guide only. Such a search as this also identifies spurious or atypical material (which have been removed from the list) due to the indexing terms. Journals associated with biomarker discovery alone will produce a different list of course and so will journals covering bone per se, so this table is a guide only

Plos One
Bone
Osteoporosis International
Journal Of Bone And Mineral Research
Journal Of Clinical Endocrinology And Metabolism
Journal Of Bone And Mineral Metabolism
Calcified Tissue International

(*continued*)

Table 3 (continued)

Clinical Calcium
Molecular Medicine Reports
Biochemical And Biophysical Research Communications
BMC Musculoskeletal Disorders
Menopause
Journal Of Cellular Physiology
European Journal Of Endocrinology
Endocrinology
Biomed Research International
Clinical Endocrinology
Clinical Oral Implants Research
International Journal Of Clinical And Experimental Medicine
Journal of Biological Chemistry

Table 4 Relevant books on biomarkers. This table lists books on biomarkers

Aptamer Handbook: Functional Oligonucleotides and Their Applications. Klussmann S. Wiley-VCH, 2006, Weinheim
Aptamers in Bioanalysis. Mascini M. Wiley-Interscience, 2009, USA
Biomarker Guide. Peters KE, Walters CC, Moldowan JM. Cambridge University Press, 2010, USA
Biomarkers: In Medicine, Drug Discovery, and Environmental Health. Vaidya VS, Bonventre JV. John Wiley & Sons, 2010, USA
Biomarkers in Oncology: Prediction and Prognosis. Lenz H-J, Springer, 2013, Germany
Biomarkers of Disease: An Evidence-based approach. Trull AK, Demers LM, Holt DW, Johnston A, Tredger JM, Price CP. Cambridge University Press, 2002, UK
Development and Application of Biomarkers. Lundblad RL. CRC Press, 2016, USA
Handbook of Biomarkers. Kewal KJ. Lippincott, 2010, USA

Table 5 Relevant books on bone disease. This table lists books on bone disease

Advances in MRI of the Knee for Osteoarthritis. Majumdar S. World Scientific, 2010, Singapore
Biochemistry and Physiology of Bone. Bourne GH. Elsevier, 2014, USA
Bone Resorption: New Insights for the Healthcare Professional. Acton QA. ScholarlyBrief, 2012
Bone and Joint Imaging. Resnick D, Kransdorf MJ. Elsevier Saunders, 2005, USA
Imaging of Arthritis and Metabolic Bone Disease. Weissman BN. Elsevier Saunders, 2009, USA
Pain in Osteoarthritis. Felson DT. Wiley-Blackwell, 2009, USA
Physiological Basis of Metabolic Bone Disease. Nordin BEC, Morris HA, Anderson P. CRC Press, 2014, USA
Primer on Metabolic Bone Diseases and Disorders of Mineral Metabolism, 8th Edition. Rosen CJ. Wiley-Blackwell, 2013, USA

Table 6 Sources and resources for emerging techniques and platforms. This table lists some emerging sources, resources platforms in biomarker discovery and application

Biobanking and Biomolecular Resources Research Infrastructure
www.bbmri.eu
Canadian Light Source – BMIT
www.lightsource.ca/beamlines/details/biomedical_imaging_and_therapy_bmitid.html
Canadian Multicentre Osteoporosis Study
www.camos.org
Creatis (bone tissue imaging)
www.creatis.insa-lyon.fr/site/en/Bone_Cellular_Imaging.html
FLUIDIGM CyTOF2 (mass cytometry)
www.fluidigm.com/products/helios
Quanterix Simoa (automated immunoassay platform)
www.quanterix.com/products/simoa-hd-1-analyzer
Multicenter Osteoarthritis Study
www.most.ucsf.edu/studyoverview.asp
Osteoarthritis Initiative
https://oai.epi-ucsf.org/datarelease/
University of Zurich Progenetix database
www.progenetix.org/cgi-bin/pgHome.cgi

Table 7 Relevant internet resources. This table lists some internet resources on biomarkers and cardiovascular disease

Arthritis Alliance of Canada
www.arthritisalliance.ca
Arthritis Research Canada
www.arthritisresearch.ca
Arthritis Society
www.arthritis.ca
Biomarkers Test (BMT)
www.biomarkers.it
Biomed Central (BMC) Biomarkers
www.biomarkerres.org
Bone and Joint Canada
www.boneandjointcanada.com

(*continued*)

Table 7 (continued)

Canadian Research and Education: Arthritis
www.carearthritis.com
EBSCO
https://health.ebsco.com/
International Osteoporosis Foundation
www.iofbonehealth.org
Medscape
www.medscape.com
News medical
www.news-medical.net/health/What-is-a-Biomarker.aspx
Endocrinology Unit, Department of Health Sciences and Mother and Child Care, University of Palermo, Palermo, Italy
www.unipa.it
World Health Organization
http://who.int/mediacentre/en/

- Biomarkers are used in risk stratification, diagnosis, prognostication, directing initial therapy, monitoring response to treatment, and guiding the choice of further treatments.
- This chapter lists the most up-to-date resources on the regulatory bodies, journals, books, professional bodies, and websites that are relevant to an evidence-based approach to the use of biomarkers of bone diseases.

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Starup-Linde J, Vestergaard P. Biochemical bone turnover markers in diabetes mellitus – a systematic review. Bone. 2016;82:69–78.

Wright NC, Looker AC, Saag KG, Curtis JR, Delzell ES, Randall S, Dawson-Hughes B. The recent prevalence of osteoporosis and low bone mass in the United States based on bone mineral density at the femoral neck or lumbar spine. J Bone Miner Res. 2014;29(S1):2520–6.

Yavropoulou MP, Tomos K, Tsekmekidou X, et al. Response of biochemical markers of bone turnover to oral glucose load in diseases that affect bone metabolism. Eur J Endocrinol. 2011;164:1035–41.

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