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Stephen J. Winters
Ilpo T. Huhtaniemi *Editors*

Male Hypogonadism

Basic, Clinical and Therapeutic Principles

Second Edition

Humana Press

Contemporary Endocrinology

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Series Editor Foreword

Controversies regarding the treatment of male hypogonadism using testosterone recently attracted much public attention. Indeed, comprehensive knowledge of male hypogonadism still leaves a lot to be desired, even among health professionals. The current volume of the Contemporary Endocrinology book series goes a long way to help correct this knowledge deficit by addressing the subject in a truly comprehensive way.

The international group of experts assembled by Drs. Winters and Huhtaniemi examines the problem from fetal life to puberty to advanced age, from brain dysfunction to dysfunction of the testis itself, and from the genetic influences to those of the environment. Fertility issues are addressed, as well as the relevant aspects of obesity, cancer, and the metabolic syndrome.

The chapters are written to stand on their own as complete reviews of a particular topic and are assembled logically to produce a comprehensive picture of the entire subject. As intended by the editors and the authors, this volume is appropriate for a wide audience of readers—from medical students to advanced investigators in the areas of male hypogonadism.

The editors and the authors are to be congratulated on their accomplishment, which will benefit both current and future generations of physicians dealing with this increasingly important problem.

Leonid Poretsky
New York, NY, USA

Preface to the Second Edition

The first edition of *Male Hypogonadism: Basic, Clinical and Therapeutic Principles* was published in 2004. Since then, our understanding of the biology and clinical management of hypogonadal men has increased substantially. Much has been learned about the cellular and molecular biology of male reproduction and about testosterone treatment from carefully performed clinical studies.

This second edition builds on the first, but has changed with some new chapters and new authors, and it is now co-edited by Stephen J. Winters and Ilpo T. Huhtaniemi. The monograph is again intended for the wide audience of scientists and clinicians who are interested in the reproductive endocrinology of males and the disorders that cause its dysfunction. Accordingly, the goal of this monograph is to link the recent advances in our understanding of the biology of hypothalamic–pituitary–testicular function to improved care for our patients. The chapters were contributed by authors from around the world, and from various scientific and clinical disciplines, who have devoted their careers to the study of the biology and pathophysiology of the male.

The format of this monograph remains unchanged. Chapters 1 – 3 review the neuroendocrine control of testicular function, provide an overview of the steroidogenic function of Leydig cells from the fetus to adulthood, and summarize what is known about spermatogenesis and spermiogenesis. Chapters 4 – 12 discuss clinical disorders that cause gonadotropin deficiency and testicular failure. Chapters 13 – 17 summarize how exercise, the environment, obesity, and aging influence how the testis functions, and Chaps. 18 – 20 present current views of the benefits and risks of androgen replacement therapy and the approach to stimulating spermatogenesis in gonadotropin-deficient men. We have tried to minimize the overlap and differences of opinion, but perhaps some is beneficial.

We thank Springer Science for supporting the publication of the second edition of *Male Hypogonadism: Basic, Clinical and Therapeutic Principles* , and we thank the authors, some for a second time, who devoted a great deal of effort to prepare these informative and well-written chapters. We learned a great deal of modern medicine from reading these reviews and hope that our readers will do likewise.

Stephen J. Winters

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1. Neuroendocrine Control of Human Testicular Function

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Overview

The major regulator of testicular function is gonadotropin-releasing hormone (GnRH) produced in neurons scattered throughout the anterior hypothalamus. When it reaches the anterior pituitary, GnRH stimulates the synthesis and secretion of the pituitary gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH are released into the circulation in bursts and activate G-protein coupled receptors on Leydig and Sertoli cells, respectively, that stimulate testosterone production and spermatogenesis. The system is tightly regulated and is maintained at a proper set-point by the negative feedback effects of testicular steroids and inhibin-B. Testicular function is also influenced by multiple internal (paracrine and autocrine) and

external (endocrine) environmental factors.

GnRH Synthesis and Secretion

GnRH is the primary neuronal link to reproduction. GnRH, a C-terminal amidated decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2), is found in a small number of neurons that are located diffusely throughout the medial basal and preoptic areas of the anterior hypothalamus in primates [1]. GnRH neurons send axons through subventricular and periventricular pathways to terminate in the capillary space within the median eminence located in the floor of the third ventricle. GnRH from these axons enters the capillaries and is transported in the hypothalamic portal blood to the cells of the anterior pituitary. The median eminence is located outside the blood brain barrier and is accessible to regulatory factors within the systemic circulation.

The amount of GnRH that is secreted is influenced by many factors. GnRH mRNA levels are determined by the rate of transcription of the pro-GnRH gene which is known to be controlled by the POU-homeodomain protein, Oct-1, adhesion-related kinase (Ark), and by retinoid-X receptors, among many other regulatory factors [2]. Studies in GT1-7 cells, a GnRH-producing murine neuronal cell line, suggest that mRNA stability also plays an important role in maintaining GnRH gene expression. GnRH mRNA levels increase in the hypothalamus of adult male monkeys following bilateral orchidectomy [3], indicating that the testis secretes endocrine hormones, presumably testosterone, that suppress GnRH gene expression. Transcription of GnRH mRNA produces a pro-GnRH precursor, and yet another level of control within GnRH neurons involves the posttranslational processing of the inactive precursor to the active decapeptide. Subsequent to its secretion, peptidases in the median eminence and pituitary inactivate GnRH and have been demonstrated to produce a biologically active fragment that can antagonize the effects of active GnRH [4] and thereby further regulate the actions of GnRH. The current GnRH analogs used clinically are not degraded in the same manner adding to the potency of their effectiveness. Recent work analyzing developmental changes in the promoter regions of the GnRH gene of the mouse and rhesus monkey has demonstrated that alterations in chromatin interactions and DNA methylation are coincident with changes in GnRH mRNA levels and supports potential epigenetic regulation of GnRH expression [5, 6].

GnRH, like most hypophysiotropic peptides, is released into the portal blood in bursts. Measurement of GnRH concentrations within third ventricular cerebral spinal fluid in non-human primates revealed that GnRH is released in a pulsatile manner and is followed by a coincident pulse in systemic LH [7]. The average concentration of GnRH in hypothalamic portal blood (in rams) is approximately 20 pg/ml (0.02 nM), and levels in conscious sheep ranged from nadir values of <5 pg/ml to pulse peak values of about 30 pg/ml [8]. In those studies, the amplitudes of GnRH pulses in intact, castrated and

testosterone-replaced castrated rams were roughly equivalent; by contrast, GnRH pulse frequency was higher in castrates than in intact animals and was reduced by testosterone replacement. The implication of those observations is that GnRH secretion rises with testosterone deficiency primarily because GnRH pulse frequency is accelerated.

Throughout the past decade, the primary discovery for the regulation of GnRH synthesis and secretion has been on unraveling the critical role of the kisspeptin-GPR54 system. Kisspeptin is encoded by the *Kiss1* gene, located on chromosome 1q32.1, and is the natural ligand of the previously orphaned receptor—GPR54 [9, 10]. In 2003, mutations in GPR54 were identified in two separate consanguineous families with selective congenital hypogonadotropic hypogonadism [11, 12]. Genetic knockout studies in mice confirmed that the kisspeptin protein and GPR54 receptor were essential for sexual maturation and fertility [11, 13]. Kisspeptin neurons are located in the infundibular nucleus and preoptic area within the human hypothalamus, and they synapse with the dendrites, axons, and/or cell bodies of GnRH neurons which express the GPR54 receptor [14, 15]. Intravenous injection of kisspeptin increases LH pulse amplitude and frequency in healthy and hypogonadal men [16, 17]. Infusion of a kisspeptin antagonist into the hypothalamus of rhesus monkeys reduced mean GnRH and GnRH pulses and blocked kisspeptin-stimulated GnRH secretion [18, 19]. Ongoing investigations are providing strong evidence that kisspeptin neurons play an important role in modulating GnRH responses to circulating gonadal steroids, stress indices and nutritional status.

GnRH pulse frequency is controlled by the "GnRH pulse generator," the term used to describe the highly synchronized firing of GnRH neurons in the mediobasal hypothalamus (MBH). The idea that changes in cell membrane potentials predispose to bursts of GnRH release follows from the finding that bursts of electrical activity in the MBH in the non-human primate coincided with pulses of LH secretion [20]. The coincident firing of multiple GnRH-expressing neurons may reflect communication by gap junctions, through interneurons, or second messengers. Recently, clustered GnRH neurons in the preoptic area of the hypothalamus have been shown to possess intermingled dendrite bundles with shared synapses [21]. The identification of GnRH receptors on GnRH neurons, and the observation that adding GnRH to GnRH neuronal cultures depresses GnRH pulsatile release, provides a possible framework for intraneuronal communication by GnRH itself [22]. Experiments using various 5' deletion constructs of the GnRH promoter-luciferase vector suggest that episodic GnRH gene expression is a promoter-dependent event that is mediated by Oct-1 and specific GATA-binding elements [23, 24].

Kisspeptin neurons have been shown to play a role in the pulsatile pattern of GnRH secretion in primates. Kisspeptin neurons are localized within the same hypothalamic regions, outside of GnRH neuronal locations, where lesions in rhesus monkeys were originally demonstrated to suppress LH secretion [25, 26]. Pulsatile administration of

kisspeptin to juvenile male monkeys stimulates a pulsatile pattern of gonadotropin release that mimics LH secretion in pubertal males and can be blocked with a GnRH antagonist [27]. Delivery of a single i.v. bolus of kisspeptin to healthy men induces an immediate pulse in circulating LH and effectively resets the GnRH pulse generator delaying subsequent endogenous pulses to the naturally occurring interval [28]. Most convincingly, measurements within the median eminence of female rhesus monkeys revealed that kisspeptin is secreted in a pulsatile manner with an interpulse interval of 60 min and that most kisspeptin pulses are accompanied by a simultaneous increase in GnRH [29]. Elaborate studies in ruminants and rodents have confirmed the important regulatory role for kisspeptin on GnRH pulsatility.

The prevailing model by which kisspeptin neurons can regulate pulsatile activity of GnRH neurons comes from the recent discovery of a subset of kisspeptin neurons that co-express the neuropeptides neurokinin B (NKB), and dynorphin (DYN) thereby known as KNDy neurons. KNDy neurons form an interconnected reciprocal network in the arcuate nucleus of rats and sheep [30, 31]. The synchronous activity of KNDy neurons is thought to be controlled by excitatory actions of NKB and the inhibitory signaling of DYN which can autoregulate KNDy neurons through DYN and NKB receptors. As illustrated in Fig. 1.1, a pulse of GnRH is initiated by a constitutive increase in NKB secretion which synchronizes a further increase in NKB by the network of KNDy neurons. The synchronized increase in NKB initiates a pulse of kisspeptin which stimulates the rapid secretion of GnRH. NKB also stimulates the secretion of DYN from KNDy neurons which initially limits kisspeptin secretion, and, within minutes inhibits KNDy neuronal activity, and effectively terminates GnRH release. The inhibitory tone of DYN signaling will gradually suppress DYN secretion and allow for constitutive NKB release to initiate the next pulse of GnRH secretion. Although this model has not been directly tested in humans, it is supported by finding inactivating mutations in kisspeptin and NKB receptors that result in hypogonadotropic hypogonadism in men [12, 32].

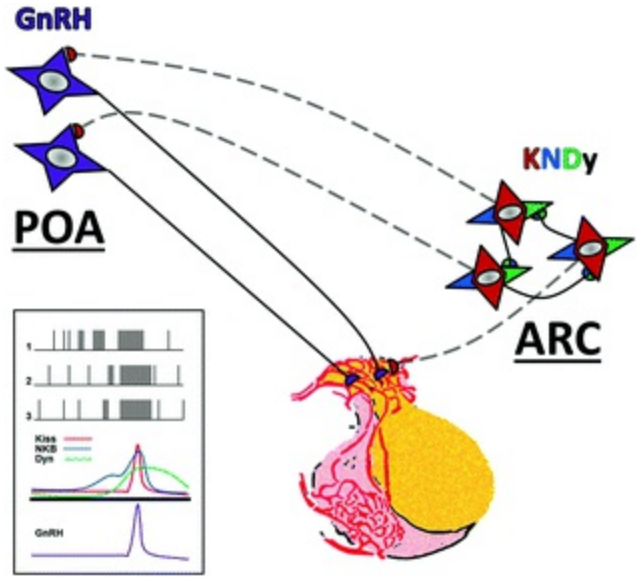

Fig. 1.1 Model depicting the role of ARC KNDy neurons in the generation of pulsatile GnRH release. KNDy neurons in the ARC nucleus form an interconnected population with their axons and/or dendrites. In the presence of low levels of Dyn, individual KNDy neurons (insert, 1–3) begin constitutively increasing NKB secretion leading to a synchronous excitation and Kiss release which stimulates GnRH secretion (*graphs* in insert). Moments later, Dyn levels begin to increase and apply a break to NKB, Kiss and GnRH secretion. Increased local concentration of Dyn will inhibit KNDy neurons, resulting in an interpulse interval, until a lower threshold is reached and the cycle can reset

As shown in Fig. 1.2, the release of GnRH is influenced by multiple neurotransmitters including glutamate, gamma-aminobutyric acid (GABA), neuropeptide Y, opiates, dopamine, norepinephrine, cAMP and nitric oxide [33]. The presence of receptors on GnRH neurons for most of these substances implies that they influence GnRH neurons directly. NMDA receptors that mediate glutamate activation of GnRH may involve the nitric oxide signaling pathway. Neurotransmitters with receptors that are not expressed on GnRH neurons may regulate GnRH via synaptic connections between GnRH neurons and other interneurons. Regulation of GnRH secretion may also occur directly on neuronal axon terminals that abut on capillaries in the median eminence.

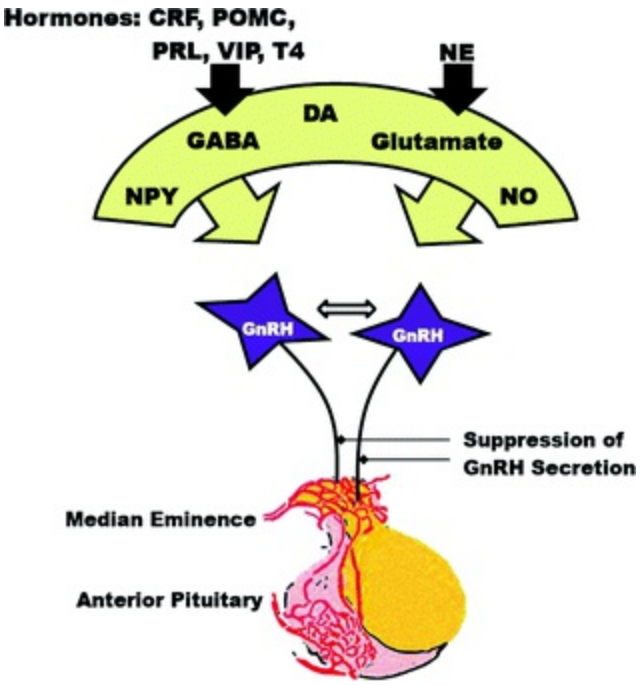

Fig. 1.2 Diagram showing activation of GnRH neurons by neurotransmitters and the relation to the anterior pituitary

A second form of GnRH, GnRH-2 [34, 35] that was initially identified in non-vertebrates, is also found in the primate brain [36]. GnRH-2 appears to activate a unique GnRH-II receptor [37], but this receptor does not appear to be expressed in humans [38]. Thus, the significance of GnRH-2 in humans is not yet known, and there is little evidence for a role in reproduction.

There has been resurgence in research on a gonadotropin-inhibiting hormone (GnIH) which appears to have a functional role in regulating GnRH neurons and gonadotropin secretion in seasonal breeders and lower mammals (for review, see [39]). However, the role of the primate GnIH ortholog, RFRP-3, has not been elucidated. In a recent study in rhesus macaque, an antagonist to RFRP-3 was demonstrated to prevent the decline in circulating testosterone levels that accompanies acute fasting [40]. This result suggests the possibility that a GnIH hormone exists in primates and may have a role in modulating reproductive function in response to varying metabolic states. Further research is necessary to validate this hypothesis.

Gonadotrophs and GnRH Receptors

Gonadotrophs account for 6–10% of the cells of the normal anterior pituitary [41]. Gonadotrophs may be small and round, or larger and ovoid, and are difficult to identify by morphological criteria. Instead, immunostaining using specific antibodies for LH-β and FSH-β proteins are used to identify gonadotrophs. In primates [42], as in rodents [41], the great majority of gonadotrophs are bihormonal, i.e., they express both LH-β

and FSH-β subunit genes. A small fraction of cells appear to express LH or FSH selectively, and some gonadotrophs also produce growth hormone. The proportions of each of the hormone-producing gonadotrophs are developmentally regulated [43]; however, the biological significance of these observations remains unclear.

GnRH activates gonadotrophs through both short-term and long-term mechanisms that are illustrated in Fig. 1.3. Upon reaching the pituitary, GnRH binds to and activates a cell-surface G-protein coupled receptor (GnRH-R) [44]. This receptor is a structurally unique member of the seven-transmembrane G-protein-linked receptor family that lacks the long C-terminal intracellular tail that is typical of most G-protein-coupled receptors. This tail is important in the rapid desensitization of other G-protein-coupled receptors, whereas down-regulation of the GnRH receptor by GnRH is a relatively delayed event that occurs over hours rather than minutes. Binding of GnRH to its receptor facilitates binding of a G-protein to the receptor's third intracellular loop. The bound G-protein exchanges GDP for GTP and dissociates into its constituent α and βγ subunits. The α-subunits are unique to each G-protein, whereas the β- and γ-subunits of the different G-proteins are similar. The dissociated G-protein α-subunit activates downstream signaling pathways [45]. $G\alpha_{q/11}$, the major G-protein that associates with the GnRH-R, activates membrane-associated phospholipase C to hydrolyze membrane phosphoinositides and increase intracellular inositol phosphates (Ips) including inositol triphosphate $(I_{1,4,5})P3$. IP3 rapidly mobilizes calcium from intracellular stores, and L-type voltage-gated calcium channels open following which extracellular calcium enters the cell [45]. The rise in intracellular free calcium is primarily responsible for the immediate release of LH and FSH [46]. GnRH receptors also interact with other G-proteins including $G\alpha_i$ and $G\alpha_s$. The GnRH receptor can couple to $G\alpha_s$ in rat pituitary cells [47] and subsequently activate cAMP production and the PKA signaling pathway [48]. Interaction with $G\alpha_i$ occurs primarily in non-pituitary tissues.

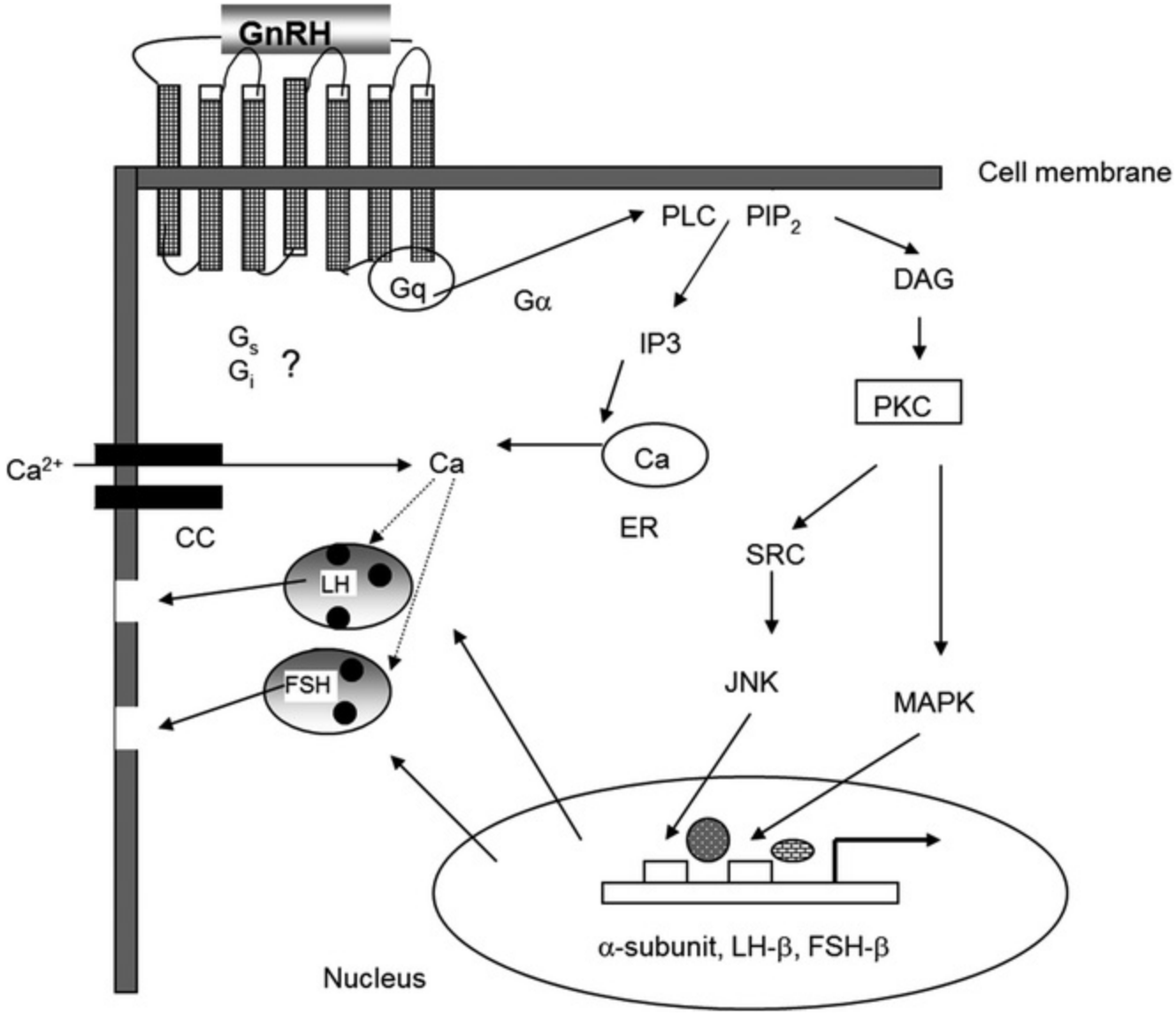

Fig. 1.3 Signaling mechanisms initiated by activation of the GnRH receptor stimulate gonadotropin synthesis and secretion

The long-term stimulatory effects of GnRH are to increase transcription of the genes for the gonadotropin subunits and the GnRH receptor, among other genes. These effects occur primarily through liberation of membrane diacylglycerol (DAG) that in turn activates protein kinase C. The subsequent phosphorylation of subfamilies of mitogen-activated protein kinases (MAP kinases), including members of the ERK and JNK families, initiates nuclear translocation of proteins that bind directly to the 5′ regulatory regions of gonadotropin subunit and the GnRH-receptor genes or serve as cofactors for promoter activation [49]. Increased intracellular calcium also causes increased activation of calmodulin-dependent protein kinases which contributes to the transcriptional effects of GnRH [50].

GnRH receptors are up-regulated by pulsatile GnRH [51]. Accordingly, when pulsatile GnRH secretion increases, as in castration or primary testicular failure, GnRH

receptors increase. Gonadotrophs become more responsive to GnRH, and the LH response to GnRH stimulation is amplified [52]. With continuous GnRH treatment on the other hand, GnRH receptors decline, followed by suppression of LH-β and FSH-β mRNAs. This "homologous desensitization" of the GnRH-R is regulated by several serine-threonine protein kinases including protein kinase A (PKA) and protein kinase C (PKC), as well as by G-protein-coupled receptor kinases (GRKs) which combine to cause a reduction in the efficiency with which $Ins(1,4,5)P_3$ mobilizes Ca^{2+} from intracellular storage [53]. GnRH receptors also decline with GnRH deficiency.

GnRH receptors undergo a low level of agonist-independent constitutive internalization which is not influenced by binding to GnRH agonists [54]. Accordingly, a wide variety of postreceptor modifications have been proposed for the observed receptor desensitization that disconnects GnRH binding from LH secretion after prolonged exposure [55]. The primate GnRH-R is different than other mammalian GnRH-R due to the presence of a primate-specific Lys191 [56], the lack of a second glycosylation site near the NH2 terminus [57] as well as the absence of any COOH tail [58]. These differences lead to a comparatively significant reduction in the percentage of membrane-bound receptors and suggest that differential expression of intracellular chaperones or membrane scaffolding proteins may play an important role in GnRH signaling and desensitization. Evidence for this is the recent discovery that the proto-oncogene SET, also called I2PP2A (inhibitor 2 of protein phosphatase 2A), interacts with the first and third intracellular loops of the GnRH receptor and leads to inhibition of calcium signaling while increasing the cAMP signaling pathway [59].

The Gonadotropic Hormones

LH and FSH are members of the glycoprotein family of hormones that also includes thyrotroph-stimulating hormone (TSH) and chorionic gonadotropin (hCG). These heterodimers are composed of a common α-subunit and unique β-subunits. The subunits have N-linked oligosaccharide chains that are associated with asparagine residues. Each subunit is encoded by a unique gene that is found on a separate chromosome: The human α-subunit gene is on 6p21.1–23, LH-β is on 19q13.3, and FSH-β is on 11p13 (see Chap. 6).

The production of LH and FSH is directly influenced by the level of the gonadotropin subunit mRNAs. This relationship seems especially strong for FSH-β mRNA and FSH secretion. Each of the gonadotropin subunit mRNAs is increased by GnRH, and stimulation of the β-subunit genes by GnRH is primarily transcriptional. Complexes of transcription factors including SF-1, EGR-1, and SP1 are activators of the LHβ-subunit gene [60], whereas the AP1 proteins fos/jun are important for up-regulation of FSH-β transcription by GnRH [61]. GnRH stimulation must be pulsatile for LH-β and FSH-β mRNA levels to be increased. Transcriptional regulatory proteins

that control α-subunit expression include cAMP response element binding protein (CREB), MAP kinase/ERK1 and GATA-binding proteins [62]. α-Subunit gene expression is increased robustly both by pulsatile and by continuous GnRH, and GnRH not only stimulates α-subunit transcription but also prolongs the half-life of the α-subunit mRNA [63]. Thus, the requirements for α-subunit mRNA up-regulation by GnRH seem less stringent than are those for the β-subunit genes. These factors partly explain why α-subunit is synthesized in excess of β-subunits and why β-subunits are rate limiting for gonadotropin synthesis.

Proteins that are destined for secretion are synthesized on ribosomes bound to the endoplasmic reticulum. During the translational process, preformed oligosaccharide chains are linked to the side chain amino group of asparagines on the gonadotropin subunits. As translation continues, sugar moieties are trimmed, and the subunits change configuration allowing for their combination. A region of the β-subunit, termed the "seatbelt," wraps around the α-subunit loop 2 [64]. Dimeric LH and FSH are segregated in the ER and are transferred to the Golgi where they are concentrated in secretory granules. These protein-rich vesicles subsequently fuse with the plasma membrane following stimulation by GnRH. This process is termed "exocytosis." There is evidence that granules containing FSH are also exported directly to the plasma membrane independent of GnRH pulsatile stimulation. This mode of secretion allows for FSH secretion between pulses. Gonadotrophs also secrete uncombined α-subunit both in pulsatile fashion and continuously.

Pulsatile Gonadotropin Secretion

Experiments in ovariectomized rhesus monkeys rendered gonadotropin-deficient with hypothalamic lesions lead to the proposal that an intermittent pattern of GnRH secretion was necessary for normal LH secretion [65]. In those animals, GnRH administered in pulses stimulated LH secretion, but GnRH administered continuously was much less effective. The pulsatile nature of LH secretion was subsequently established in all species, including man [66]. Accordingly, LH secretion is stimulated when GnRH is administered in pulses to gonadotropin-deficient patients but not when administered continuously [67, 68]. An understanding of these physiological principles leads to the use of pulsatile GnRH as a treatment to stimulate fertility and to the development of long-acting GnRH analogs that initially stimulate LH/FSH secretion but then deplete membrane GnRH-R and inhibit gonadotropin production to produce a biochemical gonadectomy that is used as a treatment for patients with prostate cancer and other androgen-dependent disorders [69].

With current assays, GnRH is undetectable in the peripheral circulation. Therefore, GnRH secretion cannot be studied directly in humans. Instead, changes in circulating LH levels are used as a surrogate marker for the activity of the GnRH pulse generator. LH

secretion is determined by the frequency, amplitude, and duration of its secretory pulses. Presumably because of its longer circulating half-life, FSH pulses are less clearly defined in peripheral blood than are LH pulses because FSH pulses are clearly evident in jugular blood (in ewes) where clearance effects are minimized [70].

Cultured pituitary cells that are perifused with pulses of GnRH are a powerful model yielding important information on the actions of GnRH and other factors that regulate gonadotropin secretion under controlled conditions. Using this experimental approach, episodes of LH as well as FSH secretion are distinct and short-lived with a rapid upstroke and abrupt termination. LH pulse amplitude is directly proportional to the dose of GnRH administered, and the median duration of an LH pulse about 25 min.

In contrast to the regularity of LH pulses produced by a constant dose of GnRH in vitro, Fig. 1.4 illustrates that LH pulses in the peripheral circulation in man vary in amplitude and that interpulse intervals are inconstant. LH pulses in vivo are also characterized by a less rapid upstroke and a slower decline from the peak than are pulses in vitro. This presumably reflects dilution of secreted hormone by plasma in the general circulation and the influence of clearance of LH by the liver and kidney. Whether LH is released in the basal interval between GnRH-initiated secretory episodes has been debated, but this mode of secretion is small and is probably not biologically important.

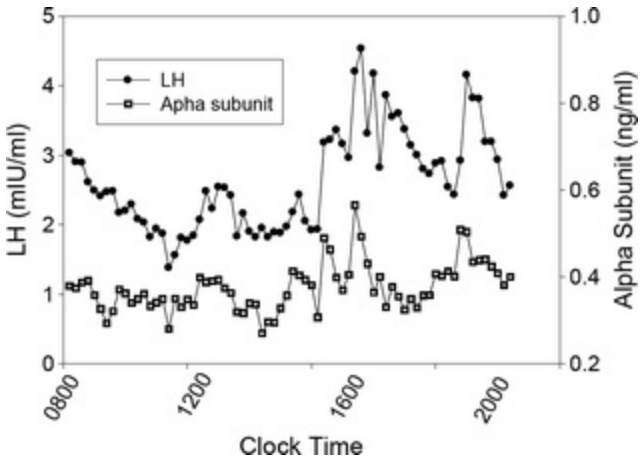

Fig. 1.4 Circulating LH and alpha subunit levels in a normal adult man. Blood samples were drawn every 10 min for 12 h beginning at 0800 h and measured for LH using Nichols Allegro LH 2-site assay, and a specific assay double antibody immunoassay for α-subunit

Hormone pulse detection has been standardized by the development of computer algorithms [71]. Using this approach, objective assessment of the frequency and amplitude characteristics of hormone pulses has been possible. Pulses of LH secretion occur throughout the day and night in normal adult men. However, estimates of the frequency of LH (GnRH) pulses in men have varied based on the intensity and duration of the blood sampling protocol, the assay used to measure LH and the algorithm used to

identify pulses. Most investigators have proposed an average frequency of 1 LH pulse every 1–2 h for normal men, but interestingly there is a large between-individual variation [72]. Because of variability in pulse amplitude and frequency, the distinction between true and artifactual pulses can be difficult. One approach is to co-analyze LH and uncombined α-subunit pulses [31] because α-subunit is released into the circulation by GnRH together with LH and FSH (Fig. 1.4). According to this logic, concordant LH and α-subunit fluctuations presumably reflect true GnRH pulsatile signals. There is generally a positive relationship between LH pulse amplitude and the preceding interpulse interval in part because a longer interval allows for the circulating level to decline to a lower baseline value.

In addition to moment-to-moment pulsatile pattern of LH secretion, there is a diurnal rhythm in circulating LH as well as testosterone levels in pubertal boys with increased LH pulsatile amplitude during sleep and increased testosterone levels in the early morning hours [73]. The diurnal variation in plasma testosterone in adults is coincident with diurnal rhythms for LH [74, 75]. The diurnal testosterone variation in men is disrupted by fragmented sleep [76], while LH rhythms are not affected [75] and therefore the mechanism for this alteration has not been established. The diurnal variation in testosterone is blunted in older men [77] and in young men with testicular failure [78].

LH Control of Testosterone Synthesis

Testosterone, a C19 3-keto, 17β-hydroxy Δ4 steroid, is synthesized from cholesterol through a series of cytochrome P450- and dehydrogenase-dependent enzymatic reactions [79]. The conversion of cholesterol to pregnenolone occurs within mitochondria and is catalyzed by P450scc, the cytochrome P450 side chain cleavage enzyme. Pregnenolone exits the mitochondria and can be converted to testosterone by two alternative routes that are referred to as the Δ^4-pathway or the Δ^5-pathway, based on whether the steroid intermediates are 3-keto, Δ^4 steroids (Δ^4) or 3-hydroxy, Δ^5 steroids (Δ^5). Classical experiments using human testicular microsomes incubated with radiolabeled steroids revealed that the Δ^5-pathway predominates in the human testis. In that pathway, C17 hydroxylation of pregnenolone to form 17α-hydroxypregnenolone is followed by cleavage of the C17–C20 bond of 17α-hydroxypregnenolone to produce dehydroepiandrosterone (DHEA). Oxidation of the 3β-hydroxy group and isomerization of the C5–C6 double bond of DHEA by 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4 isomerase (3βHSD) forms androstenedione. The C17 keto group of androstenedione is oxidized to a hydroxyl group by 17β-hydroxysteroid dehydrogenase type III (17β HSD)-producing testosterone (see Chap. 3).

LH stimulates testosterone biosynthesis in Leydig cells through a G-protein-

associated seven-transmembrane receptor [80]. LH binding to the receptor initiates a signaling cascade by activating Gs that stimulates adenylate cyclase activity and increases intracellular cAMP levels to activate cAMP-dependent protein kinase A (PKA). cAMP-dependent PKA stimulates testosterone synthesis in at least two ways. An acute response, within minutes of hormonal stimulation, is characterized by an increase in cholesterol transport into the mitochondria and is mediated by the steroidogenic acute regulatory (StAR) protein [81]. StAR acts on the outer mitochondrial membrane, where it undergoes conformational changes through interactions with protonated phospholipids causing opening and closing of a sterol-binding pocket, permitting uptake and discharge of cholesterol [82]. The chronic response to LH, which requires several hours to days, involves transcriptional activation of the genes encoding the steroidogenic enzymes of the testosterone biosynthetic pathway, P450scc, P450c17, 3βHSD and 17βHSD.

Other factors that stimulate testosterone synthesis directly include PRL, GH, T3, PACAP, VIP and inhibin, whereas glucocorticoids, estradiol, activin, AVP, CRF and IL-1 have been reported to reduce testosterone production by Leydig cells. Although controversial for many years, experiments with recombinant FSH suggest that FSH is not an important regulator of Leydig cell function [83, 84]. However, it can indirectly stimulate Leydig cells through a Sertoli cell-mediated paracrine loop. In an elegant experiment, however, selective genetic ablation of Sertoli cells in neonatal or adult mice resulted in a significant decrease in Leydig cell number with normal mean testosterone levels but fewer high level pulses of circulating testosterone and higher levels of LH [85], suggesting that a Sertoli cell factor may regulate pulsatile testosterone biosynthesis.

The blood production rate of testosterone in normal adult men has been estimated to range from 5000 to 7500 μg/24 h [86], and levels of total testosterone among normal men range from 250 to 1000 ng/dl (10–40 nmol/L) in most assays. The level of testosterone in adult men declines by more than 95% if the testes are removed. The remainder of the circulating testosterone is derived from the production of androstenedione and dehydroepiandrosterone (DHEA) by the adrenal cortex. Intratesticular testosterone concentrations in normal men are 100-fold higher than are serum levels [87]. The higher concentration is due to local production as well as sequestration by the Sertoli cell-derived androgen binding protein [88]. High intratesticular concentrations of testosterone are believed to be important for spermatogenesis; however, concentrations tenfold above serum levels are sufficient for normal spermatogenesis [89, 90].

Estrogens in Males

Normal men produce about 40 μg of estradiol and 60 μg of estrone per day. Estradiol is

produced from testosterone, and estrone from androstenedione, by aromatase P450, the product of the *CYP19* gene [91]. This microsomal enzyme oxidizes the C19 angular methyl group to produce a phenolic A ring. Aromatase mRNA is expressed in adult Leydig cells where it is activated by LH/hCG [92]. However, most of the estrogen in men is derived from aromatase in adipose and skin stromal cells, aortic smooth muscle cells, kidney, skeletal cells and the brain. The promoter sequences of the extra-gonadal and testicular P450 aromatase genes are distinct and tissue-specific due to differential splicing, but the translated protein appears to be the same in all tissues. There is evidence in vitro that cytokines such as IL-6 stimulate aromatase in stromal cells, but the factors that regulate extra-testicular aromatase are not well understood.

Estrogens are known to exert important physiological effects on reproduction as well as other organ systems in men. The two forms of the estrogen receptor, ER-α and ER-β, are each known to play a role in reproduction [93]. ER-α is the dominant form in the pituitary and hypothalamus, whereas both ER-α and ER-β are found in the testis, prostate and epididymis [94]. Studies in one man with an inactivating ER-α mutation and four men deficient in aromatase (Table 1.1) reveal effects of estrogens on bone metabolism, insulin sensitivity, hepatic lipid metabolism and reproduction. Studies in normal men using pharmacologic inhibitors and steroid add-back protocols support these findings [95]. Dilatation and atrophy of the seminiferous tubules in ER-α-deficient mice [96] imply an effect of estrogen to regulate aquaporin expression in the efferent ductules of the testis. Aromatase-deficient mice, although fertile in early life, develop infertility. Thus, estradiol is essential for male reproduction.

Table 1.1 Hormone levels in a man with a mutation of the ERα (1), and four men deficient in aromatase (2–5)

	Age (yrs)	Testosterone	Estradiol (pg/ml)	LH (mIU/ml)	FSH (mIU/ml)	Testis size	Sperm count	Reference
1	28	44 (ng/dL)	119	37 (2.0–20)	33 (2.0–15)	20–25 mL	25 mil/ml	[176]
2	24	2015 (ng/dL)	<7	26.1 (2.0–9.9)	28.3 (5.0–9.9)	R 6 × 3.5 L 5.5. × 3.2 cm	ND	[177]
3	31	523 (ng/dL)	<10	5.6 (1.4–8.9)	17.1 (1.7–6.9)	8 mL	1 mil/ml	[178]
4	27	829 (ng/dL)	<20	8.3 (1.2–8.6)	18.8 (1.3–19.3)	Ambiguous genitalia 20 mL	56 mil/ml	[110]
5	24	20.l5 (nmol/L)	UD	4.8 (<11.1)	14.4 (<20.3)	15 mL	129 mil/ml	[109]

LH and FSH levels in parentheses are the normal ranges reported in those references
UD Undetectable

Testicular Control of Gonadotropin Secretion

Gonadotropin secretion, while up-regulated by GnRH, is maintained at physiological

levels through testicular negative feedback mechanisms summarized in Fig. 1.5. Accordingly, plasma LH and FSH levels decrease following the administration of testosterone or estradiol and rise when negative feedback is disrupted by castration.

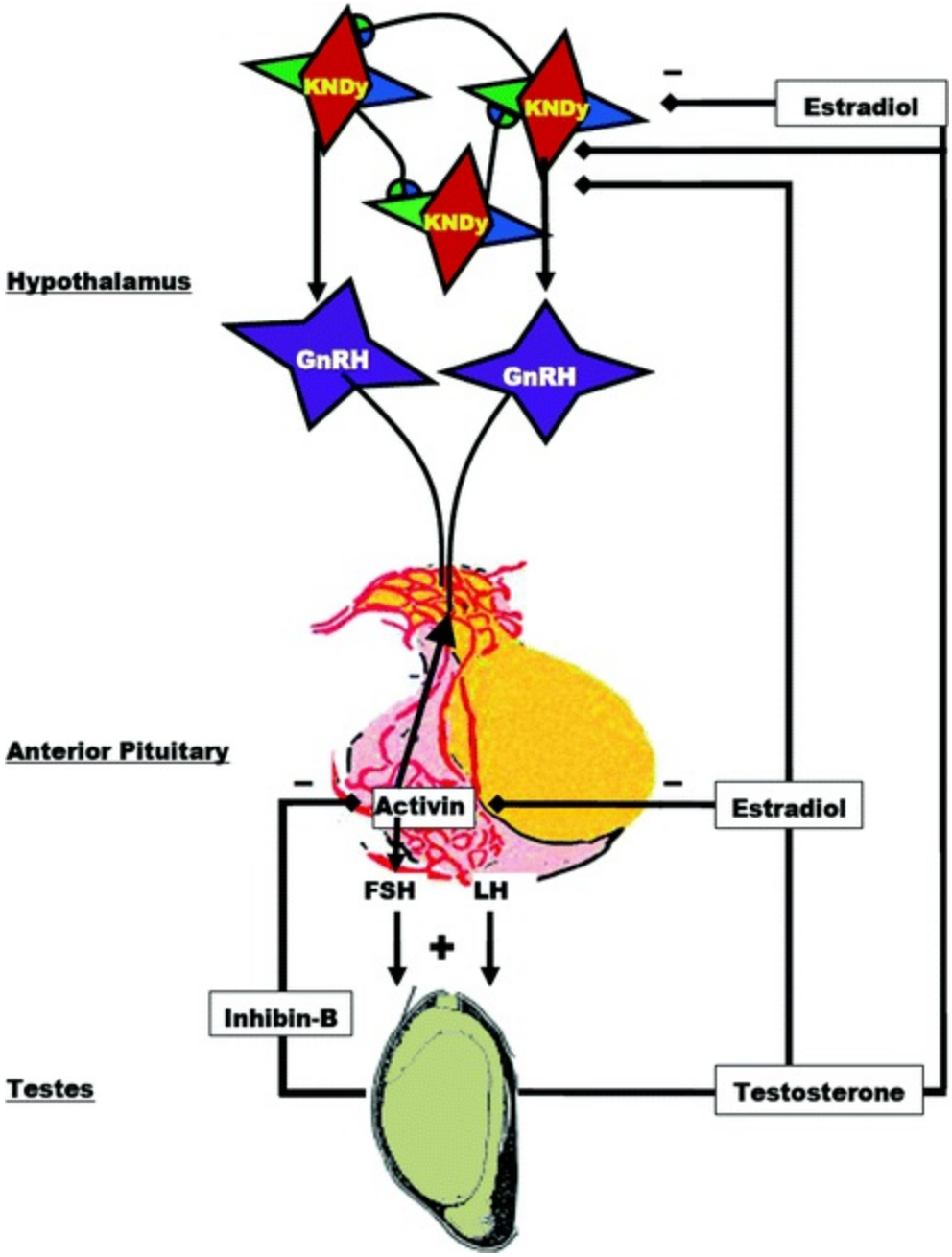

Fig. 1.5 Diagram of the negative feedback control of gonadotropin secretion by testicular hormones

The process of negative feedback control of LH and FSH by gonadal steroids in males is partly species-specific. There is considerable evidence in primates that androgens suppress LH synthesis and secretion primarily through an action on the GnRH pulse generator. For example, LH pulse frequency and amplitude are elevated in castrates and are suppressed by testosterone replacement [97]. Furthermore, when male

rhesus monkeys were rendered gonadotropin-deficient with radio-frequency lesions, and stimulated with pulses of GnRH, LH secretion increased little after bilateral orchidectomy until GnRH pulse frequency was increased [98]. In addition, expression of the mRNAs for GnRH [3], as well as pituitary GnRH receptors, and the gonadotropin subunit genes are increased in orchidectomized monkeys [99]. Moreover, when pituitary cells from adult male monkeys were stimulated with pulses of GnRH, no inhibition of GnRH-induced LH secretion by testosterone or DHT was found. In pituitary cells from rats, on the other hand, the gonadotroph is a direct site of testosterone negative feedback control since GnRH-stimulated LH pulses were suppressed in amplitude by testosterone, and α-subunit gene expression was reduced (Fig. 1.6 [100]).

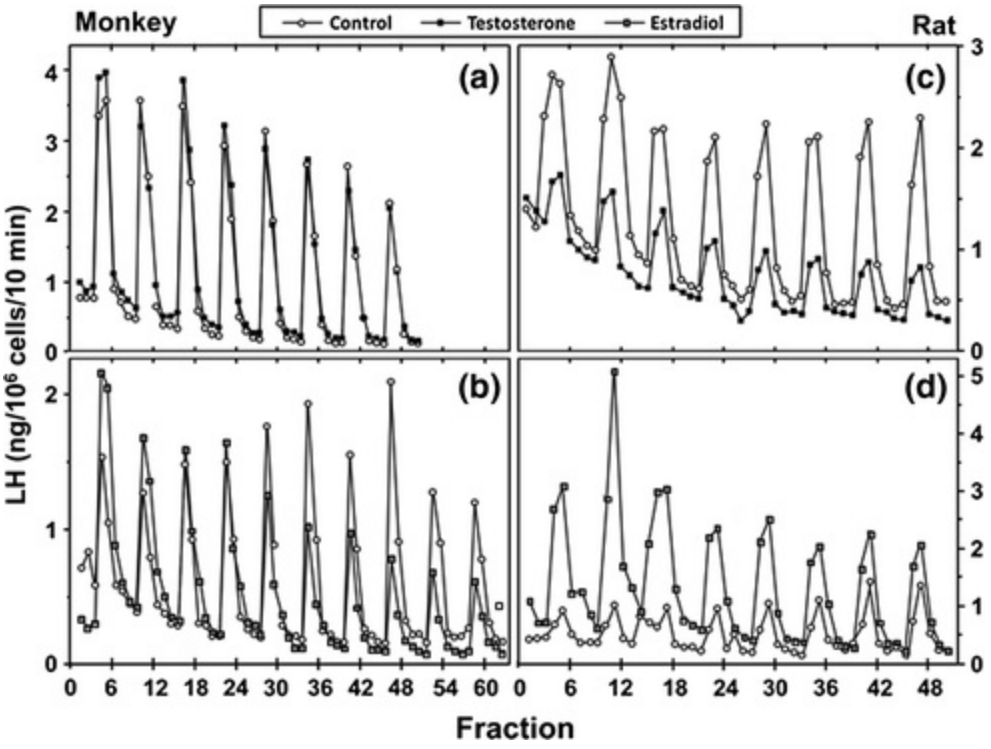

Fig. 1.6 Effects of testosterone or estradiol on GnRH-stimulated LH secretion from pituitary cell cultures from adult male monkeys and rats. From Kawakami and Winters [100]. Reprinted with permission from Endocrine Society

GnRH-deficient men represent a human model to examine the effects of testicular steroids on the hypothalamus and pituitary. When such patients were treated with fixed doses of GnRH [101], testosterone suppressed LH secretion less than in normal men implying that testosterone controls GnRH secretion. Moreover, suppression by testosterone was blocked by the aromatase inhibitor testolactone [101], and in separate experiments, dihydrotestosterone had no effect [102]. Together these data imply that the pituitary effect of testosterone to inhibit LH was through bioconversion to estradiol.

Whether it is testosterone, itself, and/or the estradiol derived from testosterone by aromatase in the central nervous system, pituitary system, or peripheral tissues that controls LH secretion, has been a subject of considerable interest. The finding that

dihydrotestosterone (DHT), a non-aromatizable androgen, decreases LH pulse frequency strongly supports a role for androgens in the regulation of the GnRH pulse generator. Furthermore, LH pulse frequency is increased in patients with non-functional androgen receptors (AR) in the complete androgen insensitivity syndrome, indicating that AR signaling regulates GnRH pulse frequency [103].

Estradiol also plays an important physiological role in the negative feedback control of gonadotropin secretion in men. This control mechanism was suggested by pharmacological studies using the estrogen antagonist clomiphene [104], or the aromatase inhibitor, testolactone [105]. When those drugs were administered to normal men, circulating LH and FSH levels rose together with plasma testosterone concentrations. This finding was confirmed and extended in studies using the aromatase inhibitor, anastrazole [106, 107]. Variable LH and FSH levels in an adult man with an inactivating mutation of the ER-α and four men with mutations of the CYP19 aromatase gene (reviewed [108–110]) are shown in Table 1.1. Estradiol treatment also decreases the LH response to GnRH stimulation in men [111] and in primate pituitary cells perifused with pulses of GnRH (Fig. 1.6, [100]) indicating an additional direct negative feedback effect of estradiol on the pituitary.

GnRH neurons appear by autoradiography and immunocytochemistry to lack both androgen and estrogen receptors and numerous animal models have shown that kisspeptin neurons mediate the negative feedback effects of gonadal steroid hormones (for review, see [112]). Both estrogen and androgen receptors are localized to kisspeptin neurons in the hypothalamus of male mice [113]. Furthermore, testosterone administration to castrated male monkeys decreased kisspeptin mRNA in the medial basal hypothalamus coincident with suppression of circulating levels of LH [114]. A comparison of kisspeptin neurons in the hypothalamus of young and elderly men dying a sudden death revealed an age-related increase in the number of kisspeptin neurons and fibers and an enhancement of afferent contacts they established onto GnRH neurons [115]. These studies support the concept that kisspeptin neurons mediate the negative feedback effects of testosterone in primates including man and are consistent with studies showing that androgen negative feedback responsiveness increases with aging [116]. However, many other hypothalamic neuromodulators are responsive to castration and testosterone replacement, and therefore, the role of kisspeptin neurons may be partial with regard to steroid hormone negative feedback.

Inhibin, Activin and FSH

Although LH and FSH are both produced within the same cell type, the synthesis and secretion of the gonadotropins are, in some circumstances, differentially regulated. Both LH and FSH are stimulated by GnRH and are suppressed by gonadal steroid hormones. However, FSH levels may rise selectively in men with seminiferous tubular failure, and

FSH synthesis, and secretion, can be selectively regulated through the actions of two paracrine factors, pituitary activin and follistatin, and by testicular inhibin-B. Activin selectively up-regulates FSH secretion by stimulating FSH-β gene expression [117] with negligible effects on LH synthesis. Inhibin and follistatin prevent activin from binding to its receptor through separate mechanisms, effectively antagonizing the stimulatory effects of activin on FSH synthesis.

Activin, a member of the TGF-β family of growth factors, is a dimeric peptide consisting of two similar subunits that were designated as β-subunits because the identification of activin followed the cloning of inhibin, an α–β heterodimer. There are at least four forms of the β subunit: β_A, β_B, β_C and β_D. Activin-B ($\beta_B\beta_B$) is the predominant form in the pituitary, whereas most other tissues produce activin-A. Activin functions in neural and mesodermal morphogenesis, wound healing, vascular remodeling and inflammation, as well as in reproduction [118–121]. Activins (and follistatin) primarily function as local paracrine or autocrine factors with no observable changes in the general circulation except in pregnancy and advanced stages in cancer patients [122, 123]. Therefore, activin-stimulated FSH synthesis and secretion is tightly and selectively regulated within the pituitary milieu.

Like other members of the TGFβ superfamily, activins signal through a serine-threonine receptor hetero-oligomeric complex that activates intracellular SMAD proteins (Fig. 1.7, [124]). Initially, the activin ligand binds to a specific type II receptor subunit, either ActRII or IIB [125–127]. Subsequent to ligand binding, the type II subunit pairs with a type I receptor subunit (either ActRI or IB) and forms a heteromeric complex at the cell surface [128–130]. The serine/threonine kinase of the type II subunit is responsible for phosphorylation of the type I subunit, thereby initiating postreceptor signaling/phosphorylation of SMAD2 and SMAD3 proteins which then dissociate from the receptor complex, bind SMAD-4 and translocate from cytoplasm to nucleus. Activated SMAD complexes interact with cofactor proteins and bind to SMAD binding elements on gene promoters, including FSH-β, driving immediate gene activation.

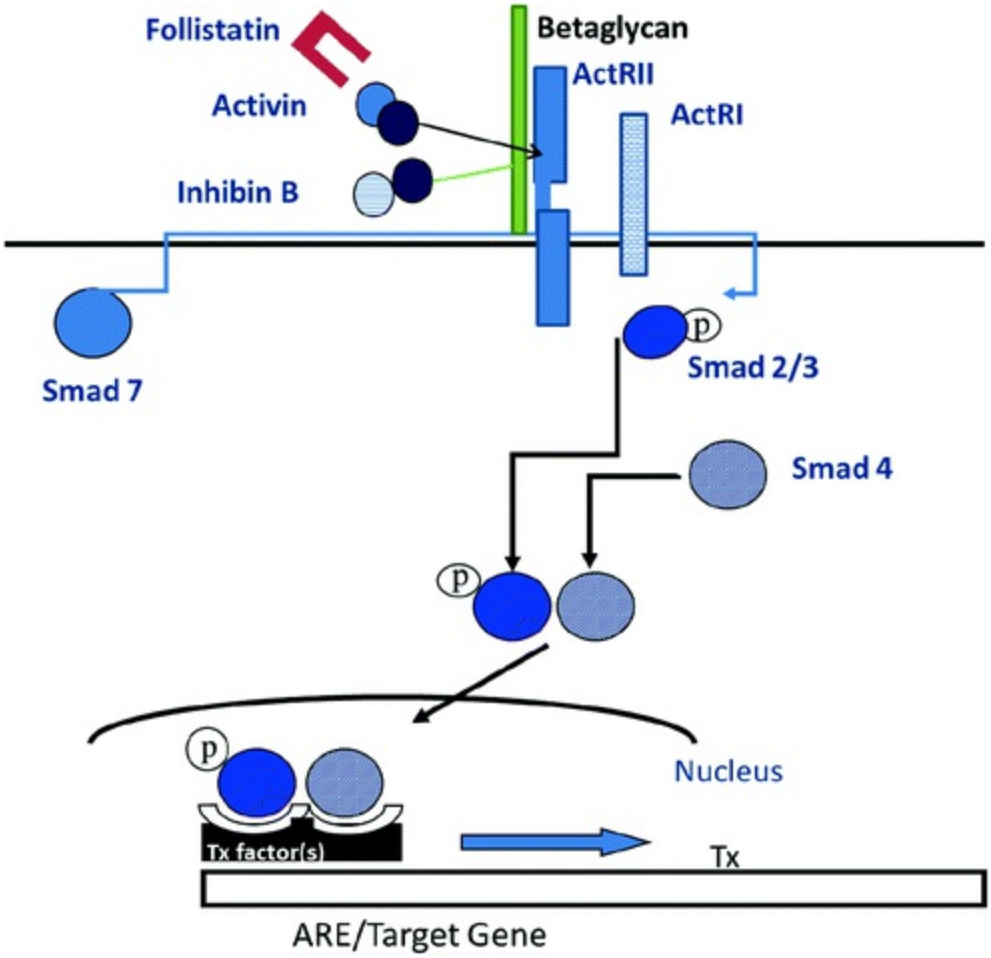

Fig. 1.7 A diagram for activin signaling through Smad proteins

The actions of activin are antagonized by follistatin that binds to and neutralizes the bioactivity of activin, as well as by inhibin that competes with activin for binding to the activin RII receptor [131]. Inhibin is an antagonist of activin because it fails to initiate intracellular SMAD signaling. Inhibin has a lower affinity for Act RII than activin, and betaglycan, a membrane proteoglycan, appears to function as an accessory receptor binding protein to potentiate inhibin binding, as well as for TGF-β and bone morphogenic protein [132]. Follistatin is structurally unrelated to activin and inhibin, but like activin, is found in all tissues examined. In the rat pituitary, follistatin is up-regulated by activin, GnRH and PACAP, and is suppressed by testosterone and by follistatin itself, no doubt through binding to activin. In primate pituitary cultures, on the other hand, GnRH is ineffective, and testosterone, as well as activin, increases follistatin expression [133]. Pituitary follistatin influences the FSH and LH response to castration. Follistatin expression increases following orchidectomy in adult male rats [134], and there is a reciprocal relationship over time between follistatin and FSH-β gene expression implying that follistatin attenuates the FSH castration response in that species. In male primates including man, by contrast, the level of follistatin mRNA is relatively unaffected by bilateral orchidectomy, and FSH-β mRNA increases about 50-

fold (Fig. 1.8, [99]). Thus, follistatin appears to function as a brake on FSH production in male rodents but not in primates.

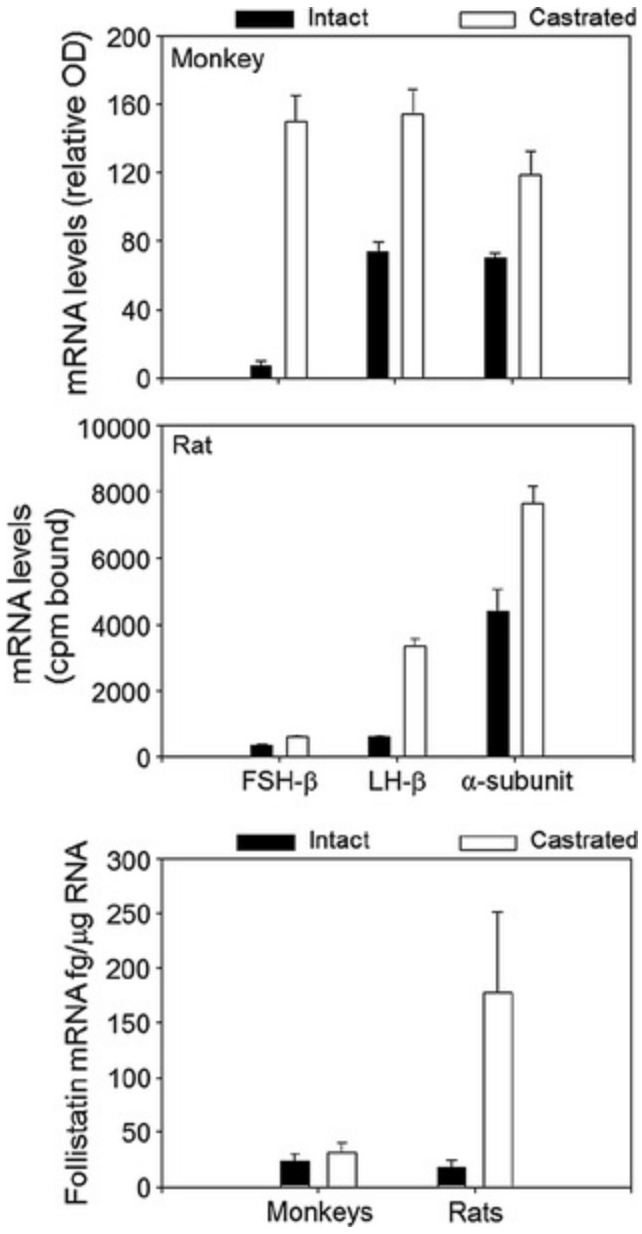

Fig. 1.8 A comparison of the effects of orchidectomy on gonadotropin subunit and follistatin mRNA levels in adult male rats and monkeys. From Winters et al. [99]. Reprinted with permission from Endocrine Society

Inhibin is produced by Sertoli cells and by fetal Leydig cells and plays a fundamental role in the selective regulation of FSH [135, 136]. Inhibin also regulates ovarian steroidogenesis and follicular development while its paracrine function in testis is not well understood. The term inhibin was first applied in 1932 [137] to the aqueous extract of bull testis that prevented the development of castration cells within the anterior pituitary, and was distinguished from androitin, that was present in the ether-extract and stimulated prostate growth, and is now known as testosterone.

Inhibin is a heterodimer of an α-subunit (20 kDa) and one of two β-subunits, β_A and β_B (13 kDa). Of these, only the β_B subunit is expressed by the testis in most species, and therefore testicular inhibin is inhibin-B. The inhibin α-subunit is expressed in Sertoli cells and somewhat in Leydig cells and is up-regulated by FSH. The βB subunit is expressed in Sertoli cells and germ cells, but the factors regulating the β_B subunit gene are not well understood. The level of inhibin/activin β_B mRNA in the rat testis is unaffected by hypophysectomy or by FSH treatment [138]. Transcription factors of the GATA-binding protein family regulate both the inhibin β- and α-subunit promoters [139]. Exogenous testosterone treatment results in suppression of FSH and a parallel decrease in circulating inhibin-B [140].

Conversely, inhibin-B levels increase in men treated with FSH for infertility [140, 141]. Control of inhibin β_B by a germ cell factor is suggested by the decline in plasma inhibin-B levels, but not inhibin-α subunit levels, that follows destruction of germ cells by cancer chemotherapy [142]. Inhibin-B is a marker of Sertoli cell function and is positively correlated with sperm count and testicular volume [143].

Inhibin-B is a selective negative regulator of FSH. While initially shown in vitro using pituitary cells from rodents [144], subsequent experiments in adult male monkeys revealed suppression of circulating FSH with no effect on the levels of LH or testosterone [145]. Inhibin can also augment the suppressive effect of testosterone on FSH synthesis, because inhibin suppresses FSH secretion [146]. On the other hand, very large doses of FSH are required to increase circulating inhibin-B levels [147]. Consequently, plasma inhibin-B and FSH concentrations are inversely related among normal men and are more strongly inversely correlated when values from men with varying degrees of primary testicular failure are included in the analysis [147, 148]. The relationship between inhibin-B and FSH differs from that between LH and testosterone which is bidirectional, i.e., LH stimulates testosterone, and testosterone suppresses LH, such that there is no comparable strong correlation between circulating LH and testosterone levels among normal men. The different relationships between plasma LH with testosterone compared to FSH with inhibin-B levels were clearly demonstrated in experiments conducted by Ramaswamy et al. [149]. These investigators removed one testis from adult male rhesus monkeys following which the plasma levels of both testosterone and inhibin-B decreased. The decline in testosterone was brief and was restored to normal by a rise in LH, whereas inhibin-B levels remained at approximately 50% of baseline values for up to 6 weeks even though FSH levels rose. Thus, LH and testosterone form a classical feed-forward/feedback loop whereas inhibin-B controls FSH but is less dependent on FSH stimulation.

Inhibin-B levels increase during the neonatal phase of development, sometimes called mini-puberty, and again at puberty. Although plasma LH and FSH levels rise at these developmental stages, the number of Sertoli cells also increases. Furthermore,

studies in adult monkeys showed a strong positive correlation between circulating inhibin-B levels and Sertoli cell number [150]. Thus, circulating inhibin-B appears to reflect the number and function of Sertoli cells and is less dependent on FSH stimulation.

Activin, inhibin and follistatin may also act locally in the testes to regulate reproductive function. Activin receptor gene expression has been detected in Sertoli cells, primary spermatocytes and round spermatids, and radiolabeled activin-A binding has been demonstrated in the latter cell types [138, 151, 152]. Both proposed inhibin receptors, betaglycan and InhBP/p120 are present in rat Leydig cells [132, 153], and betaglycan is expressed in germ cells [154]. In general, activin treatment reduces hCG-driven Leydig cell production of androgens [155] while inhibin action can prevent activin effects [155], and has been shown to stimulate testosterone release in some [156] but not all [157] studies. In terms of actions on gamete production, activin and inhibin tend to have opposing actions with activin stimulating [158] and inhibin reducing [159, 160] spermatogenesis. Inhibin-α-deficient mice have large testes and develop gonadal stromal tumors that appear to occur due to activin excess [161] and over-expression of the α-subunit in transgenic mice results in hypospermatogenesis [162]. Thus, the balance of activin and inhibin (and potentially follistatin), directly in the testes, may impact both endocrine signals via altered testosterone production as well as gamete maturation.

Neuroendocrine Mechanisms for the Differential Control of FSH and LH

In addition to the selective regulation of FSH-β mRNA levels by pituitary activin and follistatin and by testicular inhibin-B, there may be other mechanisms for the differential secretion of FSH and LH. Results from studies in rats [163] and rat pituitary cell cultures [164] revealed that the frequency of GnRH pulses regulates LH-β and FSH-β mRNA levels differently, with very rapid GnRH pulse frequencies (every 15–30 min) favoring LH-β over FSH-β gene expression. Variation in GnRH pulse frequency has been shown to activate distinct signaling pathways to affect gonadotropin subunit transcription (for review see [165]). LH-β transcription is dependent on early growth response-1 protein (Egr-1). Egr-1 expression has been shown to increase with high-frequency GnRH, while the Egr-1 corepressor, Nab-2, is increased during low-frequency GnRH delivery [166]. Other recent studies have revealed that PKA can selectively stimulate FSH-β in response to GnRH at both fast and slow pulse frequencies [167]. Low-frequency GnRH pulses stimulate PKA activity and increase FOS and JUN proto-oncogenes which stimulate the FSH-β gene promoter [167]. High-frequency GnRH stimulates corepressors of FOS and JUN (ICER, SKIL and TGIF-1)

which negatively regulate the FSH-β promoter [168, 169].

Up-regulation of pituitary follistatin by rapid GnRH pulse frequencies [164] with blockade of activin-stimulated FSH-β gene expression may also occur. Follistatin mRNA levels are inversely correlated with GnRH pulse frequency, with rapid frequencies supporting maximal follistatin expression and slower frequencies causing a selective rise in FSH-β mRNA without an increase in follistatin [163].

While GnRH pulse frequency seems to differentially regulate FSH and LH in rodents, its importance in men is less certain. For example, in men with congenital hypogonadotropic hypogonadism (e.g., Kallmann syndrome) who were treated long term with pulsatile GnRH, increasing the frequency of GnRH stimulation from every 2 h to every 30 min for 7 days increased serum LH levels threefold, but FSH levels rose by 50% [170]. In a second study in a similar patient population, increasing the GnRH pulse frequency from every 1.5 h to every 0.5 h suppressed plasma FSH but plasma LH levels were unchanged [171]. In both studies, changes in testosterone, estradiol and inhibin-B levels as a consequence of increased GnRH may have influenced the results.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that stimulates α-subunit transcription, and lengthens LH-β mRNA transcripts and presumably prolongs half-life, but suppresses FSH-β mRNA levels in part by stimulating follistatin transcription [172]. These observations in vitro suggest PACAP could play a role in the differential production of FSH and LH. Recent analysis of transgenic mice that over-express PACAP in the pituitary has provided in vivo evidence for an autocrine/paracrine role for PACAP in the differential regulation of the gonadotropins during sexual maturation [173]. PACAP can directly affect gonadotrophs as well as modulate the effects of GnRH through interactions of intracellular signaling mechanisms (for review, see Halvorson, 2014 [174]). PACAP can stimulate gonadotropin synthesis and secretion from human gonadotrophinomas [175]. The role of PACAP in the physiological regulation of gonadotropins in humans has not been fully established to date and will require future investigation.

Conclusion

This chapter provides background information on the normal function of the hypothalamic-pituitary-testicular unit in men. The recent growth of understanding of this system through the application of molecular and cellular methods is impressive. In the following chapters, conditions that disrupt these normal processes are discussed.

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2. The Human Leydig Cell

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Introduction

In all mammals, Leydig cells are the main source of the androgens required for development of the male phenotype, for germ cell production, and for male sex drive. The Leydig cells are essential, therefore, for masculinization, fertility, and reproductive health and, recent evidence would also suggest, for general adult wellbeing in the male [1]. In this chapter, the development, steroidogenic function and regulation of human Leydig cells will be summarized. Clinical aspects of aging and pathology related to Leydig cells will also be reviewed. The data presented is based, where possible, on information from the human, but there is a much larger database of rodent studies available and data from these studies is included where relevant.

Leydig Cell Development

Leydig cells were first described by the German histologist Franz Leydig in 1850 as prominent clusters of cells lying between the seminiferous tubules in a variety of mammals [2]. Subsequently, Leydig cells have been shown to be the major functional cell type in the interstitial compartment of the testis, separated from the seminiferous tubules by the peritubular myoid cells (Fig. 2.1). Human Leydig cells are ovoid or

polygonal in shape with eosinophilic cytoplasm, a euchromatic round eccentric nucleus with a peripheral distribution of heterochromatin, and a conspicuous nucleolus. The predominant cytoplasmic organelle is the smooth endoplasmic reticulum (SER), which is characteristically abundant in steroidogenic cells, with mitochondria and lipid droplets also numerous (Fig. 2.2). Crystals of Reinke are variably found in normal adult human Leydig cells, although their composition and significance remains unknown.

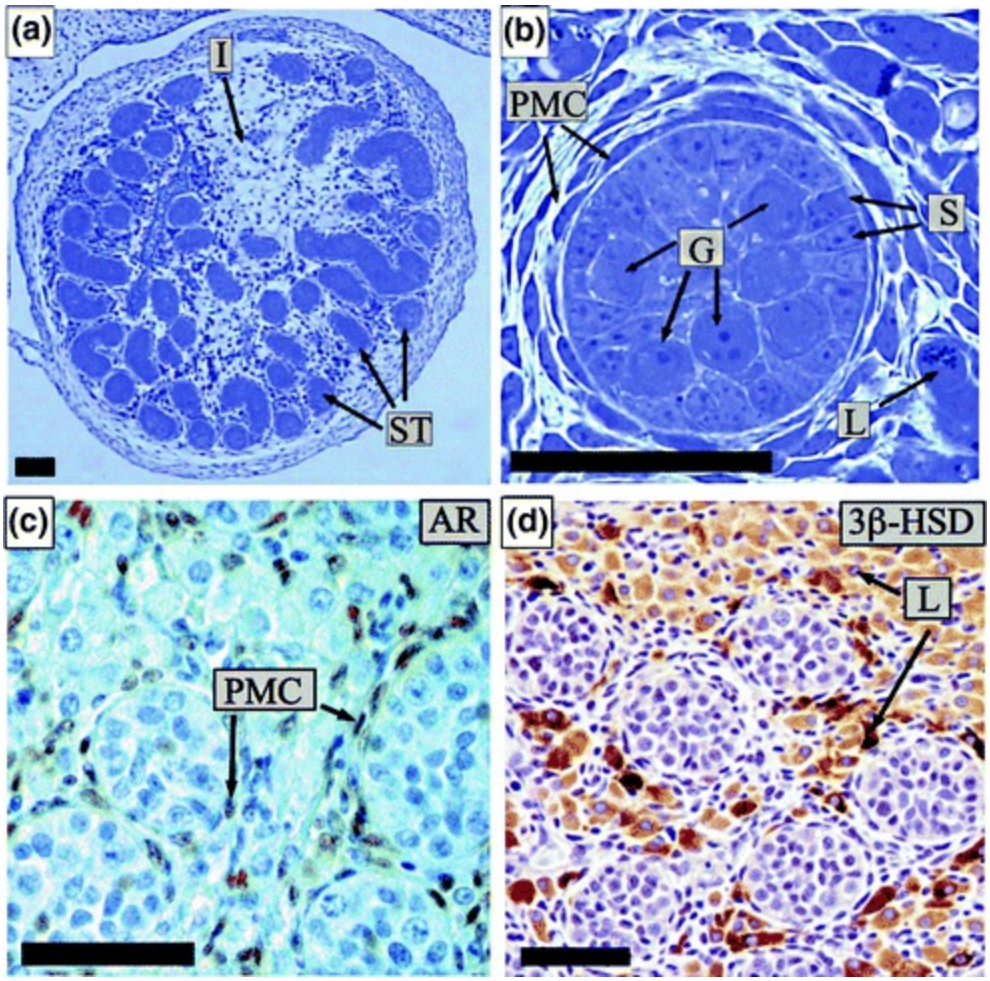

Fig. 2.1 Fetal testis. **a** Fetal mouse: low magnification of a semi-thin section showing seminiferous tubules (*ST*) and interstitial tissue (*I*). **b** Fetal mouse: higher magnification of (**a**) showing gonocytes (*G*) in the central part of the sex cord with the Sertoli cells (*S*) around the periphery. The peritubular myoid cells (*PMC*) form a concentric layer around the cord, and Leydig cells (*L*) are present within the interstitium. **c** Fetal human: immunohistochemically labeled for the androgen receptor (*AR*) which is clearly expressed in PMC and in some interstitial cells. **d** Fetal human: immunohistochemically labeled for 3β-hydroxysteroid dehydrogenase (HSD3B) which is localized exclusively in the Leydig cells. In all photomicrographs, the bar represents 50 μm, Adapted from O'Shaughnessy and Fowler [22]. (c) Society for Reproduction and Fertility (2011). Reproduced with permission

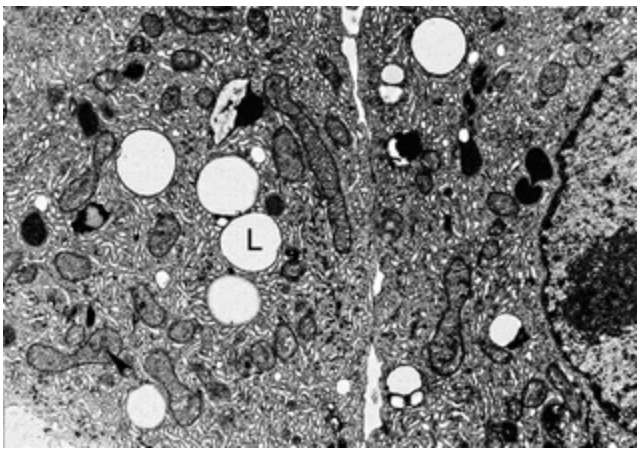

Fig. 2.2 Pubertal adult human Leydig cells. This electron micrograph of a resin section shows part of two adult human Leydig cells. Lipid droplets (*L*) can be seen along with numerous mitochondria (example is indicated with an *arrowhead*) and abundant smooth endoplasmic reticulum, Reproduced from Prince [200] with permission from the publisher John Wiley and Sons

In all mammalian species so far studied, two populations of Leydig cells have been identified. A fetal population that arises soon after testis differentiation, and an adult population which develops before puberty [3]. Until recently, it was thought that the fetal population regressed as the adult population developed, but evidence from the mouse suggests that the fetal cells remain present in the adult at about 10% of the total Leydig cell number [4]. In humans, blood levels of testosterone peak three times during development [5], and it has been suggested that this is evidence for three populations of Leydig cells in humans [6]. The first peak occurs at 12–14 weeks of gestation, during the fetal differentiation of Leydig cells [7, 8]. Testosterone levels then decline and are low for the remainder of gestation and the very early neonatal period. There is a second peak of testosterone, with associated high levels of INSL3, at 2 months postpartum that has been associated with the "extra" population of Leydig cells in the human (termed neonatal Leydig cells—see below) [9–11]. This post-natal testosterone surge is often referred to as "minipuberty" and may act to increase reproductive growth and alter neurobehavioral development in boys [12]. Beyond the neonatal period, Leydig cell numbers regress, although whether the fetal cells degenerate or remain in a morphologically unrecognized state is not clear. Either way, the interstitium contains few steroidogenically active cells during infancy [3]. The adult generation of Leydig cells starts to differentiate prepubertally but is not complete until adulthood [13] with serum levels of testosterone averaging 6 ng/ml (20 nmol/L) during adulthood [14]. Finally, there is a decline in testosterone secretion with aging, which is variable in its magnitude and time of onset. This age-related decline is multifactorial (see below), but it is likely to be associated with decreased testosterone production by the Leydig cells.

Fetal Leydig Cells

The testes begin to develop in the human fetus at around 6 weeks of gestation, with migration of the preSertoli cells from the coelomic epithelium and formation of the sex cords [15]. Fetal Leydig cells can be identified in the interstitial compartment by about eight weeks of gestation [16]. Studies in the mouse indicate that the fetal Leydig cells originate from two lineages: one arising from the coelomic epithelium and the other from cells associated with the vasculature along the gonad/mesonephros border [17]. Fetal Leydig cell numbers increase exponentially during the first half of the second trimester reaching a maximum number of about 2×10^6 around 18 weeks [7] before declining again toward birth [7, 18]. Testosterone is first detectable in the testis as early as 6–7 weeks of gestation [19] and rises toward the prenatal peak at 12–14 weeks [7, 20]. This peak in testosterone is due to the increasing number of fetal Leydig cells and to increasing levels of chorionic gonadotrophin (hCG) [21] which acts through the LHCGR to stimulate Leydig cell function. Testosterone levels decline in the second half of gestation as hCG levels drop markedly and Leydig cell numbers decline [8, 20]. Surviving Leydig cells in the second half of gestation are partly dependent on pituitary LH [22] for activity.

Neonatal Leydig Cells

Shortly after birth in the human, levels of LH rise and the number of Leydig cells increases leading to a neonatal surge in plasma testosterone levels at 2–3 months of age. At this stage, Leydig cells contain abundant smooth endoplasmic reticulum and mitochondria, as well as varying amounts of lipid droplets [13, 23, 24]. After the neonatal stage, Leydig cell numbers regress rapidly and become very scarce until six to eight years of age when they begin to increase toward adult levels. Well-differentiated Leydig cells are absent from the interstitial space during the quiescent phase, and in their place are partially differentiated Leydig cells and fibroblast-like cells. At this stage, Leydig cells are dispersed in a loose connective tissue matrix, and contain elongated nuclei with scarcely visible cytoplasm. It has been proposed that these partially differentiated Leydig cells and primitive fibroblasts are precursors of adult Leydig cells [25–27] although studies in the mouse would suggest other origins (see below). As suggested above, it has been proposed that the neonatal Leydig cells represent a third population of Leydig cells in the human, alongside the fetal and adult populations [6]. Given that fetal Leydig cells persist in the mouse, however, a simpler explanation would be that this neonatal peak of testosterone is due to re-activation of the fetal Leydig cells by the increase in LH levels which occurs at this time [4].

Adult Leydig Cells

The precursor cells for the adult Leydig cell population begin their transformation at 6–8 years of age, and the number of adult Leydig cells increases during puberty reaching a

maximum reported as 8×10^8 per testis in the young adult [13]. At this time, a third rise in testosterone concentrations occurs, and levels remain high into middle/old age. Thereafter, Leydig cell numbers may decline in men as they age past 60 years, but there is current uncertainty about this as discussed below (see Leydig cell aging). Either way, between ages 20 and 60, there is a relatively stable equilibrium in the number of Leydig cells, which make up about 4% of the volume of the mature testis [28].

Steroidogenic Function of Leydig Cells

The main function of Leydig cells is to synthesize and secrete androgens, primarily testosterone. In humans, testosterone is synthesized mainly through the Δ^5 pathway via dehydroepiandrosterone (DHEA) and androstenedione (Fig. 2.3) [29]. This requires the activities of four enzymes: cholesterol side chain cleavage (CYP11A1), 17α-hydroxylase/C_{17-20} lyase (CYP17A1), 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (HSDB3), and 17β-hydroxysteroid dehydrogenase type 3 (HSD17B3) [30]. Leydig cells are the only cells in the testis which express CYP11A1, HSDB3, and CYP17A1 and are, therefore, the sole site for cholesterol conversion to C_{19} steroids. In contrast, data from the mouse show that the HSD17B3 enzyme is expressed only in the Sertoli cells of the fetal/neonatal testis [31, 32] which means that fetal Leydig cells primarily secrete androstenedione (the Δ^4 pathway predominates in rodents) and Sertoli cells are required for testosterone synthesis. In the adult mouse, HSD17B3 is expressed solely in the Leydig cells and the Leydig cells alone produce testosterone [31, 32]. Whether this pattern of HSD17B3 expression and steroid synthesis is specific to the mouse or is also relevant to the human remains to be determined. During fetal development, testosterone from the fetal Leydig cells will masculinize the internal ducts and glands but masculinization of the external genitalia depends on the formation of DHT in the genital tubercle [33]. Formation of DHT can be from testosterone through the action of SRD5A2, as shown in Fig. 2.3, but an alternative pathway has also been described which bypasses testosterone, and this pathway may be equally important for fetal masculinization in the human [34].

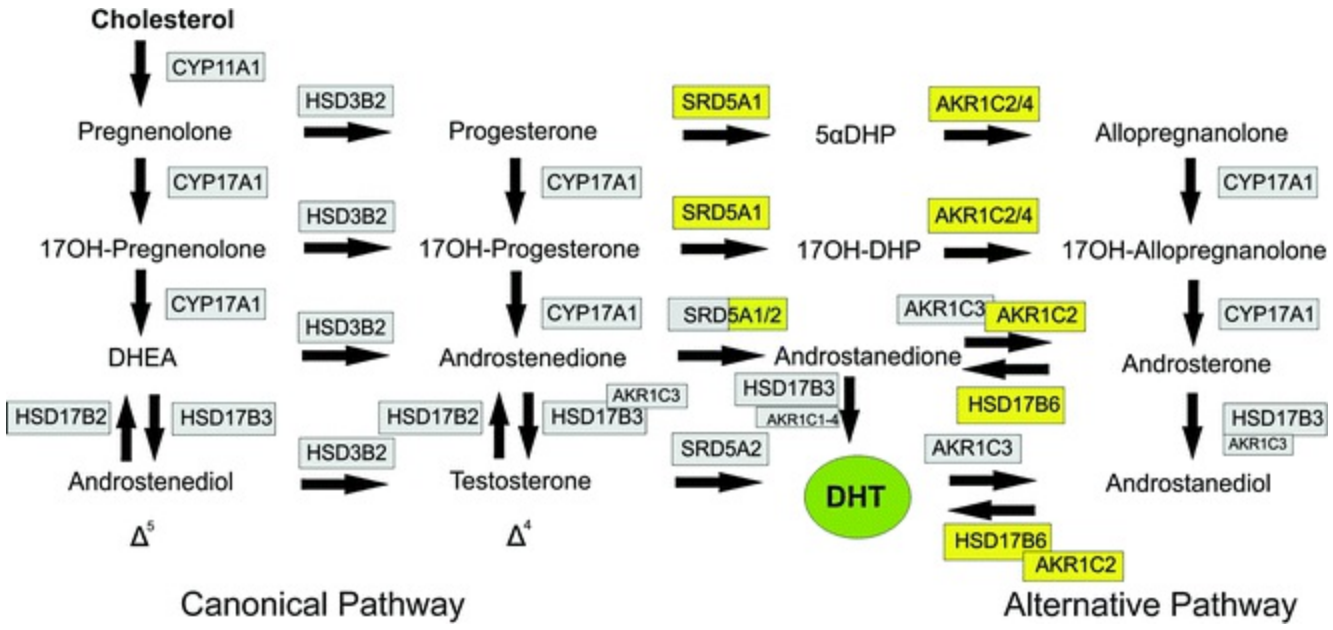

Fig. 2.3 Steroidogenic pathways leading to formation of dihydrotestosterone (DHT). The canonical pathways through Δ^4 and Δ^5 steroids are shown on the left with the alternative (backdoor) pathway on the right. Enzymes shaded in *gray* are required for the canonical pathway (with a number also required for the alternative pathway). Enzymes shaded in *yellow* are specific to the alternative pathway, Abbreviations; 5αDHP, dihyroprogesterone; 17OH-DHP, 17α-hydroxydihydroprogesterone; DHEA dehydroepiandrosterone; DHT, dihydrotestosterone, This Figure was published in Knobil and Neill's Physiology of Reproduction, Vol 1, PJ O'Shaughnessy, Testicular Development, Pages 567–594, Copyright Elsevier (2015). Reprinted with permission

Androgen Synthesis via the Canonical Pathway

Androgen synthesis from cholesterol depends initially upon the transport of cholesterol from intracellular sources to the inner mitochondrial membrane, and subsequent loading of cholesterol into the catalytic site of CYP11A1 [35]. Cholesterol is hydrophobic and cannot cross the aqueous intermembrane space of mitochondria to reach the CYP11A1 enzyme rapidly enough by simple diffusion to support acute steroid synthesis [36]. To overcome this problem, the cholesterol is transferred into the mitochondria through the transduceosome which includes the steroid acute regulator (STAR) protein, translocator protein, and voltage-dependent anion channel [37]. In the inner mitochondrial membrane, cholesterol is converted by CYP11A1 to pregnenolone and isocaproaldehyde [38] which is unstable and quickly oxidized to isocaproic acid. This reaction requires a mitochondrial electron transfer system of adrenodoxin and adrenodoxin reductase to convey electrons from NAPDH to CYP11A1 [35].

Pregnenolone can act as substrate for testosterone synthesis through two different routes in the canonical pathway—the Δ^4 and Δ^5 pathways—so called because of the position of one of the double bonds on the steroid backbone. The particular pathway taken is species-dependent and is regulated by the relative affinities of the converting enzymes for different substrates. In humans, the pathway is predominantly Δ^5 because

CYP17A1 has a higher binding affinity for pregnenolone than HDS3B [39]. In rodents, CYP17A1 has a higher affinity for progesterone, and the Δ^4 pathway predominates [40]. In human Leydig cells, therefore, the pathway is predominantly through pregnenolone, 17α-hydroxypregnenolone, DHEA, androstenedione, and testosterone.

The enzymes involved in the canonical pathway are located in the mitochondria (CYP11A1, HSD3B2) and in the smooth endoplasmic reticulum (CYP17A1, HSD17B3) [30, 41]. In humans, there are two genes encoding HSD3B (types I and II) and both enzymes show the same activity although only HSD3B2 is expressed in the Leydig cells [42, 43]. The CYP17A1 enzyme carries out two steps in the bioconversion of pregnenolone and progesterone to the C_{19} steroids, DHEA, and androstenedione, respectively, with 17α-hydroxypregnenolone or 17α-hydroxyprogesterone as transient intermediates, although human CYP17A1 fails to show detectable activity with 17α-hydroxyprogesterone as substrate [40]. The final step in the formation of testosterone is the conversion of androstenedione to testosterone, catalyzed by HSD17B3. This enzyme is part of a family of 14 enzymes that show related activities [44], and in humans, HSD17B1 and HSD17B5 (a member of the aldo/keto reductase superfamily, also known as AKR1C3) are also able to carry out the same reaction [44]. It is clear, however, that in the human fetal testis at least, the HSD17B3 is predominant since XY individuals lacking functional HSD17B3 activity have a significantly reduced ratio of testosterone/androstenedione and fail to masculinize externally during fetal development [45]. Interestingly, testosterone levels rise at puberty in these individuals and virilization does occur leading to the suggestion that HSD17B5 may be of importance in the adult Leydig cells [46].

Androgen Synthesis via the Alternative Pathway

Individuals who are genetic males but with disorders of activity in the enzymes of the canonical pathway will show disordered sex development (DSD) with incompletely masculinized external genitalia. Some cases of DSD (with normal androgen receptor signaling) cannot be explained by alterations in the canonical pathway, however, suggesting that other pathways/mechanisms are involved in androgen synthesis and masculinization [47]. In 2003, Wilson and colleagues described an alternative "backdoor" pathway of androgen synthesis in the testes of the pouch young tammar wallaby [48] (Fig. 2.3), and this pathway was subsequently shown to be active in the prepubertal mouse testis [49]. The importance of this pathway to human masculinization became apparent when unrelated individuals with DSD were shown to have disorders in one or more of the enzymes involved in the alternative pathway [34]. The non-functional/partially functional enzymes in these individuals were AKR1C4 and/or AKR1C2 which are both required for the alternative pathway but will not affect the canonical pathway (Fig. 2.3). Barring some other unknown mutation in these

individuals, this would suggest that *both* canonical and alternative pathways to DHT synthesis are required during human male fetal development to ensure normal masculinization. Transcripts encoding enzymes involved in the alternative pathway have been shown to be present in the fetal human testis [34], and it has been assumed that testes secrete DHT via this pathway [34]. This is not clear, however, since earlier studies have reported little or no DHT synthesis by the human fetal testis [50, 51] suggesting that all DHT synthesis must take place at the target organ. It is also not clear whether the testes are the only organs involved in the alternative pathway since the fetal human liver also expresses at least some of the same enzymes [52]. Further studies are needed, therefore, to identify which tissues are involved in the alternative pathway, and to measure the levels of the different pathway intermediates in the fetal circulation.

Estrogen Synthesis by Human Leydig Cells

Estrogens are formed by aromatization of androstenedione or testosterone by the enzyme CYP19A1. In the testis of most species, aromatase activity is detectable in Leydig cells, Sertoli cells, and germ cells [53], and the relative contributions of each of these testicular cell types to testicular aromatase activity varies with age and between species. In the rat and mouse, Sertoli cells contribute to testicular aromatase activity in immature animals while germ cells are a significant site of activity in the adult [53]. Sertoli cells from juvenile Rhesus monkeys are reported to express aromatase activity, [54] although, in humans, Leydig cells appear to be the only source of testicular estrogens at all ages [55, 56].

Androgen Secretion by the Leydig Cells/Testis

Once synthesized, the lipophilic androgens move out of the Leydig cells by passive diffusion, down the concentration gradient. Within the testis, testosterone and precursors diffuse freely into the interstitial space and enter the testicular blood capillaries that are immediately adjacent to Leydig cells [57]. Interestingly, it is this process of testosterone release into the testicular vascular bed which might be altered in Klinefelter syndrome leading to reduced circulating testosterone levels [58]. Once they are part of the systemic circulation, secreted testosterone binds to plasma proteins and is present in both bound and unbound forms. In adult humans, more than 95% of testosterone is complexed with proteins, both the high affinity ($K_D = 1\ nM$) sex hormone binding globulin (SHBG) and the low affinity ($K_D = 1000\ nM$) albumin. The proportion of testosterone that is unbound or loosely bound represents the biologically active fraction, which freely diffuses from capillaries into cells. The SHBG-bound fraction is thought to act as a reservoir for the steroid, although SHBG-bound steroids may also enter cells via endocytic receptors on the surface of target cells and contribute to hormone action [58]. Increasing levels of SHBG during aging contributes to reduced free plasma

testosterone during this period (see below, Leydig cell aging).

Other Functions of the Leydig Cell

The principal function of the Leydig cells is to produce androgen, but the cells are also the only source of INSL3 during fetal development [59, 60]. This hormone is essential, along with androgen, for inducing normal testicular descent although INSL3-receptors (RXFP2) are found on Leydig cells and on germ cells, and there is evidence that INSL3 can act to reduce germ cell apoptosis [61]. Any role that INSL3 plays in Leydig cell function, however, is likely to be restricted to the period around early puberty [61]. In order to identify other functions of the Leydig cells, Leydig cell-specific transcripts in the adult rat have been identified on the assumption that at least some of these transcripts are likely to be involved in cell-specific functions [62, 63]. Apart from transcripts encoding components of the steroidogenic apparatus, the most common predicted function of translated proteins from these cell-specific transcripts is endogenous and xeno-toxicant metabolism and reduction in oxidative stress [62]. The Leydig cells may, therefore, play a significant role in protecting the adult testis from damage caused by toxicants or by stress.

Regulation of Leydig Cell Development and Function

Leydig Cell Development

Most information available on the control of Leydig cell development comes from rodent models, and there is no reason to doubt that these fundamentals are significantly different in the human, but caution needs to be maintained when extrapolating between species.

Initial development of the fetal Leydig cells is dependent upon the Sertoli cells and, in particular, upon Desert Hedgehog (DHH) and Platelet-derived Growth Factor-α (PDGFA) released by the Sertoli cells [64–66]. In the absence of DHH in mice, there is a marked reduction in fetal Leydig cell numbers, reduced androgen levels and failure of masculinization, with a similar phenotype also seen in humans with a mutation in DHH [67, 68]. Similarly, reduced fetal Leydig cell numbers are seen in mice lacking PDGFA [69], and it is likely that the effect of both DHH and PDGFA is to promote expansion of the fetal Leydig cell precursor population [69]. In contrast, both NOTCH and WNT4 signaling act to inhibit fetal Leydig cell differentiation and WNT4 appears to be important in preventing Leydig cell development in the fetal ovary [70, 71]. Interestingly, downregulation of Wilm's tumor gene (WT1) may also be required for fetal Leydig cell development, with over-expression in fetal Leydig cells leading to development of a Sertoli cell-like phenotype [72, 73]. A number of homeoproteins (ARX, LHX9, PBX1, and RHOX4) are expressed in interstitial cells, and they have

been shown to be involved in testis development. Only ARX has, so far, been linked directly to fetal Leydig cell development [74], however, possibly through an action on the progenitor cells [75]. In rodents, secretion of LH by the fetal pituitary is not required for fetal Leydig cell activity [76, 77], and once formed, the cells appear to function largely independently of the fetal Sertoli cells [78]. Similarly, fetal Leydig cell activity in humans is not dependent on fetal pituitary LH [79] but, unlike rodents, activation of the LHCGR is essential [80], indicating that hCG is required in humans to stimulate Leydig cell activity in utero [22].

The adult Leydig cells develop from peritubular precursors [81–83] which may be stem cells [84]. After initial differentiation, the cells undergo one or two mitotic divisions to reach the final population size [85]. The initial process of adult Leydig cell differentiation is completely dependent on the Sertoli cells [78] since Sertoli cell ablation in the neonatal mouse leads to general failure of adult Leydig cell differentiation except in regions where Sertoli cells or Sertoli-like cells have survived ablation [78]. This failure of adult Leydig cell development is associated with apparent loss of Leydig cell precursor cells, although it is likely that the Sertoli cells are also directly involved in stimulating Leydig cell differentiation since DHH appears to be required for normal adult Leydig cell development, perhaps through inducing stem cell commitment [64, 86, 87]. The Sertoli cell-derived factor anti-Müllerian hormone (AMH) may also be involved in the regulation of adult Leydig cell development with a pubertal decrease in AMH required for normal maturation [88]. It has been shown that the orphan nuclear receptor NR2F2 is necessary for adult Leydig cell development, possibly through development of the progenitors and through maturation of the differentiated cells [89]. PDGFA is also involved in the differentiation process although the origin of this factor in the post-natal testis is not clear [90–93]. In addition to the requirement for Sertoli cells, it is clear that adult Leydig cell development is critically dependent on LH. Progenitor Leydig cells do not express the LH receptor [94], and initial Leydig cell differentiation is LH-independent [95, 96] but, in the absence of LH or the LH receptor, there is a marked reduction in the number of Leydig cells that develop in the adult [97]. Similarly, in mice with enhanced chronic exposure to LH activity, there is hyperplasia of the adult Leydig cell population [98]. Overall, therefore, the data suggest that initial Leydig cell differentiation is Sertoli cell-dependent/LH-independent, but that further development of the cells is critically dependent on LH.

One other factor of importance in Leydig cell development is androgen. In mice lacking androgen receptors, there is failure of normal adult Leydig cell development, with a reduction in Leydig cell number, and those cells which do develop lack many transcripts/proteins which are associated with the adult cell population [99, 100]. In mice with a more specific deletion of androgen receptors only in the Leydig cell, there is also inhibition of cell maturation although the effect is less marked than with complete androgen receptor deletion [101]. Leydig cell numbers are unaffected in

Leydig cell-specific knockouts and so androgen effects on Leydig cell development appear to be a mix of direct effects on the cells and indirect effects through other cells which express androgen receptors such as the peritubular myoid cells and Sertoli cells. In humans lacking functional androgen receptors, Leydig cell dysfunction appears to be less marked than in the mouse, with circulating testosterone levels in the normal range, albeit with high circulating LH levels [102–105]. This difference may be accounted for by loss of CYP17A1 in mice lacking androgen receptors [106], as the CYP17A1 enzyme levels appear to be normal, or possibly increased, in androgen-insensitive humans [107]. Interestingly, in both mouse and human, loss of androgen receptors leads to late-onset Leydig cell apoptosis, an event that is very rare in normal Leydig cell populations [101]. Androgenic stimulation also appears to be required by the adult Leydig cell stem cells during fetal life to ensure normal stem cell numbers in the adult [108] which may explain evidence suggesting that reduced fetal androgen exposure is associated with lower adult male androgen levels [109].

Regulation of Leydig Cell Activity

The main regulator of Leydig cell activity is LH, and two types of responses to LH are seen. The first, acute response triggers a rapid production of steroid within minutes [110] through the binding of LH to the receptor, stimulation of adenylate cyclase, and the formation of the second messenger adenosine 3',5'-cyclic monophosphate (cAMP) (Fig. 2.4). Increased cAMP causes subsequent phosphorylation of proteins via protein kinase A or C [111, 112] in a cascade of events leading to increased STAR phosphorylation and expression and increased testosterone synthesis. The increase in cAMP also leads to the activation of a Ca^{2+}-signaling pathway [113] and increased NR4A1-mediated hormone-stimulated STAR expression [114]. In addition, LH-induced cAMP signaling in the Leydig cell transactivates the epidermal growth factor receptor (EGFR) leading to activation and expression of STAR protein [115] (Fig. 2.4). The second effect of LH is a long-term trophic effect on the Leydig cells mediated through transcriptional regulation. LH signaling acts through phosphorylation of transcription factors to enhance transcription from cyclic AMP-response elements (CREs) in the promoters for several LH target genes including STAR, CYP11A1, and HSD3B [110, 116]. Some target genes lack a consensus CRE, and expression is modulated by interaction between different transcription factors such as NR5A1 [117]. Overall, in the longer term, LH acts to maintain the steroidogenic enzyme activity of the Leydig cell and the steroidogenic apparatus. This can be seen clearly in animals which lack LH stimulation either through gene mutation/manipulation or after treatment such as hypophysectomy. In *hpg* or LHRKO mice, which both lack LH stimulation; the Leydig cells are smaller and contain large lipid droplets; steroidogenic enzyme transcript level/activity is markedly reduced and androgen output in response to trophic

stimulation is very low [95–97, 118].

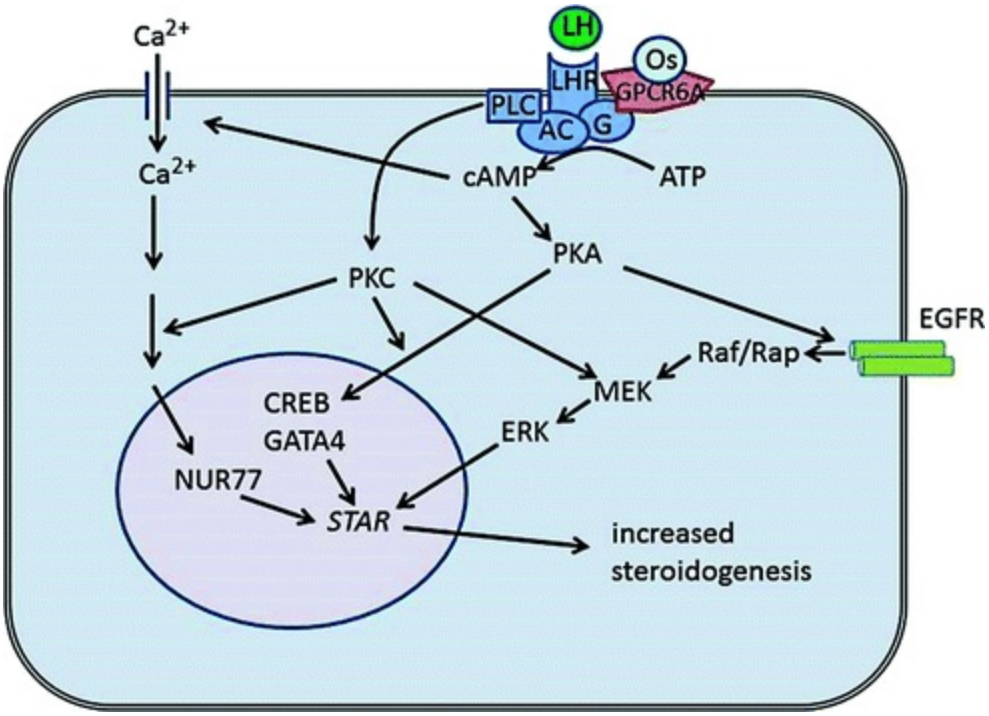

Fig. 2.4 Mechanisms of hormone-mediated steroid production in Leydig cells. LH is the major endocrine regulator of Leydig cell steroidogenesis, and binding of LH to the LH receptor (LHR also termed LHCGR) leads to activation of adenylate cyclase (AC). Osteocalcin (Os) also activates adenylate cyclase through binding to GPRC6A. Activation of adenylate cyclase leads to an increase in cAMP which, in turn, causes influx of Ca^{2+} into the cells and induces activation of various kinases including protein kinase A (PKA). This leads to activation of transcription factors (e.g. CREB, GATA4, NUR77) and induced expression of genes involved in steroid hormone synthesis, including STAR. Increased cAMP also activates the MAP kinase pathway via activation of the epidermal growth factors receptor (EGFR) which leads to increased expression and phosphorylation of STAR. The overall effect in the short term is to increase testosterone synthesis by the Leydig cells and, in the longer term, to increase transcription of the steroidogenic enzymes

In addition to LH, adrenocorticotrophic hormone (ACTH) can act to regulate fetal Leydig cell function in the mouse, although it does not appear to have any effect on adult Leydig cells [119]. Isolated fetal/neonatal mouse testes or Leydig cells will respond rapidly in vitro to physiological levels of ACTH with a marked rise in testosterone, similar to that seen in response to LH [119, 120]. It is not clear that ACTH plays a physiological role in development of the fetal Leydig cells in mice, however, since androgen levels are normal in mice lacking ACTH or ACTH and LH [121]. It remains to be seen whether ACTH is of importance to fetal Leydig cell function in species, such as the human, which require trophic endocrine support for normal fetal androgen production. Responsiveness of fetal Leydig cells to ACTH in the mouse does raise one interesting aspect of these cells and that is their similarity to adrenocortical cells. It has been reported that at least some of the fetal Leydig cells may derive from the same progenitor population as the adrenocortical cells [122], and at least some of the cells

show distinct similarities both in hormone responsiveness and steroidogenic enzyme expression [123–125]. Whether these are a subpopulation of normal fetal Leydig cells or ectopic adrenal cells which have been shown to give rise to adrenal rest tumors in the testis [126] remains to be seen.

In rodents, the fetal Leydig cells are also responsive to a variety of local and endocrine factors such as pituitary adenylate cyclase-activating polypeptide, vasoactive intestinal peptide, and natriuretic peptides [127–129]. Since fetal Leydig cells in rodents do not specifically require LH, it is possible that activation of the cells in vivo is through multiple redundant mechanisms involving LH, ACTH, and activating peptides. A redundant mechanism such as this may have evolved to ensure that sufficient Leydig cell activation occurs to induce fetal masculinization. Whether human fetal Leydig cells are also sensitive to multiple agonists is not known, although it is clear that human fetal androgen production is critically dependent on LHCGR stimulation [80].

Recently, the bone protein osteocalcin has been reported to act as a trophic hormone on adult Leydig cells via the GPRC6A receptor [130, 131] (Fig. 2.4). Osteocalcin was shown to have a direct effect on testosterone synthesis by adult Leydig cells, and circulating testosterone levels and seminal vesicle weights were significantly reduced in mice lacking osteocalcin [130]. These effects were less marked than in mice lacking LH, but the fertility of the osteocalcin-deficient mice was reduced indicating that osteocalcin is required for optimal reproductive function. Evidence from two human patients with primary testicular failure linked to mutations in GPRC6A is also supportive of the hypothesis that osteocalcin can regulate testicular function [131]. The link between steroid hormones and bone mass has been known for a number of years but these data show that the skeleton can regulate Leydig cell function in a classic endocrine feedback loop [132].

In addition to the direct effects of LH, ACTH, and osteocalcin on Leydig cell function, follicle stimulating hormone (FSH) may play an indirect stimulatory role in the regulation of Leydig cells. Evidence for an effect of FSH comes from gonadotrophin-deficient mice and rats treated with FSH, and from differences in Leydig cell function between control mice and animals lacking FSH stimulation [133–137]. In addition to mice, there is also evidence that FSH can stimulate Leydig cell function indirectly in other species including humans [18, 138–140]. FSH receptors are restricted to the Sertoli cells [141], and so FSH effects on the Leydig cells must be mediated through Sertoli-secreted factors. The effects of FSH are fairly rapid, however, with a response seen in *hpg* mice in less than 4 h [135] suggesting that whatever paracrine factors are involved they must be acting directly on the Leydig cells. These effects of FSH are consistent with recent studies which show that ablation of the Sertoli cells in the adult mouse causes a marked reduction in Leydig cell number within 30 days [142], demonstrating that Sertoli cell factors are required for maintenance of the adult Leydig cell population. The identity of these factors remains unknown but an obvious candidate

is DHH which is closely involved in Leydig cell development and continues to be secreted specifically by the Sertoli cells into adulthood.

Clinical Aspects

Leydig Cell Aging

Most studies show that plasma levels of total testosterone in men fall between 1% and 2% per year beginning at about age 40, although free testosterone declines more rapidly (~3% per year) as levels of SHBG increase at the same time [143–145] (Fig. 2.5). This decline in testosterone is multifactorial but can be divided into primary/compensated hypogonadism (low/normal testosterone with high LH) which is primarily linked to aging and secondary hypogonadism (low testosterone with low LH) which does not appear to be age-related but is clearly linked to obesity [146]. Metabolic clearance of testosterone slows with age [147] (which would tend to increase circulating testosterone), and so the increased LH seen in primary/compensated hypogonadism is evidence that the primary endocrine failure associated with aging is likely to be at the level of the testis [148]. Also, it has been shown that the circulating androgen response to increased LH declines with age in humans [148, 149] as might be expected from primary testicular failure.

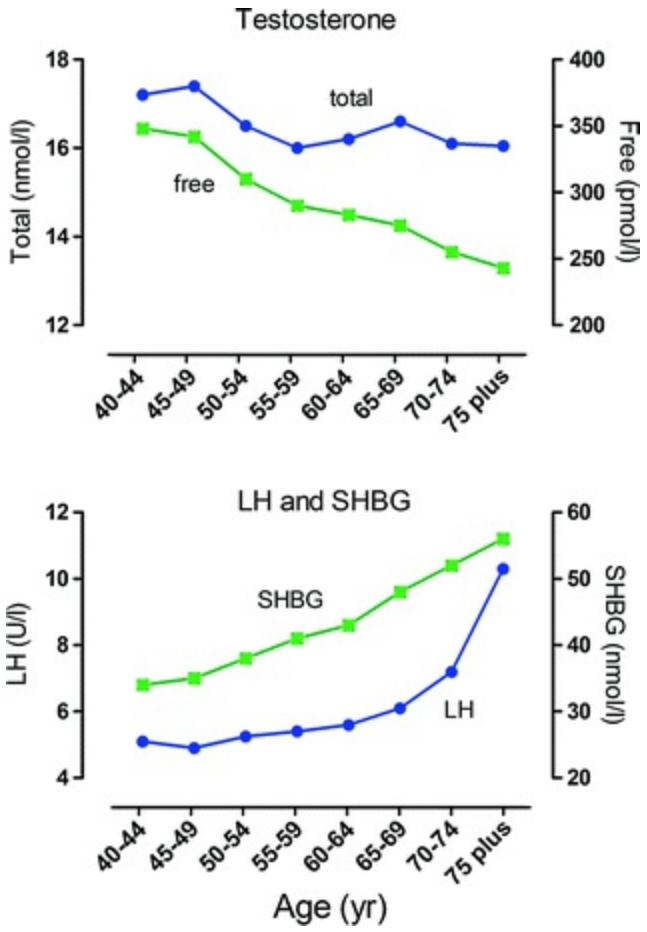

Fig. 2.5 Age-dependent changes in male hormone levels. These graphs show the relationship between age and hormone levels in men. Mean hormone values at 5-year age bands are shown based on data from 3220 men. Men with known pituitary or testicular diseases or current use of medications that could affect pituitary or testicular function or sex-steroid clearance were excluded. Total testosterone and free testosterone were significantly lower and luteinizing hormone (LH) and sex hormone binding globulin (SHBG) significantly higher in the older age groups. There was an apparent inflection point around 70 year for LH, Data shown in these figures are from [145] with permission of the authors

Age-related declines in testicular testosterone output could be caused by decreased Leydig cell numbers and/or reduced steroidogenic ability. There are a number of studies which report that Leydig cell numbers decline with age in the human population [150–153], although a more recent study found no age-related change in Leydig cell number [154]. Counting cell numbers in the testis is prone to technical problems which may have affected the older studies while the number of men over 60 in the more recent report [154] was only 4, so whether Leydig cell number declines with aging will remain uncertain until further studies are carried out.

Whether or not Leydig cell numbers decline with age, there is good evidence for degenerative changes in the cells including cytoplasmic or intranuclear crystalline inclusions, lipofuscin granules, diminished smooth endoplasmic reticulum, and smaller and fewer mitochondria when compared to young men [155–157]. Older men with higher serum LH and lower serum testosterone levels also have a large number of

abnormal Leydig cells, suggesting that Leydig cell structural changes are related to changes in steroidogenic function [155]. Studies of intratesticular steroid levels in aging men do not show a specific lesion in the steroidogenic pathway but overall levels are lower, and there is evidence of reduced mitochondrial steroid production [158]. Using rat models in which the primary aging deficit is at the level of the Leydig cells, it has been shown that Leydig cell aging in the rat is associated with multiple defects in the steroidogenic machinery from reduced LH-dependent cAMP production to reduced steroidogenic enzyme levels [159]. This deterioration of Leydig cell function may be related to alterations in the redox balance of the cells leading to increased superoxide content with aging [159]. It is also likely, however, that other factors also contribute to the decline in Leydig cell function with age, as newly formed Leydig cells in testes from aged Norway rats show a rapid decline in steroidogenic function [160]. This does not appear to be due to changes in LH but may be caused by alterations in the levels of other trophic factors, changes in cell–cell signaling in the testis or vascular re-modeling associated with aging.

Leydig Cell Tumors

Leydig cell tumors were first identified by Sacchi in 1895, and are the most common interstitial tumors although they are rare overall and account for only 1–3% of testicular tumors [161–163]. Most Leydig cell tumors are unilateral (only 3% are bilateral [164]) and can appear at any age, although there is a peak incidence before puberty (between 5 and 10 years) and a second larger peak between 30 and 60 years. The tumors produce androgens, mainly testosterone, but serum estrogen levels may be elevated either through direct production of estrogen by the tumor or by peripheral aromatization of secreted androgen [165]. In boys, Leydig cell tumors are uniformly benign, hormonally active tumors, and account for about 10% of cases of precocious puberty [166]. In adults, most Leydig cell tumors are benign, and patients present with a painless testicular mass which may be palpable but is usually an incidental finding on scrotal ultrasonography for other conditions [167]. Small non-palpable Leydig cell tumors which are not visible on ultrasonograms can be seen by magnetic resonance imaging [168]. Where there is significant estrogen production, gynecomastia may be present along with loss of libido, erectile dysfunction, impotence, and infertility [165]. In adults, approximately 10% of Leydig cell tumors are malignant [162, 163] with regional lymph nodes, liver, lungs, and bone, the most common sites of metastases [162].

Macroscopically, the lesions associated with Leydig cell tumors are generally small, yellow to brown, well circumscribed and rarely hemorrhagic or necrotic. Microscopically, four different cell types can be found, ranging from large polygonal cells to spindle-shaped sarcomatoid cells [165, 169]. The cells have round nuclei with eosinophilic granular cytoplasm containing lipid vacuoles, lipofuscin granules, and

Reinke's crystals present in about one third of the cases [170] (Fig. 2.6). Ultrastructually, the cells show features expected of steroid-secreting cells, including abundant smooth endoplasmic reticulum.

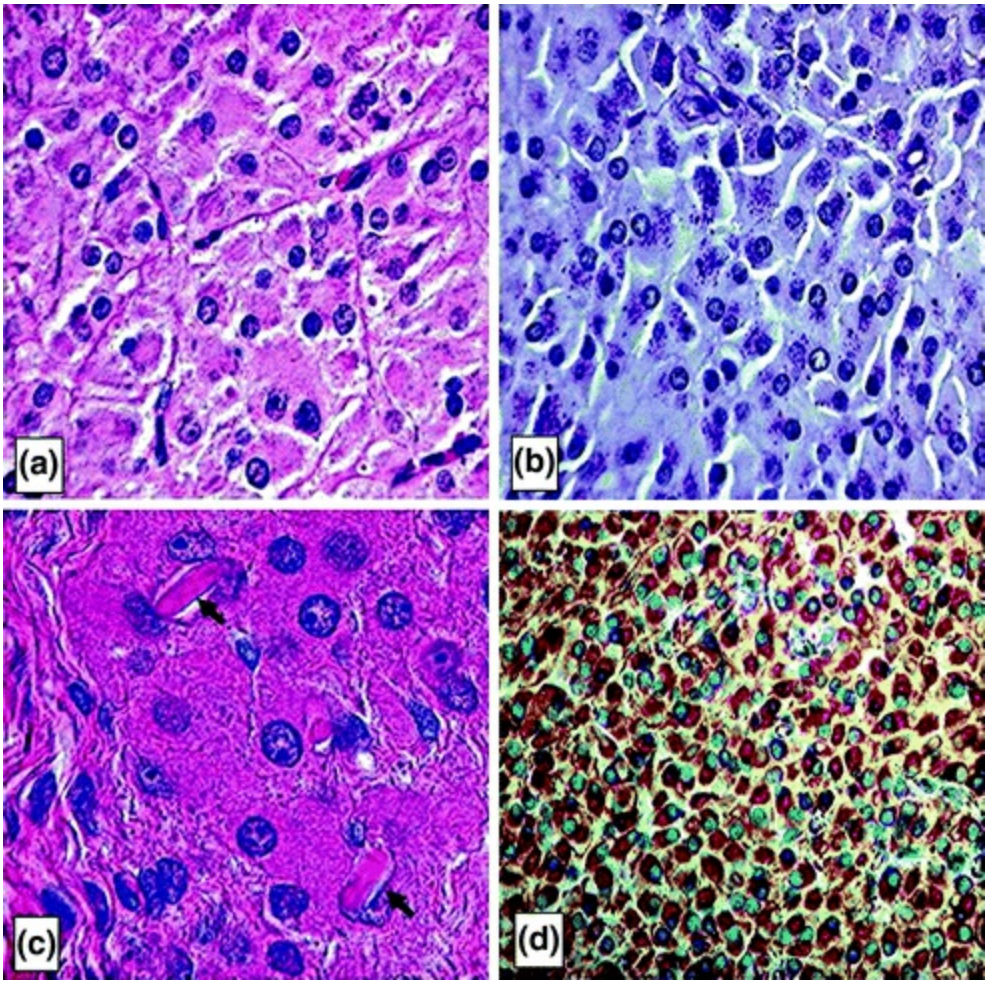

Fig. 2.6 Leydig cell tumors. Histologic sections of Leydig cell tumors. **a** Leydig cell tumor cells have abundant, eosinophilic cytoplasm with regular round nuclei, distinct cell borders and fibrovascular septa between the tumor cells. **b** Periodic acid Schiff stain which highlights the cytoplasmic lipofuscin granules in the tumor cells, a diagnostic clue to Leydig cell tumors. **c** Higher power photomicrograph showing intracytoplasmic Reinke crystals in a Leydig cell tumor (*arrows*). The crystals appear as refractile, cylindrical, rectangular, or rhomboid structures, and their identification is helpful for distinguishing Leydig cell tumors from other similar lesions (hematoxylin-eosin). **d** Leydig cell tumors typically show strong, diffuse cytoplasmic reactivity for α-inhibin (INHA) which is shown here as a brown stain. INHA is a sensitive and specific marker that can be used to separate testicular sex cord–stromal tumors, including Leydig cell tumors, from germ cell tumors, Reprinted from Al-Agha & Axiotis, An In-Depth Look at Leydig Cell Tumor of the Testis. Arch Pathol Lab Med. 2007; Vol 131 (issue 2): pp 311–317 with permission from Archives of Pathology & Laboratory Medicine. Copyright 2007 College of American Pathologists [165]

Classically, the primary treatment for Leydig cell tumors has been radical orchiectomy, and it remains in use for malignant cases. Testis-sparing surgery, with enucleation of the mass, has proved to be a feasible and safe choice, however, and is increasingly being reported for benign cases. A recent study of patients with Leydig cell

tumors found a 100% disease-free survival with no local recurrences or metastases following testis-sparing surgery [171]. Testis-sparing surgery should also be considered for children who present with the clinical and biochemical findings typical of Leydig cell tumors, and an ultrasonographically defined encapsulated intratesticular mass [172]. Malignant Leydig cell tumors are radio-resistant and chemo-resistant, and have a poor prognosis with median survival time of 2 years [173].

The etiology of Leydig cell tumors remains uncertain, particularly in adults, but somatic activating mutations of the LHCGR have been found in a number of these tumors in boys [174]. This is consistent with the gonadotropin-independent nature of these tumors and with the development of fetal Leydig cell tumors in mice exposed to persistently high levels of hCG [175]. Other somatic mutations are also likely to be involved although a link to activating mutations in genes such as the Gs α-subunit of the stimulatory G protein (GNAS) have not been shown [174]. Leydig cell tumors in adults may be derived from the adult population of Leydig cells which would be consistent with the presence of Reinke's crystals in some tumors as fetal Leydig cells lack these structures [176]. This may mean that the etiology of adult tumors differs from tumors in boys. The tyrosine kinase inhibitor imatinib has been reported to show chemotherapeutic activity in animal models [177], but this activity has not been demonstrated in an adult human trial [178].

Leydig cell hyperplasia shares the same clinical presentation as a Leydig cell tumor, including painful gynecomastia and decreased libido in adults, precocious puberty in children, and infertility or palpable testicular masses [179]. It should be noted, however, that many cases of apparent Leydig cell hyperplasia reported in the literature, in both humans and rodent models, are due to loss of germ cells (e.g., through cryptorchidism) causing shrinkage of the seminiferous tubules and an apparent increase in relative interstitial volume. Stereological measurement of Leydig cell numbers in these cases will often show no change in Leydig cell number per testis [180]. It is significant, therefore, that reported clinical Leydig cell hyperplasia is always associated with spermatogenic failure [179]. However, there is no doubt that true Leydig cell hyperplasia can occur if LH (or hCG) levels are elevated [179], and the hyperplastic Leydig cells are generally arranged in diffuse, multifocal, small nodules and show frequent mitoses, necrosis, and vascular invasion. Hyperplastic Leydig cells usually infiltrate between seminiferous tubules while benign Leydig cell tumors form nodules that compress surrounding tubules.

Leydig Cell Toxicology

As described above, the Leydig cells express a number of metabolic enzymes which might be expected to inactivate xenotoxicants and thereby protect the spermatogenic and steroidogenic function of the testis [62]. At the same time, however, the Leydig cells

themselves are the potential target of a number of possible toxicants. This is an area which has seen a marked increase in publication activity in the last 10 years, with a considerable number of potential toxicants identified, and some of the better characterized/most relevant substances include phthalates, bisphenol A, statins, and ethanol. Phthalates are present in food packaging, cosmetics, and medical devices such as tubings and catheters, and so human exposure is significant with particular concern about fetal exposure [181]. Inhibitory effects of phthalates on rodent Leydig cell development and function are well documented, particularly with respect to the fetal Leydig cells, and may be related to altered expression of NR2F2 [182]. Changes to fetal Leydig cell development would be likely to affect normal masculinization of the fetus and are, therefore, potentially serious, but the effects of phthalates may be species-dependent with no clear effect seen in human fetal testis organ culture [181, 183], although there may be effects on adult human Leydig cells [184]. Bisphenol A is ubiquitous in the environment and is used primarily to manufacture polycarbonate plastic or as a non-polymer additive to other plastics and to epoxy resins. Bisphenol A has estrogenic activity and is classed as an endocrine disrupting compound, but it is also reported to directly inhibit fetal Leydig cell function in both rats and humans [185, 186] and is of ongoing concern. Statins act to inhibit cholesterol synthesis, and are taken daily by an estimated 20 million men, many of whom are more than 60 years old, and so with aging Leydig cells. Leydig cells need cholesterol as substrate for androgen synthesis (Fig. 2.3), some of which comes from circulating lipoproteins and some from *de novo* synthesis. A recent meta-analysis reported that statins reduce circulating testosterone concentration in men [187] which would be consistent with reported inhibitory effects in vitro on rat Leydig cells [188, 189], although no studies have yet been reported using isolated human Leydig cells. Long-term abuse of ethanol reduces circulating testosterone levels which may be a combination of direct effects on the Leydig cells [190, 191] with changes in circulating LH. Most studies report normal or elevated LH following alcohol abuse but the gonadotrophin response to pituitary stimulation is reduced suggesting altered hypothalamic pituitary function [190, 192, 193]. Finally, it has been shown that the alkylating agent ethane dimethane sulfonate (EDS) can act as a specific cytotoxicant in Leydig cells. This effect is species-specific with complete Leydig cell ablation seen in rats within 24 h but with little effect seen in the mouse, dog, and monkey [194]. It is still not known why the cytotoxic effect is specific to Leydig cells in the rat, but EDS has proven particularly useful in the study of Leydig cell biology [194].

Conclusions—Future Work

The importance of testosterone to adult male health is increasingly recognized, with an established link now made between low testosterone and the onset of conditions such as

obesity, metabolic syndrome, and type 2 diabetes [1, 195–197]. It is clear, therefore, that we need to understand how Leydig cells are regulated in the adult human, and why testosterone levels are low in some individuals. In general, our knowledge and understanding of testis biology and Leydig cell function are expanding quickly due largely to the availability of transgenic mouse models which allow hormonal control and cell–cell interactions to be dissected and examined. With the important exception of fetal development, specific studies on the human Leydig cell are limited, however, and it is clear that more human-specific information is needed, particularly for important areas such as Leydig cell aging and toxicology. There has been increased availability of human fetal tissues for research in recent years which means that our understanding of this phase of human testicular development is improving, although use of human fetal material remains controversial in the USA (http://tinyurl.com/hrxj29r). It is known that fetal programming can have a marked effect on adult health [198], and there is evidence that low testosterone in the fetus is associated with reduced adult testosterone levels [109, 199] perhaps through changes in Leydig stem cell development [108]. The recent identification of factors which appear to be required for Leydig stem cell differentiation in the rat [87] highlights an area which is likely to be of considerable focus in coming years as interest develops in the manipulation of this process.

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3. Human Spermatogenesis and Its Regulation

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Introduction

In human spermatogenesis, undifferentiated spermatogonia derived from spermatogonial stem cells give rise to diploid spermatocytes which undergo meiosis I/II to produce haploid spermatids [1–3]. Spermatids, in turn, undergo spermiogenesis to form functional spermatozoa [3–5]. After puberty at ~12 years of age, a healthy man produces upwards of 200 million sperm daily from his 20 s through 50 s [6, 7], which declines

gradually with a reduction of ~50% by his 80 s [8, 9]. Spermatogenesis is composed of a series of cellular events which includes (i) self-renewal of spermatogonial stem cells and spermatogonia via mitosis, (ii) transformation and differentiation of spermatocytes, (iii) generation of haploid spermatids via meiosis I/II, and (iv) final morphological maturation of spermatids to become spermatozoa via spermiogenesis. These processes thus involve multiple cellular events in the testis and are under complex controls by regulatory hormonal and signaling axes. The most important of these regulatory pathways is the hypothalamic–pituitary–testicular axis involving both FSH and LH, and testosterone and estradiol-17ß as key regulators [10–14]. Since the subject of hormonal regulation of spermatogenesis in men has been extensively reviewed [10–15], we focus our discussion herein on other topics in which advances have been made in recent years. A better understanding of human spermatogenesis is necessary since about 15% of married couples are infertile, and half of these cases are attributed to male factors, indicating that about 7% of men in the general population are infertile [16]. In this context, it is of interest to note that male reproductive health can be considerably altered by the environment and life style factors [17–21]. Semen quality is believed to be declining [22–25], illustrating the global trend of impaired male reproductive health. While 6–18% of non-obstructive azoospermia or severe oligozoospermia cases can be attributed to Y chromosome microdeletions [26], as many as 25–40% of infertile men have no identifiable cause for hypospermatogenesis [27, 28]. In fact, infertility is an emerging global public health issue right after cancer and cardiovascular diseases. Interestingly, experiments using mouse genetic models, including knockout/knockin/gene-trapped, transgenic, and chemical-induced point mutant mice, have identified more than 400 genes pertinent to spermatogenesis [28, 29], and the use of proteomics, epigenomics, and genomics have identified >2300 genes pertinent to spermatogenesis and/or testis function. However, how this information applies to humans remains largely unknown [30]. Future directions in “omics” research pertinent to human spermatogenesis have been recently reviewed [30, 31]; herein, we focus on the local regulation of spermatogenesis and highlight areas of research which can serve as helpful examples to guide future investigations.

Structure and Composition of the Seminiferous Epithelium

In adult humans, as in rodents, the functional unit that produces sperm via spermatogenesis is the seminiferous tubule. Spermatogenesis is supported by testosterone produced by Leydig cells in the interstitium, and by estradiol-17ß produced by Sertoli and germ cells [10–15], although there is evidence that human Leydig cells also produce estradiol-17ß [32]. In men, each testis has ~400–600 seminiferous tubules, the length of all the tubules combined per pair of testes is ~400 meters, and each tubule has a diameter of ~200 μm [3, 33]. Spermatogenesis occurs as shown in the cross

section of a tubule (Fig. 3.1), consisting of Sertoli cells and germ cells at different stages of development. These germ cells include undifferentiated spermatogonia, dark type A spermatogonia, pale type A spermatogonia, type B spermatogonia; preleptotene, leptotene, zygotene and pachytene primary spermatocytes, and secondary spermatocytes; as well as Sa, Sb, Sc, Sd1, and Sd2 spermatids until Sd2 transform into spermatozoa [34]. Sertoli and germ cells near the base of the seminiferous epithelium are in close contact with the basement membrane, which is a modified form of extracellular matrix and is composed of mostly collagen (type IV) and laminins, heparin sulfate proteoglycan, and nidogen/entactin [35, 36]. Behind the basement membrane is the type 1 collagen layer, to be followed by the myoid cell layer, lymph, and then lymphatic endothelium. Collectively, they constitute the tunica propria [35, 36] (Fig. 3.1). During spermatogenesis, germ cells, in particular post-meiotic spermatids, rely almost solely on Sertoli cells for structural, nutritional, and paracrine support since haploid spermatids are metabolically quiescent cells with relatively little cytosol to support their cellular functions [37, 38]. Thus, the Sertoli cell is known as the “mother” or “nurse” cell, with each Sertoli cell supporting ~30–50 developing germ cells based on morphometric analysis in the rat testis [39].

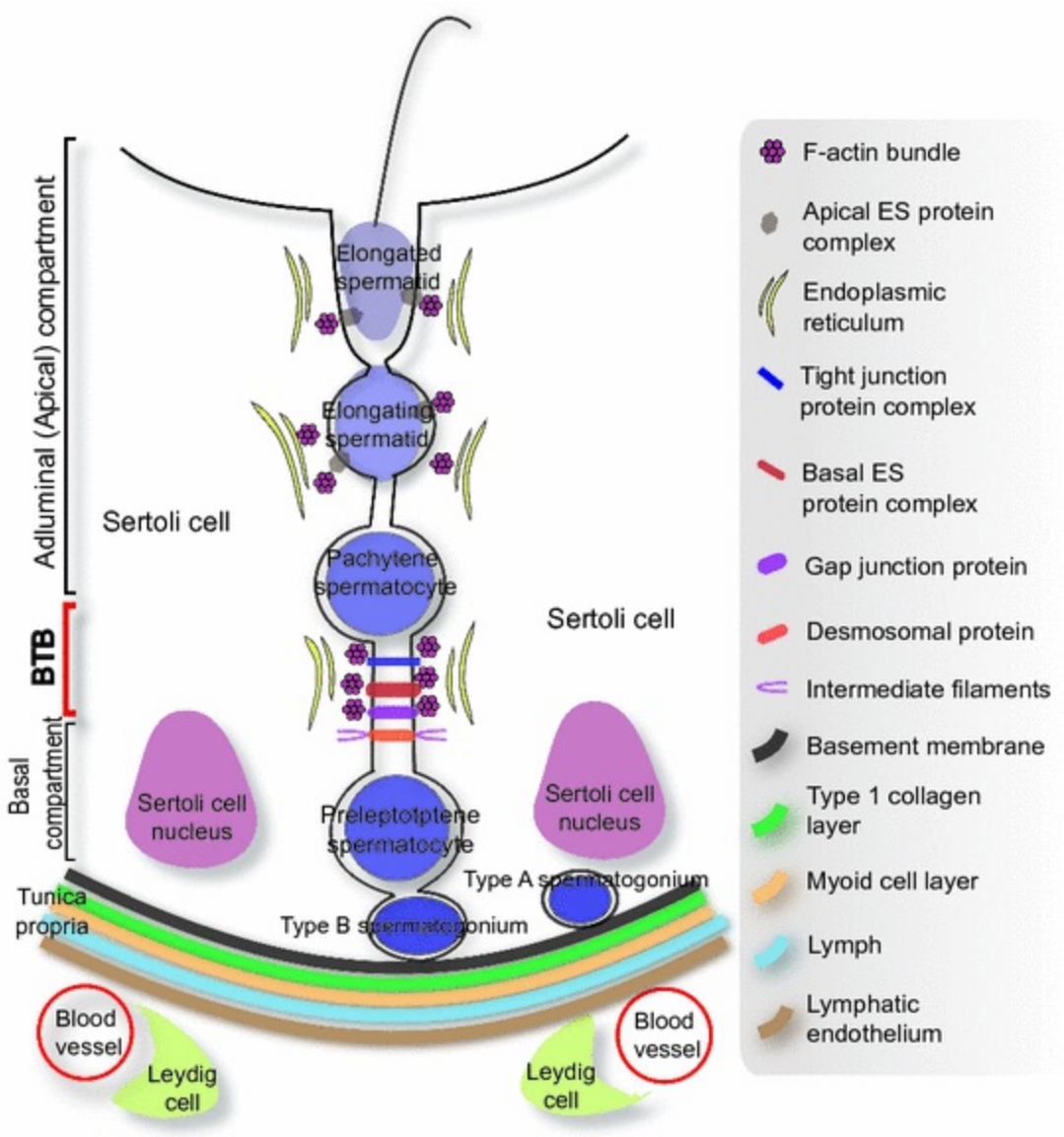

Fig. 3.1 Structure and cellular composition of the seminiferous epithelium in the human testis. The seminiferous epithelium, as seen in this schematic drawing of a cross section of a seminiferous tubule, is composed of Sertoli and germ cells. The BTB is constituted by actin-based tight junctions, basal ectoplasmic specialization (ES), and gap junctions, as well as the intermediate filament-based desmosome. As one of the tightest blood–tissue barriers in the human body, the BTB divides the seminiferous epithelium into the adluminal (apical) and basal compartments. Spermatogonia (both type A, B and undifferentiated) and preleptotene spermatocytes reside in the basal compartment, whereas the other primary and secondary spermatocytes and post-meiotic spermatids are found in the apical compartment. The most notable junction in the mammalian testis including humans is the ES, which is typified by the presence of actin microfilament bundles sandwiched in-between cisternae of endoplasmic reticulum and the apposing Sertoli cell–cell plasma membranes. The ES at the BTB is called basal ES while the ES at the Sertoli–spermatid interface is designated apical ES. Since developing germ cells (e.g., preleptotene spermatocytes) and developing spermatids are being transported across the seminiferous epithelium, Sertoli cell–Sertoli cell and Sertoli–germ cell junctions are continuously remodeling throughout the 6 stages of the epithelial cycle of spermatogenesis in the human testis

In humans, Sertoli cells undergo mitotic proliferation in two separate phases. The first phase takes place shortly after birth during the neonatal-infantile period when the

Sertoli cell population increases via mitotic proliferation [40]. During puberty, Sertoli cells again rapidly divide mitotically [40], giving rise to ~500 × 10^6 Sertoli cells per testis (each adult human testis weighs ~18–19 gm). The Sertoli cell number declines to ~300 × 10^6 by 50–85 years of age, associated with a reduction in daily sperm output [8]. Each testis has ~4.5 × 10^6 Sertoli cells in mice [41] and 30−40 × 10^6 Sertoli cells in rats [42–44] compared to 500 × 10^6 in men [8], and a daily sperm production of 8x [41], 70x [45], and 200x10^6 [46], respectively. The number of Sertoli cells is determined by FSH, thyroid hormones, growth hormone, and several paracrine growth factors [40]. Outside the tubules in the interstitial space lay the Leydig cells, fibroblasts, and some macrophages and microvessels (Fig. 3.1). Leydig cells produce testosterone (T) under the influence of luteinizing hormone (LH) since LH receptors are limited to Leydig cells in human and rodent testes. Interestingly, the T level in the interstitial fluid and seminiferous tubule fluid is at least 70- and 50-fold higher than the T level in the systemic circulation in the rat [47]. In humans, the intratesticular T (e.g., interstitial fluid) level is also 100-fold higher than the systemic circulation [48–50], illustrating a considerably higher T level is maintained in the testis to support spermatogenesis.

Sertoli Cells and the Blood–Testis Barrier (BTB)

The seminiferous epithelium of the mammalian testis is further divided into two functional compartments: (1) the basal, and (2) the adluminal (apical) compartments (Fig. 3.1), due to the presence of the Sertoli cell blood–testis barrier (BTB) located near the basement membrane. The BTB is constituted by coexisting actin-based basal ectoplasmic specialization (basal ES) and tight junction (TJ), as well as coexisting basal ES and gap junction, and intermediate filament-based desmosome [51, 52]. As such, undifferentiated spermatogonia, both type A and B spermatogonia, and preleptotene spermatocytes differentiated from type B spermatogonia reside in the basal compartment. More advanced primary spermatocytes, secondary spermatocytes, and all haploid spermatids including spermatozoa reside in the adluminal compartment (Fig. 3.1).

Briefly, the BTB provides a unique microenvironment in the adluminal compartment for meiosis I/II and post-meiotic spermatid development so that these events are segregated from the systemic circulation, behind an immunological barrier. Studies have shown, however, that the BTB contributes minor significance to the immune privilege of the testis since germ cells residing in the basal compartment are equally immunogenic, containing multiple testis-specific antigens [53, 54]. Instead, the testis immune privilege is maintained mostly by Sertoli cells through their secretory immunosuppressive biomolecules: cytokines, bioactive lipids and peptides, and androgens from Leydig cells [55]. The BTB in humans is established at puberty by ~12–13 years of age when Sertoli cells cease to divide and become fully differentiated, concomitant with the onset

of meiosis I/II [40]. It is of interest to note that human Sertoli cells, similar to Sertoli cells in rodents, can be de-differentiated, becoming mitotically active when they are exposed to fetal bovine serum [40], such as cultured in media containing 5–10% fetal bovine serum (v/v) [56]. Under such conditions, Sertoli cells can be maintained through multiple passes in vitro without detectable changes in their functional and physiological properties [57].

BTB function in rodents is dependent on testosterone [58–61]. However, treatment of normal men with testosterone plus levonorgestrel (LNG), which is capable of suppressing spermatogenesis and causing azoospermia or oligospermia with suppression of intratesticular androgen levels, did not affect BTB function since the distribution of claudin-3 remained relatively unaltered [62]. However, in a more recent study in which healthy men were treated with T plus LNG together with the GnRH antagonist acyline or the 5α-reductase inhibitor dutasteride (in order to provide added suppression of spermatogenesis), there was considerable down-regulation of claudin-11, connexin-43, and vinculin at the BTB compared to men treated wth T + LNG alone [63]. Collectively, these findings suggest that human BTB is regulated in a manner similar to rodents. Some species differences exist, however, such as the extent of androgen-dependency, since it appears that a considerable reduction in the intratesticular T level is necessary to suppress the BTB integrity in humans.

Due to the limited access to normal human testes for functional analysis, it is difficult to compare the regulation of the human BTB integrity with that of rodents. With advances in cell and tissue culture techniques, however, human Sertoli cells, using media containing fetal bovine serum, have been cultured and maintained successfully for several weeks in vitro [56, 57, 64–66], with a functional tight junction-permeability barrier [56, 64]. Using this approach, the organization of F-actin that supports BTB integrity has been investigated at the human Sertoli cell BTB. It is now known that human Sertoli cell F-actin organization is maintained, similar to that of rodents, by actin binding proteins, including Arp2/3 (actin related protein 2/3) complex and Eps8 (epidermal growth factor receptor pathway substrate 8) that confers branched actin network and bundled actin microfilaments, respectively [57]. Furthermore, exposure of human Sertoli cells to environmental toxicants, such as $CdCl_2$, or bisphenol A (BPA), rapidly perturbs F-actin organization in these cells. Changes in the distribution of cell adhesion proteins at the BTB, including the TJ-protein ZO-1, and the basal ES proteins N-cadherin and ß-catenin, from the cell surface to the cell cytosol [57], likely reflect degradation via an endosome-dependent degradation pathway. These changes disrupt integrity and function of the BTB. It is expected that much information will be obtained using this in vitro system to gain insight into the biology of the human BTB.

Cell–Cell Interactions in the Testis

Due to the presence of the BTB, germ cell development, in particular post-meiotic spermatid development, relies almost exclusively on the structural, nutritional, and paracrine support of Sertoli cells. This is mediated via cell junctions at the Sertoli–germ cell interface, involving multiple genes and their proteins [37, 38, 67, 68] (Fig. 3.2). Even though undifferentiated spermatogonia [e.g., spermatogonial stem cells (SSCs)], type A and type B spermatogonia, and preleptotene spermatocytes reside outside the BTB, they also rely on the Sertoli cell for functional and structural support through the expression of unique genes (Fig. 3.2). This is particularly true for SSCs which are located at the stem cell niches—usually at the base of the seminiferous epithelium wherein three seminiferous tubules meet, adjacent to the microvessels in the interstitium. In short, SSCs rely on Sertoli cells for structural and functional support, besides biomolecules from the microvessels [69, 70].

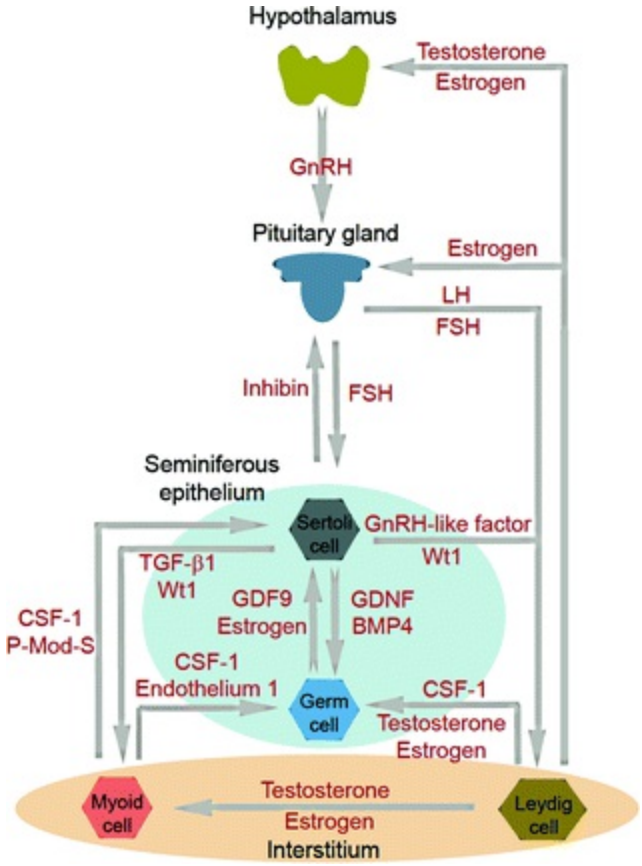

Fig. 3.2 Cell–cell interaction within the testis and its functional relationship to the hypothalamic–pituitary–testicular axis. The classic hormonal regulatory axis that exerts its regulatory effects on the testis through the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, which in turn regulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland. Testosterone produced by interstitial Leydig cells provides the feedback loop to modulate the production of GnRH from the hypothalamus and thereby LH and FSH from the pituitary gland. In humans and primates in contrast to rodents, testosterone has no direct feedback effect on the pituitary [240]. Inhibin produced by Sertoli cells provides selective feedback to the production of FSH. There is cross talk among germ cells (including spermatogonial stem cells) and Sertoli cells in the seminiferous epithelium, as well as Leydig cells and peritubular myoid cells in the interstitium. Studies have shown an array of growth factors, genes and hormones are involved. Abbreviations: CSF1, colony stimulating factor 1; BMP4, bone

morphogenetic protein 4; GDF9, growth differentiation factor-9; GnRH, gonadotropin-releasing hormone; P-Mod-S, peritubular myoid cell modulates Sertoli cell factor; TGF-β1, transforming growth factor-1; Wt1, Wilms' tumor 1

Sertoli cells also modulate spermatogenesis through cross talk with Leydig cells. Sertoli cells stimulate Leydig cell differentiation but inhibit Leydig cell steroidogenesis [71–73]. One of the paracrine factors produced by Sertoli cells that modulates Leydig cell function has been partially characterized [74]. Studies using genetic models have shown that Sertoli cells maintain Leydig cell number (both fetal and adult Leydig cells) and support Leydig cell development [75, 76], as well as determine Leydig cell differentiation status and cell fate through the *Wt1* gene [77]. On the other hand, Sertoli cells also interact with germ cells to support spermatogenesis through multiple paracrine factors [37, 78, 79] (Fig. 3.2).

There is also functional cross talk between Sertoli cells and the tunica propria, in particular peritubular myoid cells (Fig. 3.2). For instance, Sertoli cells work in concert with peritubular myoid cells in the production and deposition of extracellular matrix (ECM) components [80] to produce and maintain the basement membrane, which is a modified form of ECM [35, 36]. Studies have also suggested that peritubular myoid cells modulate Sertoli cell function wherein myoid cells produce a paracrine factor known as P-Mod-S that regulates Sertoli cell function [81]. However, the identity of this factor remains to be clarified. Another report has shown that while peritubular myoid cells do not secrete clusterin or α_2-macroglobulin (a wide-spectrum protease inhibitor [82]), co-cultures of myoid cells with Sertoli cell promote Sertoli cell α_2-macroglubulin and clusterin secretion [83]. It must be noted, however, that unlike Sertoli cells, which can be maintained in culture in serum-free medium (e.g., F12/DMEM with several growth factors [84]) for up to ~2-wk, peritubular myoid cell cultures require the presence of serum proteins, such as ~5–10% fetal calf serum for their survival [83].

The most conclusive study thus far to illustrate the importance of peritubular myoid cells in spermatogenesis is the generation of a peritubular myoid cell-specific androgen receptor (AR) knockout (PTM-ARKO) mouse model [85]. PTM-ARKO male mice have reduced seminiferous tubule fluid production and are azoospermic and infertile, with reduced expression of certain androgen-dependent Sertoli cell genes [85]. These findings illustrate that ARs in Sertoli cells fail to assume the function of ARs in myoid cells to sustain spermatogenesis. Furthermore, these findings demonstrate that Sertoli cell androgen-dependent gene expression is modulated by peritubular myoid cells, unequivocally demonstrating that Sertoli and peritubular myoid cells are functionally connected. Interestingly, studies using Amh-Cre to induce expression of the Diphtheria toxin receptor in Sertoli cells to cause controlled, cell-specific, and acute ablation of the Sertoli cell population in adult mice have shown that Sertoli cells also control peritubular myoid cell fate, including its differentiation status and cell population [76].

Using this approach, Sertoli cells were shown to modulate testicular vascular network development, including modulating circulating testosterone levels in adult mice [86]. It should be noted that much of the information stated above and depicted in Fig. 3.2 is derived from studies in rodents. Clearly, further studies are needed to understand the cell–cell interactions between Sertoli cells, Leydig cells, and peritubular myoid cells in the human testis.

Spermatogonia and Self-renewal

Spermatogonia are the progenitor cells of all germ cells and are designated type A and type B [87, 88]. In humans, type A spermatogonia are further categorized into progenitor dark type A spermatogonia (A_d) and pale type A spermatogonia (A_p) which are capable of undergoing mitotic proliferation for self-renewal [89] (Fig. 3.3). A_p are the predominant spermatogonia that survive radio- and chemotherapy while A_d are largely eliminated during such treatment, illustrating that A_p are the potential spermatogonial stem cells [90]. At present, it is generally accepted that A_d constitute a pool of reserve spermatogonia which remain quiescent but can give rise to A_p when needed and are considered to be the 'true' spermatogonial stem cells in humans [4, 91]. The molecular basis for the regulation of human spermatogonia remains largely unknown. cKIT, a transmembrane protein tyrosine kinase receptor (also known as stem cell factor receptor or CD117), and its ligand cKIT ligand (also known as stem cell factor (SCF)) are known to be involved in the differentiation of spermatogonia in humans, rodents, and primates [91]. cKIT is expressed in spermatogonia, round spermatids, and spermatozoa as well as in Leydig cells whereas its ligand or SCF is primarily expressed in Sertoli cells in humans [92]. However, cKIT is not expressed in spermatocytes or elongating spermatids [93]. Expression of cKIT in type A spermatogonia varies across the seminiferous tubules, being highest in proliferating A_p spermatogonia and type B spermatogonia [93–95], illustrating the involvement of cKIT/SCF in spermatogonial self-renewal and differentiation. The reduced staining of cKIT in type A spermatogonia has been shown in men with subfertility, and its reduced expression is associated with an increase in apoptosis in type A spermatogonia [96].

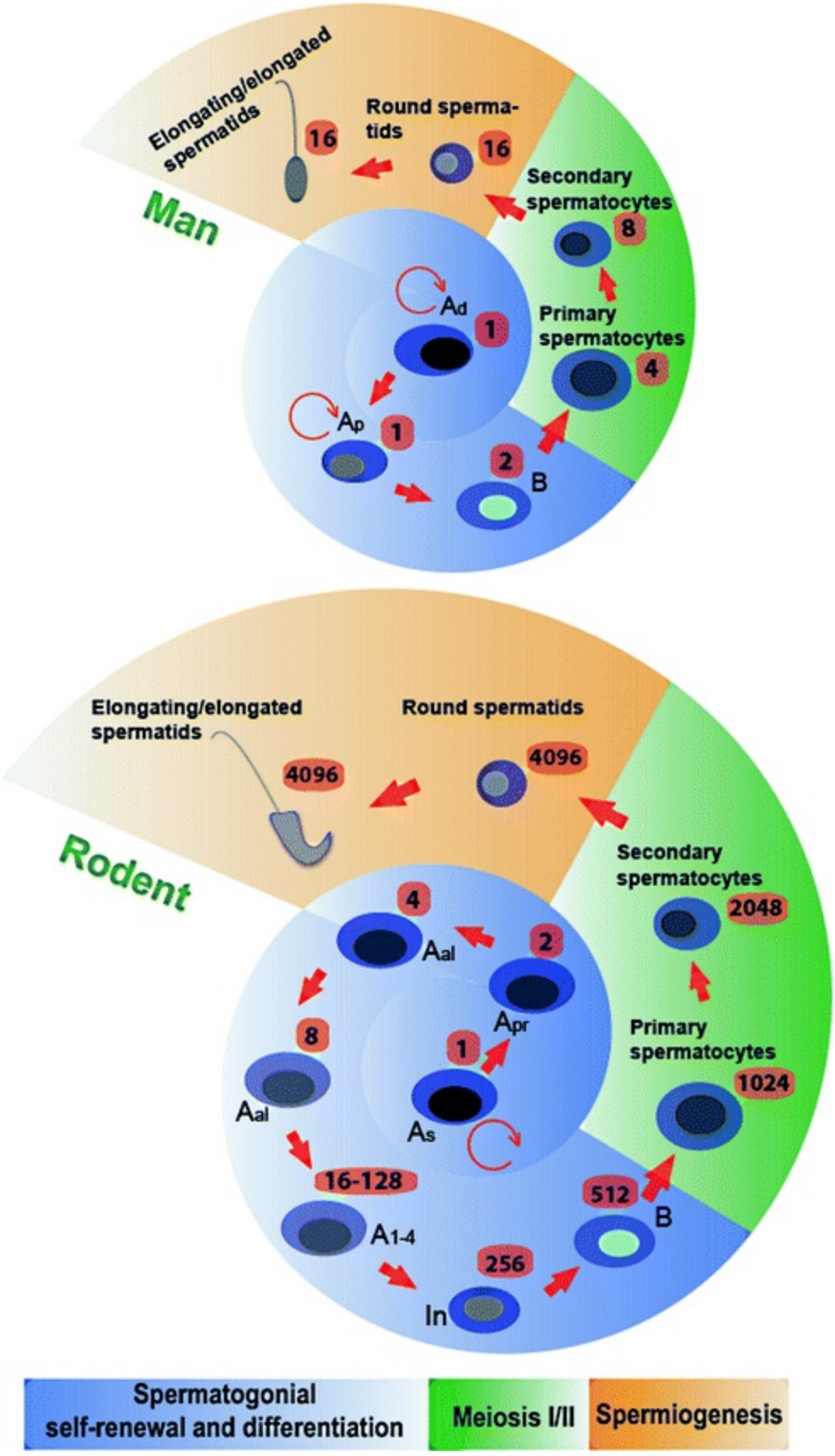

Fig. 3.3 Comparison of germ cell development in the human *versus* the rat testis. While the development of germ cells through spermatogenesis is similar between the two species, there are some notable differences such as during

spermatogonial self-renewal and differentiation. In the rat, Asingle spermatogonia (A_s) divide and form Apaired spermatogonia (A_{pr}), which further divide to form Aaligned spermatogonia (A_{al}). The differentiating A_{al} undergo 5 cell divisions (A_{1-4} and In) to form type B spermatogonia. In humans, both Adark spermatogonia (A_d) and Apale spermatogonia (A_p) are capable of undergoing self-renewal through mitosis, and A_p also give rise to type B spermatogonia, the latter of which are then differentiate to form preleptotene spermatocytes. In the human testis, preleptotene spermatocytes formed in stage III (concomitant with spermiation that takes place at late stage II) of the epithelial cycle are transported across the BTB to enter the adluminal compartment to prepare for meiosis I/II which takes place at stage VI of the epithelial cycle, and Sa spermatids, the equivalent of round spermatids in the rat, are found in stage I. In the rat, a single diploid A_s can give rise to 4096 haploid sperm; whereas in the human, only 16 sperm are derived from one type A spermatogonium. The number of different types of germ cells that can be derived from a single committed type A spermatogonium is indicated. This figure was prepared based on the information of earlier reports [1, 51, 127] and in the model of rat spermatogenesis it is noted that A_{pr} and A_{al} spermatogonia have been shown to be able to revert to A_s [241]

GFRA1, α6-INTEGRIN, PLZF, GPR125, SALL4, and THY1 are undifferentiated spermatogonia markers in mouse, monkey, and man, whereas UTF1 (undifferentiated embryonic cell transcription factor 1) and FGFR3 (fibroblast growth factor receptor 3, also known as CD333) are molecular markers specific for human type A spermatogonia [91]. The molecular basis for the transition of undifferentiated spermatogonia to type A spermatogonia has been established in rodents and involves retinoic acid (RA), the active metabolite of vitamin A, which is also involved in the initiation of meiosis [97]. Indeed, treatment of mice with the pan-retinoic acid receptor antagonist, BMS-189453 that blocks the action of RA in the mouse testis, leads to reversible infertility due to meiotic arrest [98, 99]. The notion that RA is involved in spermatogenesis in humans is based on findings that bisdichloroacetyldiamine WIN 18,446, an inhibitor of retinoic acid synthesis, inhibits ALDH1A2 and induces reversible infertility in mice, rats, monkeys, and men [100–103]. More study is needed to define the role of retinoic acid in human spermatogonial differentiation.

Spermatogonial differentiation is a gonadotropin-dependent event in primates [104] since FSH treatment increases the number of A_p and type B spermatogonia in monkeys [105, 106]. Emerging evidence suggests that FSH plays a role in spermatogonial differentiation in men [107]. However, in normal adult men, gonadotropins (FSH and LH) serve as spermatogonial survival factors by regulating the intrinsic apoptotic pathway to promote germ cell survival but have no effect on germ cell proliferation [108].

Spermatocytes and Meiosis

Spermatocytes, namely leptotene, zygotene, pachytene, and diplotene spermatocytes are found in the human seminiferous epithelium [109] following the initiation of meiosis in humans at puberty [110] (Figs. 3.3 and 3.4). Diplotene spermatocytes undergo meiosis I to form secondary spermatocytes (with haploid number of chromosomes but 2n content

of DNA), which rapidly progress to meiosis II so that a single secondary spermatocyte produces two haploid round Sa spermatids. In this context, it is of interest to note that, unlike other somatic cells, neither spermatocytes nor spermatids metabolize glucose; instead, they rely on lactate supplied by Sertoli cells as their energy source [111, 112]. In brief, glucose is taken up by Sertoli cells via the specific glucose transporter GLUT1 and is processed into lactate glycolytically [113] by testis-specific lactate dehydrogenase C4 (LDHC4). Lactate is transported out of Sertoli cells by a monocarboxylate transporter MCT1 and taken up by meiotic and post-meiotic germ cells via their specific monocarboxylate transporter, MCT2 [111]. Studies have shown that energy metabolism of Sertoli and germ cells is regulated by FSH, steroids, insulin and paracrine factors, and their disruption leads to male infertility [112, 114]. It is also noted that defects in meiosis, such as meiotic maturation arrest (or early maturation arrest) is found in ~10% of men with non-obstructive azoospermia (NOA), with the histopathological features of reduced tubule diameter, reduce germ cell number, and degenerating spermatocytes [115].

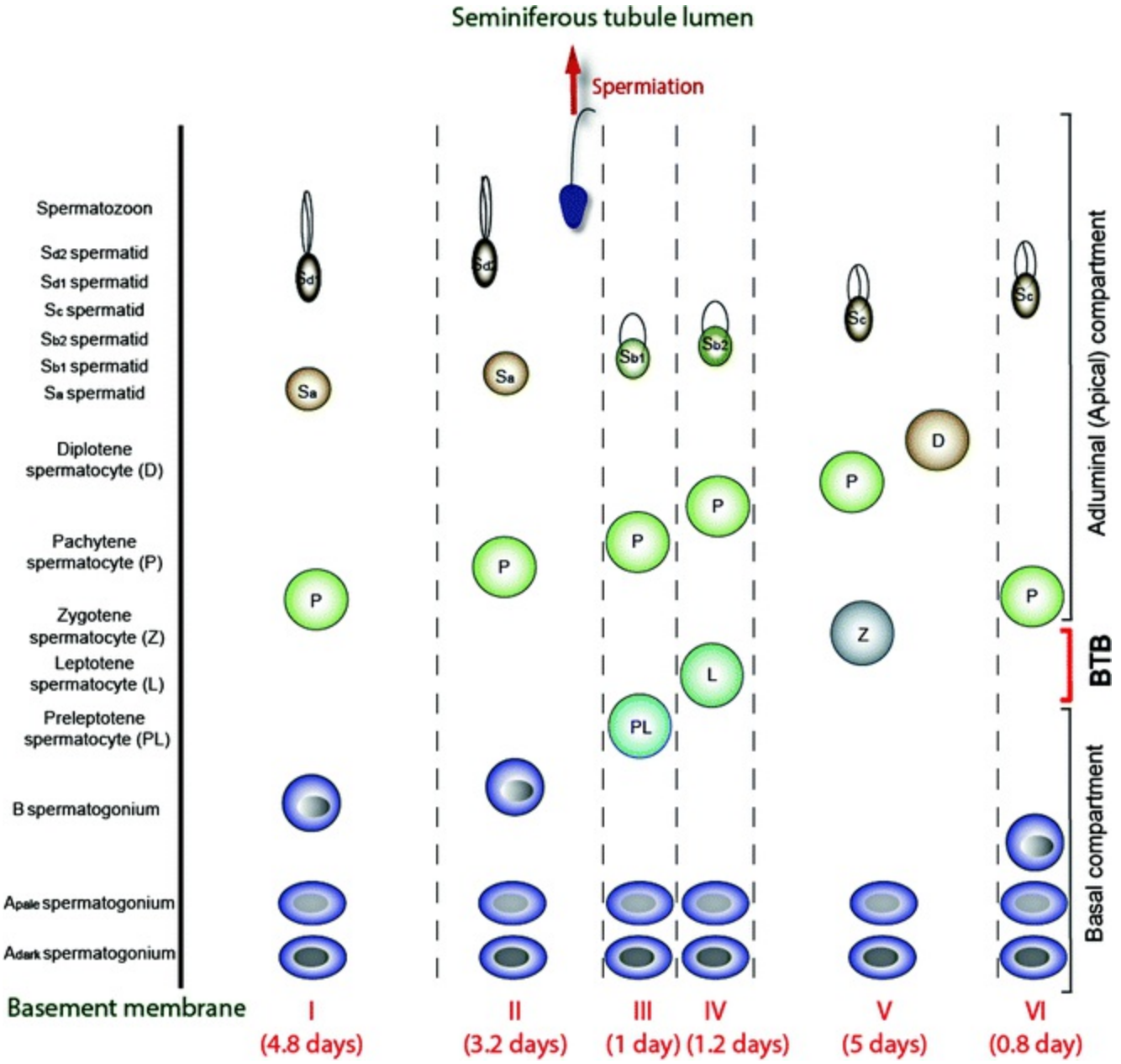

Fig. 3.4 A schematic drawing illustrating the six stages of the epithelial cycle (I-VI) in the human testis, and the associated germ cell types in the seminiferous epithelium in each of these stages. It is noted that spermiation takes place in late stage II, and preleptotene spermatocytes arise in stage III, which are being transported across the BTB in stage III. Meiosis takes place at stage VI of the cycle, and the entire epithelial cycle takes 16 days to complete. The duration of each stage, from I to VI, is also annotated. This drawing was prepared and modified from earlier reports [4, 34, 242], and recent advances in the staging of human spermatogenesis have recently been discussed [243]

Spermatids and Spermiogenesis

The process during which spermatids undergo extensive morphological transformation through round Sa spermatids, Sb1, Sb2, Sc, Sd1, and Sd2, and eventually to spermatozoa is known as spermiogenesis [116] (Figs. 3.3 and 3.4). During this process, no further cellular divisions occur, newly formed round spermatids from secondary

spermatocytes are characterized by a small spherical nucleus with the usual array of cytoplasmic organelles, including the Golgi apparatus, mitochondria, and centrioles [116]. Each spermatid undergoes considerable morphological changes that begin with a large granule called the acrosomic granule which is generated by several small proacrosomic granules in the area of the Golgi apparatus that grows over the nucleus. As spermiogenesis continues, the acrosome transforms further, and nuclear chromatin condensation begins [117]. At the final steps, the spermatid nucleus completes its chromatin condensation, and the majority of the cytoplasm eventually detaches from the spermatid to form the residual body, which is engulfed by the Sertoli cell and processed into a phagosome. The phagosome is then transported to the base of the Sertoli cell for lysosomal degradation [118]. In humans, spermiogenesis can be disrupted, leading to: (i) late maturation arrest, manifested by an arrest in development in early spermatids with dark round nuclei, and (ii) hypospermatogenesis, with an arrest in further development of condensed oval spermatids [115]. Mature spermatozoa, once formed, are released into the tubule lumen via the final cellular process of spermatogenesis at spermiation [5, 119]. While the molecular mechanism underlying spermiation remains relatively unexplored, studies in rodents have shown that spermiation is tightly regulated by the spatiotemporal expression of signaling molecules such as p-FAK-Tyr^{407}, as well as actin binding/regulatory proteins (e.g., the Arp2/3 complex, Eps8), involving degeneration of apical ES and generation of laminin fragments [5, 119]. However, this process is poorly understood in humans except that it is likely dependent on FSH and testosterone. So far, human male infertility has not been attributed to defects of spermiation alone [3].

Cycle of the Seminiferous Epithelium

In human as well as rodent testes, the distinctive cellular associations in the seminiferous epithelium along the tubule can be defined into different stages which appear cyclically throughout spermatogenesis, known as the epithelial cycle of spermatogenesis [88, 120, 121]. In the testis of rats, mice, and humans, an epithelial cycle is composed of I-XIV, I-XII, and I-VI stages, respectively [4, 122, 123]. It is of interest to note that spermiation and meiosis I/II take place at stage VIII and XIV, VIII and XII and II and VI, in the rat, mouse, and human testis, respectively. It takes about 16 days to complete an epithelial cycle in the human [124] versus 8.6 days in the mouse [125] and 12.8 days in the rat [126]. This is the time it takes for a given spot in a seminiferous tubule at a specific stage of the epithelial cycle, such as at stage II, when observed under the stereomicroscope, to undergo cyclic staging and become stage II again. However, the duration of spermatogenesis, i.e., from type A spermatogonia to spermatozoa, is ~68 days (4.2 cycles) in humans versus ~35 days (4 cycles) and ~58 days (4.5 cycles) in mice and rats as noted in Fig. 3.4 for humans (for reviews, see

[1, 127, 128]). This is the time it takes for a single human diploid Ap spermatogonium (progenitor germ cell, wherein Ad spermatogonium is the regenerative reserve stem cell) to develop into multiple haploid spermatozoa which requires ~4.2 cycles. In this context, it is noted that the staging of the epithelial cycle is largely defined according to changes in the Golgi region of developing spermatids, namely the acrosome, when it is visualized by the periodic acid Schiff's reaction (PAS). In mouse, rat, and human, spermatids can be divided into 16, 19, and 6 steps, respectively, throughout spermiogenesis before they develop and transform into spermatozoa. The classification of human spermatids into 6 steps of Sa, Sb1, Sb2, Sc, Sd1, and Sd2 was based on the use of osmium-dichromate as fixative for the human testis, which was then stained with osmium and examined by transmission electron microscopy [117].

Factors that Regulate Spermatogenesis

Numerous factors are known to affect human spermatogenesis. Below, we highlight regulators that are well established or are rapidly developing in the field.

Hormonal Regulation

Hormonal regulation of spermatogenesis in humans is a complex biological event involving both testosterone and estradiol-17ß [10, 13, 14, 129, 130] and is tightly regulated by the hypothalamic–pituitary–testicular axis by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that exert their effects on Sertoli and Leydig cells, respectively. Besides these hormones, inhibins, activins, follistatin, and other paracrine factors are also involved. Since the hormonal regulation of spermatogenesis in humans has been eminently reviewed recently [11, 13, 131–136], interested readers are encouraged to seek further information from these earlier reviews. Below is a summary of recent findings pertinent to the hormonal regulation of human spermatogenesis.

FSH

FSH is an important regulator of spermatogenesis through effects on Sertoli cells where FSH receptors are expressed. FSH activates at least five signaling pathways in Sertoli cells: cAMP-PKA, MAPK, PI3 K-AKT, intracellular Ca, and phospholipase A2 [137–139]. Gene expression profiles indicate that FSH regulates a panoply of Sertoli cell genes. FSH promotes Sertoli cell proliferation before puberty, while after puberty, FSH activates Sertoli cell to support germ cell development. Despite the important role of FSH in spermatogenesis, this hormone, unlike testosterone (see below), is not considered essential to, but rather fine-tunes spermatogenesis. For instance, deletion of the FSH-ß subunit in mice led to infertility in FSH-deficient females due to a block in folliculogenesis, but FSH-deficient males were fertile even though the testes of these

mice were reduced in size [140, 141]. Furthermore, men with inactivating mutations of the FSH-β gene or the FSH receptor have impaired spermatogenesis but generally remain fertile—an observation explained in part by the finding that the FSH receptor possesses low-level constitutive activity in the absence of FSH [129, 142–144] (see Chap. 6). On the other hand, it is of interest to note that men with congenital complete hypogonadotropic hypogonadism (CHH) are usually treated with hCG (human chorionic gonadotrophin) together with FSH to induce testis development, spermatogenesis, and fertility (see Chap. 20). Also, men with idiopathic oligoasthenoteratozoospermia treated with recombinant FSH for 3 months were found to have considerable improvement in seminal parameters vs. untreated controls [145, 146].

LH

The primary function of LH is to stimulate the production of testosterone by Leydig cells [147]. LHCG-receptor (luteinizing hormone/choriogonadotropin receptor, also called LHCG-R or LHR) expression begins in fetal Leydig cells [148]. Human males with inactivating mutations of the LHCG-R develop ambiguous genitalia and are testosterone-deficient indicating that hCG/LH signaling is essential for testosterone production in the fetus and adult [149–151]. LH stimulates testosterone production by stimulating the expression of key steroidogenic enzyme genes as well as transcription factors that are required for testosterone biosynthesis to support spermatogenesis and other male reproductive function [152]. Ligand binding to the LHCG-R stimulates adenylate cyclase, increases cAMP production and phosphorylates target proteins through protein kinases A and C, activates ERK1 and 2, and increases calcium signaling [148]. Interestingly, accumulating evidence supports the notion that steroidogenesis in human fetal testes is highly sensitive to environmental toxicants or elected lifestyle (e.g., cigarette smoking) which are disruptive to LH-mediated testosterone production by Leydig cells [17, 153].

Intratesticular Testosterone (ITT) Microenvironment

Testicular aspirations of normal men have shown that T is the predominant intratesticular sex steroid [48, 49, 154]. ITT concentration in men averages 609 ± 50 ng/ml, which is much higher than the average serum T level of 3.7 ± 0.3 ng/ml [48–50]. This gradient between the testis and serum is similar to findings in rodents, wherein ITT in rat testes is ~100-fold higher than the serum T level [47, 155]. Collectively, these findings support the notion that a high ITT level is necessary to maintain spermatogenesis. For instance, the high level of T in the testis is known to support spermatid adhesion, spermiogenesis, and BTB function in rodent testes [5, 51, 156]. In this context, it is of interest to note that the ITT concentration at ~2000 nM vesurs ~12 nM of T in serum in normal men is considerably higher than that of

SHBG/ABP (sex hormone binding globulin/androgen binding protein, ~50 nM; note: SHBG/ABP reduces androgen bioavailability), suggesting that most ITT is bioavailable [154]. Studies by Jarow et al. found that ~70% of the total ITT is bioactive based on a novel androgen bioactivity assay [154]. Interestingly, neither ITT concentrations nor intratesticular bioactive androgen levels were strongly correlated with serum T concentration (correlation coefficients, $r = 0.38$) nor serum bioactive androgen level ($r = 0.46$). Yet SHBG/ABP levels in the testis and serum in humans were strongly correlated [48, 154]. These data thus fail to explain the disparity between intratesticular and serum bioactive androgen levels [48, 154, 157]. In contrast, Roth et al. found that T levels in testicular aspirations (i.e., to obtain ITT) and serum of fertile men in samples obtained simultaneously were strongly and positively correlated ($r = 0.67$) [158]. The different results in the two studies may be due to different assay approaches. In this context, it is of interest to note that a recent report has shown that high ITT is not necessary to support spermatogenesis in humans [159], and proposed that the ITT level may be high only because T is synthesized locally by Leydig cells in the interstitial space of the testis. Thus, the physiological significance of the ITT level to support spermatogenesis requires additional further studies.

Importantly, the earlier report of Roth et al. also demonstrated considerable variations in ITT levels in fertile men which correlated very strongly with serum LH levels ($r = 0.87$). It was hypothesized that LH secretory pulsatility might cause pulsatility in ITT concentrations [158]. In fact, ITT pulsatility was suggested by an earlier study that quantified T and estradiol concentrations over 4 h in cannulated gonadal veins of men with varicocele [32]. While the importance of ITT pulsatility to spermatogenesis remains to be established, it may influence the cyclic nature of spermatogenesis during the epithelial cycle. For instance, the requirements of the seminiferous epithelium to support androgen-dependent cellular events at late stage II during spermiation are quite different from stages VI when meiosis takes place. As such, it is likely that the ITT level required at stage II is different from stage VI and other stages to support spermatogenesis. Nonetheless, the ITT concentration necessary to support spermatogenesis in humans has not been established by quantifying T levels in intratesticular fluid obtained during micro-TESE (microdissection testicular sperm extraction) [160]. Furthermore, studies in rodents have shown that T, besides playing a crucial role in maintaining germ cell adhesion in particular developing spermatids, modulates endocytic vesicle-mediated protein trafficking [161]. Due to the high level of cellular activities in the epithelium to maintain the daily sperm production rate, many cellular proteins, in particular those found at the Sertoli–Sertoli or Sertoli–germ cell interface, are being re-cycled [162]. Studies have shown that the relative ratio of ITT/cytokines within the micro-environment of the seminiferous epithelium may be crucial to govern these protein recycling events during the epithelial cycle [163]. These studies, while recently performed in rodents, can now be extended to humans by using

the human Sertoli cell in vitro system [57].

Androgen Receptor (AR)

T exerts its effects through AR signaling [156, 164–166]. In humans, AR expression is restricted to Sertoli cells [167, 168]. Studies using murine models have shown that specific deletion of AR in Sertoli cells (SC-specific AR KO) leads to meiotic arrest and early spermatogenic maturation arrest [169, 170], and also terminal differentiation of haploid spermatids [171]. Collectively, these findings illustrate the significance of AR-mediated action in spermatogenesis, in particular murine meiosis and spermiogenesis. In men, the AR expression level visualized by immunohistochemistry in Sertoli cells was higher in those with NOA (non-obstructive azoospermia) *vs.* OA (obstructive azoospermia), and a significant positive correlation was observed between FSH levels and Sertoli cell AR expression in OA patients [167]. However, efforts to relate AR function or distribution to infertility in humans have been unsuccessful. For instance, no correlation was found between AR expression in Sertoli cells with serum T levels, or serum LH, and FSH levels in men with NOA [167]. Also, exogenous FSH was shown to trigger an increase in AR expression in the human testis [167]. However, hCG therapy had no apparent effect, thereby underscoring the significance of FSH-dependent Sertoli cell AR expression [167].

Exon 1 of the AR contains a CAG trinucleotide repeat that encodes a polyglutamine tract in the N-terminus of the AR. This region is required for AR interaction with transcriptional co-regulators, and variation in CAG repeat length, even within normal alleles, influences AR function in that shorter repeats are associated with a more transcriptionally active receptor. Furthermore, an expanded polyglutamine tract confers toxic properties responsible for neuronal and non-neuronal degeneration in the neurological disorder spinal and bulbar muscular atrophy in which affected men have small testes and gynecomastia (SBMA) [172]. Attempts to correlate the severity of spermatogenic dysfunction with CAG-encoded polyglutamine length polymorphism in the AR gene have, however, yielded inconsistent and conflicting results [173–176]. Interestingly, an insertion mutation near the beginning of the CAG repeat in exon 1 of the AR gene was found in an azoospermic man [177]. Treatment of an infertile man who had a point mutation of p.Val686Ala in the AR ligand-binding domain with prolonged high-dose testosterone therapy was found to produce marked improvement in sperm count and semen quality [178]. Combined with intracytoplasmic microinjection, testosterone treatment resulted in fertility. A point mutation in the transactivation domain (TAD) of the AR gene was found in an infertile man with gynecomastia with a high FSH level, small testes, and Sertoli cell-only syndrome [179]. However, a fertile man with gynecomastia was found to have a p.Pro69Ser mutation within the AR ligand-binding domain but to display no considerable defects in semen quality [180]. Taking these data

collectively, it is anticipated that more AR mutation-associated male infertility cases will be identified. As more data are available, a systemic analysis is warranted to relate mutation(s) of the AR gene with idiopathic male infertility. At this point, based on the available data, it is likely that FSH-dependent AR expression is crucial for spermatogenesis. However, more accurate functional assays are necessary to pin-point the importance of AR in male infertility.

Estrogen

Testes are known to produce a considerable amount of estradiol-17ß via aromatase, which is essential to maintain spermatogenesis [10, 12, 181]. In humans, the role of estrogen in spermatogenesis remains a subject of debate. Emerging evidence, however, supports the notion that estrogen is involved in testis development, fluid resorption in the rete testis, maintenance of spermatogenesis, and in the maturation of spermatozoa [10, 181, 182]. Most importantly, aromatization of T to estradiol-17ß in the hypothalamus provides an important negative feedback signal to gonadotropin secretion [10]. Aromatase is expressed by human Leydig cells, Sertoli cells, spermatocytes, spermatids, and spermatozoa (its presence in spermatogonia remains unknown) [181] while estrogen is produced mostly by Leydig cells in the adult human testis [10].

Estrogen exerts its effects via estrogen receptors ERα (ESR1) and ERß (ESR2) [10, 12]. Studies in rodents have shown that ERα is expressed by Leydig cells and peritubular myoid cells; whereas ERß is found in some Leydig cells, but mostly in Sertoli cells and germ cells [183, 184]. In short, ERα is predominant in the interstitium and ERß in the seminiferous epithelium. Subsequent studies, however, have demonstrated that ERα is also expressed in Sertoli and germ cells in the rat testis [185]. In human testes, the binding of estradiol-17ß to human sperm was first reported in 1981 [186]. In 1998, human sperm were shown to express both the ERα mRNA and protein [187]. Subsequent studies have confirmed the presence of ERß as well as ERα in human sperm [12]. It is now generally accepted that ERα is expressed in spermatogonia, pachytene spermatocytes, and early round spermatids; whereas ERß is expressed in pachytene spermatocytes, early round spermatids, Sertoli cells, and Leydig cells in human testes [188, 189]. A genetic analysis study in 300 infertile Indian men *and* 255 fertile normal subjects identified single nucleotide polymorphisms (SNPs) (found in 4 subjects) and mutations (found in 8 subjects) in the ERß gene, suggesting ERß gene mutations are a cause of spermatogenesis failure in men. These infertile men displayed normal reproductive tract and serum hormone levels [190]. The few men identified with aromatase deficiency due to autosomal recessive inheritance of mutations in the *CYP19A1* gene tend to have small testes and an abnormal semen analysis. Additionally, hormone levels in men with mutation in ERα and deficient in aromatase have considerable changes in serum T, estradiol, LH, and/or FSH compared to normal

subjects (see Chap. 1). While the number of patients is small, these findings illustrate the significance of estrogen in human spermatogenesis. Furthermore, genetic and pharmacologically induced estrogen deficiency leads to reduced libido in men [191, 192]. In summary, estrogen is known to be involved in the development of the testis, maintenance of male reproductive tract, fluid resorption in the rete testis, and in particular sperm maturation in humans [12, 181, 193, 194].

Small Non-coding RNAs (sncRNAs)

As noted above, spermatogenesis is a highly complicated process that requires the intriguing participation of multiple genes at the transcriptional and post-transcriptional levels to produce spermatozoa. Studies have shown that germ cells, including spermatogonia, spermatocytes, and post-meiotic spermatids all contain abundant levels of non-coding RNAs, such as siRNA (small interfering RNA, 20–25 nucleotides), miRNA (microRNA, 21–24 nucleotides), and piRNAs (Piwi-interacting RNA, 26–31 nucleotides) [195–197]. The most important sncRNAs that are involved in spermatogenesis are miRNAs and piRNAs and the associated pathways based on studies of genetic models in rodents [198–201]. These small RNAs are short single-stranded non-coding nucleotides that are known to directly disrupt target mRNAs through degradation, causing translation repression to block protein synthesis [20, 202]. Since miRNA can bind to more than one mRNA, a specific miRNA can regulate the function of more than a single gene. Small RNAs including miRNAs and piRNA are mostly stored in the chromatoid body which is a dense structure in the germ cell cytosol composed of mainly RNAs and RNA-binding proteins and are involved in the regulation of germ cell apoptosis, proliferation, and differentiation, as well as spermatogonial stem cell self-renewal [200, 203, 204]. Studies have shown an alteration in the expression patterns of small RNAs in infertile men [205–207]. For instance, miR-141, miR-429, and miR-7-1-3p were up-regulated in men with idiopathic non-obstructive azoospermia (NOA) [206] while miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p, and miR-513a-5p were markedly down-regulated in men with NOA. These miRNAs are possibly involved in regulating germ cell apoptosis [207].

In this context, it is of interest to note that miRNAs and siRNAs are processed by an RNase III endonuclease Dicer, the deletion of which in mice leads to infertility. Dicer is essential for haploid spermatid differentiation [208] and for the assembly and maintenance of cell junctions in the seminiferous epithelium during spermatogenesis [209]. Specific deletion of Dicer in Sertoli cells also leads to infertility with complete absence of spermatozoa in seminiferous tubules and progressive testicular degeneration [210]. While the role of Dicer in human fertility remains to be elucidated, it is obvious that small regulatory RNAs represent a tempting target for non-hormonal male

contraception. Also, much work is needed to better understand the role of small RNAs in human spermatogenesis, and their use as diagnostic markers to monitor infertility, for example, men who are at risk because of industrial exposure to toxicant-induced infertility.

Obesity

According to NIH guidelines (see http://www.nhlbi.nih.gov/health/health-topics/topics/obe/diagnosis), adults with a body mass index (BMI) >30 (kg/m^2) are obese. Based on the latest statistics in 2014, the obese adult population in the U.S. is at 27.7% (http://www.gallup.com/poll/181271/obesity-rate-inches-2014.aspx), which is double the world's average of 13% (http://www.who.int/mediacentre/factsheets/fs311/en/), illustrating an alarming trend given the health risks associated with obesity. A lower sex hormone binding globulin (SHBG) level is found in men with obesity and/or diabetes, which in turn lowers the total testosterone level (see Chap. 16 and [211]). Emerging evidence suggests a negative correlation between rising BMI and sperm count, sperm concentration, and motile sperm, which impedes male fertility [212–214]. Interestingly, while other studies suggest that obesity has no major impact on fertility, semen quality, gonadotropin levels, or other sperm parameters despite reduced testosterone levels [215, 216]. The reason(s) behind the different conclusions is not immediately known. Nonetheless, studies have demonstrated possible mechanism(s) by which obesity might lead to reduced spermatogenesis capacity and daily sperm output, as well as reduced sperm count and sperm quality. One proposed mechanism is hyperestrogenism in which serum estrogens are considerably higher in obese men due to an increase in peripheral conversion of testosterone to estrogens by aromatase in adipose tissue [217–219], coupled with a reduced testosterone production [214]. The excessive estrogens in the systemic circulation thus inhibit the release of LH and FSH from the pituitary gland via the negative feedback on the hypothalamus, thereby reducing T production, intratesticular T levels, and the testosterone/estrogen ratio. The reduced T and FSH levels result in suppressed spermatogenesis. However, the correlation between high levels of estradiol and obesity is controversial and is further affected by low SHBG [220], suggesting the need for additional studies. Nonetheless, this mechanism is supported by findings that obese men have considerably lower levels of inhibin B *than* healthy men [221–223]. Elevated levels of cytokines are another potential cause of hypospermatogenesis in obese men [224]. Other mechanisms that might cause defects in human spermatogenesis in obesity include reduced levels of SHBG, insulin and leptin resistance, sleep apnea, and adiponectin deficiency [225–229]. Additionally, factors involved in the pathogenesis of obesity, such as high calorie diet, genetic, and epigenetic disorders, might also play a role in perturbing sperm production [230, 231].

Besides the disruptive effects of obesity on spermatogenesis, there is emerging

evidence that obesity also affects the molecular structure of testicular germ cells and mature spermatozoa, such as an impairment of acrosome reaction [232], leading to altered growth in offspring, increased susceptibility to disease in adults [233], and erectile dysfunction [234]. Studies also suggest that high-fat diets can affect the epigenetic content of sperm or the endocrine content of seminal fluid, which in turn affects early fetal development [213].

Bariatric surgery [235], such as Roux-en-Y gastric bypass surgery [236], has been reported to increase T levels and improve sexual function in obese men. Much of the increase in T, however, results from an increase in the level of SHBG [236]. Interestingly, there are case reports in which bariatric surgery was followed by impaired semen parameters, possibly by perturbing absorption of vitamins and trace elements [237, 238]. However, one study of six men showed no disruptive changes of bariatric surgery on semen parameters, coupled with an increase in urinary total T levels [239].

Summary

Spermatogenesis is a series of cellular events that take place in the seminiferous tubules of the testis. In this review, we provide a brief overview of human spermatogenesis, from spermatogonial self-renewal via mitosis, meiosis, post-meiotic spermatid development via spermiogenesis, to the release of sperm at spermiation. We discuss the role of Sertoli cell and especially the role of the BTB in spermatogenesis. We also highlight some specific areas of research that deserve future attention. Due to the lack of human testis samples for analysis, in particular those from normal subjects, the study of human spermatogenesis lags far behind studies in rodents. However, human Sertoli cells obtained at biopsy can now be cultured in vitro and maintained up to weeks and months where they remain mitotically active. These cells can be subcultured for creative experiments. We also briefly discuss some emerging fields of research that focus on factors affecting human spermatogenesis. These factors may deserve more attention by investigators in future years.

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4. Normal and Delayed Puberty

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Normal Puberty

Physiology

Puberty is the maturational process of the reproductive endocrine system that results in final adult height and body proportion as well as the development of the genital organs and the capacity to reproduce. The onset of puberty is driven by an increase in the frequency and amplitude of pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. This activation results in increased luteinizing hormone (LH) and follicular-stimulating hormone (FSH) production by the anterior pituitary which acts on the gonads to stimulate their development, gametogenesis, and sex steroid secretion.

Clinical Markers of Puberty

In boys, the first physical marker of pubertal onset is an increase in testis volume above the prepubertal volume of 3 mL (Tanner stage G2) [1] (Fig. 4.1). There is a classic pattern of pubertal progression with the onset of testicular enlargement followed by

pubic hair development and penile growth. The testes increase in size from a prepubertal volume of 1–2 to 4–8 mL even before pubic hair appears and reach 20–30 mL in adulthood [2]. Development of mature spermatogenesis is called spermarche, at which time mature spermatozoa are found in the urine. This hallmark of gonadal maturation occurs at a mean age of 13.5 years, when the mean testis volume reaches 11.5 mL [3]. The tempo and process of puberty are well conserved across ethnicities and populations (Table 4.1).

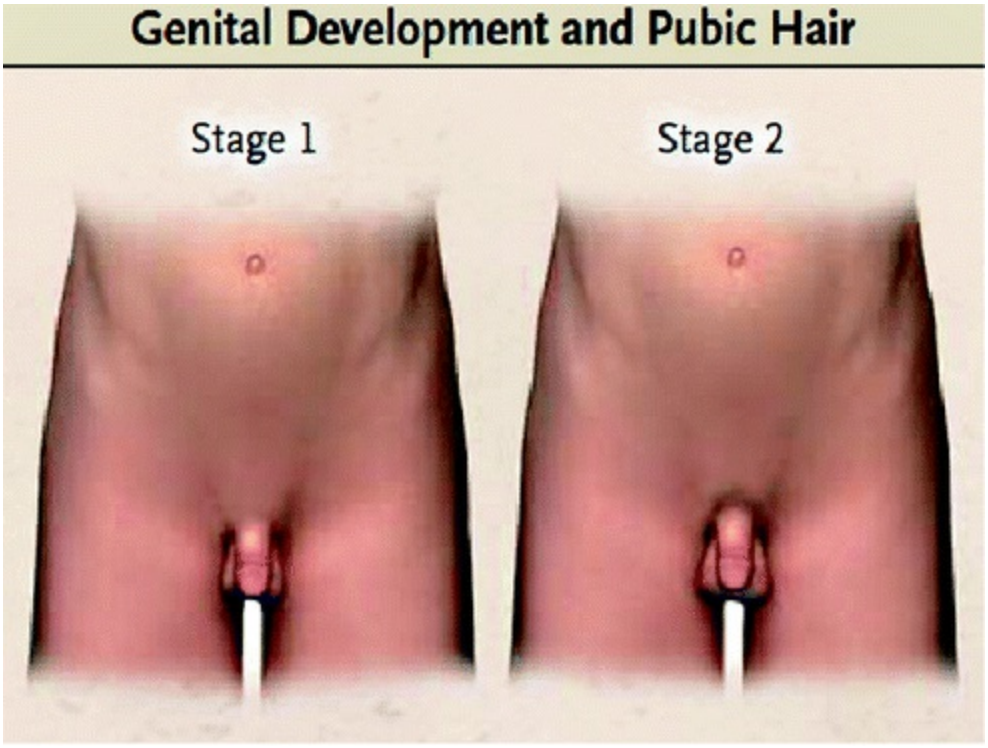

Fig. 4.1 Tanner staging of puberty onset in boys from Palmer and Dunkel [4]. Copyright © (2012) Massachusetts Medical Society. Reprinted with permission

Table 4.1 Details of the Tanner stages of puberty

Tanner stage	Males	
	Genitalia	**Pubic hair**
1	Testis 1–3 ml	None
2	Testis >3 ml Scrotal enlargement	Sparse, lightly pigmented
3	Testis continue to enlarge, penis lengthens	Increases in amounts, darkens, and starts to curl
4	Scrotum darkens, widening of glans penis	Resembles adult type but not spread to medial thighs
5	Adult size and morphology	Spreads to medial thighs, adult distribution

Timing of Puberty

In the general population, there is a near-normal distribution of the timing of puberty,

with the mean age of onset of G2 at 11.5 years in boys (Fig. 4.2). In healthy boys, the normal age limits for G2 development are between 9.5 and 14 years [4]. While a large variability in the timing of pubertal onset exists, clear age cutoffs for normal pubertal development have been established. However, the age limits for identifying children who need evaluation for precocious or delayed puberty may vary among different ethnic groups. Within this distribution, there has been in recent years an increasing degree of skew at both ends of the spectrum, as an increasing prevalence of an earlier age of pubertal onset (G2) has been documented in some populations as well as an increase in the number of children completing puberty at a later age [5].

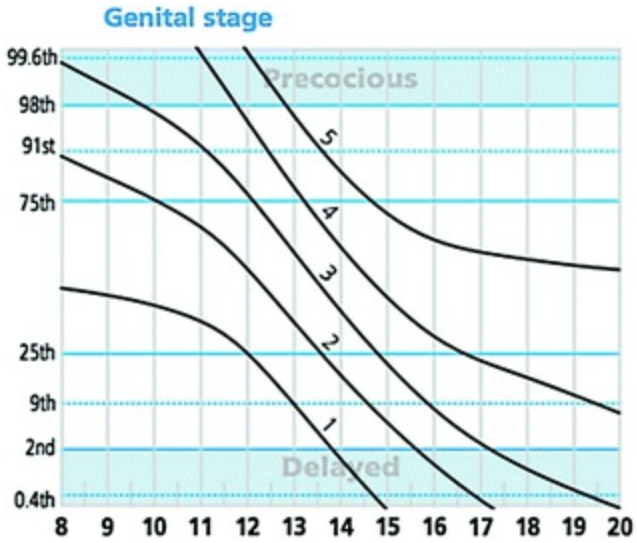

Fig. 4.2 Puberty normogram of the timing of genital Tanner staging in healthy boys. This *staged line* drawing gives age in years on the x-axis and centiles on the y-axis. Each *curved line* represents one genital tanner stage from 1–5 and illustrates the distribution of the timing of each stage in the normal population. These data have been incorporated into UK growth charts and are available at www.growthcharts.rcpch.ac.uk. Original concept and data from van Buuren [167]

Peak height velocity (PHV) and peak pubertal growth hormone production coincide approximately with the midpoint of pubertal development (Fig. 4.3). In boys, the PHV coincides with G3-4 at an average age of 13.5 years and achieves an incremental rate of 9.5 cm/yr [6]. Up to 25% of total adult height is achieved from growth during puberty, but the amplitude and peak velocity of the pubertal growth spurt are not fixed and vary with age at onset of puberty [7]. Early puberty is associated with a large pubertal growth spurt, while late maturers, who have a longer prepubertal period of growth, in turn experience a less pronounced pubertal growth spurt [8]. Therefore, although extremes of pubertal timing may lead to a small degree of final height reduction due to the reduced overall period of growth in precocious puberty, or poor PHV in delayed puberty [9], in general within wide limits, the timing of puberty does not influence adult height.

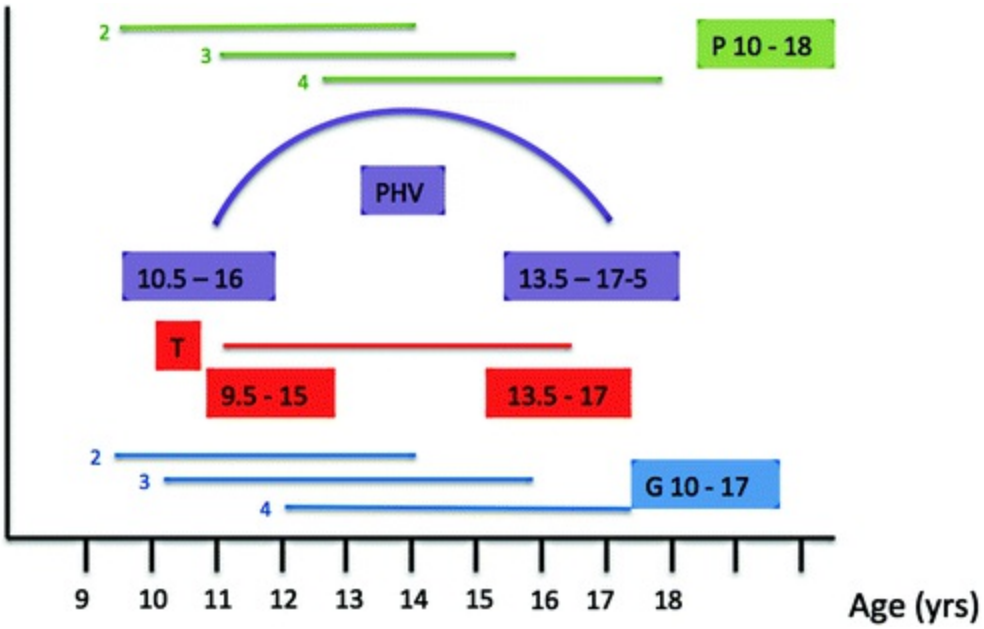

Fig. 4.3 Relationship between peak height velocity and pubertal development (male). *P* pubic hair stage; *PHV* peak height velocity; *T* testicular growth; *G* genital stage. Age ranges presented: in linear format—3rd–97th centiles for each Tanner stage, e.g., G2; in colored boxes—age range in years for each parameter

Regulators of the Timing of Puberty

Variability in the timing of puberty in healthy adolescents is governed by complex regulatory mechanisms involving genetic, environmental, and other factors [10]. Nutritional status, adoption, geographical migration, and emotional well-being have all been shown to affect pubertal timing [11–13]. While the timing of pubertal onset in girls in most countries in the developed world exhibited a rapid decrease in the first half of the twentieth century [14, 15], in boys, these trends are less clear. A small but significant change in the normal spectrum of timing of G2 development was documented in a European cohort [16] and in the USA [17] but remains controversial [18]. Much has been postulated about this observed secular trend toward an earlier age of pubertal onset in the developed world [19]. It also follows that it is difficult to understand the pathophysiology of delayed puberty before we can interpret this population level advancement in pubertal age.

Nutritional changes clearly have an important role, as shown by the positive correlation between age at puberty onset and childhood body size [20], most markedly in girls [11]. He and Karlberg demonstrated in a large dataset (n = 3650) that one BMI unit increase between the ages of 2 and 8 years is associated with a 0.11 year advancement in the timing of puberty as measured by peak height velocity in both genders [21]. In boys, however, the data are less consistent, with some studies documenting an earlier onset of puberty with greater adiposity, and some a later onset. In particular, more European studies have noted the former trend, while US studies have

more often shown the latter [22]. A recent study from the USA reported a far more complex relationship between fat mass and pubertal timing, with overweight status being associated with earlier pubertal onset, while obesity was associated with later onset. These effects also vary between ethnic groups [23]. Thus, one hypothesis in boys is that greater BMI leads to earlier pubertal timing up to the threshold at which obesity occurs. Obesity may delay pubertal timing in boys due to the suppression of the HPG axis with adiposity leading to excess aromatase activity and thus increased estrogen production.

Additional data point to an earlier trend in the age of puberty onset that is independent of BMI [24]. As detailed above, some studies suggest that over the last decade, the age of completion of puberty in males in some populations has become skewed toward later ages [5]. The effect of possible endocrine-disrupting chemicals (EDCs) on the timing of puberty is also an ongoing concern [5, 25]. Polybrominated biphenyls, bisphenol A, atrazine (herbicides), and phthalates, among others, have been suggested as possible EDCs responsible for contributing to this observed trend [26]. For example, children migrating for international adoption, and previously exposed to the estrogenic insecticide DTT in their country of origin, displayed early or precocious pubertal timing [27]. However, a mechanism of action for EDCs through the early initiation of the pulsatility of GnRH has not been conclusively demonstrated. Studies are complicated by the likely differing and possibly divergent influence of variable doses and mixtures of EDCs, and differing effects depending on age and length of exposure [5, 27, 28].

Epigenetic regulators are potential mediators of the effects of the environment on the hypothalamic control of puberty. However, while experimental data from rats provide evidence for changes in histone acetylation and gene methylation leading to altered gene expression during sexual development, the link between environmental factors and the epigenetic control of puberty has not been established. Although the window of opportunity for the effects of EDC exposure was historically considered to occur in the late prepubertal period, evidence of a fetal and neonatal origin for changes in pubertal timing counters this dogma. A multicenter US study found that prenatal exposure of boys to EDCs such as phthalates, based on the concentrations of four phthalate metabolites in a sample of urine, was associated with a short anogenital index, a marker for reduced masculinization of genital structures [29] (see Chap. 14). Epigenetic changes during fetal life are a potential mechanism for the effects of EDCs in utero [5]. Recent evidence suggests that effects of EDCs may persist in pregnant rats in not only their unborn fetus but into the next generation [30].

Despite the demonstrated importance of environmental factors, the genetic influence on pubertal timing is clearly fundamental. While the timing of pubertal onset varies within and between different populations, it is a highly heritable trait. The timing of sexual maturation is highly correlated within families and in twin studies, suggesting

strong genetic determinants [31]. Previous epidemiological studies and genetic approaches estimate that 60–80% of the variation in pubertal onset is under genetic regulation [14, 32, 33]. Despite this strong heritability, however, little is known about the genetic control of human puberty either in the normal population or in cases of disturbed pubertal timing [4]. A lack of clear understanding of the genetic factors that control and trigger the onset of puberty is an important barrier to deciphering the mystery of EDC and other environment/external cues to understand the secular trend toward earlier puberty [34].

Endocrine Changes During Puberty

Gonadotropin-Releasing Hormone and Gonadotropins

The hypothalamus–pituitary–gonadal (HPG) axis is already functional in the late fetal and neonatal period. The development of the HPG axis is exceptional in that GnRH is produced by hypothalamic neurons that develop outside of the central nervous system. The embryonic migration of GnRH neurons from the posterior wall of the nasal cavity to hypothalamus is key for the creation of the neuroendocrine pathways that allow normal pubertal development [35]. The whole process of migration involves no more than a few hundred neurons per hemisphere in the mouse (several thousand in primates or humans) [36]. The absolute number of GnRH neurons required for pubertal development is not known, but there appears to be a degree of redundancy in the system [37]. In addition, adult reeler mice have significantly fewer GnRH neurons in the hypothalamus and display a phenotype of delayed pubertal maturation and low fertility [38].

During gestation, there is an increase in GnRH content, which peaks at 34–38 weeks in the male fetus [39]. At mid-gestation, there is a striking rise in circulating gonadotropin levels in both male and female fetuses which fall to low levels in late gestation. This change in gonadotropin secretion is thought to result from the development of sex steroid negative feedback and from inhibiting influences from the CNS to GnRH neurons.

LH and FSH secretions rise during the first month after birth, probably because the negative feedback effect of placental estrogens is withdrawn. LH is secreted in pulses during this postnatal period indicating control by GnRH [40]. While the HPG axis is active during this ‘mini-puberty,’ it becomes dormant in young children between the age of 2 and 8–9 years [41]. Suppression of the axis is not absolute, however, as LH pulsatility is detectable during this stage using ultrasensitive assays, but pulses are infrequent, of low amplitude and occur mostly at night [42].

The clinical features of puberty are initiated by reactivation of the HPG axis after this relative quiescence during childhood. During this reactivation, there is a gradual development of a dynamic interplay between the central production of GnRH and gonadotropins, and gonadal sex steroid production, with progressive maturation of

negative and positive feedback loops. The central suppressant drive from the CNS gradually abates, and intensifying positive feedback results from the increase in sex steroid production by the gonads. The gonadal inhibition of the hypothalamic–pituitary system occurs later, becoming operative only at mid-puberty, and eventually becomes dominant over the central inhibitory feedback drive (Fig. 4.4). Both mean LH and FSH levels increase through pubertal development, although LH rises to a greater extent, probably due to mechanistic differences in feedback by estradiol and inhibin for these two hormones [43]. These rises are due to both an increase in basal levels of LH and FSH, and to a greater number and amplitude of LH pulses.

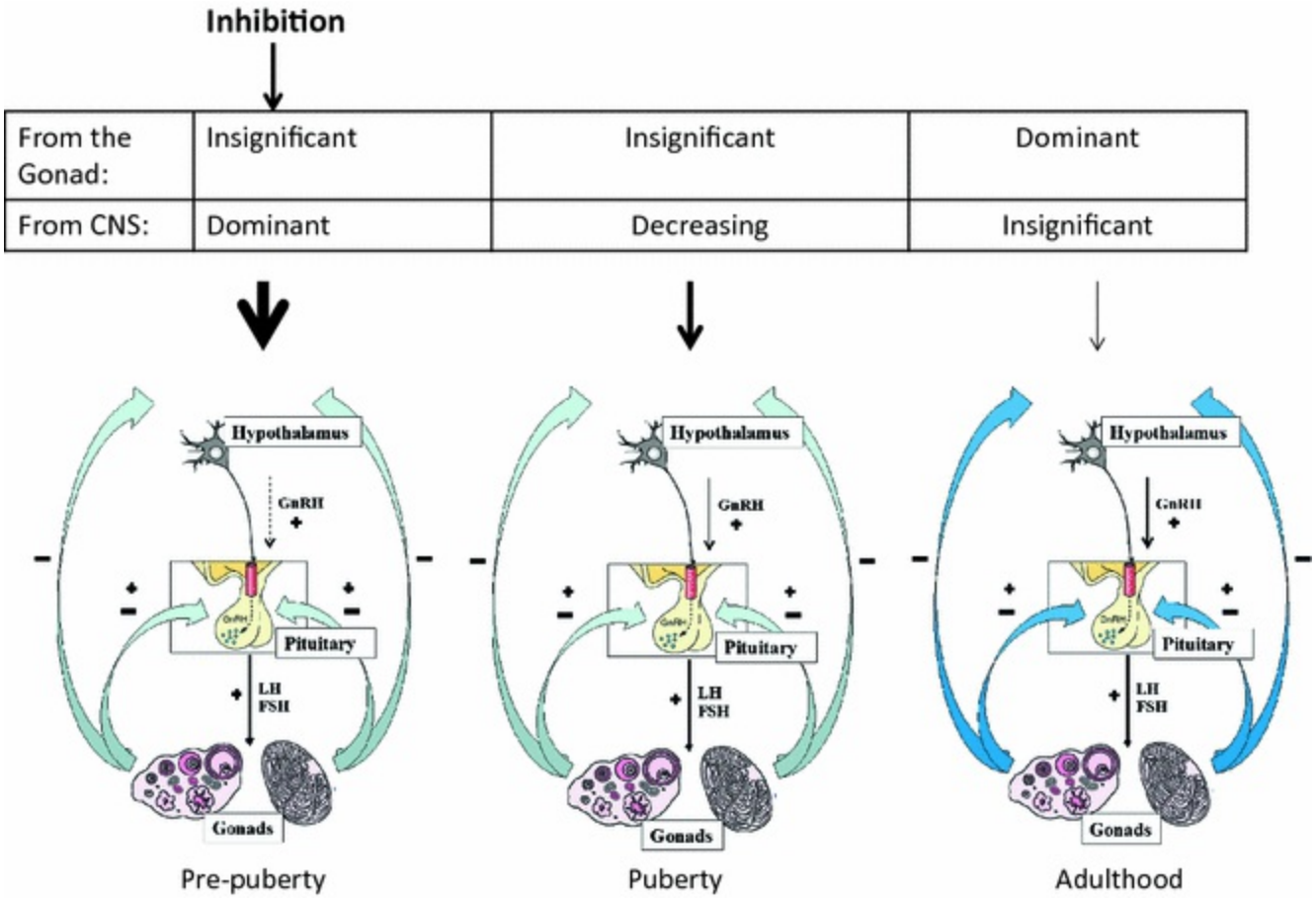

Fig. 4.4 A model showing the regulation of the hypothalamic–pituitary–gonadal axis during pubertal development

Sex Steroids and Inhibin-B

LH controls sex steroid production by upregulating expression of the steroidogenic enzymes and by controlling metabolic activity of the steroid-producing Leydig cells (see Chap. 2). LH may also stimulate Leydig cell proliferation and differentiation [44]. During puberty, plasma testosterone levels increase dramatically. Table 4.2 summarizes the levels of testosterone at various developmental stages with respective testis sizes. The pubertal increase in testis size results primarily from an increased number of

proliferating and differentiating germ cells and to an increase in the number of Sertoli cells [45]. In early and mid-puberty, there is a pronounced diurnal rhythm with a morning peak in testosterone levels, but this is less pronounced in later puberty, and declines gradually with age [46], probably due to decreased day–night ratios of gonadotropins [47].

Table 4.2 Pubertal stages (according to Tanner) with respective testis volumes and plasma testosterone concentrations

Pubertal stage (Tanner)	Testicular volume (mL)	Plasma testosterone (ng/dL)	Plasma testosterone (nmol/L)
1	<4	<10	<0.3
2	4–8	12–69	0.4–2.4
3	8–10	60–275	2.1–9.5
4	10–20	142–515	4.9–17.9
5	20–25	319–775	11.1–26.9

Values adapted from Knorr et al. [2]

Inhibin-B is a heterodimeric glycoprotein produced by Sertoli cells beginning in the fetus. Several studies show that serum inhibin-B levels in children change in concert with the secretion of gonadotropins [48–50]. During the 'mini-puberty,' serum inhibin-B levels increase to similar [51, 52] or even higher [49] levels than in adolescent boys and adult men. This early inhibin-B secretion is sustained until the age of 18–24 mo; thereafter, serum concentrations decline to lower but readily measurable levels [49]. Early in puberty, between Tanner stages G1 and G2, serum inhibin-B concentrations again increase to reach peak levels at the Tanner stage G2, but then the levels plateau [48, 50].

Central Control of Puberty via GnRH Upstream Pathways

GnRH pulsatility is coordinated by a balancing act between a number of inhibitory and excitatory neuronal and glial inputs (Fig. 4.5) [53]. Inhibitory inputs are primarily from GABAergic and opiatergic neurons, while glutamate and kisspeptin are the central excitatory neuronal signals. Glial cells additionally facilitate GnRH secretion via growth factor-derived signaling [54]. The onset of puberty is triggered by a decline in these inhibitory signals and an amplification of excitatory inputs, leading to increased frequency and amplitude of GnRH pulses [55]. Before the onset of puberty, GABA release in the preoptic area decreases in female rats [56], and in the rhesus monkey, GABA release into the median eminence decreases concomitant with the pubertal increase of GnRH secretion [57]. In female rats, glutamine synthase is downregulated, and glutamate dehydrogenase becomes more abundant in the hypothalamus at puberty, both leading to increased availability of glutamate [58]. Glutamate agonists are potent

stimulators of GnRH secretion, and administration in prepubertal primates can stimulate LH and testosterone secretion [59].

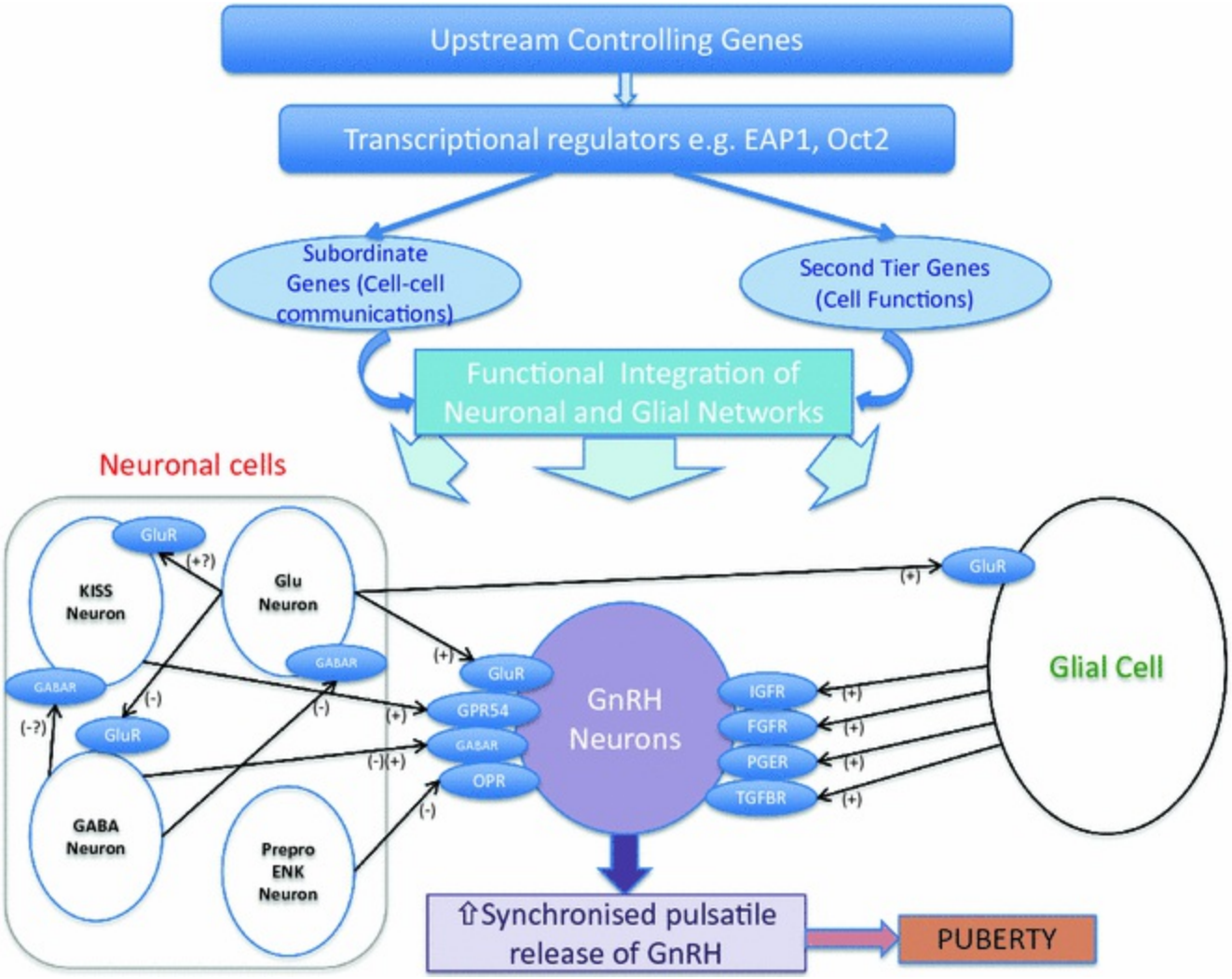

Fig. 4.5 Genetic regulators in the trans-synaptic and glial control of GnRH neurons during puberty. Adapted from Ojeda et al. [53]

Another vital piece in the puzzle of the central control of GnRH release came with the discovery of Kisspeptin. This excitatory neuropeptide was identified as a permissive factor in puberty onset by the discovery of patients with GnRH deficiency who had loss-of-function mutations in the *KISS1* receptor, *KISS1R* (previously known as *GPR54*) [60, 61]. Mice with knockout of *kiss1r* were simultaneously discovered to be infertile despite anatomically normal GnRH neurons and normal hypothalamic GnRH content [61]. Their phenotype can be rescued by exogenous delivery of GnRH. *Kiss1* knockout mice also have a phenotype consistent with normosmic GnRH deficiency. However, to date, only very rarely have mutations in *KISS1* been found in patients with delayed or absent puberty [62], although two potential activating mutations were identified in patients with central precocious puberty (CPP) [63].

Kisspeptin signals directly to GnRH neurones to control pulsatile GnRH release. Kisspeptin is upregulated in both primates and mice in the peri-pubertal period, and its administration in prepubertal rodents advances the onset of puberty [64]. Kisspeptin also appears to be downregulated in functional hypogonadism, suggesting its role as a mediator of the action of environmental factors such as nutritional status and emotional well-being on puberty and reproductive capacity. Kisspeptin signaling is an important element of both positive and negative feedback loops in the HPG axis. While kisspeptin has been identified as a pivotal upstream regulator of GnRH neurons, whether kisspeptin is the key factor that triggers the onset of puberty remains unclear.

An additional excitatory neuropeptide, neurokinin B, has been implicated in the upstream control of GnRH secretion. Identification of this pathway was via the discovery of loss-of-function mutations in *TAC3*, encoding neurokinin B, and its receptor *TACR3*, in patients with normosmic GnRH deficiency and pubertal failure [65, 66]. Both *KISS1* and *TAC3* are expressed by neurons in the arcuate nucleus of the hypothalamus that project to GnRH neurons, and their expression is downregulated by estrogen [67]. However, studies of the effects of neurokinin B administration have provided conflicting results. While central administration of neurokinin B agonists failed to stimulate GnRH release in rodents, and *Tacr3* knockout mice have grossly normal fertility [68, 69], primate studies showed that neurokinin B can act to stimulate GnRH release via kisspeptin signaling [70]. Additionally, neurokinin B is expressed more widely in the central nervous system than in kisspeptin, suggesting some differences in the roles of these two neuropeptides in the control of pubertal onset.

Dynorphin, an opioid peptide that is coexpressed with kisspeptin and neurokinin B in so-called KNDy neurons, inhibits the release of GnRH, and together, these peptides are currently believed to play a fundamental role in the GnRH pulse generator. Other neurons regulate GnRH including GABAergic signaling pathways which function in the stress-induced suppression of LH, and *RFamide-related peptide* gene (*RFRP*), the mammalian ortholog of the avian peptide *gonadotrophin-inhibiting hormone* (*GnIH*) [71].

Glial inputs appear to be predominantly facilitory during puberty, acting via growth factors and small diffusible molecules, including TGFβ1, IGF-1, and neuregulins that directly or indirectly stimulate GnRH secretion [72]. Glial cells in the median eminence regulate GnRH secretion through the production of growth factors that activate receptors with tyrosine kinase activity. FGF signaling is required for GnRH neurons to reach their final destination in the hypothalamus [73], as well as for GnRH neuronal differentiation and survival [74]. Additionally, GnRH neuron secretory activity is facilitated by IGF-1 and by members of the epidermal growth factor family such as neuregulin 1β [54, 75]. Moreover, plastic rearrangements of glia–GnRH neuron adhesiveness, mediated by soluble molecules such as neuronal cell adhesion molecule (NCAM) and synaptic cell adhesion molecule (SynCAM), coordinate the controlled delivery of GnRH to the portal

vasculature [72], a process that is subject to sex steroid regulation [76].

The interplay between puberty and metabolism has been the focus of intense study. The role of fat mass in pubertal timing is thought to be mediated, at least in part, through the permissive actions of the metabolic hormone leptin, a key regulator of body mass, secreted from white adipose tissue (WAT) [77]. Absence of leptin signaling results in obesity and infertility, whereas leptin treatment decreases food intake and restores reproductive functions. During fasting, leptin levels decrease and gonadotropin secretion is suppressed. These findings imply that nutritional status, especially fat tissue and leptin, could contribute to pubertal development. Clinical observations have further supported a role for leptin signaling in the onset of puberty, e.g., humans and mice lacking leptin (Lep ob/ob) or the leptin receptor (LepR db/db) fail to complete puberty and are infertile [78], and in a 12-yr-old girl with congenital leptin deficiency, treatment with recombinant leptin was followed by a pubertal pattern of LH release [79].

GnRH neurons do not express the LepR. Instead, leptin appears to indirectly regulate GnRH via actions on cells that are afferent to GnRH neurons such as LEPR-expressing GABA neurons from the arcuate nucleus [80], or via cells that interact morphologically with GnRH neurons, partly through nitric oxide signaling, which is required for leptin action [81], and via kisspeptin/neuropeptide Y neurons [82, 83]. Cross-sectional studies showed that serum leptin levels rise in girls in early puberty [84] but not in boys. In careful longitudinal studies in male rhesus monkeys, leptin levels decreased during the juvenile period and were unchanged during puberty [85, 86], and leptin gene expression in the hypothalamus is unchanged during the development in the rat [87, 88]. Therefore, leptin is probably not the requisite metabolic trigger for the onset of puberty, but rather is a permissive factor signaling healthy energy balance.

Neuropeptide Y (NPY) is involved in many CNS functions, including appetite control and reproduction. Evidence from primate studies suggests that NPY may also have a contributory role in the break restraining the onset of puberty in primates [68, 89]. Ghrelin and other gut-derived peptides may also form part of the mechanism by which energy homeostasis regulates reproductive development [90]. Both low birthweight and prematurity are associated with earlier onset of puberty [91, 92], particularly in those children with rapid increase in length or weight in the first two years of life [93]. It remains unclear, however, whether childhood obesity, insulin resistance, excess androgens, or other factors may explain this association [94, 95].

While GnRH pulsatility is clearly the central driver of pubertal onset, and aberrant GnRH neuronal development and function lead to hypogonadotropic hypogonadism (HH), our collective understanding of the upstream neurocircuitry regulating GnRH neurons remains incomplete. Data pointing to hypothalamic regulation via a hierarchical network of genes (Fig. 4.6) have mainly come from a systems biology approach and from animal models, with little data from human subjects [96]. Candidate

transcriptional regulators that have been identified via these approaches include *Oct-2*, *TTF-1,* and *EAP1* [97–99].

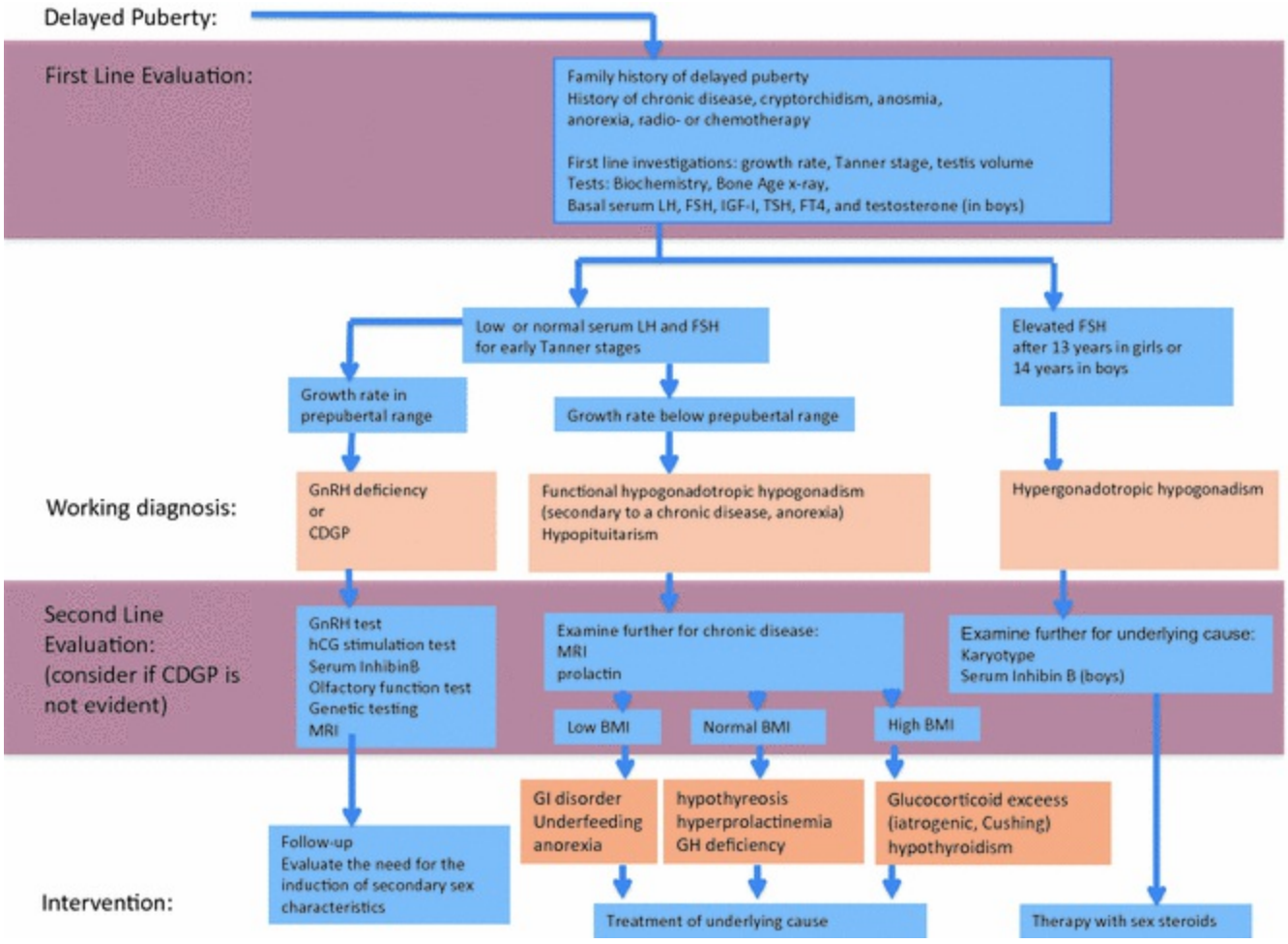

Fig. 4.6 Algorithm for the evaluation of boys with delayed puberty. *CDGP* constitutional delay of growth and puberty, *GI* gastrointestinal, *GH* growth hormone, *GHD* GH deficiency, *PRL* prolactin, and *IGF-1* insulin-like growth factor 1. Adapted from Palmer and Dunkel [4]. Copyright © (2012) Massachusetts Medical Society. Reprinted with permission

Delayed Puberty

Presentation

Disturbances of puberty encompass an important group of pathologies within the field of pediatric endocrinology. First, they are common, affecting over 4% of adolescents. In addition, abnormal timing of pubertal development is associated with adverse health and psychosocial outcomes [100–102]. This has importance for the individual, but also has a potential major impact on public health, especially in view of the secular trend toward an earlier age of puberty onset [25]. Early puberty, in particular, is associated with adverse health outcomes, including breast [103] and endometrial cancer [104], obesity [105], type 2 diabetes [102], cardiovascular disease [106, 107], short stature

[108], and even increased mortality [106]. However, persons with delayed puberty are also at risk of short adult height [109–112], decreased bone mineral density [113, 114], and psychological sequelae [115, 116].

Self-limited Delayed Puberty

Self-limited delayed puberty (DP), also known as constitutional delay of growth and puberty (CDGP), represents the most common cause of delayed puberty in both sexes. Up to 63% of boys with delayed puberty have self-limited DP [117]. Self-limited DP segregates within families, with the majority of families displaying an autosomal dominant pattern of inheritance (with or without complete penetrance) [31]. Fifty to 75% of subjects with self-limited DP have a family history of delayed puberty onset [118].

Patients with self-limited DP represent the extreme tail of normal puberty, defined as the markers of the onset of puberty occurring two or more standard deviation (SD) later than the population mean age for gender. In addition, self-limited DP may encompass older children with delayed pubertal progression, a diagnosis that is aided by the use of puberty nomograms (Fig. 4.2) [119]. The absence of a pathological medical history, signs and symptoms, and a positive family history of delayed puberty in one or both of the parents suggests a diagnosis of self-limited DP; however, before making that diagnosis, pathological conditions must be excluded.

In DP, probably because of a low estrogen concentration for chronological age (but not for bone age), growth hormone (GH) secretion is functionally and temporally reduced for age, and when this functional GH deficiency is prolonged, it may also impact adult height. After the onset of puberty or the initiation of appropriate treatment, growth velocity and GH secretion return to normal. Additionally, in about half of the subjects with constitutional delay, there is delayed maturation during early childhood, and consequently, they are shorter than their peers even before the mean age for the pubertal growth spurt [119].

It has been shown that those self-limited DP subjects who also have reduced growth in childhood may not fully exploit their genetic height potential, resulting in an adult height below their mid-parental target height [112, 120–122], with an average loss of 4.2 cm if untreated [122]. However, the majority of the patients seen at pediatric endocrinology clinics are short because their condition is compounded by two independent factors: short genetic height potential and DP. Other studies showed only a negligible difference in final height, even in DP subjects who have received no intervention [109, 123–128]. This may imply a pathophysiological mechanism in addition to lack of sex steroids contributing to the growth phenotype in some patients with DP, but not in others [128].

In self-limited DP, adrenarche may also occur later than usual [4]. Bone age in self-

limited DP is retarded compared to chronological age, but the developmental milestones are achieved at a normal bone age, that is, onset of signs of pubertal development by a bone age of 13.5 years in boys. Gonadotropin and sex steroid concentrations increase in concert with the progression of the bone age. Thus, all stages of pubertal development occur at an age that is later than average.

There are three main differential diagnoses of self-limited DP [4, 117] (Table 4.3): hypergonadotropic hypogonadism, with primary gonadal failure leading to elevated gonadotropin levels (approximately 7% of males with delayed puberty); functional hypogonadotropic hypogonadism, where late pubertal development is due to maturational delay in the HPG axis secondary to a chronic disease [129] (found in 19–20%), malnourishment [130], excessive exercise [131, 132], or psychological or emotional stress [133]; and permanent hypogonadotropic hypogonadism (HH), characterized by low LH and FSH levels (9% of boys).

Table 4.3 Differential diagnoses of male self-limited delayed puberty

	Hypergonadotropic hypogonadism	**Hypogonadotropic hypogonadism**	**Functional hypogonadotropic hypogonadism**
Common causes:	Klinefelter syndrome Gonadal dysgenesis Chemotherapy/radiation therapy	CNS tumors/infiltrative diseases Isolated hypogonadotropic hypogonadism Kallmann syndrome Combined pituitary hormone deficiency Chemotherapy/radiation therapy	Inflammatory bowel disease Celiac disease Anorexia nervosa Hypothyroidism Excessive exercise Cystic fibrosis

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Hypogonadotropic Hypogonadism

HH is often diagnosed during the second or third decades of life. Common presenting signs are delayed onset of puberty, poorly developed secondary sexual characteristics, eunuchoid body proportions, or infertility [134]. This condition can be due to a congenital hypothalamic or pituitary disorder or to an acquired central dysfunction secondary to irradiation, surgery, tumor, or a vascular lesion. Tumors causing delayed puberty most commonly interfere with GnRH synthesis or secretion, the most common being craniopharyngioma, germinoma, and Langerhans cell histiocytosis. A deficiency of other anterior pituitary hormones, diabetes insipidus, and visual disturbance is common. Congenital malformation of the pituitary, hypothalamic, and other midline structures may cause HH, often in conjunction with other pituitary hormone deficiencies.

A picture of ‘idiopathic’ hypogonadotropic hypogonadism (IHH) with no associated

anatomical or functional defect in the hypothalamic–pituitary–gonadal axis occurs in 1–10 cases per 100,000 births. Because of different causes and incomplete penetrance, there is a wide spectrum of phenotypes, ranging from complete hypogonadotropic hypogonadism, with lack of pubertal development, to partial hypogonadism with an arrest of pubertal development, and even reversible HH in some patients post treatment [135]. Despite recent advances, with over twenty genes linked with this disorder having been identified, the pathophysiological basis of hypogonadotropic hypogonadism in the majority of individuals remains unclear [136] (see Chap. 5). The condition may be due to the failure of the development of gonadotropin-releasing hormone (GnRH) neurons, lack of activation of GnRH secretion, or disrupted GnRH signaling (including mutations in the *GNRHR* gene). Kallmann syndrome (hypogonadotropic hypogonadism associated with anosmia) is the most common form of isolated HH, accounting for 60% of cases.

Downstream mutations in the GnRH signaling pathway can also present as delayed puberty. LH and FSH are encoded by a common α-subunit gene and a specific β-subunit gene. Mutations of the β-subunits genes of LH or FSH are extremely rare causes of HH [137, 138] (see Chap. 6).

Functional Hypogonadotropic Hypogonadism

Functional HH is seen in malnutrition and chronic diseases, as exemplified by patients with inflammatory bowel disease, celiac disease, chronic kidney disease, cystic fibrosis, and sickle cell anemia. Undernutrition in states of starvation such as anorexia nervosa, or an imbalance due to excess energy consumption in extreme athletic training, can also cause central GnRH suppression. The factors contributing to these states of functional HH include low fat mass, inflammation, and stress [139, 140].

Hypergonadotropic Hypogonadism

Hypergonadotropic states (chromosomal alterations, syndromes, genetic disorders, and radiotherapy/chemotherapy) are covered elsewhere in this volume (*see* Chaps. 9 and 10) and may present as pubertal delay. Testicular abnormalities are characterized by elevated gonadotropin and low inhibin-B concentrations, and patients with these abnormalities sometimes have specific physical features.

Delayed Puberty—Diagnosis

The cutoff age for boys who need an evaluation for delayed puberty may vary in different ethnic groups, but in most populations, early signs of secondary sexual development should be present by age 14 years. The evaluation of testicular volume is vital, as an accurate diagnosis of delayed puberty cannot be made without assessment of Tanner genital stage. A thorough medical history should note the symptoms and signs of

anorexia nervosa, the intensity of athletic training, and the timing of puberty of both parents, as there is often a family history of DP in cases of self-limited DP (Fig. 4.6). A history of chronic illness, such as celiac disease or inflammatory bowel disease, suggests a temporary or secondary delay of puberty. Stature and height velocity should be evaluated using appropriate growth charts. Bone age (X-ray film of left hand and wrist read according to standards such as Greulich and Pyle) delay provides useful information in the growth analysis but contributes little to the differential diagnosis. It may be very difficult to distinguish clinically between the diagnosis of self-limited DP and congenital HH in the teenage years [141]. While gonadotropin levels are generally increased in primary testicular failure or in Klinefelter syndrome, single basal serum LH and FSH determinations are not useful in the differential diagnosis of self-limited delay vs hypogonadotropic hypogonadism.

In some cases, the diagnosis of permanent HH can be suspected before the age of pubertal onset, and importantly, if this suspicion arises in the first six months of life, it can be confirmed on the basis of low testosterone and gonadotropin levels, indicating an absence of the normal 'mini-puberty.' The presence or absence of 'red flag' features remains the strongest discriminator between self-limited DP and IHH. These red flags include cryptorchidism or micropenis, or the presence of the other components of the Kallmann syndrome which include anosmia or hyposmia due to hypoplasia of the olfactory bulbs, as well as cleft lip and palate, unilateral renal agenesis, short metacarpals, sensorineural hearing loss, synkinesia, and color blindness.

Investigation of the differential diagnosis of the two conditions may involve a number of physiological and dynamic tests including assessment of LH pulsatility by frequent blood sampling [42], the prolactin response to provocation [142, 143], the gonadotropin response to GnRH [144, 145] and analogs [146], the testosterone response to hCG [146–148], and first morning-voided urinary FSH and LH levels [149]. Boys with self-limited delay who are destined to undergo spontaneous pubertal development within 6 to 12 months may have a pubertal pattern of response to GnRH (post-GnRH maximum LH levels higher than maximum FSH levels). However, a low prepubertal LH response to GnRH is usually found in boys with self-limited DP who will develop later than that, as well as in boys with permanent HH. Most recently, a single measurement of inhibin-B <35 pg/mL has been shown to help discriminate prepubertal boys with IHH from DP with high sensitivity [150]; nevertheless, patient follow-up is necessary for a definitive diagnosis in the majority of cases. With the discovery in the last two decades of genes causing HH, genetic testing will no doubt modify the management of many of our male delayed puberty patients [151].

Male Delayed Puberty—Therapeutic Indications

Induction or progression of puberty is indicated for adolescents who have either

significantly delayed or arrested puberty, or have been diagnosed with permanent hypogonadism. Appropriate treatment modalities are directed according to the underlying diagnosis.

A management strategy of 'watchful waiting' may be appropriate in self-limited DP, where pubertal onset is late but expected to occur spontaneously. However, this decision should be taken in conjunction with the patients, taking into consideration their concerns and expectations. One major concern often raised by patients and their families is the effect of delayed puberty on both current and final height, particularly for patients with concurrent familial short stature. However, patients can be reassured that adult height in DP is usually only slightly below genetic height potential (target height), although there may be large individual variation [121, 152]. If height is not a major concern, reassurance with accurate adult height prediction is frequently sufficient, especially if puberty has already started. DP in adolescents can, however, be associated with significant anxiety about body image in terms of physical size and pubertal immaturity, decreased self-esteem with social isolation, withdrawal from sporting activities, and psychosocial and peer relationship difficulties. In these circumstances, there is evidence that hormonal therapy can be beneficial [153, 154]. The link between DP and reduced academic performance, substance misuse, and behavioral difficulties is less well established.

In contrast, if 'red flag' markers of hypogonadism are present, or if endogenous gonadotropin-dependent puberty has not started after one year of treatment, then permanent HH and other diagnoses should be reconsidered, and a brain MRI should be performed. In such instances, treatment should be initiated promptly in order to optimize skeletal growth and induce secondary sexual characteristics and, therefore, minimize the psychosocial difficulties faced by adolescents with hypogonadism.

Management of Delayed Puberty—Therapeutic Principles

The options for the management of male patients with self-limited DP include monitoring with reassurance, or therapy with low-dose testosterone to augment the growth rate and induce secondary sexual characteristics (Table 4.4). There are numerous published studies of treatment of DP in boys, but most are observational, with a few small randomized controlled trials [154–156]. Most report treatment with short courses of low-dose androgens with outcomes of increased height velocity without advanced bone age, advanced sexual maturation, and often improvement in psychosocial parameters.

Table 4.4 Medications used for the treatment of delayed puberty

Drug and formulation	Induction of puberty in boys		Side effects and cautions

	Self-Limited DP	Hypogonadism	
Testosterone (T)[a]			Erythrocytosis, weight gain, prostatic hyperplasia. High doses can cause premature epiphyseal closure. Not for use in boys with bone age <10 years
T enanthate, cypionate, and propionate. T enanthate has longer duration of effect than T propionate. IM injection	Not recommended before 13.5 years of age. Initial dose 50–100 mg every 4 weeks for 3–6 months. After review of response: repeated treatment with 25–50 mg increment in dose (not exceeding 100 mg)	Can initiate after age 12 years at 50 mg/month. Increase with 50 mg increments every 6–12 months. After reaching 100–150 mg monthly, decrease interval to every 2 weeks. Adult dose 200 mg every 2 weeks	All IM preparations: local side effects (pain, erythema, inflammatory reaction, and sterile abscess). Priapism can occur in patients with sickle cell disease
T undecanoate IM/PO	Starting dose of 40 mg PO OD increasing rapidly to 40 mg BD and subsequently 80 mg BD. Rx paused and then stopped if significant increase in basal LH and/or testicular volume observed (120)	Adult dose is 1000 mg IM every 10–14 weeks	Very rarely, paroxysms of coughing and dyspnoea post-IM injection, ascribed to lipid embolism from the vehicle, hence not licensed in USA
T gel. Transdermal preparations, applied topically at bedtime	No data available	Can be started when approximately 50% adult dose with IM T has been achieved. Adult dose is 50–80 mg daily	Local irritation. After applying, avoid close skin contact with children and women

[a]Anabolic steroids are not recommended for the induction of secondary sexual characteristics

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The most commonly used treatment regimen for low-dose testosterone for boys with DP is supplementation using intramuscular depot preparations of a testosterone ester [157], at a starting dose of 50 mg each month for 3–6 months; a further 3–6 months of treatment may be given, with dose escalation as required. Oral testosterone undecanoate (where available) at a dose of 40–160 mg daily has recently been shown to be safe and efficacious for pubertal induction in DP, without compromise in final height [119] (Table 4.4). Topical testosterone gels may also be suitable for this indication [158]. Monitoring via serum testosterone increase (to mid-reference range one week post injection), basal LH increase, height velocity, testicular volume, and extent of virilization is appropriate. GH deficiency must be excluded if height velocity does not increase with testosterone therapy. Testosterone esters should be avoided in patients with hepatic impairment or hypercalcaemia and used with caution in chronic kidney

disease [159]. Preparations are generally well tolerated, but side effects may include headaches, depression, and androgenic effects such as acne. Although anabolic steroids such as oxandrolone have been used historically for short-term increase in height velocity, they are less effective in stimulating pubertal development and therefore are not recommended for the management of boys with delayed puberty.

As discussed above, self-limited DP is commonly seen in combination with idiopathic short stature (ISS). After exclusion of GH deficiency, for example, by the use of a primed GH provocation test, the treatment of GH-replete DP patients with growth hormone remains controversial: It has been approved by the US Food and Drug Administration for the treatment of ISS and height SDS $\leq$2.25 for age, but leads to only a modest increase in adult height, and its use is not recommended by the authors [160, 161].

A further potential pharmacological target in short boys with DP is the inhibition of estrogen biosynthesis from androgens using aromatase inhibitors [162, 163]. Epiphyseal closure is dependent on estrogens, and thus, aromatase inhibitors (AIs) can potentially act to extend the time period in which long bone growth occurs and therefore increase adult height. Some published data support this use of AIs to delay bone maturation and increase adult height in boys with short stature and/or delayed puberty [162, 163]. However, there remains much uncertainty in terms of efficacy, optimization, and safety profile of AI therapy [164], and the optimal dose, timing, and duration of treatment in ISS and DP remain uncertain. Controlled trials have pointed to potential adverse effects, in particular compromised trabecular bone health in the form of vertebral body deformities in boys with ISS treated with letrozole [165]. Until more information from ongoing studies of these agents is available, active management should not involve aromatase inhibitors unless used within the setting of a clinical trial.

Concluding Remarks

In healthy boys, the normal age limits for the first signs of secondary sexual development are between 9.5 and 14 years. There are multiple genetic and environmental influences on the timing of puberty in the general population, and appropriate age cutoffs for delayed puberty in different ethnic groups may vary.

DP is a frequent problem, and the most common underlying condition is self-limited (or constitutional) DP, which is usually accompanied by delayed growth. However, the differential diagnoses include hypogonadotropic and hypergonadotropic hypogonadism, and these conditions must be considered in boys with pubertal delay. Distinguishing between self-limited DP and permanent hypogonadotropic hypogonadism remains difficult.

Management of male adolescents with DP is dependent on the underlying cause. Treatment of isolated DP involves expectant observation or short courses of

testosterone in low doses, while more complex and involved management is required for males with permanent hypogonadism. However, a proportion of young men with DP will remain adversely affected by their delayed pubertal development and/or short stature in adolescence, which may have long-term consequences. It is not known whether pubertal delay has a negative impact on adult bone mass [166], and whether potentially compromised bone health is a reason to initiate sex steroid replacement. Additionally, the optimal management of males with severe hypogonadotropic hypogonadism (cryptorchidism, micropenis, and lack of spontaneous increase in testicular size in puberty) remains uncertain. Whether such patients would benefit from prepubertal FSH treatment to improve potential for future fertility is another unanswered question (see Chap. 20).

The genetic and environmental basis for both DP and HH is an area of research where there is still much to be discovered, and may bring future benefits for patient management. Our understanding of the key controllers of pubertal onset and its timing is advancing but remains a complex puzzle to be unlocked. Mechanistic discoveries in DP and hypogonadism patients are also likely to shed light on the changes in pubertal timing in the general population.

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5. Congenital Hypogonadotropic Hypogonadism in Males: Clinical Features and Pathophysiology

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Normal Sexual Development in the Human Male

Ontogeny of GnRH Secretion

Gonadotropin-releasing hormone (GnRH) is one of the key master regulators of sexual development and reproductive function in humans. This hypothalamic peptide is secreted by a unique network of hypothalamic neurons in a pulsatile pattern that is critical for its "downstream" biological effects. GnRH-induced pituitary gonadotropin secretion in the human changes dynamically across the development starting in utero, through infancy, puberty, and adulthood (Fig. 5.1). Therefore, understanding the developmental ontogeny of GnRH secretion is essential for assessing normal physiology of sexual maturation as well as pathologic disorders of this process like congenital hypogonadotropic hypogonadism (CHH).

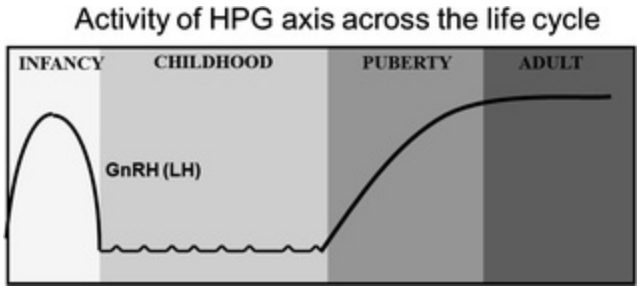

Fig. 5.1 Schematic presentation of the activity of the hypothalamic–pituitary–gonadal axis across the life cycle. During the neonatal window, pulsatile GnRH stimulates LH and FSH secretion which induce adult levels of testosterone and inhibin B. Childhood is marked by a low-amplitude LH secretion and very low T levels. Pubertal reactivation of the HPG axis triggers the onset of sexual maturation, initially in a sleep-entrained pattern

GnRH neurons originate outside the central nervous system (CNS), primarily from the neuro-ectodermally derived embryonic olfactory placode within a smaller subset of these pioneer cells that have recently been shown in murine studies to be of neural crest origin [1]. These developing GnRH neurons then migrate along the olfactory sensory axonal tracts as they enter the forebrain through the cribriform plate and eventually reach their final destination in the medial preoptic area of the hypothalamus.

GnRH neurons have been observed to be in place within the human hypothalamus by the ninth week of gestation, and secreted gonadotropins can be detected in the fetal circulation by 12–14 weeks of gestation [2]. In the neonatal period, the hypothalamo–pituitary–gonadal (HPG) axis is fully active when its pulsatile pattern of GnRH secretion initiates an initial rise in gonadotropin secretion that in turn initiates the first wave of systemic secretion of hormones from the gonads of fetuses of both sexes [3]. In male infants, this initial wave of HPG axis activity, termed the mini-puberty of infancy, declines by 6 months [3].

This initial neonatal HPG activity in male infants serves two key physiological functions: rapid proliferation of the Sertoli cells [4] and increase in germ cell number [5], both of which ultimately determine future successful spermatogenesis. If in-utero/neonatal activation of the HPG axis is defective, male infants may present with micropenis and cryptorchidism as seen in patients with CHH (see below). Following this early HPG axis activity in both sexes, the secretory activity of the HPG axis is dampened during most of childhood. However, the HPG axis is not completely quiescent since the pulsatile gonadotropin release, albeit at extremely low-amplitude pulses, has been shown to occur in prepubertal boys using ultrasensitive LH assays [6].

The onset of puberty is subsequently marked by a gradual “reawakening” of the dormant GnRH pulse generator, initially in a sleep-entrained manner, with a striking increase in the amplitude of LH pulses and less change in pulse frequency, resulting in stimulation of the gonads [7, 8]. As puberty progresses, GnRH-induced gonadotropin pulses occur during both the day and night, eventually concatenating in the development of secondary sexual characteristics and the changes in body composition characteristic

of puberty. In adult males, GnRH-induced gonadotropin secretion averages one pulse approximately every 2 h (Fig. 5.2), but there is considerable variability among men in both the frequency and amplitude of LH pulses as well as in the ensuing T levels. Indeed, 15% of normal men exhibit long interpulse intervals between LH secretory pulses, resulting in a transient decrease in serum testosterone (T) levels to as low as 3.5 nmol/L (Fig. 5.3) [9].

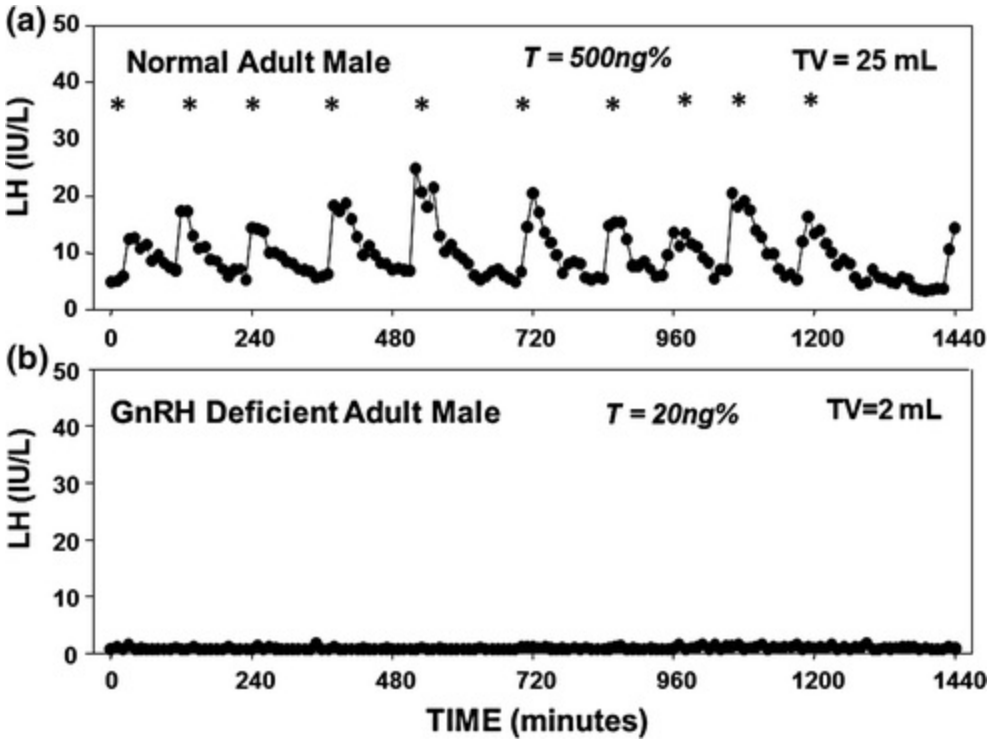

Fig. 5.2 Frequent blood sampling (every 10 min for 24 h) for LH in a normal adult male and a man with CHH. **a** Normal adult male pattern of GnRH secretion with high-amplitude LH pulsations (10 pulses in 24 h), normal serum T level, and normal testicular volume; **b** Apulsatile pattern of GnRH secretion in an CHH male as assessed by a complete absence of endogenous LH pulsations, low serum T levels, and prepubertal-sized testis

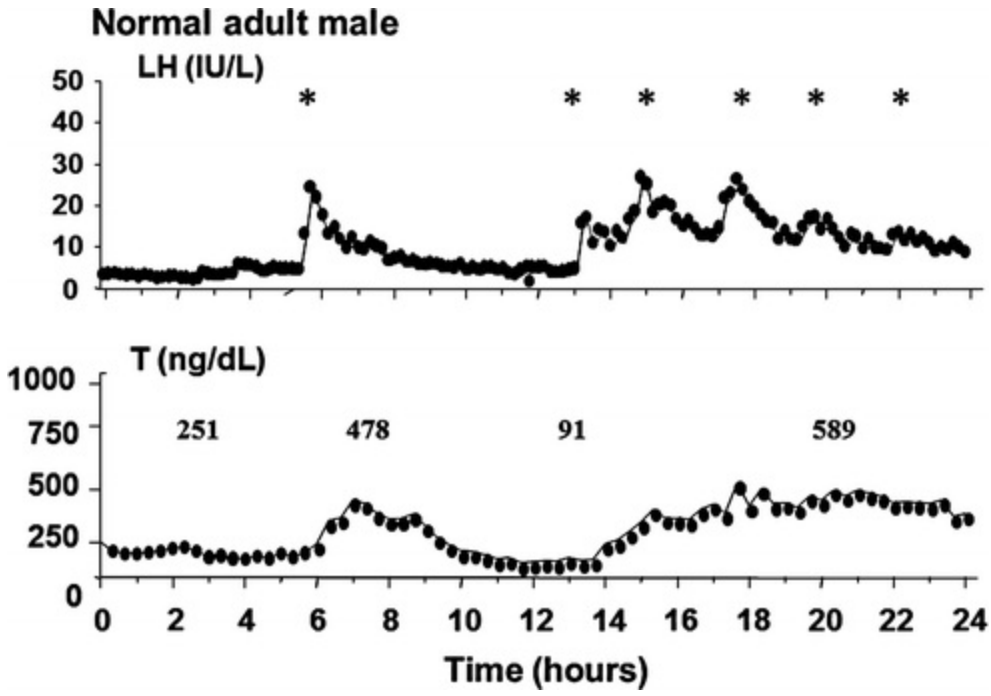

Fig. 5.3 Frequent blood sampling (q 10 min for 24 h) of serum T and LH in a normal adult man revealing a nadir T level of 91 ng/dl (to convert to nmol/L, multiply by 0.03467) after a long interpulse interval of LH secretion. Adapted from Spratt et al. [9]

Normal Gonadal Development in the Male

The gonadal sex, i.e., initial development of the fetal testes from undifferentiated gonad, is initiated by the expression of the *SRY* (sex-determining region Y) gene. Fetal testicular Leydig cells, stimulated by placental hCG, produce T that induces the growth, stabilization, and differentiation of the gonadal Wolffian structures (epididymis, vas deferens, and seminal vesicles). The 5α-reductase enzyme converts T to the more potent androgen, 5α-dihydrotestosterone, which induces the formation of the prostate and the external male genitalia. Secretion of anti-Müllerian hormone (AMH) from fetal testicular Sertoli cells causes regression of the Müllerian structures. During the later stages of gestation and in the "neonatal window of mini-puberty," GnRH-induced LH secretion drives T and insulin-like factor 3 (INSL3) secretion by the Leydig cells (T levels reaching adult levels in the neonate), directs the completion of the inguino-scrotal phase of testicular descent, and induces further growth of the phallus [10]. This first wave of postnatal Sertoli cell proliferation in response to GnRH-induced FSH secretion results in increases in Sertoli cell number during infancy [11], and this developmental phase is critical for setting the stage for future spermatogenic success in adulthood. Prior to the onset of puberty, there is a gradual threefold increase in testis size as evidenced by postmortem studies in childhood trauma victims [12]. This increase is caused by a lengthening of the seminiferous tubules, due mainly to intense proliferation of Sertoli cells and, to a lesser degree, of immature germ cells [11, 12].

A second phase of Sertoli cell proliferation occurs early in puberty with further increase in number after which gonadal cellular proliferation ceases, Sertoli cells mature and the blood–testis barrier is established, the seminiferous tubular lumen forms, and spermatogenesis is initiated [13, 14]. Both the neonatal and pubertal Sertoli cell maturation phases are marked by discrete developmental windows of neuroendocrine activity [15]. Ultimately, this final complement of mature Sertoli cells is the major determinant of testes size, seminiferous tubular development, and sperm count, as each Sertoli cell can only support a finite number of germ cells [16–18]. Thereafter, a dramatic increase in meiotic germ cells promotes the most significant increase in testis size that occurs during puberty. Finally, the improvement of spermatogenic efficiency leads to complete maturation of the seminiferous tubules. While intratesticular T plays a central role in stimulating spermatogenesis both directly and in concert with FSH (see Chap. 3), the subsequent increase in systemic T levels induces the secondary sexual characteristics of the adult male. The specific role of FSH in adult males is not yet well established. However, based on the phenotype of the males with mutations in either the FSHβ or FSH receptor (see Chap. 6), it appears that FSH is necessary to maintain both qualitative and quantitative normal spermatogenesis.

Idiopathic or Congenital Hypogonadotropic Hypogonadism

HH (i.e., secondary hypogonadotropic hypogonadism) is differentiated from primary testicular disease (i.e., primary hypergonadotropic hypogonadism) by the demonstration of low/inappropriately normal gonadotropin levels in the setting of low sex steroid levels and impaired spermatogenesis. Congenital abnormalities that lead to HH are rare but are well described and result from either hypothalamic GnRH deficiency or pituitary disorders that affect the gonadotropes.

Differential Diagnosis of Congenital Hypogonadotropic Hypogonadism
Hypothalamic Causes
- Idiopathic Hypogonadotropic Hypogonadism (IHH)
- Anosmic form: Kallmann syndrome
- Non-ansomic form: Normosmic IHH (nIHH)
- Variant forms of IHH:
- Fertile eunuch syndrome
- Reversible form
- Adult-onset HH
- *Complex syndromic forms of IHH*
- Adrenal hypoplasia congenita and IHH
- Morbid obesity syndromes: Prader–Willi syndrome; leptin pathway mutations
- CHARGE syndrome

TUBB3 E410 K syndrome
Waardenburg syndrome
Gordon-Holmes syndrome Boucher-Neuhauser syndrome
Bardet-Biedl syndrome
Pituitary Causes (see also Chap. 8):
HH associated with other pituitary hormone deficiencies
Genetic defects of the gonadotropin subunits and pituitary transcription factors

Congenital HH (CHH), also referred in the literature as idiopathic hypogonadotropic hypogonadism, results from genetic mutations that lead to abnormalities in GnRH development, secretion, and/or action. CHH presents in two major phenotypic forms: (i) Kallmann syndrome [KS], a syndromic form characterized by GnRH deficiency accompanied by an absence of olfaction (anosmia) with or without other non-reproductive features such as midline defects, skeletal abnormalities, and other somatic features; and (ii) normosmic CHH, characterized by GnRH deficiency with normal sense of smell [19, 20]. Until the discovery of GnRH, CHH was thought to be secondary to pituitary gonadotropin deficiency. However, when GnRH became available, increases in the serum levels of LH and FSH following administration of a single bolus of GnRH in these subjects suggested a hypothalamic defect [21–23] which was subsequently unequivocally confirmed when administration of exogenous GnRH to these patients in a pulsatile fashion completely restored a physiologic pattern and levels of gonadotropins and sex steroids [24, 25].

CHH can also be a component of complex, severe syndromes with multiple somatic abnormalities in which the reproductive phenotype is but a component of a larger spectrum of phenotypes (see Complex Syndromic Forms of CHH section below). GnRH deficiency may also result from functional causes (e.g., critical illness, excessive exercise, nutritional deprivation, and drugs—especially opiates) (see Functional Cause of HH section below). CHH resulting from impaired production of pituitary gonadotropins with or without other pituitary hormone deficiencies includes genetic mutations in genes encoding LHβ (*LHB*) or FSHβ (*FSHB*) subunits (see Chap. 6). In addition, mutations in pituitary transcription factors such as *POU1F1, GLI2, FGF8, PROP1, LHX3, LHX4, HESX1, SOX3, OTX2, TBX19, TCF7L1* and *SOX*2 and structural lesions/tumors involving sellar/pituitary anatomy can also present with hypogonadotropism as part of their complex syndromes. Pituitary disorders causing hypogonadism are discussed in Chap. 8.

Idiopathic Hypogonadotropic Hypogonadism (CHH)

Definition and Incidence

CHH is characterized by an isolated defect in the secretion or function of GnRH.

Typically, CHH is diagnosed in adolescence due to a failure of puberty, although rarely it may be possible to diagnose this condition in the male neonates (see below). Diagnostic criteria for CHH in males include (i) absence or failure of completion of puberty by age 18 years, (ii) decreased T levels in the hypogonadal range (e.g., <3.5 nmol/L; 100 ng/dL), (iii) low/normal gonadotropin levels representing absent or reduced GnRH-induced gonadotropin secretion, (iv) otherwise normal hormonal testing of the anterior pituitary, (v) a normal ferritin level to eliminate hemochromatosis, and (vi) normal radiographic imaging of the hypothalamic-pituitary region.

Two distinct clinical forms of CHH have been recognized historically. Franz Kallmann reported a familial form of this condition in 1944, in which CHH was seen in males who also had congenital anosmia, and this simple syndromic form has since been referred to as Kallmann syndrome (KS) [26]. However, it was subsequently recognized that some patients with CHH presented with a normal sense of smell, and these patients are termed as normosmic CHH (nCHH). The KS form of CHH accounts for nearly 60% of patients while the remaining 40% present with nCHH [27, 28]. Since CHH is a rare clinical condition, definitive data on its prevalence are limited. However, a recent population-based epidemiological study from Finland showed a minimal prevalence estimate of the KS to be 1:48,000 with a clear difference between males (1:30,000) and females (1:125,000) [29]. The prevalence of the normosmic form is likely to be similar with a similar M:F ratio, but epidemiological data will be required to confirm this assertion.

Clinical Features

While the dichotomous classification of CHH into KS and nCHH based upon the presence or absence of sense of smell is frequently used in the literature, a substantial clinical phenotypic overlap is seen between these two forms [30]. Moreover, different family members within a single sibship may manifest either KS, nCHH, or isolated anosmia without reproductive failure [31]. Therefore, stratifying patients according to reproductive phenotypes that relate to the timing of onset of HH and the severity of HH provides greater insight into the clinical spectrum of CHH [30]. Similarly, the presence of specific non-reproductive phenotypes often provides developmental insights into the various organ systems affected by specific genetic forms of CHH [32].

Reproductive Features of CHH Relating Timing of Onset of GnRH Deficiency and Severity of GnRH Deficiency

- Neonatal Period

As mentioned above, the diagnosis of CHH is typically delayed until adolescence. However, severe GnRH deficiency disrupting the normal neonatal period of HPG

activity (i.e., mini-puberty) may provide early diagnostic clues for the diagnosis of CHH in newborn male infants. The presence of cryptorchidism and/or micropenis (also referred to as microphallus) in a male infant may indicate CHH, although GnRH deficiency may still be present even if these two phenotypic features are absent. Typically, gonadotropin, sex steroid, and inhibin B levels are inappropriately low in such infants reflecting their inability to normally activate the HPG axis during the neonatal window [33]. Notably, these neonates have otherwise normal sexual differentiation without any urogenital sinus malformations such as hypospadias since early fetal testicular T production from fetal Leydig cells (see Chap. 2) and testicular fetal AMH production are normal [34]. However, cryptorchidism and micropenis in CHH patients indicate that during the late fetal/early neonatal period, endogenous secretion of GnRH is necessary for inguino-scrotal descent of the testes and full growth of the external genitalia [10].

- Adolescence

In the absence of the hallmark neonatal reproductive features of micropenis and/or cryptorchidism, a definitive clinical diagnosis of CHH can only be made when isolated gonadotropin deficiency is manifested at the time of adolescence when boys present with delayed puberty. The clinical hallmark of CHH is the failure of onset of puberty. However, it is important to recall that CHH is rare, and that most adolescents with pubertal delay will represent those with constitutional delay of growth and puberty [CDGP] in whom spontaneous endogenous GnRH activity eventually ensues and puberty is subsequently initiated and completed (see delayed puberty section below and Chap. 4). While distinct from CHH, CDGP is frequently reported among the family members of CHH patients [31]. In our series of 106 patients with CHH, 12% had relatives with a history of delayed puberty [3] versus 1% in the general population [35]. These observations suggest that delayed puberty may represent the mildest end of the phenotypic spectrum of CHH. In keeping with this notion, recent studies have also reported a shared genetic basis between the two conditions [36].

Considerable clinical heterogeneity is observed in CHH patients at the time of presentation. The vast majority (~75%) of CHH patients are severely testosterone deficient and fail to develop any signs of puberty (Fig. 5.4). Their clinical features include no signs of virilization, (lack of any secondary sex characteristics), absence of an adolescent growth spurt but the presence of normal childhood growth but with delayed epiphyseal fusion resulting in eunuchoidal body proportions (upper/lower body ratio <1; with an arm span exceeding standing height by >6cm) [37], a high-pitched voice, slight anemia, delayed bone age, and prepubertal-sized testes (<4 mL). A large proportion of these severe GnRH-deficient subjects also report a history of cryptorchidism (40%) or microphallus (20%), providing evidence for the lack of activity of the HPG axis during the late fetal/neonatal window [38]. It is, however,

important to recognize that the prevalence of microphallus among these severely affected CHH men with no prior puberty might be underestimated given the reported successful induction of phallus growth with androgen therapy during childhood [39].

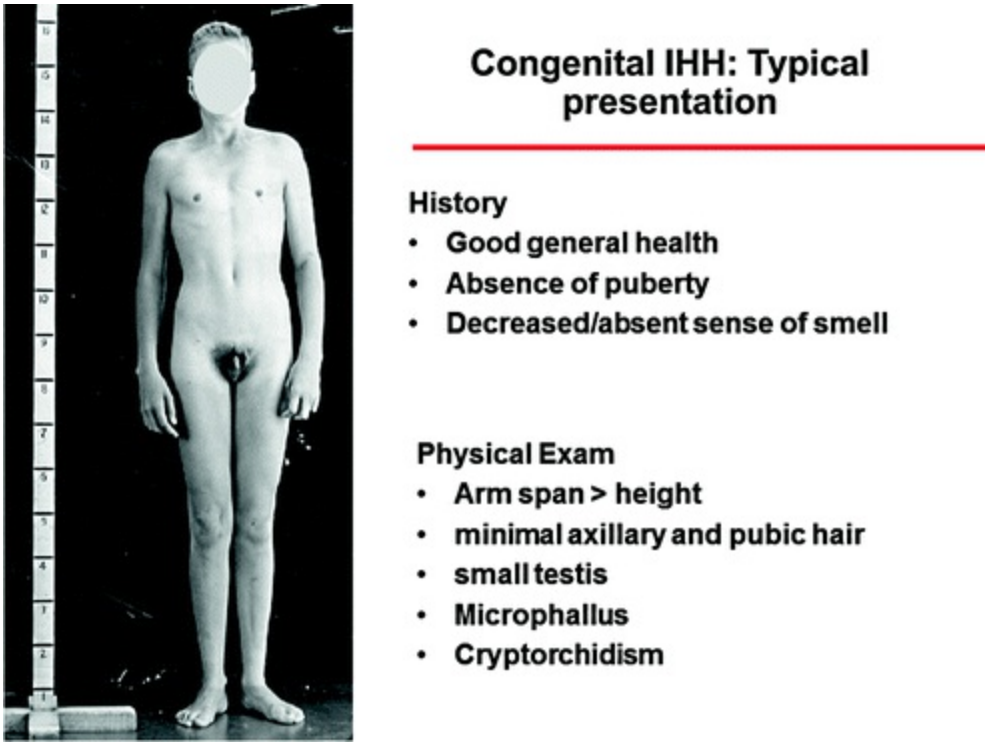

Fig. 5.4 Typical presentation of a CHH man with the most severe phenotype, including absence of puberty, eunuchoidal proportions, prepubertal testis, microphallus, and cryptorchidism

The remainder of CHH subjects (~25%) present with signs of partial puberty, displaying some degree of spontaneous puberty as assessed by at least two of these criteria: (i) some testicular growth, (ii) a growth spurt, (iii) an increase in the number of erections, and (iv) the initiation of shaving. However, these subjects, in contrast to boys with CDGP, fail to complete sexual maturation and remain hypogonadal. Low libido, lack of erectile function, low energy, and poor muscle bulk are the common somatic symptoms reported by CHH men. Gynecomastia is seen in 20–30% of CHH men and is often related to prior therapy with gonadotropins [30]. The key clinical features are depicted in Fig. 5.4.

Non-reproductive Features of CHH

Many CHH subjects, especially those with KS, may also exhibit a spectrum of non-reproductive features which may indicate a more complex syndromic genetic etiology (see below). One of the common non-reproductive phenotypes commonly encountered in KS subjects is bimanual synkinesia (also called “mirror movements”) [28, 40]. Bimanual synkinesia is detected when voluntary movements of one hand are associated

with similar involuntary movements of the opposite hand. Abnormal fast-conducting ipsilateral corticospinal tract projections are reported in these patients and are hypothesized to underlie the observed mirror movements [41, 42]. Defects in spatial perception have also been found in KS patients with synkinesia [43]. KS patients may also display unilateral renal agenesis [28, 44] and midline craniofacial and palate abnormalities [45]. Other common non-reproductive features include short metacarpals (short fingers, especially the fourth finger) [46], split hand/foot malformation [46], dental agenesis [47], congenital sensorineural hearing loss [32, 48], skin pigmentation anomalies [48], eye movement abnormalities [49], and rarely, cardiovascular defects [50, 51].

Variant Forms of CHH

Fertile Eunuch Syndrome

The first report of a man with the fertile eunuch variant of CHH was in 1950 by McCullagh [52]. These subjects display eunuchoidal proportions and are undervirilized but have normal gonadal size and preserved spermatogenesis. In this disorder, endogenous GnRH secretion, although decreased and insufficient to stimulate full virilization, appears adequate to produce sufficient intra-gonadal T levels to support spermatogenesis and testicular growth. Indeed, these patients can be fertile either spontaneously or with T or hCG therapy without the addition of FSH [53, 54]. The clinical picture of the fertile eunuch thus resembles that of normal mid-pubertal boys; indeed, a nocturnal rise of LH and T secretion synchronous with sleep, as seen normally in mid-puberty, was demonstrated in two men with the fertile eunuch syndrome [54]. These cases may have resulted from defects in GnRH regulation. In contrast, a patient with the fertile eunuch variant has also been described who had detectable, but apulsatile LH secretion associated with a partially inactivating mutation of the GnRH receptor (GnRH-R) [55]. These data underscore the clinical, genetic, and pathophysiological heterogeneity that exists in this syndrome.

Adult-Onset Hypogonadotropic Hypogonadism

A more delayed adult-onset presentation of nCHH, termed adult-onset hypogonadotropic hypogonadism, has been recognized in men with otherwise completely normal sexual development at puberty but in whom secretion of GnRH subsequently ceased during adulthood [56]. The robust responsiveness of these adult-onset subjects to exogenous GnRH administration demonstrates the hypothalamic nature of their defect. With long-term follow-up, these men fail to recover suggesting that they have a permanent defect in GnRH secretion [57].

Reversal of CHH

Although congenital CHH has been thought to be a lifelong condition, spontaneous recovery of endogenous GnRH secretion, as heralded by spontaneous testicular growth and normalization of sex steroid levels off therapy, has been demonstrated to occur in 10–15% of CHH men [58]. This reversal phenotype was seen following the therapy with a variety of treatments that effectively normalize sex steroid levels, suggesting that this prior exposure to sex steroid hormones is a key element to the milieu in which these "reversals" occur. The precise mechanisms behind this reversal process remain unknown. This phenomenon of "reversal of CHH," even in those with severe GnRH deficiency such as KS subjects with absent olfactory structures, strongly suggests a hitherto underappreciated plasticity of the GnRH neuronal network that can override developmental and neuroendocrine genetic defects that resulted in the initial hypogonadotropic state at the time of puberty.

In one recent single-center study, nearly 45% of reversal subjects had demonstrable CHH- related gene mutations but a significant enrichment was seen for mutations that disrupt neurokinin B signaling suggesting that the neurokinin B pathway, although critical for puberty, may be dispensable later in life [59]. Based on other reports from the literature, mutation in the GnRH receptor (*GNRHR*) also seems to be overrepresented in reversal subjects. More recent longitudinal review of these reversal subjects has also revealed a fragility of this reversal phenomenon as some patients slip back into a hypogonadotropic state requiring the resumption of therapy. Hence, it is important for clinicians to periodically reassess CHH subjects for evidence of reversal but also to assess the persistence of this reversal state and reintroduce therapy if required.

Pathophysiology of Congenital CHH

Biochemical Studies

Despite variation in the clinical phenotype of CHH men, their biochemical findings are very similar. By definition, all patients have T levels in the hypogonadal range. Gonadotropins are low/normal in all subjects, which in the setting of low T levels indicates hypogonadotropic hypogonadism (Fig. 5.5). Interestingly, mean LH and FSH levels are significantly higher in those patients with some degree of spontaneous puberty (as reflected by larger testicular sizes, for example) as compared with those men with no prior puberty [30].

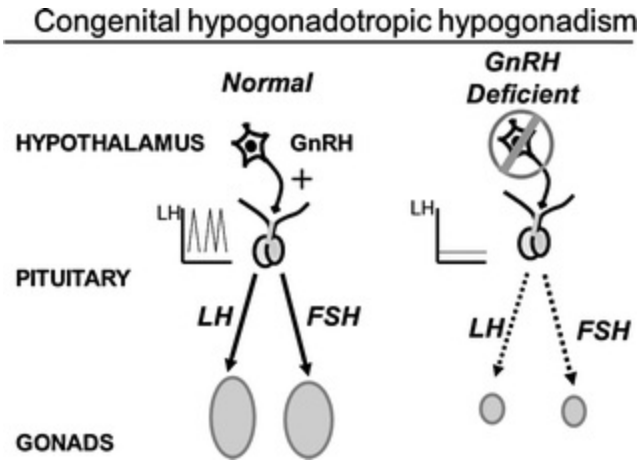

Fig. 5.5 Schematic of the hypothalamic–pituitary–gonadal axis in a normal adult male and in congenital hypogonadotropic hypogonadism

Serum levels of the Sertoli cell products, inhibin B and AMH, provide additional gonadal markers of the onset and extent of GnRH deficiency. In the normal ontogeny of inhibin B secretion, levels rise during the neonatal activation of the HPG axis [60] and then decline, but remain readily measurable throughout childhood despite low levels of FSH. During the early stages of puberty, inhibin B levels increase, plateau at stage II of puberty, and remain constant unless spermatogenesis is disrupted [61]. In contrast to normal men, CHH men with absent pubertal development display low/undetectable inhibin B levels [62] which are well below those of normal children [61]. Therefore, prior gonadotropin exposure appears to be required for normal inhibin B production during childhood. Thus, inhibin B levels represent a marker of reproductive axis activity during the fetal/neonatal period among patients with no sexual maturation. In CHH men with partial puberty, baseline inhibin B levels reach the normal range despite low gonadotropins, presumably reflecting adequate Sertoli cell proliferation during the neonatal window and early puberty. AMH, the first detectable secretory product of fetal Sertoli cells [63], is another biochemical marker of pubertal onset [64, 65]. Indeed, AMH levels are high throughout fetal and postnatal life and decline thereafter with the onset of spermatogenesis and the activation of LH-Leydig cell function [65]. Accordingly, AMH levels are high among CHH men with no pubertal development and are lower in those with partial GnRH deficiency [30]. Leydig cells also secrete INSL3 during the fetal and immediate postnatal periods, and INSL3 secretion ceases in childhood until puberty, peaking during adulthood [66]. Similar to inhibin B, baseline INSL3 levels are low in male CHH subjects and increase under gonadotropin stimulation [67]. It remains to be seen whether INSL3 levels and/or other physiological stimulation tests (e.g., kisspeptin stimulation) are able to distinguish adolescent subjects with CDGP from those who may progress to CHH.

Patterns of GnRH Secretion in CHH Subjects

Due to low circulating levels and a short half-life (2–4 min), GnRH in the peripheral circulation cannot be measured, and levels do not accurately reflect GnRH secretion [68]. Consequently, the study of GnRH secretion is limited to inferential approaches. Traditionally, LH is the most commonly used surrogate marker of the GnRH pulse generator [69, 70]. A spectrum of abnormalities in the neuroendocrine pattern of GnRH secretion is observed among CHH men. In the MGH's retrospective study of 78 CHH men, using 10-min blood sampling for LH, 75% of CHH patients failed to exhibit any detectable LH pulses, implying a lack of GnRH pulse generator activity (Figs. 5.2 and 5.6) [30]. The remaining 25% demonstrated abnormal but detectable LH pulses. Among the latter group, the majority of patients displayed a normal LH pulse frequency with a low LH pulse amplitude (Fig. 5.6). Although a higher prevalence (80%) of apulsatile LH secretion is found among those who lack sexual development, detectable LH pulses were identified in up to 20% of subjects in this group [71]. It may be that the initiation of puberty requires higher levels of GnRH stimulation than that are required to maintain a neuroendocrine axis after puberty [72]. Also, 50% of CHH patients with some pubertal development do not exhibit any LH pulses [30]. This finding suggests that transient pubertal activation of the HPG axis may have occurred that was followed by a failure of the GnRH secretory program in these patients. Alternatively, this may reflect a failure to detect a suppressed pulsatile pattern of GnRH because the LH assay was not sufficiently sensitive. From these data, it appears that neither mean gonadotropin levels nor the current pattern of LH secretion is a reliable surrogate marker for complete versus partial CHH.

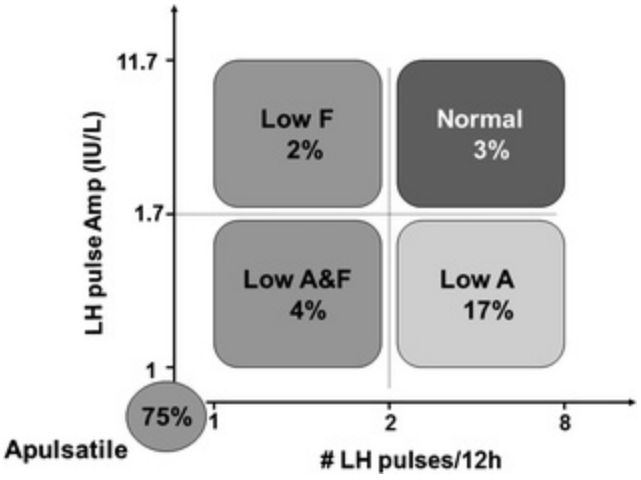

Fig. 5.6 Spectrum of GnRH-induced LH secretion abnormalities in men with CHH based on our retrospective study on 78 CHH men. 75% of CHH men exhibit no detectable LH pulses. 17% had a normal LH frequency but a low LH amplitude. 3% have a low LH amplitude and low frequency, 2% have a normal LH amplitude but low frequency. Finally, 2% have normal LH amplitude and frequency, deemed pathologic in the setting of hypogonadal T levels

The responsiveness of LH and FSH to 7 days of GnRH stimulation has been studied in CHH men and has been shown to correlate with the extent of seminiferous tubular development [73]. CHH men with no evidence of prior spontaneous pubertal development (TV ≤3 ml) showed sharp increases in serum FSH compared to men with some prior evidence of partial puberty (TV >3 ml), and inhibin B levels remained much lower in the former group. Conversely, the same group exhibited a decreased LH response to GnRH compared to the latter group who displayed robust LH responses to GnRH throughout the 7-day study. These observations underscore the role of negative feedback by inhibin B with increasing seminiferous tubule maturity on FSH responsiveness to GnRH while the LH response to GnRH is governed primarily by the extent of GnRH exposure.

Genetics of Congenital KS and NCHH

Patterns of Inheritance

Mirroring the phenotypic heterogeneity detailed above, the underlying genetic architecture of CHH is equally heterogeneous with mutations in several developmental genes causing KS, and mutations in neuroendocrine genes causing nCHH. Interestingly, some genes participate in the etiology of both KS and nCHH, and some subjects with KS have family members with nCHH, suggesting that the stratification between KS and nCHH might be oversimplified, and in fact may share common genetic mechanisms. The genetic complexity of CHH is also reflected in its different inheritance patterns. Given the reproductive failure associated with CHH, nearly two-thirds of CHH patients initially present as isolated or sporadic cases [27, 74]. Among familial cases, CHH can be inherited as an X-linked, autosomal dominant, or autosomal recessive trait [27]. Interestingly, in our cohort, the familial cases display a more severe phenotype with 95% of affected subjects presenting with absent pubertal development and a higher prevalence of cryptorchidism (71%) and microphallus (55%) [30]. While the excess of sporadic cases may suggest high rates of spontaneous de novo mutations causing the disease, closer review of their pedigrees, with detailed reproductive and non-reproductive phenotyping, often reveals subtle phenotypes that suggest familial inheritance patterns with variable penetrance [27]. In addition, as more subjects are successfully treated to enhance fertility, the incidence of familial cases may increase. In addition to these Mendelian modes of inheritance, a complex genetic architecture for CHH (occurring in 10–15% of cases) has now been documented wherein mutations in two or more CHH genes can be identified in CHH patients [75]. Almost all of the known CHH genes partake in the oligogenic inheritance mode. These oligogenic mutations presumably act in a synergistic manner, potentially accounting for some of the variable expressivity and incomplete penetrance that is characteristic of CHH.

Over the last three decades, several research groups have used a combination of

clinical investigational strategies and contemporary genetic approaches to unravel the growing genetic complexity of CHH and to expand our understanding of the neurobiology of reproduction. To date, nearly 25 causal/contributory genes for the non-syndromic forms of CHH have been identified for this condition with varied modes of inheritance that affect either the development and/or functional integrity of the GnRH neurons (Fig. 5.7).

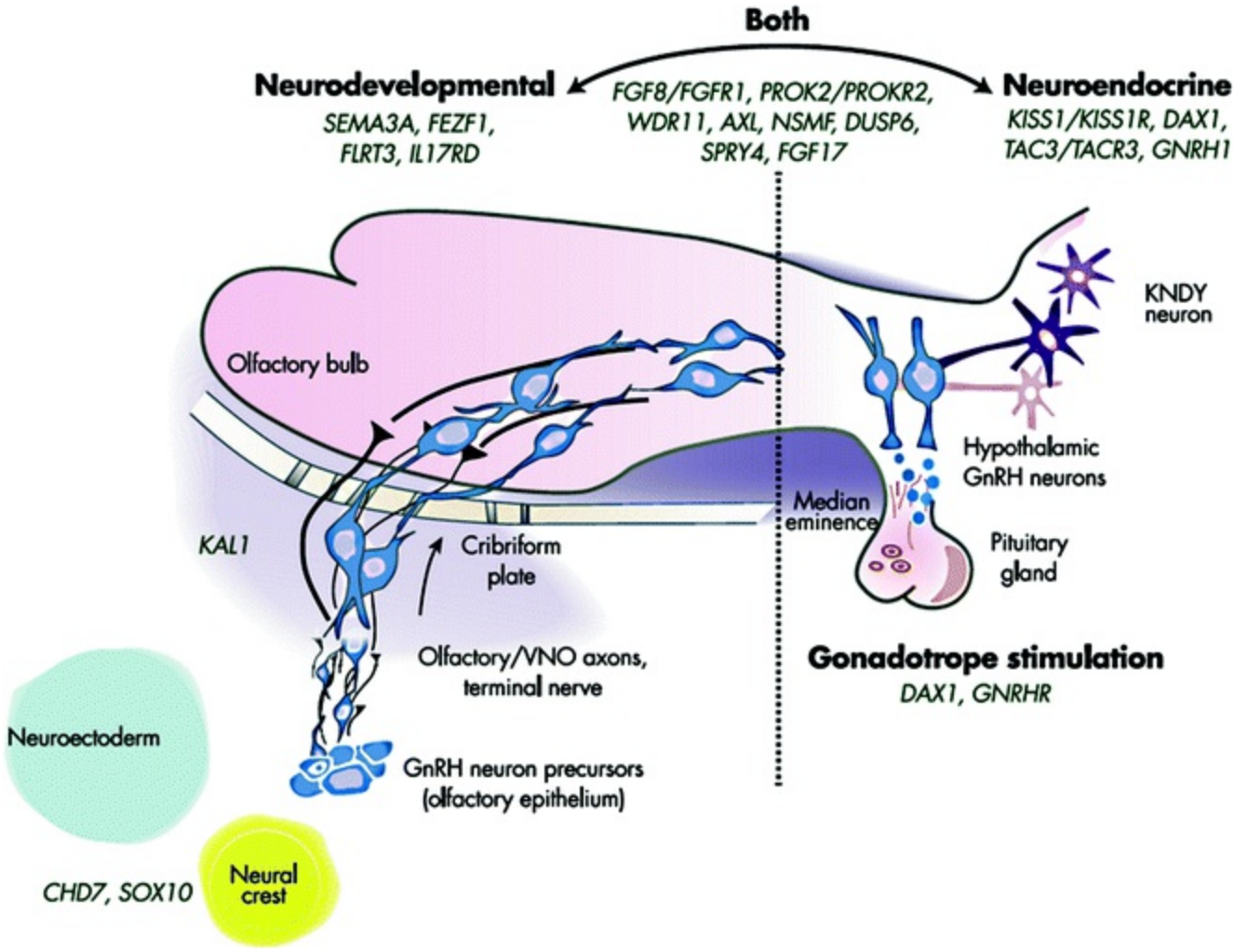

Fig. 5.7 Genetic causes of CHH showing neurodevelopmental and neuroendocrine regulation of GnRH neurons. Genes to the left are critical to GnRH neuronal migration, whereas those on the right are involved in proper GnRH function. A number of IGD genes (in the center) are involved in both processes. Adapted from Stamou et al. [213]

Genes Exclusively Causing KS Form of CHH

(i) X-linked recessive inheritance

ANOS1 (previously called *KAL1*), encoding anosmin-1 protein

Since the description of affected men by Kallmann et al. [27] in 1944, both X-linked

(X-KS) and autosomal KS pedigrees have been recognized. Though the majority of familial cases of KS are inherited autosomally [76], initial attention was focused on several X-KS pedigrees with a complex syndromic presentation that included KS accompanied by ichthyosis, chondroplasia punctata, and choanal atresia. Karyotype analysis revealed a deletion in the tip of the X-chromosome at Xp22.3, suggestive of a contiguous gene deletion syndrome [77]. Eventually, the first KS gene, *KAL1* (now called *ANOS1*), encoding an extracellular matrix protein, anosmin-1, was identified as the cause of KS using positional cloning of Xp22.3 and reported simultaneously by two independent groups [78, 79]. Subsequently, several groups have described many *ANOS1* point mutations and deletions in non-syndromic KS patients [80].

Anosmin-1 is a secreted multi-domain protein consisting of an N-terminal signal peptide followed by a cysteine-rich region (cys-box), a whey acidic protein (WAP)-like four disulfide core motif, four tandem fibronectin type III (FnIII) repeats, and a C-terminal histidine-rich region. *ANOS1* mutations occur across the length of the gene, and most mutations are loss of function (LOF) in nature (alteration of essential splicing, frameshift, or stop codons) resulting in the lack of synthesis of biologically active anosmin-1 protein. Missense ANOS1 mutations are rare [80]. The pathophysiology of disruption of anosmin-1 function in humans was studied in a 19-week gestation KS fetus known to harbor a deletion from Xp22.31 to Xpter, i.e., including the entire *ANOS1* gene [81]. Histologic studies revealed arrested migration of GnRH neurons and olfactory neurons at the cribriform plate [81]. This defect of GnRH neuronal migration results in the failure of normal GnRH neuronal axonal communication with the median eminence that is imperative for the activation of the HPG axis. In phenotypic studies of men with *ANOS1* mutations both in our cohort and in others, a severe pubertal phenotype was evident with complete absence of pubertal development, a high frequency of cryptorchidism and microphallus, an apulsatile pattern of LH secretion, low inhibin B levels, and histologically immature testes [30]. Despite the critical developmental role played by anosmin-1 in GnRH neuronal migration, mutations in *ANOS1* account for only 10–14% of familial KS [76] and 8–11% of sporadic KS patients [28]. Human subjects with *ANOS1* mutations, in addition to KS, display several specific non-reproductive features including, unilateral renal agenesis and synkinesia or mirror movements [28, 82]. In keeping with these clinical signatures, anosmin-1 is expressed in non-reproductive tissues such as mesonephric tubules, ureteric bud, digestive tract, large blood vessels, and inner ear [83]. Although several mutations of the *ANOS1* gene cluster in the four fibronectin type III repeat domains [84, 85], no phenotype–genotype correlation has been demonstrated between the non-reproductive phenotypes and location of the mutation.

(ii) Autosomal dominant KS, with variable penetrance in some pedigrees

a. *SOX10* , encoding a transcription factor, SRY (sex-determining region Y) box 10

The candidacy of *SOX10* as a genetic cause of KS came from radiological observations in humans with *SOX10* mutation-related Waardenburg syndrome (WS), a heterogeneous human "neurocristopathy" (i.e., disorders of cells from the neural crest origin) characterized by variable sensorineural deafness, abnormal skin and iris pigmentations, and Hirschsprung's disease [86]. Imaging of temporal bone abnormalities of WS patients revealed an unexpected frequency of olfactory bulb agenesis, a finding reminiscent of KS [86]. In addition, one male with WS-related olfactory bulb agenesis also exhibited KS (anosmia and CHH). In 2013, Pingault et al. [87] studied 17 KS subjects of both sexes with at least one WS-related feature and 86 random KS cases, and identified eight subjects with a spectrum of *SOX10* mutations (1 initiator codon, 1 frameshift, 1 splice site, and 6 missense mutations), all subsequently shown to be loss-of-function *SOX10* mutations. In addition, study of *Sox10* $^{-/-}$ mutant mice revealed a developmental defect in olfactory ensheathing cells, the specialized glial cell population normally found along the olfactory nerve pathway [87, 88]. In their absence, these mice misrouted their olfactory fibers, with impaired migration of GnRH cells and disorganization of the olfactory nerve layer of the olfactory bulb [55]. A third of the subcohort of KS with hearing loss was enriched for *SOX10* mutations similar to the WS patients who also exhibit hearing loss [56]. This phenotypic overlap between KS and WS is also strongly suggestive of the presumptive role for neural crest cells in GnRH neuronal ontogeny. These observations are in keeping with murine [89] and zebra fish [90] data previously suggesting a critical role for neural crest cells in GnRH neurogenesis. Thus, this important gene provides compelling evidence for a subset of KS to be considered a human neurocristopathy disorder.

b. *SEMA3A*, encoding semaphorin 3A protein

Semaphorin 3A is a secreted guidance cue of the class 3 semaphorin family that had been initially shown to be important for the development of the GnRH neuron system in mice [91]. Loss of SEMA3A signaling alters the targeting of vomeronasal nerves and the migration of GnRH neurons into the brain, and hence recapitulates the human KS phenotype [91]. Subsequently, *SEMA3A* human mutations have provided validating evidence linking SEMA3A signaling and GnRH ontogeny in humans [92, 93]. In a single pedigree, heterozygous deletion of 11–17 *SEMA3A* exons was first identified to cause an autosomal dominant form of KS [92]. Independently, heterozygous *SEMA3A* mutations (1 frameshift mutation and 7 different missense mutations [R66W, N153S, I400V, V435I, T688A, R730Q, and R733H]) were found in a total of 24/386 (6.2%) KS subjects [93]. These mutations either showed impaired secretion of SEMA3A protein or reduced signaling of the secreted protein in GN11 cell lines (a neurodevelopmental

GnRH cell line) [93]. Taken together, these observations suggest that mutations in *SEMA3A* cause KS via a loss-of-function (LOF) mechanism. In addition, in 5/24 patients with *SEMA3A* missense mutations, additional CHH gene mutations were identified, again confirming an oligogenic pattern of inheritance in CHH [93]. While several non-reproductive features were present, no significant enrichment for any specific non-reproductive feature was apparent in this group of patients.

c. *IL17RD* , encoding interleukin 17 receptor D protein

Once *FGF8/FGFR1* mutations were documented in both KS and nCHH forms of CHH patients (see below), a bioinformatic interactome search-based association study (IBAS) to identify other gene mutations in a "FGF8 synexpression group" revealed 2 genes, *IL7RD*, and *FGF17* as top candidate CHH genes [94]. In 8 KS subjects, seven *IL17RD* missense mutations (2 subjects with homozygous mutations and 6 subjects with heterozygous mutations) were found [94]. The functional effect of the *IL7RD* mutations was tested in a cell-based reporter assay in which cells co-expressing the mutants either displayed loss-of-function activity or decreased cell surface expression. IL17RD is a single transmembrane glycoprotein that is one of the major antagonists of FGF downstream signaling in vivo and in vitro [95–97]. High IL17RD expression occurs in the olfactory epithelium at E10.5, i.e., a time frame coinciding with GnRH neuronal fate specification thus suggesting a potential role for this gene in the early development of both GnRH and olfactory neurons [94]. Intriguingly, the *IL17RD* mutations identified were shown to cause loss of function. Given that IL17RD is an inhibitor of the FGF8 signaling system, these mutations should putatively increase FGF8 signaling in vitro. While this observation might appear counterintuitive given that *FGF8* and *FGFR1* LOF mutations cause CHH, it has been previously shown that both complete LOF and overexpression of FGF8 increase apoptosis in olfactory progenitor cells whereas a lesser decrease in functional FGF8 expression results in cell survival, suggesting nonlinear dosage effects for FGF8 signaling. Six out of eight (75%) IL17RD mutation subjects also displayed hearing loss, an observation consistent with *FGF8's* role as a morphogen for otic development in both chickens and mice [98]. In 2 heterozygous IL17RD mutation subjects, additional missense variants in *FGFR1* and *KISS1R*, two known CHH genes, were identified, thus attesting to an oligogenic mode of inheritance in these pedigrees [94].

(iii) Autosomal recessive KS

FEZF1, encoding FEZ family zinc finger 1
CCDC141, encoding a coiled-coil domain containing protein 141
In a consanguineous family with two affected KS subjects, whole exome sequencing

(WES) and autozygosity mapping discovered 2 different homozygous mutations in *FEZF1* (Missense: His278Tyr) and *CCDC141* (Stop-codon: R724X) [99, 100]. In another consanguineous family, an additional homozygous frameshift mutation leading to a premature stop (Ala327fsX13) was identified whose transcript is likely to be sensitive for nonsense-mediated decay [99]. The transcriptional activity of the missense FEZF1 His278Tyr mutation was studied using a HEK293 cell assay with a Hes5p-DsRed reporter that was previously known to be repressed during cortical neurogenesis. This missense mutation resulted in intermediate accumulation of the mutated reporter compared to WT FEZF1 suggesting a partial LOF [99].

FEZF1 is a zinc-finger gene encoding a transcriptional repressor selectively present during embryogenesis in the olfactory epithelium [101–103], and *Fezf1*-deficient mice have impaired axonal projection of pioneer olfactory receptor neurons (ORNs) exhibiting a failure of their neurons to penetrate the basal lamina of the CNS [101–103]. These mice have smaller olfactory bulbs and have complete absence of GnRH neurons in the brain [102]. When these experiments were repeated with the basal lamina surgically removed, GnRH neurons were able to reenter the brain suggesting that the WT FEZF1 product may promote a protease expression that will enable the olfactory sensory neurons to make synaptic connections with the olfactory nerve layer of the olfactory bulbs, providing a conduit for the migration of the GnRH neurons into the brain [102]. The presence of two homozygous mutations in each of *FEZF1* and *CCDC141* in the first family suggested an oligogenic inheritance especially since the His278Tyr mutation was only a partial LOF mutation [99]. In a subsequent study, the same authors showed in murine models that Ccdc141 is expressed in GnRH neurons and olfactory fibers, and that knockdown of Ccdc141 reduces GnRH neuronal migration, suggesting that CCDC141 participates in the embryonic migration of GnRH neurons enabling them to form a hypothalamic neuronal network [95].

(iv) Indeterminate inheritance/oligogenic KS

SEMA3E, encoding semaphorin 3E protein

Similar to SEMA3A, semaphorin 3E is another secreted guidance cue of the class 3 semaphorin family [104]. Using a combination of exome sequencing and computational modeling, a recent study implicated a shared point mutation in exon 16 of the *SEMA3E* gene leading to a missense SEMA3E R619C Ig-like domain substitution in 2 KS siblings [105]. While recombinant wild-type SEMA3E protected maturing GnRH neurons from cell death by triggering a Plexin D1-dependent (PLXND1-dependent) activation of PI3 K-mediated survival signaling, recombinant mutant SEMA3E carrying the KS-associated mutation did not protect GnRH neurons from death [105]. In the same study, SEMA3E or PLXND1 deficiency in mice was shown to increase apoptosis of GnRH neurons in the developing brain, reducing GnRH-positive neurite innervation of

the adult median eminence with phenotypic recapitulation of the human hypogonadal state. In addition to the *SEMA3E* mutations, a heterozygous p.F1019C mutation substitution in *CHD7*, a known CHH gene, was also present in the affected siblings, suggesting an oligogenic interaction [105]. The results from this single study strongly suggest a novel neurotrophic function for SEMA3E/PLXND1/PI3 K signaling in GnRH neuron development and a role for *SEMA3E* mutations in oligogenic inheritance of KS. Additional *SEMA3E* mutational screening in KS subjects will be required to evaluate the prevalence of *SEMA3E* mutations in CHH.

Genes Exclusively Causing nCHH

(i) Autosomal recessive nCHH

a. *GNRHR*, encoding the GnRH-receptor protein

A functional GnRH receptor (GNRHR) is crucial for both pubertal development and reproductive function. Indeed, hypothalamic GnRH secreted into the hypophyseal portal blood interacts with its high-affinity cognate GNRHR expressed in the cell membranes of gonadotrophs. Defects in the GNRHR emerged as the first autosomal cause of CHH [106, 107]. GNRHR is a G protein-coupled receptor with seven transmembrane segments that activates phospholipase C, leading to the intracellular increase in inositol phosphate [108]. While patients with a *GNRHR* mutation were expected to present with complete HH and unresponsiveness to GnRH stimulation, milder variants have been described. In the first family with a partially inactivating *GNRHR* mutation [106], the affected male had limited testicular growth (8 mL testes), detectable gonadotropins, and a normal response to a single pharmacologic dose of GnRH. Genetic analysis revealed a compound heterozygous mutation (Gln106Arg and Arg 262Gln substitutions). The parents were phenotypically normal, and each was heterozygous for one of the mutations. In CHO cells, Gln106Arg substitution (localized in the first extracellular loop of the GnRH receptor) markedly reduced the level of GnRH binding and stimulation of IP_3 activity. In contrast, in the Arg262Gln substitution (localized in the third intracellular loop of the GnRH receptor), hormone binding was normal, but IP_3 activation was impaired [106]. Several additional GnRH-R mutations (either homozygous or compound heterozygous) have been found which significantly impair GnRH binding and/or signaling to varying degrees [109]. Interestingly, a spectrum of pubertal development has been observed among CHH males with *GNRHR* mutations depending upon the genotype, ranging from the fertile eunuch syndrome [55] to the most severe form of GnRH deficiency characterized by cryptorchidism, microphallus, undetectable gonadotropins, and absent pubertal development [109]. In addition to

autosomal recessive inheritance, the prevalence of heterozygous rare sequence variants in *GNRHR* has been shown to be significantly higher in CHH subjects. However, as expected, biallelic recessive *GNRHR* mutations resulted in more severe phenotypes when compared to the monoallelic heterozygous *GNRHR* mutation individuals who exhibit variable penetrance of phenotypes suggesting as-yet-unidentified genetic and/or environmental factors may combine with heterozygous LOF *GNRHR* alleles to produce reproductive phenotypes [109].

b. *GNRH1*, encoding gonadotropin-releasing hormone

The GnRH gene is located at 8p21–8p11.2 and is the most obvious autosomal candidate gene in CHH. Indeed, the hypogonadal (*hpg*) mouse, in which the GnRH gene has been homozygously deleted, presents with CHH [110]. Surprisingly, after several previous failed attempts to identify *GNRH1* mutations, and 25 years after the discovery of the human sequence, homozygous frameshift mutations have now been reported in the *GNRH1* gene. Bouligand et al. identified an insertion of a single base pair between the 6th and 7th codons of the *GNRH1* gene that resulted in a frameshift that disrupted the primary sequence of the GnRH preprohormone starting at the 7th amino acid [111]. Indeed, cells transfected with the mutant GNRH1 allele failed to produce measurable amounts of GnRH [111]. Independently, Chan et al. [112] reported a single base pair deletion in the codon for the 6th amino acid of the GnRH decapeptide, that leads to a frameshift that disrupts the C-terminus of GnRH, a region essential for its function.

In both reports, *GNRH1*-deficient patients were born healthy at term countering the earlier speculation that GnRH may be essential for placental function and fetal survival. However, both patients had severe pubertal delay as well as micropenis and/or cryptorchidism, confirming the severity of these mutations. Moreover, no significant non-reproductive phenotypes were seen arguing against a major function for GnRH in other organ systems.

c. *KISS1R*, encoding the kisspeptin-1 receptor

In 2003, using linkage analysis and homozygosity mapping in inbred families, two groups independently identified inactivating mutations in KISS1R (then called GPR54), a G protein-coupled receptor, in nCHH patients [113, 114]. This observation, coupled with complementary mouse genetics, revealed that *Kiss1r* $^{-/-}$ mice were a faithful nCHH mouse model [114], providing compelling evidence that the KISS1R receptor and its cognate ligand, kisspeptin, were upstream gatekeepers of GnRH neurons. Since then, a number of mutations in the *KISSR1* have been described in nCHH patients [115]. *KISS1R* is a member of the rhodopsin family of G protein-coupled receptors, and kisspeptin is the endogenous ligand for *KISS1R* and a potent stimulator of gonadotropin

release in all mammalian species studied [116, 117]. Several key observations have emerged from the study of human GnRH-deficient subjects with KISS1R mutations. Neuroendocrine profiling of probands with *KISS1R* mutations reveal low-amplitude LH pulses [114, 118], suggesting some degree of persistence of endogenous but dampened GnRH secretion. In addition, in a subject with a *KISS1R* compound heterozygous mutation, exogenous GnRH induced a striking leftward shift in its GnRH-LH dose–response relationship suggesting some degree of endogenous pituitary priming by intact but dampened GnRH pulsatility [114]. Mutations in KISS1R account for ~2% of nCHH patients with the presence of cryptorchidism and/or micropenis in the majority of men with biallelic *KISS1R* mutations [119]. These observations strongly suggest that the kisspeptin signaling system is essential for prenatal GnRH secretion and early sexual development [119]. This observation is in keeping with a another published report of a male with *KISS1R* mutation who presented with cryptorchidism and micropenis in infancy, in whom neuroendocrine evaluation at 2 months of age revealed undetectable gonadotropins also suggesting a role for *KISS1/KISS1R* system in the "mini-puberty" of infancy [120]. While *KISS1R* inactivating mutations result in CHH, a single heterozygous activating KISS1R was shown to potentially contribute to central precocious puberty [121].

d. *KISS1*, encoding kisspeptin 1 peptide

The kisspeptin peptide is coded by the *KISS1* gene, and its proteolytic processing results in different length kisspeptins, all of which retain the amidated carboxy terminal decapeptide and are potent KISS1R agonists [122, 123]. Kisspeptin-1 is a 54 amino acid peptide, initially termed metastin due to its ability to inhibit tumor metastasis, represents the longest kisspeptin [124]. While *Kiss1* $^{-/-}$ mice have been known to recapitulate the human nCHH phenotype [125], only recently was a human consanguineous Kurdish pedigree with biallelic p.N115 K inactivating missense mutation in 4 sisters reported [126]. No male biallelic patients with *KISS1* mutations have been so far reported. All four sisters had significant pubertal delay. IP accumulation in COS-7 cells expressing human WT *KISS1R* was significantly impaired with the mutant KISS1-10 peptide compared to the WT KISS-10 peptide, suggesting a LOF mechanism for the mutation [126]. While no other mutations in the *KISS1* gene have subsequently been reported, this single family and the previously reported *KISS1R* mutations strongly reiterate the critical importance of the KISS1 signaling pathway in the neuroendocrine control of GnRH release in humans without affecting GnRH migration or GnRH synthesis.

e. *TAC3*, encoding the neurokinin B peptide and

f. *TACR3*, encoding the neurokinin B receptor

In 2009, using homozygosity mapping in consanguineous Turkish families, Topaloglu et al. [127] reported missense loss-of-function mutations in both *TACR3*, the gene encoding a G protein-coupled receptor, neurokinin B receptor (NK3R), and *TAC3*, the gene encoding neurokinin B (NKB), the endogenous ligand for TACR3, unearthing a previously unrecognized role of the TAC3/TACR3 pathway in the regulation of the GnRH secretion. Neurokinin B belongs to a phylogenetically conserved family of proteins that also include substance P, neurokinin A, and hemokinin-1 [128]. NK3R has preferential binding to NKB [129] and is predominantly expressed in the brain, specifically the hypothalamus [130, 131] with expression on GnRH neurons as well as co-expression with kisspeptin and dynorphin in a specific subset of neurons termed KNDy (kisspeptin-neurokinin B-dynorphin) neurons found in the arcuate nucleus [132]. Subsequently, homozygous splice site mutations in *TAC3* and *TACR3* were reported and shown to cause alternative splicing and premature truncation of the respective proteins [133, 134]. Gianetti et al. [135] by reviewing the phenotypes of CHH men harboring variants in *TAC3/TACR3* (n = 15) found that >90% of male probands characteristically displayed micropenis, and although they failed to enter puberty as teenagers, longitudinal review showed that 5/15 subjects exhibited ongoing evidence of endogenous HPG axis activity, suggestive of reversal later in life.

These observations strongly suggest that TAC3/TACR3 signaling, while imperative for neonatal mini-puberty and pubertal onset at adolescence, may be relatively less important in adult life. In terms of deciphering the precise mechanism by which NKB signaling regulates GnRH secretion, accumulating evidence suggests a stimulatory role of NKB that is likely mediated by autosynaptic inputs of NKB on KNDy neurons to induce the secretion of gonadotropin-releasing hormone (GnRH) in a kisspeptin- and sex hormone-dependent manner [136].

Genes Causing Both KS and NCHH

(i) <u>Autsomal dominant with variable penetrance with oligogenic inheritance in some pedigrees</u>

a. *FGFR1*, encoding the fibroblast growth factor 1 receptor protein

b. *FGF8*, encoding fibroblast growth factor 8

c. *FGF17*, encoding fibroblast growth factor 17

It has become clear that FGF signaling, known to play multiple roles in central nervous and skeletal system development including brain patterning, branching morphogenesis, and limb development [137], is an important cause of both KS [138] and nCHH forms of CHH [139, 140]. There are 23 mammalian fibroblast growth factors that act by binding and activating one of the four FGFR tyrosine kinase receptors in a heparan sulfate proteoglycan-dependent manner [141]. In two individuals with overlapping interstitial deletions with KS and congenital spherocytosis, Dode et al. [138] first implicated *FGFR1* gene as the underlying cause of KS. Since then, a large number of individual point mutations and deletions of the *FGFR1* gene have been shown to cause both KS and nCHH [139]. These mutations span all the functional domains of the *FGFR1* receptor. Structural and biochemical studies using recombinant FGFR1 proteins show that mutations in the D2 and D3 loops of the receptor cause misfolding and impaired cell surface expression of the *FGFR1* receptor and/or ligand–receptor interactions and binding [139]. Mutations in the cytoplasmic tyrosine kinase domain appear to cause structural perturbations and reduce catalytic activity of this domain [139].

At least 11 different FGFs can activate FGFR1 [137, 141]. However, the specific ligand(s) implicated in GnRH neuron ontogeny were unknown. In a proband with KS and cleft palate with a L342S mutation in the FGFR1c gene functional analysis of this mutation showed a selective and dramatic loss of binding affinity of the FGF8b isoform to the *FGFR1* receptor [142]. Integrating this observation with the described overlapping patterns of *FGF8* and *FGFR1* expressions in the brain and defective olfactory bulb neurogenesis seen in *FGF8* hypomorphic mice, a candidate gene approach revealed six *FGF8* gene mutations in GnRH-deficient unrelated probands [142]. Two of the probands also carried additional *FGFR1* mutations confirming oligogenicity in the *FGF8* signaling pathway in human GnRH deficiency [142]. The structural impact of these *FGF8* mutations was confirmed by a crystal structure analysis of the FGF8b/FGFR2c/heparin model, and functional studies revealed reduced FGF8 function in vitro confirming their LOF mechanisms [142]. Both anosmin-1 and FGFR1 bind to heparan sulfate proteoglycans. This association has led to the postulation that CHH in X-linked *ANOS1* mutations may be secondary to a FGF signaling defect on GnRH neuronal development [143]. The importance of the FGF signaling pathway in GnRH neuron ontogeny is further supported by the murine models with targeted ablation of *FGFR1* in the telencephalon [144], and *FGF8* hypomorphic mice both of which lack olfactory bulbs and have reduced GnRH neurons [145]. Phenotypic analysis shows significant variability of the penetrance of reproductive phenotypes in patients with FGF pathway mutations but significant enrichment of digit/limb abnormalities [46], midline facial defects, and dental agenesis in CHH patients with FGF pathway mutations [47].

More recently, as described for IL17RD gene discovery, bioinformatic analysis also revealed *FGF17* as a putative CHH candidate gene [94]. *FGF17* shares 61% overall sequence identity with FGF8b, signals through FGFR1c, and exhibits the same receptor-binding-specificity profile as FGF8b [146]. Consistent with the homology between FGF17 and FGF8b, the p.Leu342Ser *FGFR1* mutation also abolished FGF17-stimulated signaling through *FGFR1c,* and candidate gene sequencing revealed three unrelated probands which harbored heterozygous missense mutations in *FGF17*, one of whom also had oligogenic mutations in *FGFR1* and *HS6ST1* [94]. Moreover, mouse studies showed that Fgf17 was robustly expressed in the medial olfactory placode where GnRH neurons emerge, and importantly, there was significant reduction in Fgf17 expression in Fgf8 hypomorphic mice [94].

d. *HS6ST1*, encoding heparan sulfate 6-O-sulfotransferase 1 protein

Multiple lines of evidence suggested that *HS6ST1* was a likely candidate gene for CHH: (i) The enzyme encoded by hst-6 introduces a sulfate specifically in the 6-O-position of the glucosamine sugar moiety within heparan sulfate (HS). Notably, HS with specific 6-O sulfate modifications is required for anosmin-1 and FG8/FGFR1 signaling pathways to exert their function in vivo [147]; (ii) A genetic modifier screen uncovered mutations in the *C. elegans* HS 6-O-sulfotransferase gene (hst-6) as suppressors of this *ANOS1* (i.e., *kal1*) gain-of-function phenotype [148]. For these reasons, a CHH cohort (n = 338) that was screened for mutations in *HS6ST1* uncovered 7 subjects with KS and nCHH with sequence variants: one homozygous [p.R296W/R296W] and four heterozygous [p.R296Q; p.R313Q; p.R372W (seen in 3 unrelated patients); and p.M394V], indicating that 2% of CHH patients harbor *HS6ST1* mutations and, in some pedigrees, in oligogenic epistasis with other CHH genes [149]. Functional studies revealed that the human mutations have distinct effects in an ANOS1-dependent *C. elegans* assay, implying that *HS6ST1* may be required in vivo for *ANOS1*-dependent pathways [149].

e. *CHD7,* encoding chromodomain helicase DNA-binding protein 7 [Note: Mutations in this gene also cause the syndromic form of CHH (see below)]

Inactivating mutations in *CHD7* are known to cause a severe syndromic form of HH referred to as CHARGE syndrome (**C**oloboma, **H**eart defects, choanal **A**tresia, **R**etardation of growth and development, **G**onadal defects, and **E**ar/hearing abnormalities) [150, 151]. The CHD7 protein belongs to a family of highly conserved proteins and is one of 9 CHD proteins that have in common the ability to hydrolyze ATP to modify chromatin and nucleosome structure [152]. It is known to regulate transcription and has several functional domains including HELICc, SNF2-related

helicase/ATPase, BRK, and chromodomains that play significant roles in early embryonic development by controlling target gene expression through its activity in chromatin remodeling [152, 153].

The "gonadal defect" in CHARGE patients is hypogonadotropic hypogonadism and fulfills the clinical definition for CHH, as these patients are typically responsive to exogenous GnRH administration [150]. While truncating LOF mutations in *CHD7* typically cause the full CHARGE phenotype, recent studies show that missense mutations in *CHD7* are enriched in CHH patients (accounting for 5–6% of cases) [154, 155]. In one study, using a surrogate otolith assay in zebrafish, of a representative set of CHD7 alleles seen in CHH, 11 hypomorphic, null (revealed by rescue experiments), and dominant (revealed by overexpression experiments) alleles were identified [154]. In two families, pathogenic mutations in *CHD7* were seen with mutations in other known IGD genes. Taken together, these data suggest that rare deleterious missense *CHD7* alleles contribute to the mutational burden of patients with both KS and nCHH in the absence of full CHARGE syndrome. These findings strongly implicate a unique sensitivity for *CHD7 i*n the ontogeny of GnRH neurons and imply that mutations in *CHD7* contribute to the oligogenic basis of CHH. The question of where *CHD7* fits into the developmental biology of GnRH neurons remains unclear. It is also known that *CHD7* regulates genes involved in neural crest guidance, and there is increasing evidence that the KS form of IGD may also result from impaired neural crest guidance as seen in *SOX10* mutation patients [see above] [156]. In addition, the observation that rare pathogenic *CHD7* variants are associated with nCHH also suggests that, in addition to its neurodevelopmental role, *CHD7* may well also influence subsequent neuroendocrine development pathways of GnRH neurons.

f. *WDR11*, encoding the WD repeat domain 11

The chromosome 10q26 region has been associated previously with abnormal male genital development and function (micropenis, cryptorchidism, and hypogonadism) resulting from interstitial or terminal deletions as well as a balanced translocation [157]. In addition, trisomy involving 10q26 was reported in a KS patient with an unbalanced chromosome translocation, strongly implying the presence of a new KS gene in the 10q26 region [158]. Kim et al. studied a KS patient with a de novo chromosomal translation, 46,XY, t(10;12)(q26.3;q13.1), and by using a customized oligonucleotide array and cloning the junction fragments, they delineated the precise break points of the de novo translocation to exactly 547 kb away from the 5′ end of the *WDR11* gene [159]. In addition, by candidate gene sequencing, five further missense *WDR11* mutations were identified in one KS and in five nCHH patients. WDR11's function is unknown, but it contains twelve WD domains and is highly conserved throughout vertebrate evolution. Kim et al. [159] also identified the transcription factor EMX1 as a novel interactor with

WDR11. EMX1 is a homeobox transcription factor involved in specifying cell fates in the developing central nervous system and has been shown to be involved in the development of olfactory neurons. Analysis of the expression patterns of WDR11 in human embryonic olfactory GnRH neuroblasts, as well as in mouse and zebrafish development, revealed overlapping patterns of expression with EMX1 in regions critical for the formation of the hypothalamus, supporting the opportunity for the two proteins to interact in vivo and to act together during development [159]. Deletion analysis revealed that WDR11 interacts with EMX1 via both its N-terminus and its central region, where four of the identified missense mutations (R395W, A435T, R448Q, and H690Q) were located. While R448Q reduced binding to EMX1, both A435T and H690Q abolished binding to EMX1, physically decreasing the opportunity for productive interaction. Taken together, these genetic and functional data provide strong evidence for WDR11 as a cause of CHH and KS. Since segregation analysis was not fully possible, the precise mode of inheritance of these mutations remains unclear but the de novo nature of the deletion and heterozygous nature of the mutations indicate an autosomal dominant and an oligogenic inheritance model [160].

(ii) Autosomal recessive; indeterminate/oligogenic in some pedigrees

a. *PROK2* gene, encoding prokineticin 2

b. *PROKR2* gene, encoding prokineticin receptor 2

The candidacy of the *PROK2/PROKR2* mutations in CHH came initially from unanticipated hypogonadotropism in murine deletions *Prok2* $^{-/-}$ (162) and *Prokr2* $^{-/-}$ [162] that phenocopied human KS with olfactory anomalies, decreased GnRH neuronal migration to the hypothalamus, and produced hypogonadotropic hypogonadism responsive to exogenous gonadotropins. *PROK2* encodes prokineticin 2 (named as prokineticin in view of its stimulation of GI motility [163]), a secreted ligand with highly conserved cysteines and a basic N-terminal amino acid sequence, AVITGA, that is essential for its biological activity [164]. *PROK2* signals through both PROKR1 and PROKR2, two G protein-coupled receptors, but has higher affinity for PROKR2. In contrast to the more ubiquitously expressed *PROKR1, PROK2* and its cognate receptor, *PROKR2*, have a unique expression profile within the CNS including the olfactory system, arcuate nucleus, suprachiasmatic nuclei (SCN) and median eminence [165–167]. *PROK2* is also known to function as a chemoattractant for neural progenitor cells that ultimately populate the olfactory bulb and assist in its dynamic function during life [168]. It has been hypothesized that this olfactory developmental role of *PROK2* is the major determinant of its role in GnRH neuronal ontogeny, and that this effect may be

indirect, as both developing and mature GnRH neurons fail to express the ligand PROK2 or the PROKR2 receptor.

In keeping with these observations, biallelic LOF mutations in both *PROK2* and *PROKR2* were identified in KS subjects [169] and, surprisingly, in nCHH patients as well [161, 170]. In addition, rather intriguingly, >90% of human mutations in this pathway occur in the heterozygous state [171, 172]. Many *PROKR2* heterozygous pedigrees display variable penetrance suggesting a complex inheritance pattern [170–172]. While oligogenic mutations in other CHH genes account for some of these observations, recent evidence suggests that some of these heterozygous variants may act in a dominant-negative manner [173]. In addition, comparison of biallelic CHH patients to those harboring heterozygous variants, the latter group had a more variable expression of reproductive and non-reproductive features, suggesting that other genetic or non-genetic modifiers may be present in these patients. Although a circadian role for PROK2 has been identified in rodents, human patients with biallelic mutations in PROK2 do not exhibit any circadian phenotypes [174]. While the human mutations reveal an imperative role for prokineticin 2 signaling for GnRH neuronal integrity, the precise cellular and molecular mechanisms that link prokineticin 2 to GnRH neurons remain elusive.

c. *NSMF*, encoding NMDA receptor synaptonuclear signaling and neuronal migration factor

NSMF, previously called NELF (nasal embryonic LHRH factor), emerged as a strong candidate gene for KS in view of its putative role as an extracellular guidance molecule on growth cones, which is essential for axon growth and routing of GnRH-positive cells along vomeronasal olfactory-derived axons and eventually migration to the hypothalamus [175]. To date, several rare variants in NSMF have been identified but several were intronic variants [176, 177] or variants that were seen along with other CHH gene mutations [160, 178], suggesting that NSMF variants are unlikely to be causal monogenic variants, but may act as modifiers that synergize with other pathogenic genes to produce the observed human phenotype. In keeping with this notion, mouse knockout studies have revealed variable reproductive phenotypes. While female *Nsmf*$^{-/-}$ mice were reported to have delayed puberty and are subfertile [179], in a recent mouse study, *Nsmf*$^{-/-}$ mice did not display any reproductive phenotype but rather had substantial hippocampal hypoplasia [180].

Complex Syndromic Forms of CHH

While non-reproductive phenotypes are often seen in CHH patients with mutations in several genes described above, there are other genetic forms of CHH that occur in the

constellation of specific syndromic somatic phenotypes including (i) morbid childhood obesity syndromes (e.g., Prader–Willi syndrome [181]; syndromic obesity related to leptin pathway mutations [182–184]), (ii) adrenal hypoplasia congenita [185, 186]; (iii) CHARGE syndrome [150, 154]; (iv) cranial nerves palsies and peripheral neuropathy (TUBB3 E410 K syndrome [atypical Moebius]) [187]; (v) sensorineural hearing loss, pigmentation defects, and Hirschsprung disease (Waardenburg syndrome) [87]; (vi) neurodegenerative syndromes: Gordon-Holmes syndrome; Boucher-Neuhauser syndrome [188, 189]; and (vii) ciliopathies (Bartlett-Biedl syndrome) [190, 191]. The reproductive phenotypes in patients with these disorders can be either KS or nCHH or both, depending on the developmental or neuroendocrine defects. While most of these patients have secondary hypogonadism responsive to GnRH, some (e.g., Prader–Willi Syndrome) also display gonadal defects. Table 5.1 lists these commonly recognized syndromic forms of CHH, their recognized/putative genetic etiology, and mode of inheritance.

Table 5.1 Syndromic forms of IHH

Syndrome	Gene/locus name/mode of inheritance	Key phenotypes
Prader–Willi syndrome	Paternal loss of 15q11.2/imprinting disorder	Hypotonia in infancy, developmental delay, cryptorchidism/microphallus in males, abnormal satiety, and intellectual disability
Leptin deficiency	*LEP* mutation/autosomal recessive	Obesity
Leptin receptor deficiency	*LEPR* mutation/autosomal recessive	Obesity
PCSK1 deficiency	*PCSK1* mutation/autosomal recessive	Morbid obesity, hypocortisolism, and hypoinsulinemia
X-linked adrenal hypoplasia congenita	*NROB1* mutation/X-linked recessive	Adrenal insufficiency
CHARGE syndrome	*CHD7* mutation/autosomal dominant	**C**oloboma, **H**eart defects, choanal **A**tresia, **R**etardation of growth and development, **G**onadal defects, and **E**ar/hearing abnormalities
TUBB3 E410 K syndrome (atypical moebius)	*TUBB3* mutation/autosomal dominant	Cranial nerve dysinnervation (III, IV, VI, VII nerves) Peripheral neuropathy
Waardenburg syndrome	*SOX10* mutation/autosomal dominant	Sensorineural hearing loss, pigmentation defects, and Hirschsprung disease
Gordon-Holmes syndrome Boucher-Neuhauser syndrome	*RNF216/OTUD4/PNPLA6* mutations/oligogenic inheritance	Ataxia Neurodegeneration
Bardet-Biedl syndrome (BBS)	Multiple BBS genes/varied inheritance including oligogenic (see Ref. [199])	rod-cone dystrophy, truncal obesity, postaxial polydactyly, cognitive impairments, and renal abnormalities

Functional Cause of HH

Functional forms of HH are characterized by a transient decline in GnRH secretion. Severe weight loss and both acute and chronic illness with calorie deficit result in gonadotropin deficiency. While anorexia nervosa is mostly a disease of women, it also affects males. Hypothalamic amenorrhea (HA) is a well-recognized reversible hypogonadotropic state in females in response to energy deficit, but to date, a clinical syndrome of functional GnRH deficiency in men, that is analogous to HA, has not been well characterized. However, in healthy men, moderate to severe dietary restriction has been reported to be associated with a mild decrease in T levels because GnRH secretion is impaired [192, 193]. In addition, some [194–196] but not all [197, 198] studies have shown that strenuous physical exercise causes a transient decrease in T levels. Clinically, reversible hypogonadotropism in men is recognized in conjunction with drugs and critical illness and is discussed below.

Drug-induced HH

The use of anabolic steroids may result in reversible HH. This is more commonly seen in men and is manifested by a decrease in gonadotropins, gonadal steroids, and, if sufficiently prolonged, suppressed spermatogenesis [199, 200]. Suppression of the HPG axis following androgen therapy may last more than 16 weeks following discontinuation of the drug [199, 200]. Chronic treatment with pharmacologic doses of glucocorticoids may also cause hypogonadism [201]. Chronic use of narcotic analgesics may also suppress LH secretion and result in reversible HH [202].

HH Associated with Critical Illness

Any severe chronic illness [203], or acute critical illness [204], surgery [205], myocardial infarction [206], or burn injury [207] may result in decreased T levels. Acute injury also causes immediate suppression of Leydig cell function [208]. Prolonged severe stress may also inhibit pulsatile LH secretion, resulting in HH [208].

Evaluation of Congenital HH (See also Chap. 4)

Clinical criteria for the definition of CHH have been described in the Clinical Features section above. A full clinical examination should be done with special emphasis on linear growth, growth chart review, and measurements for evaluating eunuchoid proportions. Sexual examination for the evaluation of Tanner stages for pubic hair, penis length, and testicular size should be performed. Additional system examination for identifying other non-reproductive features of CHH (see Clinical Features section above) should be performed. The biochemical diagnosis should be made on the basis of

basal concentrations of T and pituitary gonadotropins. Although it is clinically important to distinguish secondary hypogonadism from hypothalamic versus pituitary causes, the serum LH response to a single bolus of exogenous GnRH cannot determine whether the defect is localized to the pituitary or hypothalamus, because a subnormal response may occur in both settings. Indeed, patients with complete GnRH deficiency are likely to have had no prior exposure to endogenous GnRH, and in this setting, repeated administration of GnRH is needed to prime the gonadotropes and induce a gonadotropin response. The pretreatment inhibin B level may be a useful predictor of therapeutic outcome for CHH men who desire to conceive [30, 71]. Finally, while frequent blood sampling to characterize the pulsatile pattern of LH secretion may help refine the diagnosis in a research study, it is not practical in the clinical setting. Sense of smell can be evaluated by history and by formal diagnostic smell tests, such as the University of Pennsylvania smell identification test (UPSIT) [209]. This "scratch and sniff" test evaluates an individual's ability to identify 40 microencapsulated odorants and can be easily performed in most clinical settings. Identification of anosmia, hyposmia, or normosmia is based on the individual's score, age at testing, and gender and is interpreted using a standard nomogram in the UPSIT manual.

A semen analysis should be obtained in patients with CHH who are able to produce an ejaculate and should include an assessment of the volume, sperm count, motility, and morphology. Since the seminal vesicles, an exquisitely androgen-dependent organ is the major source of seminal fluid volume, many hypogonadal men may be unable to ejaculate any semen, however. The most recent WHO criteria [210] for normal semen analysis parameters are a volume >1.5 mL, a sperm count ≥15 million/mL of ejaculate with progressive motility ≥32%, and normal morphology in ≥4%. Because of the variability in sperm counts, it is worthwhile to obtain a second semen specimen if the first sample is abnormal. Finally, in the case of secondary hypogonadism, it is critical to assess other pituitary functions (see Chap. 8), including a prolactin level, to ensure that the defect is isolated to the HPG axis. Serum iron/TIBC or ferritin levels should be measured to exclude hemochromatosis. Patients with HH typically undergo adrenarche at a normal age and should therefore have normal adult male levels of DHEAS. Additional tests include a comprehensive metabolic panel, and inflammatory markers may be needed to rule out severe systemic illness that may cause functional HH. Radiographic evaluation should include a bone age determination, MRI of the pituitary and hypothalamic area, renal ultrasound, and a DEXA scan to assess bone mineral density. In patients with an adult-onset presentation of hypogonadotropic hypogonadism, other secondary causes such as obesity-related hypogonadism must be considered in the differential diagnosis. Additional investigations should be guided by the presence or absence of other syndromic clinical features.

Genetic testing is useful for diagnosis, prognosis, and genetic counseling of patients with CHH. Genetic inheritance patterns (e.g., X-linked vs. autosomal) and the presence

of specific clinical features, specifically non-reproductive features, may allow the prioritization of genetic screening [211]. However, given the variable penetrance and expressivity associated with CHH and demonstration of oligogenic inheritance, multiple gene testing may be required, and several gene testing panels are available for screening for the common genetic causes of CHH [212].

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6. Male Hypogonadism Resulting from Mutations in the Genes for the Gonadotropin Subunits and Their Receptors

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Keywords Human chorionic gonadotropin (hCG) – Follicle-stimulating hormone (FSH) – Follicle-stimulating hormone receptor (FSHR) – Gonadotropin signaling – G protein-coupled receptor (GPCR) – Hypogonadism (hypergonadotropic – Hypogonadotropic – Idiopathic) – Luteinizing hormone (LH) – Luteinizing hormone/chorionic gonadotropin receptor (LHCGR) – Mutation – Polymorphism

Introduction

The unraveling of the structure of the human genome has allowed for major advances in the diagnostics of inherited diseases. Besides clear disease-causing mutations, the emerging knowledge about polymorphisms concerning single nucleotides (SNPs), DNA repeats (microsatellites), deletions, and copy number variation in the human genome adds a new level of complexity to genomic function, providing the structural basis for individual variability of the genome and its phenotypic expression. All mutations can, in principle, be classified as inactivating, activating, or neutral, i.e., with no effect on function of the encoded protein. The hormone ligand mutations that are known today are almost invariably inactivating. The polymorphisms, by definition, are neutral, or they have only minor effects at the functional level. In some cases, they may even offer a

functional advantage to their carriers. In the case of hormone receptors, the inactivating mutations impair receptor function through a variety of mechanisms, whereas with activating mutations, the receptor becomes constitutively activated in the absence of hormone, or it acquires novel functions not present in the wild-type (WT) receptor.

Reproductive functions, like all functions on the human body that are under genetic control, are affected by specific mutations and polymorphisms of key genes. The focus of this chapter is to review the currently known mutations in the three gonadotropins, pituitary luteinizing hormone (LH), its placental analog, human chorionic gonadotropin (hCG), and pituitary follicle-stimulating hormone (FSH), as well as their cognate receptors (R), the LHCGR and FSHR. Specific forms of male gonadal dysfunction are caused by these mutations. Common polymorphisms exist in these genes as well, and they may be responsible for borderline alterations of gonadal function. This review concentrates on the role of gonadotropin and gonadotropin receptor mutations and polymorphisms in male gonadal dysfunction. Relevant genetically modified animal models will be described when they enhance our knowledge of the molecular pathogenetic mechanisms underlying the mutations. More information is available on some gonadotropin and gonadotropin receptor mutations found in women, so, for the sake of a more complete presentation, I will also review them briefly.

Structure of the Gonadotropin Subunit and Gonadotropin Receptor Genes and Proteins

Gonadotropin Subunits

FSH, LH, and hCG, together with thyroid-stimulating hormone (TSH), comprise the family of glycoprotein hormones. Each hormone is composed of two subunits that are coupled by noncovalent interactions: the common α-subunit of 92 amino acids (aa, encoded by the *CGA* gene) and the hormone-specific β-subunit; 111 aa in FSHβ (encoded by *FSHB*), 121 aa in LHβ (encoded by *LHB*), and 138 aa in hCGβ (Encoded by *CGB*) (see Fig. 6.1). hCG is structurally similar to LH, and both hormones have similar actions by activating the common receptor, LHCGR. Gonadotropins are glycosylated through *N*-linked bonds (see Fig. 6.1), and the carbohydrate content of LH is approx. 15%, of FSH 20%, and of hCG 30% [1, 2]. Most conspicuously, the carbohydrate moieties increase the circulating half-lives of the gonadotropins, which are approx. 20 min for LH, approx. 2 h for FSH, and 12–24 h for hCG. The long half-life of hCG is primarily explained by the 24-aa C-terminal extension of its β-subunit, in comparison to LHβ, which is heavily glycosylated through four O-linked glycosylation sites (see Fig. 6.1). The short half-life of LH is a function of the high proportion of terminal sulfate groups in its carbohydrates; a specific hepatic receptor accelerates the elimination of this type of glycoprotein from the circulation [3].

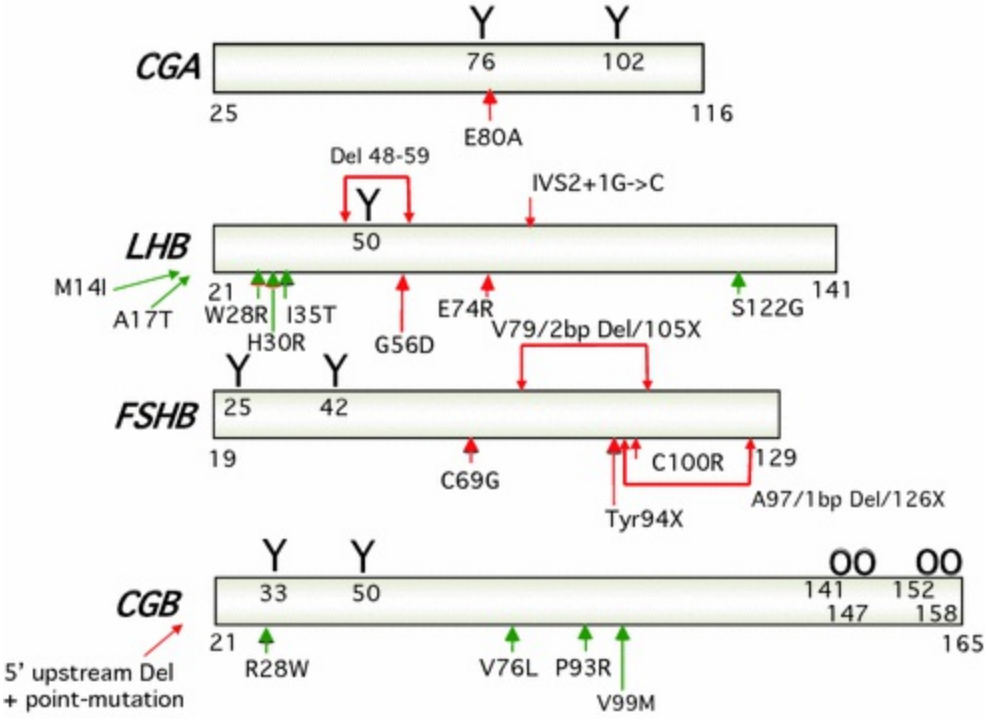

Fig. 6.1 Schematic presentation of sizes, locations of the carbohydrate side chains, and currently known mutations (*red arrows*) and functionally significant polymorphisms (*green arrows*) in the gonadotropin subunits, i.e., *CGA*, *LHB*, *FSHB,* and *CGB*. The numbers below the right ends of the bars indicate the numbers of amino acids in the mature subunit proteins. Symbols "Y" and "O" indicate the locations of *N*-linked and *O*-linked carbohydrate side chains, respectively. The amino acid numbering includes the signal peptides (24 amino acids for CGA, 20 for LHB, 18 for FSHB and 20 for CGB), but the bars only depict the mature peptides. For references, see Table 6.1 and the text

The *CGA* gene is located on chromosome 6q12.21, *FSHB* is found on chromosome 11p13, and the *CGB/LHB* gene cluster, consisting of one *LHB* and four *CGB* encoding genes and two apparent pseudogenes, is located on chromosome 19q13.32 [4, 5]. *CGA* consists of four exons, and *LHB*, *CGB* and *FSHB* are each composed of three exons. The crystal structures of deglycosylated hCG [6] and FSH [7] are similar and reveal that both subunits contain so-called "cystine knot structures" that are similar to some remotely related growth factor-type signaling molecules. Each subunit has an elongated shape, with two β-hairpin loops on one side of the central cystine knot and a long loop on the other side. The noncovalent interaction between the two subunits is stabilized by a segment of the β-subunit that extends like a "seatbelt" around the α-subunit and is "locked" by a disulfide bridge.

Gonadotropin Receptors

Like their ligands, the gonadotropin receptors are structurally related glycoproteins, belonging to class A of the large family of G protein-coupled receptors (GPCRs) (see Figs. 6.2 and 6.3) [8–10]. They each have a transmembrane domain that traverses the plasma membrane as seven α-helices connected by three extracellular and three

intracellular loops. The glycoprotein hormone receptors form a subgroup within this gene family with a distinct long *N*-terminal extracellular domain, comprising approximately half the size of the receptor. The C-termini of the receptor proteins form short intracellular tails. Whereas the FSHR binds only FSH, LH, and hCG are structurally so similar that they bind to the same LHCGR. Both gonadotropin receptor genes are localized on chromosome 2p21 [8, 10], and they carry considerable structural similarity, especially in their transmembrane domains. The clearest difference is that the first 10 exons encode the extracellular domain of LHCGR, whereas this domain is encoded by 9 exons in the FSHR. The last long eleventh exon in *LHCGR* (tenth in *FSHR*) encodes the transmembrane and intracellular domains.

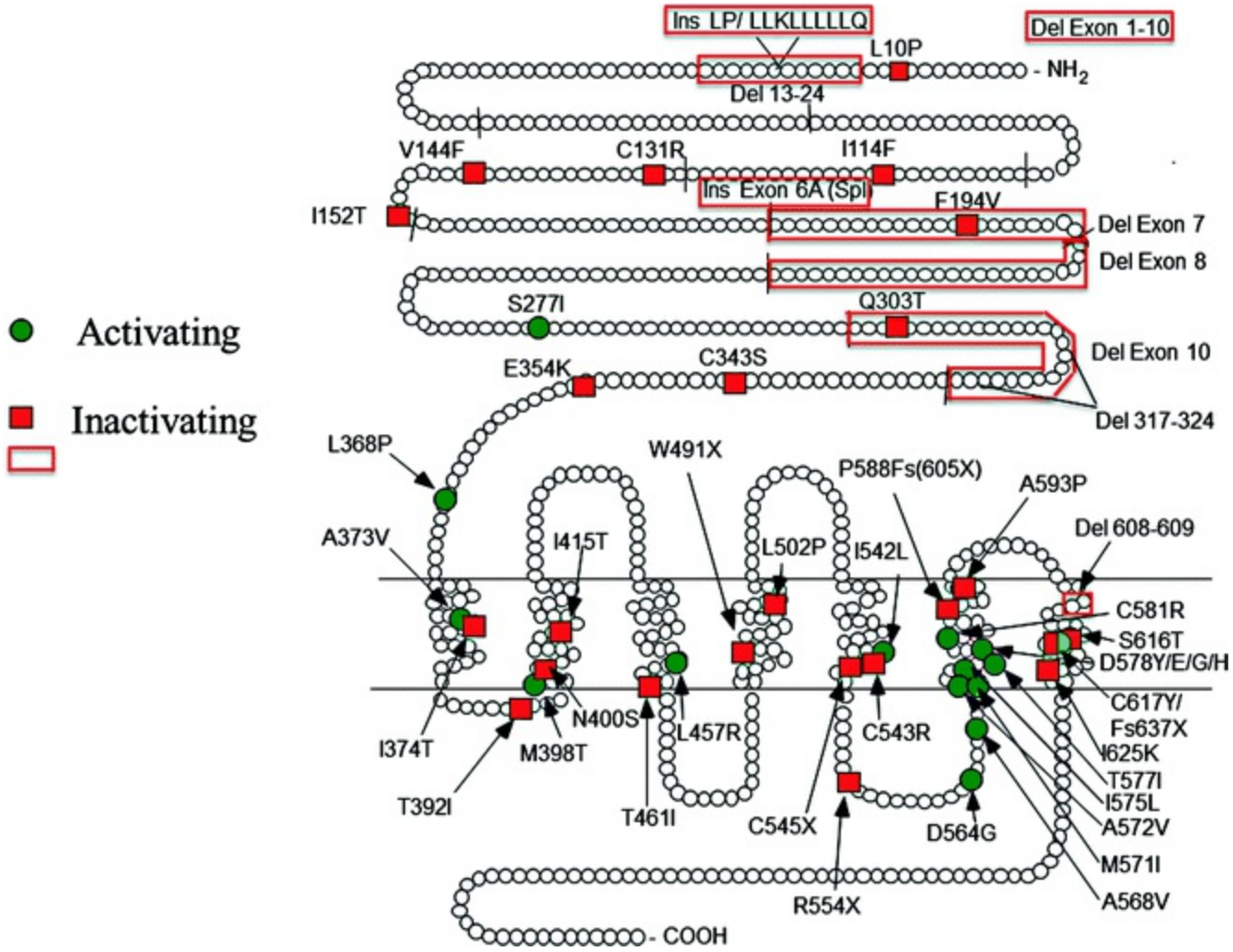

Fig. 6.2 Locations of the currently known mutations of *LHCGR*. *Red squares and boxes* indicate inactivating mutation and *green circles* activating mutations. The short lines across the peptide chain depict the exon boundaries. The original references are found in earlier reviews on the topic [9, 97]. The mutations detected thereafter are described in Refs. [99, 102, 147–149]. *Fs* frame shift, *Ins* insertion, *Del* deletion, *Spl* splice variants, *X* premature stop codon

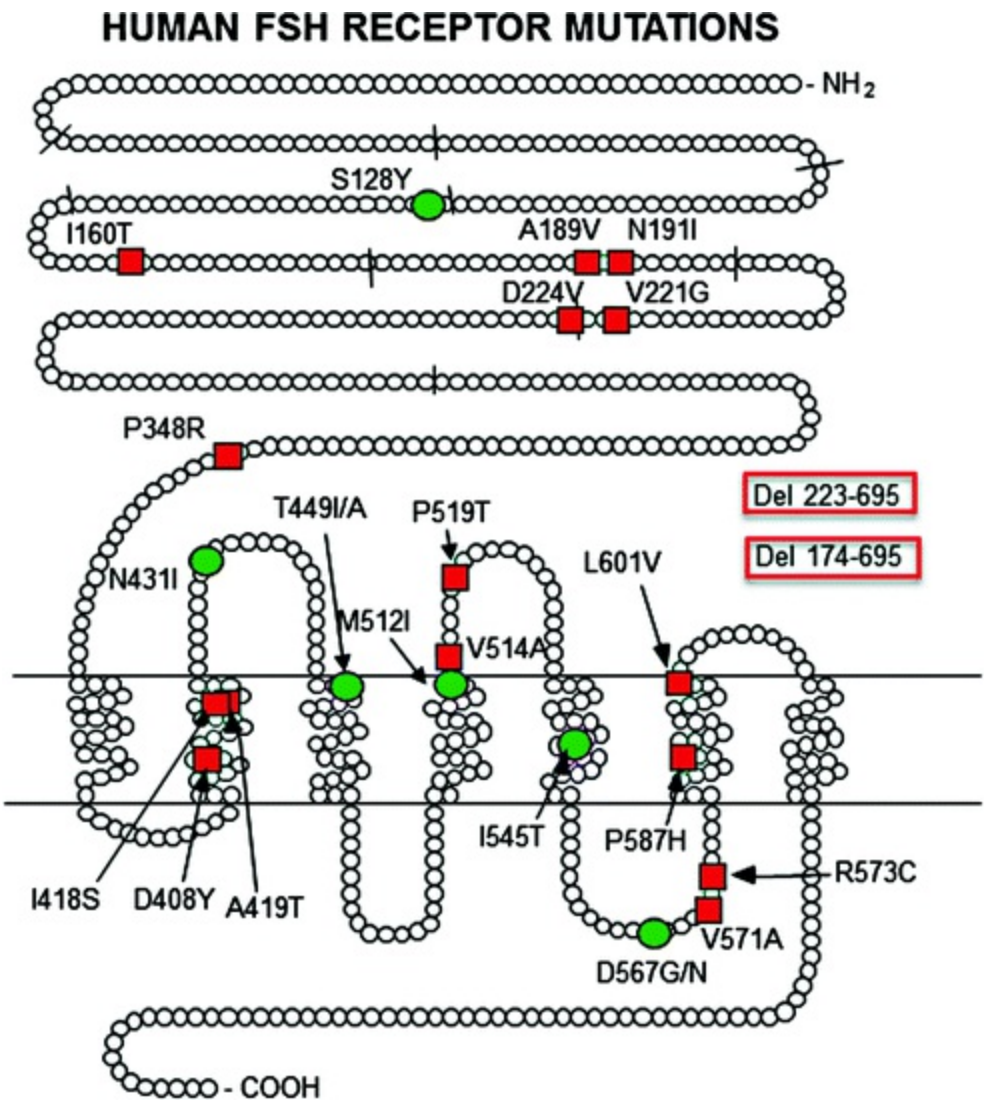

Fig. 6.3 Locations of the currently known mutations of *FSHR*. Red squares and boxes indicate inactivating mutation and *green circles* activating mutations. The short lines across the peptide chain depict the exon boundaries. The original references are found in earlier reviews on the topic [9, 117]. The mutations detected thereafter are described in Refs. [125, 130–157]. *Del* deletion

Gonadotropins bind to the extracellular domain of their cognate receptors. This domain contains several leucine-rich repeats that are found in various proteins responsible for protein–protein interactions. The domain is glycosylated in an *N*-linked fashion, but the role of these structures in receptor function is not clear. The extracellular and transmembrane domains are connected by a short hinge region that may influence ligand specificity, as shown with a human mutation of *LHCGR* lacking this region [11] and subsequent in vitro mutagenesis studies [12]. The transmembrane domain, with its seven membrane-spanning α-helices and intracellular and extracellular loops, resembles other GPCRs and is crucial for signal transduction. In particular, the third intracellular loop, the sixth transmembrane domain, and the cytoplasmic tail are closely involved in G protein coupling and signal transduction. The crystal structure of

the gonadotropin receptors (FSHR, and structurally related TSHR) has been partially unraveled [13–15].

The gonadotropin receptors are primarily coupled to a G protein that activates adenylyl cyclase and elevates intracellular cyclic adenosine monophosphate (cAMP) levels [8, 10]. These receptors also activate other signaling pathways, however, including phosphatidylinositide turnover, intracellular Ca^{2+}, mitogen-activated protein kinases, and activation of the G_i protein [8, 10]. The alternate signaling pathways are often activated at higher hormone concentrations and receptor densities in target cells, and their functions may be limited to special circumstances in which gonadotropin levels are elevated, e.g., ovulation, pregnancy, or constitutively activating receptor mutations (see sections on activating mutations). A schematic presentation of the signaling cascades mediating gonadotropin action is shown in Fig. 6.4.

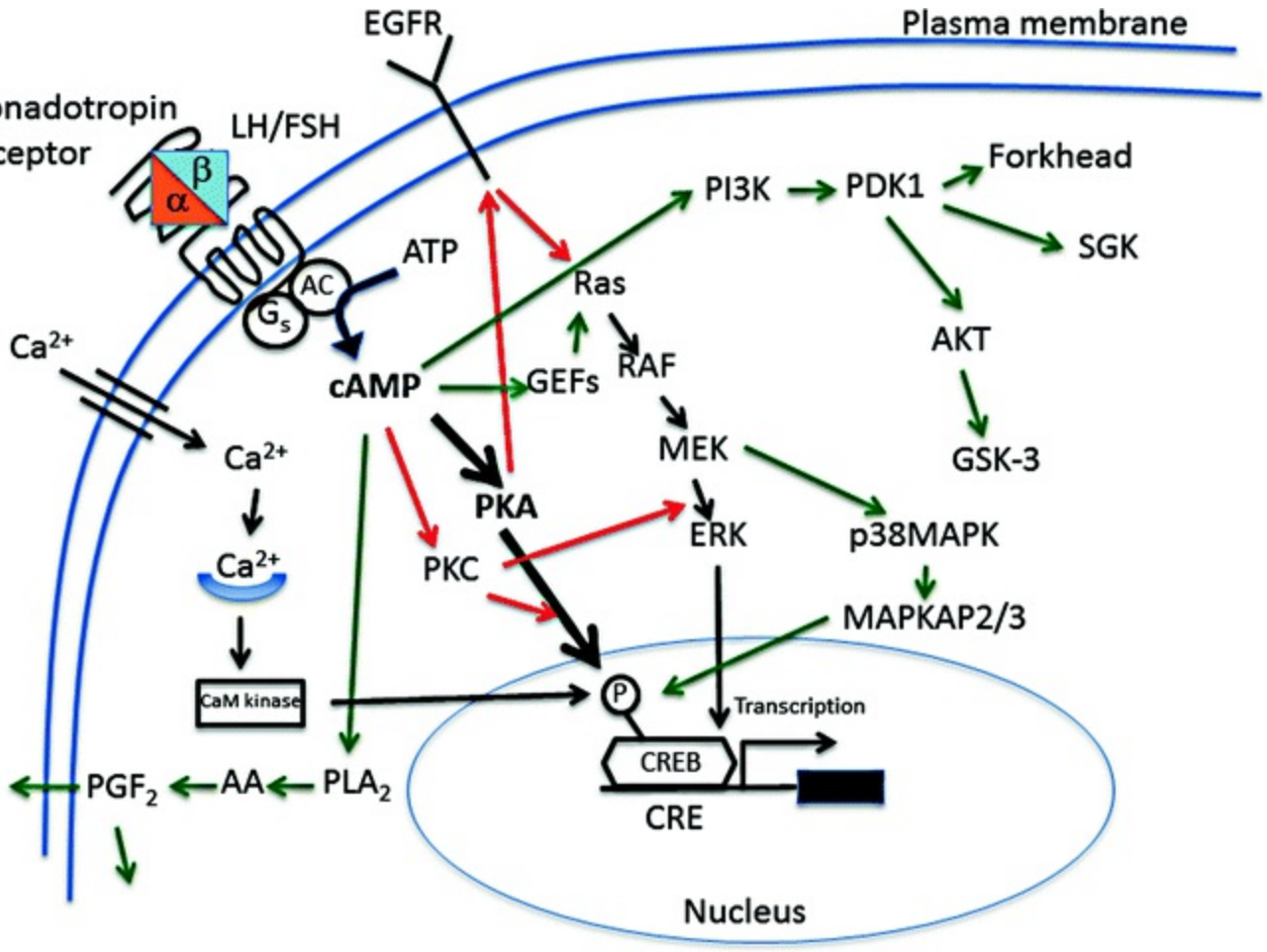

Fig. 6.4 Main signaling cascades employed in LH/hCG and FSH action. *Black arrows* depict pathways common for both hormones, *red arrows* those specific for LH/hCG and green arrows those specific for FSH. *AA* arachidonic acid, *AC* adenylyl cyclase, *ATP* adenosine trisphosphate, *CaM* calmodulin, *cAMP* cyclic adenosine-3':5'-monophosphate, *CRE* cAMP response element, *CREB* cAMP response element binding protein, *EGFR* epidermal growth factor receptor, *ERK* extracellular signal-regulated kinase, *Forkhead* transcription factor, *GEF* guanine nucleotide exchange factor; G_s stimulatory guanine nucleotide binding protein; *GSK-3* glycogen synthase kinase-3, *MEK*

mitogen/extracellular signal-regulated kinase, *PDK1* phosphoinositol-dependent protein kinase, *MAPK* mitogen-activated protein kinase, *MAPKAP, MAPK*-activated protein kinase; *PGF* $_2$ prostaglandin F_2, *PI3K* phosphoinositol 3 kinase, *PKA* protein kinase A, *PKC* protein kinase C, *PLA* $_2$ phospholipase A_2, *RAF* rapidly accelerated fibrosarcoma–ser/thr specific protein kinase, *Ras* small GTPase family, *SGK* serum glucocorticoid-regulated kinase

Gonadotropin Secretion and Actions Throughout the Male Life Span

Physiological studies have clarified in great detail the role of gonadotropins in the development and mature functions of male reproduction. In the fetal period, placental hCG, a superagonist of pituitary LH, is responsible for the stimulation of testosterone production by fetal Leydig cells, which is essential for masculinization of the male reproductive organs [16]. This occurs in collaboration with the Sertoli cell product, anti-Müllerian hormone (or Müllerian-inhibiting substance), and another Leydig cell product, insulin-like factor-3 (INSL3) that regulate, in the male fetus, the involution of the Müllerian ducts and the transabdominal phase of testicular descent, respectively [17, 18]. Although FSH secretion begins in the fetus, and fetal testes express FSHR [19], FSH's role in the fetal period is uncertain. Data from animal models indicate that its first role is the stimulation of Sertoli cell proliferation in the prepubertal testis [20]. Fetal pituitary gonadotropin secretion begins at the end of the first trimester of pregnancy, with high levels in mid-pregnancy, followed by a decline toward the end of gestation, apparently resulting from development of feedback regulation [21]. A secondary increase in gonadotropin levels (often termed "minipuberty", see Chap. 4) occurs during the first 4–6 mo of postnatal life, which is accompanied by a peak in testosterone production [22] and by Sertoli cell proliferation [23]. Spermatogenesis, however, remains quiescent, apparently because of lack of androgen receptors in Sertoli cells of the postnatal testis [24]. No functional role has been demonstrated for this period of the hypothalamic–pituitary–gonadal activation, and it may represent an adaptation to extrauterine life and the hormonal milieu following elimination of placental hormones.

The prepubertal phase of male development is typified by low circulating gonadotropin levels, but they are not absent, and ultrasensitive assays can detect pulsatile LH secretion especially at night [25]. Prepubertal testes also express both gonadotropin receptors, as shown indirectly by their functional responses to stimulation by hCG or FSH [26].

Puberty is the period of reawakening of gonadotropin secretion (see Chap. 4). At this time, both hormones reach adult levels and the pulsatile secretion pattern. LH activates Leydig cell growth, proliferation, and steroid production. FSH stimulates Sertoli cell proliferation and the metabolic functions (in concert with testosterone) that are needed for the initiation and maintenance of spermatogenesis. Whether FSH is

mandatory for the pubertal initiation and adult maintenance of human spermatogenesis is controversial, but it is clear that qualitatively and quantitatively normal spermatogenesis is possible only in the presence of all endocrine and paracrine factors, including FSH, that are functioning in the pituitary–gonadal axis [27].

Human testicular function is maintained through old age. However, mean testosterone levels and spermatogenesis decline with aging [28–30]. This phenomenon of "andropause" or "late-onset hypogonadism" (LOH) and the controversies of its testosterone replacement therapy has recently received a great deal of attention (see Chap. 17).

When considering the influence of gonadotropin or gonadotropin receptor mutations on male reproductive functions, specific phenotypic effects can be identified during all stages of the male life span.

Gonadotropin Subunit Mutations

Common α-Subunit (CGA) and hCGβ-Subunit (CGB)

Mutations in the gonadotropin subunit genes are extremely rare, most likely because of the deleterious effects they would have on reproduction (see Table 6.1). Probably for the same reason, not a single patient with a germ line mutation in the *CGA* has been detected. Such mutations would lead to defective hCG, LH, FSH, and TSH function, a condition that would probably be lethal, particularly because of lack of hCG. Nevertheless, *CGA* knockout mice are viable, though hypogonadal and hypothyroid [31], but because this species does not produce chorionic gonadotropin, the gestation regulation may be under different hormonal control. Only one somatic *CGA* point mutation (E56A) in the ectopically produced hCG of a carcinoma has been described in the literature (see Fig. 6.1) [32]. Rare non-synonymous substitutions have been detected in *CGB5* and *CGB8* at higher frequency in women with recurrent miscarriages [5]. Despite the phenotypic association, only mild functional alterations were detected in the mutant hormones in vitro [33]. One relatively common (4.5% allelic frequency) amino acid-altering point mutation, V79 M, has been detected in heterozygous form in apparently asymptomatic subjects (see Fig. 6.1) [34]. This mutation hampers α/β dimer formation in vitro and, if present in homozygous form, could block sufficient bioactive hCG synthesis and be lethal. Curiously, additional studies have been unable to find this polymorphism in 5 European and Mexican populations [35, 36]. Milder forms of *CGB* mutations could, if they exist, cause intrauterine undermasculinization of male fetuses because of hCG's crucial role in the stimulation of fetal testicular testosterone synthesis. However, because CGB is expressed by 4 duplicate genes, mutation in a single *CGB* gene may not be functionally significant.

Table 6.1 List of currently known mutations and polymorphisms in human LHB and FSHB genes

Gene	Location	Type	Amino acid change	Symptoms in patient(s)[a]	Functional effect	Reference
LHB	Exon 3	**Inactivating** missense	Q74R	*Infertility, delayed puberty*	No binding to receptor	[37]
LHB	Exon 2	**Inactivating** missense	G56D	*Infertility, delayed puberty*	Disrupted dimerization and secretion	[38]
LHB	Exon2/Intron 2 boundary	**Inactivating** splice site mutation	Inhibition of intron 2 splicing	*Sexual infantilism* Secondary amenorrhea, infertility	Disrupted dimerization and secretion	[39]
LHB	Exon 2	**Inactivating** deletion	Deletion of amino acids 30–32 (HPI)	*Testosterone-induced puberty, normal spermatogenesis off-treatment*	Reduced stability and secretion, low bioactivity	[40]
LHB	Exon 2 (signal peptide)/Intron 2	**Inactivating** compound heterozygous splice site mutation/deletion	Inhibition of intron 2 splicing/Deletion of amino acids 7-10 (LLLL)	*Hypogonadism, infertility* Secondary amenorrhea, infertility	Disrupted secretion	[41]
LHB	Exon 2	**Polymorphism** with 2 missense mutations	W28A/I35T	*Delayed tempo of puberty* Slightly suppressed fertility	Increased bioactivity in vitro Short half-life in vivo	[45, 46, 51]
				Association with PCOS?		
LHB	Exon 3	**Polymorphism,** missense	G122S	Endometriosis	Not detected	[68]
LHB	Exon 3	**Polymorphism**, missense	A17T	Not studied	Slight decrease in signaling activity	[69]
LHB	Exon 2	**Polymorphism,** missense	M14I	Not studied	Unknown	[70]
LHB	Exon 2	**Polymorphism,** missense	H10R	Not studied	Unknown	[70]
FSHB	Exon 3	**Inactivating**, 2-bp deletion → frame shift	V79fs105X	Prim. amenorrhea, infertility *Azoospermia*	Truncated protein, disrupted secretion	[71, 77]
FSHB	Exon 3	**Inactivating**, missense	C69G	Delayed puberty, hypogonadism	Faulty tertiary structure and disrupted dimerization	[72]
FSHB	Exon 3	**Inactivating**, missense	C100R	*Azoospermia*	Faulty tertiary structure and disrupted	[78]

					dimerization	
FSHB	Exon 3	**Inactivating,** nonsense	Y94X	Prim. amenorrhea, infertility *Azoospermia*	Disrupted secretion and loss of bioactivity	[73, 74]
FSHB	Exon 3	**Inactivating,** nonsense	Y94X	Prim, amenorrhea, infertility	Disrupted secretion and loss of bioactivity	[75]
FSHB	Exon 3	**Inactivating,** 1-bp deletion → frame shift	A97fs126X	Prim, amenorrhea, infertility	Disrupted stability and secretion	[76]
FSHB	Exon 3	**Polymorphisms,** silent	No change (Y94)	High FSH, association with PCOS+obesity	Studied	[158]

The amino acid numbers are counted from the translation start site, i.e., containing the signal peptide

[a]Findings on male subjects in *italics*

LHβ Subunit(LHB) Mutations

Five reports describing inactivating mutations of the *LHB* gene have so far been published, comprising a total of 6 affected men and 2 women [37–41] (Table 6.1 and Fig. 6.1). The male subjects have similar phenotypes with delayed puberty, sexual infantilism, and infertility. The only exception is the male described by Achard et al. [40], who appeared to have normal spermatogenesis (see below). The men have undetectable LH, elevated FSH and anti-Müllerian hormone, low testosterone, and normal or low-normal inhibin B and estradiol levels. There is an elevated FSH but no LH response to GnRH stimulation. Testicular size is reduced (5–12 mL) and histological examination revealed [38, 41] tubular hypoplasia and greatly reduced spermatogenesis. Normal pubertal maturation can be induced in these men by testosterone treatment. hCG treatment induces a normal serum testosterone response, and long-term hCG treatment results in testicular enlargement and normal virilization, but spermatogenesis appears to recover only partially, and the men may remain infertile.

The man described by Achard et al. [40] represents a remarkable exception. Despite profound inactivation of LH through a 3-amino acid deletion (Table 6.1), the man had normal spermatogenesis. His other features, such as absent virilization, undetectable serum LH, and low serum testosterone levels, were as in the other men with LH inactivation. It was concluded that in this man, the residual LH activity was able to stimulate intratesticular testosterone levels sufficiently to maintain quantitatively normal spermatogenesis. Upon testicular biopsy, intratesticular testosterone was 20-fold

reduced, suggesting that a high intratesticular testosterone concentration may not be necessary for spermatogenesis. The low residual testosterone, in conjunction with elevated FSH, may be sufficient to maintain spermatogenesis. Similar observations have been made on mice with disrupted *LHCGR* function [42, 43]. Perhaps the only reason why the intratesticular testosterone concentration is so high may be because this organ is the site of testosterone synthesis.

Different types of *LHB* mutations have been detected in affected individuals, including point mutations, deletions in the signal peptide and mature protein encoding regions, and splice site mutations. The severity of the phenotype is in good agreement with the severity of the inactivation of LH function when studied in vitro by expression of the mutated gene product (Table 6.1). Heterozygous family members, both men and women, seem to be asymptomatic, indicating that one functional *LHB* gene can encode sufficient LH synthesis.

These rare cases clarify some points about pituitary LH's developmental role. Because the affected men were apparently normally masculinized at birth and had descended testes, pituitary LH is not needed for the stimulation of testicular testosterone or INSL-3 production in utero. Indeed, fetal testicular testosterone production is initiated seemingly autonomously but becomes subsequently dependent on placental hCG [16, 44], and fetal pituitary LH plays a minimal role in utero. In contrast, the endocrine function of the postnatal testes is critically dependent on pituitary LH, as demonstrated by the total absence of mature Leydig cells in testicular biopsy samples and of spontaneous puberty in its absence.

The two women with homozygous *LHB* inactivation [39, 41] have normal puberty, followed by secondary amenorrhea and infertility. Like men, their LH is undetectable, but FSH is normal to high, estradiol and progesterone are at the follicular level, and testosterone, androstenedione, dehydroepiandrosterone sulfate, and prolactin levels are normal. Apparently theca cell androgen production without LH stimulation is sufficient to provide substrate for FSH-stimulated granulosa cell estrogen production, which explains the normal puberty of the affected females. However, LH stimulation is crucial for ovulation, which explains the infertility of the affected women. In fact, the phenotype of the female *LHB* inactivation is identical with inactivating *LHCGR* mutation (see below).

LHB Polymorphisms

Several polymorphisms have been detected in the *LHB* gene (see Table 6.1 and Fig. 6.1), and their occurrence has been studied in relation to various phenotypic signs of male and female gonadal dysfunction, including hypogonadism and infertility [9, 45]. We concentrate below on findings that concern the effects on male reproductive function.

Of particular interest is the W8R/I15 N polymorphism resulting from two point mutations that are in linkage disequilibrium [46–48]. This variant (V) LH is common worldwide, with considerable ethnic variability in carrier frequency, from 0% in western India to 53.5% in aboriginal Australians [49, 50]. It was originally detected as an immunologically anomalous form of LH in a woman with normal reproductive function but undetectable LH using a specific combination of monoclonal antibodies to detect immunoreactive LH [51]. It has been shown to display increased bioactivity in vitro but to have a shortened circulating half-life [52–54]. However, there is also a report indicating a longer half-life of the variant in the circulation [55]. The discrepancy is likely due to differing experimental conditions used in the studies.

The polymorphism affects variably circulating LH concentrations. No difference was found in LH levels in 73–94-year-old Dutch men who were WT or *V-LHB* carriers [56], but significantly lower serum LH concentrations were detected in *V-LHB* carriers in a large cohort of 40–79-year-old men from 8 European countries [57]. In another study on young Estonian men (mean age 20.1 yrs), *V-LHB* carries were reported to have higher LH levels than WT controls [58]. Likewise, in women, both higher and lower concentrations of serum LH have been reported in *V-LHB* carriers [48, 59–61]. The best explanation for the discrepant findings is provided by the fact that *V-LHB* has eight additional point mutations in its promoter region [62]. They increase the transcriptional activity of the variant promoter by approximately 50% in vitro. However, the promoter could respond in vivo differently to various physiological and pathophysiological conditions, resulting in differences in the relative amounts of the two LH forms [63].

The significance of V-LH as a contributing factor to the variability of reproductive functions remains elusive. Most of the existing studies associate the LH variant with various mild reproductive disorders in men and women [reviewed in 9, 45]. In women, it has mild, although statistically significant, associations with fertility problems and ovarian steroidogenesis, including certain types of polycystic ovary syndrome, pregnancy complications, premature ovarian failure, and breast cancer occurrence. Many of these findings have been made in Japan, which, along with its lower frequency in Asian populations [49, 50] may indicate ethnic differences in its phenotypic expression.

In the male, several findings associate V-LH to reproductive function. When pubertal progression was compared in WT boys and heterozygotes for *V-LHB,* there was no difference in the age of onset of puberty, which is mainly determined by the reawakening of hypothalamic gonadotropin-releasing hormone (GnRH) secretion. Instead, the tempo of pubertal progression was significantly slower in boys who were heterozygous for the *V-LHB* allele [64]. When its occurrence was studied in cryptorchidism, the allele frequency in cryptorchid and healthy boys was similar, but when the frequency in cryptorchid boys was correlated with gestation duration, it increased significantly with increasing gestation duration, from 6% in boys born before

week 30–45% in boys born beyond week 42 of pregnancy [65]. The final phase of testicular descent, from the inguinal region to the scrotum, depends on testicular testosterone production in late gestation. The tropic stimulus for this activity is provided by the combined action of hCG and pituitary LH, and when the duration of pregnancy is prolonged, the role of pituitary LH becomes more important. If this hormone is less active, as V-LH is, then testosterone production may be compromised, predisposing the post-term boys carrying the *V-LHB* allele to cryptorchidism. There is also a report describing an association of V-LH with familial and sporadic prostate cancer [66], but no association of V-LH has been found with male infertility [67]. Finally, V-LH is more prevalent in older men who are obese and have low testosterone levels [56].

These findings, together with those in women, indicate that V-LH represents a genetic variant of the hormone with borderline significant effects on a variety of reproductive functions in men and women. Most importantly, the clinician must be aware of its occurrence, because some immunoassay kits for LH are unable to detect V-LH, which may explain unexpectedly low LH levels.

Four other amino acid-altering polymorphisms have been detected in the coding sequence of *LHB,* A17T, M15I, H30R, and G122S [68–70]. Their phenotypic effects are largely unknown, and the functional alterations of some of these mutant hormones have been found marginal.

FSHβ Subunit (FSHB) Mutations

Six women [71–76] and three men [73, 74, 77, 78] with a total of five different inactivating mutations of the *FSHB* gene have so far been described in the literature (Fig. 6.1 and Table 6.1). All women had similar phenotypes, with slightly variable severity, including sexual infantilism, lack of follicular development, and infertility, which are readily explained by the lack of FSH action on granulosa cell estrogen production and progression of follicular maturation beyond the early stages.

Of the reports on the 3 men with inactivating *FSHB* mutations, the one from Sweden described a 32-year-old man of Serbian origin who presented with azoospermia, normal puberty and male physical characteristics, and a selective FSH absence [78, 79]. His serum testosterone level was low–normal (11 nmol/L), and LH was slightly elevated (12 IU/L). These findings reflect the small size of his testes (3 mL/6 mL) and the missing FSH effect on their growth at puberty. Genetic analysis demonstrated a homozygous T to C mutation, predicting a C100R substitution in the FSHB protein. It was postulated that the elimination of a cysteine would impair formation of the proper intramolecular disulfide bond of FSHB. This would result in abnormal tertiary structure during FSHB synthesis, with extensive intracellular degradation, inability to dimerize with the CGA, defective glycosylation, and, finally, inability to form biologically active hormone.

The second male, reported from Israel [77], was an 18-year-old with slight delay of puberty, small testes, azoospermia, and a plasma FSH concentration below 0.5 IU/L. Total testosterone was low (4.5 nmol/L), and LH was increased (24.5 IU/L), indicating a defect in testosterone biosynthesis. DNA sequencing revealed the same homozygous 2-bp deletion in codon 79 that was found previously in female patients [71–73]. The mutation gave rise to a completely altered amino acid sequence between codons 79 and 105 of the FSHB chain, which was followed by a premature stop codon, and lack of translation of amino acids 106–129. Consequently, the translated FSHB protein was truncated and unable to associate with common α-subunit to form bioactive or immunoreactive α/β dimers.

The third man with inactivating *FSHB* mutation, described from Brazil [73, 74], had had normal puberty but presented with infertility resulting from azoospermia. His serum testosterone at the age of 30 yr was normal (26 nmol/L), FSH was low, and LH was elevated. His testes were small (12 mL), and testicular biopsy revealed Leydig cell hyperplasia and sparse, small seminiferous tubules with germinal cell aplasia, peritubular fibrosis, and few Sertoli cells. The C to A mutation in codon 94 of his *FSHB* gene brought about a premature stop codon (TAA). Because the phenotype of the female patient reported with this mutation was less severe than those of women with the other *FSHB* mutations [72–74], it was hypothesized that the missing amino acids 94–129 of FSHB may not bring about complete inactivation of FSH action.

Conspicuously, all 3 men with the *FSHB* mutation were azoospermic. As will be elaborated in the section on *FSHR* mutations, this is not the case with the five men reported to carry homozygous inactivating *FSHR* mutations [80]. They each displayed variable suppression of spermatogenesis but were not azoospermic. Neither are the knockout mice for *FSHB* [81] or *FSHR* [82, 83] azoospermic, and, moreover, the phenotypes of women with inactivating *FSHB* and *FSHR* mutation were practically identical [71–76, 84]. The more severe phenotype of the ligand mutation in men is curious also for the reason that animal data show the opposite [85], most likely because of the slight constitutive activity of FSHR in the absence of ligand. Why then are the three men with *FSHB* mutation azoospermic? This will be discussed in more detail in connection with *FSHR* mutations.

FSHB Polymorphisms

One functionally significant single nucleotide polymorphism (SNP) of *FSHB*-211G/T [86] has been recently identified and appears to affect both male and female reproductive function. In a study from Estonia, The TT phenotype carrier men had highly significantly lower serum FSH concentrations compared to GG men [87], and in infertile men, FSH was even lower [88]. A similar attenuating effect of the T allele was observed with the high FSH levels in Klinefelter syndrome patients [89]. The apparent

mechanism for the reduced *FSHB* expression is attenuated transcriptional activity through an impaired LHX3 binding site [90]. The association of the T allele with lower FSH levels has been confirmed in other populations [91–93] and is associated with lower serum inhibin B and testosterone levels, smaller testes, and a lower sperm concentration. This polymorphism has been suggested as a pharmacogenomics tool to predict the response of men with idiopathic oligozoospermia to FSH therapy [94]. Indeed, significant improvement in sperm parameters following FSH treatment has been demonstrated in the minor allele carriers [91]. In women, curiously, both increased and decreased levels of FSH have been associated with the T allele [95, 96]. The combination of the -211G/T polymorphism with a common FSHR polymorphism in improving the pharmacogenetic prediction will be discussed later.

Gonadotropin Receptor Mutations

Gonadotropin receptor mutations are also quite rare. There are both activating and inactivating mutations in these genes, with widely varying phenotypic effects. Several mechanisms can underlie receptor inactivation: (1) decreased synthesis of receptor protein; (2) aberrant intracellular processing; (3) reduced or absent ligand binding activity; (4) impaired or absent signal transduction; (5) inability to anchor to the plasma membrane; (6) inability to dimerize, if needed, for signal transduction; (7) dominant negative effect, i.e., formation of non-functional dimers, or (8) increased degradation. Unlike the activating mutations, the types of inactivating mutations vary from point nonsense and missense mutations to various size insertions and deletions. In this case, only homozygotes or compound heterozygotes for two different inactivating mutations produce a clear phenotype because the normal function of a single receptor allele supplies a sufficient number of functional receptors to sustain normal gonadotropin action.

Activating receptor mutations can be classified into four categories: (1) receptor activation in the absence of ligand hormone; (2) increased sensitivity of the receptor to its normal ligand; (3) relaxed specificity of the receptor to ligands; and (4) acquired novel functions of the mutated receptor. All activating gonadotropin receptor mutations detected have been missense point mutations that are almost invariably localized to the transmembrane region or its vicinity (see Figs. 6.2 and 6.3). These mutations produce a structural alteration, activating constitutively the signal transduction machinery of the transmembrane region. Most clearly, receptor activation without ligand explains the altered function of these mutations. Abnormal phenotypes occur in heterozygous individuals because activation of the translation product of a single allele is sufficient to initiate the altered functional response.

LHCGR Mutations

Inactivating Mutations

About 30 different LHCGR mutations have been reported (Fig. 6.2). In the human male, LHCGR function is essential at all ages for testicular androgen production. Therefore, these mutations influence male sexual differentiation, development, and mature functions from the fetal period through adulthood. *LHCGR* mutations thus form one etiological subclass of 46, XY disorders of sexual development (DSD). The receptor inactivation varies in completeness, and the phenotype of affected males depends on its extent [9, 10, 97]. In the mildest forms, the phenotype is hypospadias and/or micropenis (Leydig cell hypoplasia, LCH Type 2), and in the completely inactivating form, the male phenotype is complete male to female sex reversal (LCH Type 1) [9, 97]. The latter group presents with near-total to total lack of male type sexual differentiation in utero and during postnatal development. The phenotype resembles the complete form of androgen insensitivity syndrome but notably differs by the lack of pubertal breast development, which, in androgen insensitivity, is explained by the conversion of testosterone to estradiol. On histological examination, the testes of the individuals with complete LHCGR inactivation are totally devoid of mature Leydig cells, which explain the lack of testosterone production and absent masculinization. However, it is apparent that even in complete LHCGR inactivation, some testicular steroidogenesis is possible, because epididymides and vasa deferentia are present [9]. The explanation for these structures may be low, but functionally sufficient, constitutive steroidogenesis in precursor fetal Leydig cells. It could induce in paracrine fashion, partial masculization of the Wolffian ductal structures. This has also been shown with *LHCGR* knockout mice in which low basal androgen synthesis in their testes can stimulate spermatogenesis, a response which can be eliminated by antiandrogen treatment [42].

In women, LHCGR inactivation causes a milder phenotype with normal intrauterine female sex differentiation and a seemingly normal FSH-dependent component of ovarian function [9]. Affected women present with primary amenorrhea and normal primary and secondary sex characteristics, increased gonadotropin levels, and a lack of ovarian response to hCG treatment. Histological study of ovarian tissue revealed all stages of follicular development, with the exception of preovulatory follicles and corpora lutea [98]. These observations emphasize the importance of LH for ovulation and formation of corpora lutea. LH may also be needed for the maturation of antral to preovulatory follicles. A very interesting recent study reported on successful pregnancy of a woman with inactivating *LHGCR* mutation following ovum donation [99], indicating that the extragonadal *LHCGR* expression, demonstrated in a variety of male and female organs, is redundant for fertility.

Activating Mutations

The first gonadotropin receptor mutations detected were those activating constitutively the *LHCGR* [100, 101], perhaps because of their striking phenotype. The syndrome caused by this mutation, i.e., familial male-limited precocious puberty (FMPP), is a gonadotropin-independent form of precocious puberty, also called "testotoxicosis". Boys with FMPP begin to exhibit signs and symptoms of puberty between 0.5 and 3 yr of age. If they remain untreated, puberty progresses rapidly, resulting in premature epiphyseal closure, compromised adult height and associated behavioral problems of premature puberty. For the treatment, various antihormonal therapies have been used (medroxyprogesterone, ketoconazole, cyproterone acetate, anastrozol, bicalutamide) [102] to reduce virilization and advancement of bone age. Men with this syndrome appear to be fertile.

FMPP has been known for a long time and was originally assumed to result from a circulating nongonadotropic factor that induces premature LH-independent activation of Leydig cell function. However, the subsequent detection of point mutations in the *LHCGR* gene, which, in transfection studies, caused constitutive activation of the LH signaling pathway in the absence of ligand, provided a logical molecular pathogenesis for the syndrome. Today, more than 10 activating mutations of the *LHCGR* gene are known (see Fig. 6.2), and they all (except for one) are localized in or near the transmembrane region of the LHCGR, which is pivotal for LH signal transduction. The mutations change the conformation of the transmembrane region of the receptor such that it assumes, at least partially, an activated conformation in the absence of ligand. The consequence is premature activation of Leydig cell testosterone production before the pubertal onset of LH secretion. In cell transfections, the mutated receptor protein usually displays an approx. tenfold increase in basal cAMP production, whereas maximal stimulation is often suppressed. The latter alteration has no physiological importance, because at the circulating levels of LH, receptor occupancy remains relatively low. Whether other signaling pathways that are initiated by LHCGR activation (see Fig. 6.4) are also activated has not been systematically studied.

Despite the distinct phenotype in males, no phenotype is known for females with activating LHCGR mutation. One explanation is that the LH action initiation in the ovary requires FSH priming, and FSH-dependent paracrine factors may be needed to induce theca cell LHCGR responsiveness. Because there is little FSH secretion in these women before the normal age of puberty, the constitutively activated LHCGR would not be expected to be prematurely functional. The lack of LHCGR in prepubertal granulosa cells is evident, because this receptor is acquired during the later stages of follicular maturation. Therefore, the presence of an activating mutation in the *LHCGR* is functionally unimportant if the gene is not expressed before the age of normal puberty.

A specific Leydig cell tumor phenotype of activating *LHCGR* mutations associated

with Leydig cell tumors (LCTs) has been described in six reposts on the D578H mutation of *LHCGR* in boys with isosexual precocious puberty without a family history of this condition [103–108]. Besides the symptoms and findings of FMPP, a unilateral testicular mass was found by ultrasonography in all boys. In each case, well-circumscribed tumors were removed that were composed of nested polygonal cells with abundant eosinophilic cytoplasm and round ovoid nuclei. Mitotic activity was low and Reincke's crystals were absent. When genomic DNA was extracted from the tumor tissues and peripheral blood leukocytes, DNA sequencing revealed that in each reported case the tumor tissues, but not leukocytes, contained a heterozygous somatic mutation encoding a D578H replacement in *LHCGR*. When the mutated receptor was transfected into COS-7 cells, Liu et al. [103] found it to initiate constitutive activation of cAMP and inositol phosphate production, of which the latter response has not been made with other activating *LHCGR* mutations. Besides the tumor phenotype, these subjects differ from FMPP because their *LHCGR* mutation is somatic. This particular mutation has not been detected in the germ line. It remains to be determined whether this mutation is the only one that causes Leydig cell tumorigenesis.

Another report described nodular LCH in a boy with FMPP, which is not a typical finding in this syndrome [109]. The genomic heterozygous D564G mutation detected in this boy caused only partial activation of cAMP production, and similar Leydig cell alterations were not found in other patients affected with the same mutation. Therefore, the mechanism of the nodular hyperplasia appears to differ from that of the D758H mutation.

A third type of testicular tumor reported in a 36-year-old patient with FMPP due to a germ line D578G mutation is testicular seminoma [110]. Although no causative relationship was established for the *LHCGR* mutation and seminoma, the possibility remains that a prolonged high intratesticular testosterone concentration resulting from LHCGR stimulation could be oncogenic. However, transgenic mice that overexpress hCG [111] or knockin mice with *LHCGR* activating mutation [112] do not show signs of testicular tumors beyond elevated testosterone production and mild Leydig cell hypertrophy.

The situation with activating *LHCGR* mutations and testicular tumors is somewhat analogous to familial nonimmunogenic hyperthyroidism and sporadic thyroid adenomas caused by germ line and somatic mutations of the *TSHR* gene, respectively [113, 114]. As in these thyroid disorders, the somatic *LHCGR* mutations brought about greater constitutive activity of the receptor than do germ line mutations. The severe forms would probably be eliminated from the genetic pool because of their strong deleterious effects. The mutation detected occurred in a position where three other amino acid-substituting mutations have been detected in D578 to Y/E/G (see Fig. 6.2). The other forms cause milder receptor activation and, at most, patchy Leydig cell hyperplasia but no tumors. Hence, the high level of basal stimulation of the receptor is important for

adenoma formation. Whether high cAMP production alone or additive or synergistic effect of the concomitantly stimulated phospholipase C pathway are involved in tumorigenesis remains to be established. In fact, the analogous mutation in the TSHR, D633H, has been found in a patient presenting with insular thyroid carcinoma with metastases [115].

These data emphasize that the same initial phenotype of FMPP may be observed in cases without and with subsequent Leydig cell tumors. Therefore, long-term follow-up with testicular ultrasonography is advised for boys with FMPP to exclude Leydig cell adenoma. Genetic analysis of the *LHCGR* mutation is also advisable if testicular biopsy material is available. Finally, another genetic cause for LCTs is activating mutation in the stimulatory G protein [116].

FSHR Mutations

Inactivating Mutations

Several inactivating mutations have been detected in the *FSHR* gene; most of them in women with hypergonadotropic hypogonadism [9, 117] (see Fig. 6.3). The complete form of *FSHR* mutation in women causes total to near-total arrest of follicular development [84], a process which is dependent on FSH action. The incomplete forms cause a partial phenotype that is responsive to high-dose gonadotropin treatment [118, 119]. In addition to the Finnish-type FSHR inactivation (C → T transition, causing A189 V mutation), which has been found in multiple families [84], all other *FSHR* mutations detected have been sporadic [9, 117].

Five men with totally inactivating mutation of *FSHR* have been described from Finland [80]. These men were identified because they were homozygous brothers of women with hypergonadotropic hypogonadism caused by *FSHR* mutation. The men were normally masculinized, with normal puberty and virilization. Their testes were mildly or severely reduced in size, and all had pathological semen samples, although conspicuously, none was azoospermic (Table 6.2). Moreover, two of the men each had fathered two children. As expected, the men had high FSH levels, low inhibin B, normal or slightly elevated LH, but normal testosterone concentrations [80]. Both testicular sizes and the endocrine parameters of the five men displayed considerable variability. The large age range of the subjects (29–55 yr) may reflect the individual variability in the importance of FSH in maintaining testicular function. More detailed conclusions are limited by the small sample size. These findings were rather surprising because of the concept of a fundamental role for FSH in spermatogenesis, particularly the pubertal initiation of this process. Findings in these men demonstrated that FSH action per se is not necessary for qualitatively complete spermatogenesis. However, FSH is needed for qualitatively and quantitatively normal spermatogenesis but not necessarily for fertility. Subsequent experiments in mice in which *FSHR* or *FSHB* gene expression was

disrupted produced similar results [81–83]; the animals were fertile, but their testes were reduced in size and their spermatogenesis was qualitatively and quantitatively suppressed. The mild phenotype of the men with *FSHR* mutation indicated that they cannot be readily distinguished phenotypically from other men with idiopathic oligozoospermia. In fact, when this was studied in a region with high allelic frequency (1%, northern Finland) for the inactivating *FSHR* mutation, the frequency of the mutation was the same in a cohort of men with idiopathic oligozoospermia and in normal controls [80]. There was no apparent disturbance in pubertal development in these individuals. The sperm quality was highest in the youngest of the five men suggesting that the spermatogenic capacity in the absence of FSH action may be compromised—these men may be fertile only as young adults.

Table 6.2 Semen analyses and hormone levels in men homozygous for the inactivating Ala189Val FSHR mutation

Subject	Age (yr)	Fertility	Testis size ml (right/left)	Semen analysis			FSH (IU/L)	LH (IU/L)	Testosterone	Inhibin B
				Sperm count/mL	Vol. mL	(at age)			(nmol/L)	(ng/L)
1	47	Two children	16.5/16.5	5×10^6	3.0	(47)	12.5	5.6	8.8	<15
2	55	Two children	13.5/15.8	$<0.1 \times 10^6$	3.3	(55)	15.1	4.2	15.8	33
3	45	infertile	4.0/4.0	$<0.1 \times 10^6$	4.8	(30)	23.5	16.3	14.5	62
4	29	unknown	8.6/6.0	42×10^6	1.5	(29)	39.6	11.1	14.7	54
5	42	unknown	8.0/8.0	$<1.0 \times 10^6$	2.5	(42)	20.6	16.2	26.2	53
Reference range				$>20 \times 10^6$			1–10.5	1–8.4	8.2–34.6	76–447

From Ref. [80]

The discrepancy between the azoospermia of the three men with *FSHB* mutation (see above) and the oligoasthenozoospermia, but not azoospermia, in the men with *FSHR* mutation is puzzling. One explanation is that azoospermia is the real phenotype of total elimination of FSH action, and the receptor mutation described is only partial, with partial inactivation of FSH action that does not totally impede spermatogenesis. All evidence collected thus far about the inactivating A189 V *FSHR* mutation shows, however, that it is near-totally inactivating because of the sequestration of the mutant receptor inside the cell [120]. A minute fraction of the mutated receptor may reach the cell surface but is unable to mediate the signal properly even at high FSH levels. Cells transfected with the mutated *FSHR* gene are devoid of the basal constitutive adenylyl cyclase activity that can be displayed in cells transfected with WT *FSHR* [120].

Likewise, women with this mutation are totally resistant to FSH treatment [121].

A second explanation is that oligozoospermia is the true phenotype of FSH deficiency, and the men with inactivating *FSHB* mutation (see above) have a second disturbance in the regulation of spermatogenesis predisposing to azoospermia. This is quite likely because spermatogenesis is a process that is regulated by a complex network of endocrine, paracrine, and autocrine mechanisms. Two key players in this process with paramount importance are testosterone and FSH. The importance of FSH may be relative; if all other factors function properly, FSH is not vital, and, therefore, men with *FSHR* mutation have qualitatively complete spermatogenesis. If, on the other hand, there is a failure in some other mechanism, then the function of FSH becomes critical. The Israeli patient [77] with *FSHB* mutation had an additional failure in testosterone production that might explain the critical role of loss of FSH function. The Swedish patient was treated for extended periods of time with FSH, but there was no spermatogenic response [78, 79]. It is possible in his case that key paracrine mechanisms had failed, and FSH replacement could not compensate for those deficiencies. Furthermore, these two patients were found because of azoospermia, whereas the *FSHR* mutation patients were detected because they were the brothers of women homozygous for this mutation. Hence, the outcome of genotype/phenotype correlations partly depends on which end of the cascade the search is initiated. The third explanation is that we are dealing here with a genuine species difference (man versus mouse) of FSH action.

In conclusion, the majority of information available implies that FSH action per se is not mandatory for the pubertal initiation of spermatogenesis or for male fertility. However, FSH improves spermatogenesis both qualitatively and quantitatively. The phenotype of men with defective FSHR function varies from severe to mild impairment of spermatogenesis, in the face of apparently normal Leydig cell androgen production. The azoospermia of men with *FSHB* mutation may result from additional contributing factors and not solely from FSH deficiency. However, it is apparent that additional cases of genetically proven FSH deficiency are needed before the existing discrepancy between the phenotypes of the ligand and receptor inactivation can be reconciled.

Activating Mutations

Only two reports of activating *FSHR* mutation in men exist in the scientific literature. The first [122] describes a German man who had undergone hypophysectomy and radiotherapy because of a pituitary tumor and, despite panhypopituitarism and unmeasurable serum gonadotropin levels, displayed persistent spermatogenesis during testosterone treatment. Detailed studies of this patient were undertaken because androgen treatment is not sufficient to maintain spermatogenesis in the absence of gonadotropins. A heterozygous D567G mutation was found in the third intracellular

domain of exon 10 of his *FSHR* gene (see Fig. 6.3), and it was shown in vitro to have marginal constitutive activity. Further evidence for the constitutive activation of this mutation has been obtained in transgenic mice expressing the mutant gene [123]. The mutant mice had no phenotype in the wild-type genetic background, but when crossed to the gonadotropin-deficient *hpg* genetic background, a functional Sertoli cell response was observed. These findings indicate that activating *FSHR* mutation may have no phenotype when the LH-testosterone arm of testicular endocrine regulation is normal, but its effect can be seen when LH stimulation of testosterone production is absent. It also explains why the phenotype of the German patient was observed when his gonadotropin secretion was eliminated by hypophysectomy. In line with the above discussion, male transgenic mice expressing a strongly activating FSHR mutation [124] have no apparent phenotype (Oduwole, Peltoketo and Huhtaniemi, unpublished data).

The second activating *FSHR* mutation in a male was detected in an asymptomatic Argentinian man originally referred for pain during intercourse. He had normal spermatogenesis, profoundly suppressed serum FSH, and normal biochemical markers of FSH action except for twofold elevated AMH [125]. Normal spermatogenesis in the absence of FSH raised the suspicion of *FSHR* mutation, which was detected (N431I) in heterozygous form. This was apparently a neomutation because it was absent in the parents. With different experimental strategies on cells transiently expressing the mutant, it was found that despite decreased cell surface expression, the mutant receptor conferred a low level of constitutive activity associated with markedly altered agonist-stimulated desensitization and internalization. The latter features may explain the persistent activation of the receptor in the absence and presence of FSH.

The search for *FSHR* mutations in candidate diseases, such as premature ovarian failure, ovarian tumors, megalotestes, precocious puberty, and twin pregnancies, has yielded negative results [126–130]. It also remains possible that activating *FSHR* mutation has no abnormal phenotype in otherwise normal individuals. This was the case with the serendipitous discovery of the two males so far described, one with unexpected spermatogenesis following hypophysectomy, the other with unexpected normal spermatogenesis in the face of very low FSH level.

Another surprise concerns the activating FSHR mutations detected in women [117, 131]. The phenotype is pregnancy-associated ovarian hyperstimulation, which is explained by the combination of marginal constitutive activity of the mutant receptor and/or relaxed ligand specificity, where hCG is able to activate FSHR and cause ovarian hyperstimulation during pregnancy.

Gonadotropin Receptor Polymorphisms

Several polymorphisms have been detected in the gonadotropin receptor genes, but relatively little is known thus far about their functional significance. The polymorphisms

T307/N680 and A307/S680 in the *FSHR* gene are in linkage disequilibrium in most populations, and with almost equal frequency, at least in individuals of European ethnicity [132]. It was reported from Germany that in women undergoing ovulation induction, the basal FSH concentration of the 680 N/N genotype (6.4 ± 0.4 IU/L) was significantly lower than that of the S/S genotype (8.3 ± 0.6 U/L), and that the latter group required approx. 50% more exogenous FSH to stimulate follicular growth [132]. A subsequent study, quite surprisingly, found that ovarian hyperstimulation syndrome was associated with the poor responder S/S genotype of the previous study [133]. A large body of information on the effect of this polymorphism on ovarian function has appeared thereafter, and a recent review concluded that the 680S allele conveys poorer response to FSH upon ovarian stimulation [134].

Several studies have addressed the associations of the FSHR T307/N680 and A307/S680 polymorphisms on male reproductive function. The initial studies of small size detected no difference in reproductive phenotype for either genotype of men; neither were there functional differences between the two receptor types [135]. When combined with the-29A/G polymorphism, it was found that certain SNP combinations showed significant association with azoospermia in Germany [136], and with testis cancer [137], but there was no association with azoospermia in Italy [138]. When oligozoospermic men were treated with FSH only, those with at least one serine in position 680 showed a statistically significant increase in these sperm parameters, whereas subjects with homozygote T307/N680 showed no difference in any seminal parameters evaluated [139]. Studies from Sweden and Estonia have reported the best sperm parameters in this genotype [140, 141]. Two studies, from Japan and India, have reported that heterozygosity for the polymorphisms is associated with infertility [142, 143]. An interesting approach was to assess the joint effect of the -211G/T *FSHB* polymorphism and the N680S FSHR polymorphism on male reproductive parameters [93]. The TT *FSHB* carriers were more prevalent amongst men with low sperm counts. Although not effective alone, the *FSHR* 680S genotype amplified the effect of the *FSHR* polymorphism. A similar combined effect of the *FSHR*-28A/G, *FSHB*-211G/T, and FSHR N680S polymorphism was confirmed by another study [90]. It remains to be seen whether *FSHB* and *FSHR* polymorphism analyses will be a clinically useful pharmacogenomics test to predict which infertile men will benefit from FSH treatment.

Relatively little is known about the possible functional effects of *LHCGR* polymorphisms in men. One early study suggested the relationship of the known *LHCGR* polymorphisms (LQ insert between amino acids 8 and 9) with male undermasculinization [144]. It was found that the polymorphism encoding insertion of leucine and glutamine between codons 8 and 9 of the LHCGR protein was significantly associated with those undermasculinized individuals that had a long polymorphic polyglutamine repeat (>26) in their androgen receptor, which is associated with suppressed androgen receptor activity. Also, two single nucleotide polymorphisms of

the *LHCGR,* both altering the amino acid sequence (N291S and N312S), were found, both alone and in combination, to be associated with undermasculization. Another study reported significant association between the N312S polymorphism in exon 10 with spermatogenetic damage [145]. A recent study on *LHCGR* polymorphisms compared the endocrine responses to testis cancer chemotherapy with several endocrine gene polymorphisms and found that the *LHCGR* exon 1 LQ insert protected from cisplatin-induced suppression of testosterone [146].

Inconsistent findings on the phenotypic effects of polymorphisms are not unusual. For example, inconsistencies were observed when the frequency of the common LH polymorphism (see the section on *LHB* Polymorphisms) was studied in women with polycystic ovary syndrome; the frequency and correlation with the syndrome varied according to ethnicity of the patient cohorts [45]. This is understandable because the phenotypic effects of mild alterations of function of gene products, such as the polymorphisms depend on the genetic background and combined effects of other genes, and, therefore, the phenotypic expression may differ between populations. Overall, the studies on phenotypic effects of the polymorphisms in gonadotropin and gonadotropin receptor genes are still in their infancy and more information about their potential role in the individual variability in pituitary–gonadal function will undoubtedly be obtained in the future. They may provide in the future important targets for pharmacogenomic approaches to treatment.

Concluding Remarks

No germ line mutations of the glycoprotein hormone *CGA* have been reported, apparently because of the dramatic phenotypic effects they would have (possibly embryo lethality). Five inactivating *LHB* mutations have been described in men with normal prenatal masculinization but arrested pubertal development. The common polymorphism in *LHB* affects bioactivity of the hormone and has multiple mild phenotypic effects, including slow tempo of puberty in boys, and is enriched in post-term boys with cryptorchidism. The three men so far described with *FSHB* mutation were azoospermic, but additional patients are needed to confirm the phenotype, particularly because this phenotype conflicts with the findings in FSHR inactivation as well as animal models for FSH and FSHR inactivation.

Constitutively activating mutations of the *LHCGR* gene give rise to FMPP. Inactivating *LHCGR* mutation results in an array of male phenotypes ranging from micropenis and hypospadias to complete sex reversal (XY, disorder of sexual development), depending on the completeness of inactivation of the receptor. Inactivating *FSHR* mutations in men cause a decrease in testicular size and suppressed quality and quantity of spermatogenesis but not azoospermia, and some affected men may be fertile. Since the original discovery in 1997, no additional cases have been

reported. Only two cases of activating *FSHR* mutations have been detected, and they suggest that the mutation does not have phenotype in men with otherwise normal endocrine function.

The discrepancy between the phenotypes of men with inactivating *FSHB* (azoospermia) and *FSHR* (no azoospermia) mutations must be clarified with additional subjects. Information about the genotypic effects of common polymorphisms in gonadotropin and gonadotropin receptor genes is gradually mounting, and they may form important targets for the pharmacogenetic evaluation of gonadotropin treatment in infertility.

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7. Hypogonadism in Males with Congenital Adrenal Hyperplasia

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Introduction

Congenital Adrenal Hyperplasia: Impaired Steroid Synthesis in the Adrenal Gland

Congenital adrenal hyperplasia (CAH) is a group of inherited conditions in which the synthesis of cortisol by the adrenal gland is impaired as a result of an enzyme deficiency. Consequently, the secretion of adrenocorticotropin (ACTH) by the pituitary

gland is increased, resulting in hyperplasia of the adrenal cortex and excess production of steroids that do not require the specific deficient enzyme for their synthesis [1]. Figure 7.1 depicts the pathway for normal adrenal steroid synthesis. Mutations in the genes encoding the enzymes 21-hydroxylase, 17-hydroxylase/17,20-lyase, 11-hydroxylase, 3β-hydroxysteroid dehydrogenase, or the protein StAR (intracellular cholesterol transport protein) lead to CAH. 21-hydroxylase deficiency is by far the most frequent cause of CAH (more than 90% of all cases). This deficiency results in cortisol and aldosterone deficiency and an overproduction of 17-hydroxyprogesterone and androstenedione, leading to androgen excess. Recent publications describe also transcription factors (PAPPS and cytochrome B5) resulting in insufficient enzymatic activity [2, 3].

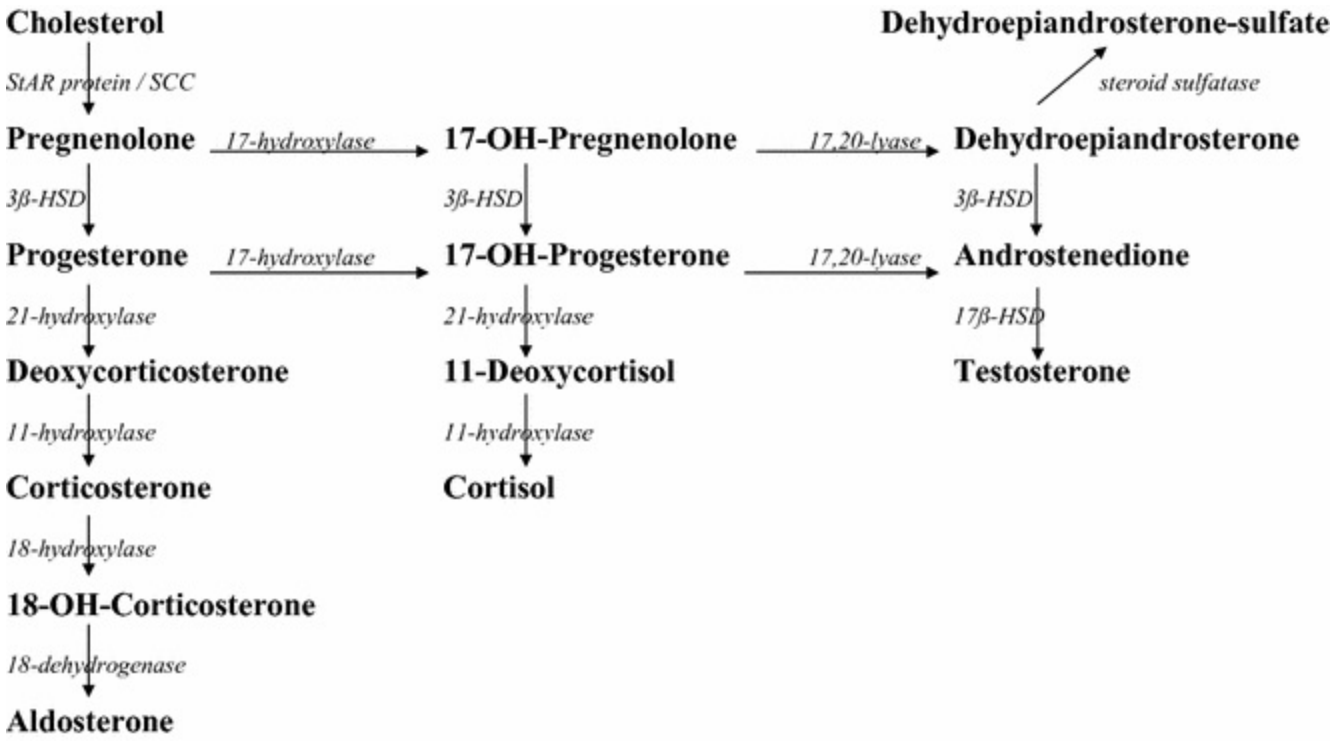

Fig. 7.1 Adrenal steroid synthesis. *StAR* steroidogenic autoregulatory protein; *SCC* (cholesterol) side-chain cleavage enzyme; *3β-HSD* 3β-hydroxysteroid dehydrogenase; *17β-HSD* 17β-hydroxysteroid dehydrogenase

Prevalence

Most enzyme deficiencies that cause CAH are extremely rare, except for 21-hydroxylase deficiency and 11-hydroxylase deficiency. The prevalence of classic 21-hydroxylase deficiency worldwide is 1:10.000 to 1:18.000, as derived from results of neonatal screening. Non-classic 21-hydroxylase deficiency is estimated to be more prevalent (1:600), but this diagnosis can easily be missed in males because signs of adrenal androgen excess may go unrecognized. The estimated prevalence of 11-hydroxylase deficiency is 1:100.000 [1].

Presentation and Diagnosis

CAH represents a broad spectrum of phenotypes depending on the severity of the enzymatic defect. In clinical practice, however, CAH is divided into 3 forms. The most severe, classic CAH phenotype is subdivided into a salt-wasting form (SW-CAH, no residual enzymatic activity) and a simple virilizing form without aldosterone deficiency (SV-CAH, residual enzymatic activity of 1–2%). Non-classic CAH (NC-CAH) has a less severe phenotype with a residual enzymatic activity of 30–50%. Patients with the mildest forms present with symptoms are caused by androgen excess only: premature adrenarche, hirsutism, menstrual irregularities, and infertility, most of which are limited to women (non-classic form) [1].

Genetics

All forms of CAH are characterized by an autosomal recessive inheritance. The genes that encode the various enzymes have all been elucidated [1]. 21-hydroxylase is encoded by the CYP21 gene. CYP21 and the homologous pseudogene CYP21P are located in the HLA major histocompatibility complex on chromosome 6, a region with a high frequency of genomic recombination. CYP21 and CYP21P are 98% identical, which explains the high mutation rate of the CYP21 gene. One-third of the described mutations are deletions and large gene conversions that were caused by recombination between the two homologous loci. The other mutations are small defects that are transferred from the CYP21P pseudogene to the active CYP21 gene, resulting from non-homologous pairing. The genotype–phenotype correlation is reported to be 80–90%, with the best predictions in the most severe and the mildest deficiencies [1, 4].

Of all types of congenital adrenal hyperplasia, 21-hydroxylase deficiency is the most frequent and the most extensively studied. Therefore, this chapter on male hypogonadism in CAH will focus predominantly on 21-hydroxylase deficiency.

Fertility in Males with 21-Hydroxylase Deficiency

Reported Fertility in Males with 21-Hydroxylase Deficiency

Reports on child rate in CAH males show a reduced fertility. Jääskeläinen et al. [5] found a child rate of 0.07 in the complete Finnish male CAH population, compared to 0.34 in age-matched Finnish males. Other authors reported parenthood only as additional information in smaller and selected patient populations. Urban et al. [6] found apparently normal fertility in men with CAH (some even untreated): They described 14 fathers among 20 male patients (aged 18–37 years), and Cabrera et al. [7] reported paternity in 2 of 30 patients (aged 17–43 years). Three more recent reports in large patient cohorts show paternity in 41, 67, and 51%, respectively, in patients who

had sought fertility [8–10].

Several recent studies reported results of semen analysis in CAH patients [8, 10–13]. In most of these recent studies, only a subgroup of the study population consented to provide semen for analysis (32–91% of study participants). The combined results of these studies show azoospermia in 5–20% of cases and oligozoospermia in 20–54% of cases. In the studies in which the upper reference limit of the WHO consensus (>15 million sperm per ml) was used [14], normospermia was found between 34 and 68% [10, 12, 13]. Although reports of semen analysis are hampered by low participation rates and ambiguous use of reference values, we could conclude that spermatogenesis is impaired in a substantial group of CAH males.

In summary, there is substantial evidence that fertility can be impaired in male CAH patients. This finding raises the question to what extent male subfertility in the general population is caused by undiagnosed CAH. Ojeifo et al. [15] investigated basal and ACTH-stimulated serum 17-hydroxyprogesterone levels in a population of 50 males with idiopathic infertility, and compared the results with those of 25 controls. No differences in basal or stimulated levels were found, and they concluded that 21-hydroxylase deficiency is a rare cause of idiopathic male infertility. Pinkas et al. [16] studied a cohort of 484 healthy men attending a fertility clinic and likewise found no patient with CAH (defined as an ACTH-stimulated level of 17-hydroxyprogesterone (17OHP) of ≥45 nmol/l, performed in patients whose random mid-morning level of 17OHP was ≥6 nmol/l).

Causes of Subfertility in CAH Males

The most frequently reported cause of subfertility in CAH males is the presence of adrenal rest tumors in the testes [7, 17, 18]. These tumors may interfere directly (mechanically or paracrine) or indirectly (endocrine) with testicular function [19, 20]. Subfertility can also be caused by hypogonadotropic or hypergonadotropic hypogonadism. Psychological problems may also be important. The next sections will focus on these topics separately, although, in practice, these factors may not be separated easily.

Testicular Adrenal Rest Tumors (TART)

Introduction

The presence of testicular tumors in male patients with CAH due to 21-hydroxylase deficiency was first described in 1940 by Wilkins et al. [21]. Since then, these tumors have been described in several case reports and patient cohort studies. It is now generally accepted that testicular adrenal rest tumors are the most important cause of infertility leading to gonadal dysfunction and primary gonadal failure [22]. Because of

their histological and functional resemblance to adrenocortical tissue, they are generally called *testicular adrenal rest tumors* (TART). These lesions are often found in both testes of adult men with CAH with a typical location within the rete testis. The tumors are benign, although case reports also describe malignant Leydig cell tumors in CAH patients that can be difficult to diagnose.

Due to the central location within the rete testis, the tumors may lead to the obstruction of the seminiferous tubules and consequently infertility. Long-standing chronic obstruction may also lead to the damage of testicular tissue, and thereby impaired Sertoli and Leydig cell function.

Prevalence of TART in Adult CAH Patients

The reported prevalence varies between 0 and 100% [5–7, 12, 17, 18, 23]. It strongly depends on patient selection (prepubertal, adolescent, or adult patients) and on the method of tumor detection (physical examination or imaging techniques). Because of the central location of the tumors, small lesions <2 cm can be easily missed. Therefore, additional techniques such as MRI or ultrasound are necessary to detect smaller lesions.

The first report of a larger patient cohort was by Urban et al. [6] who found no testicular tumors by physical examination in 30 adult patients. Using ultrasonography, however, adrenal rest tumors were described in several studies reporting TART in 2/14 adult patients [5], 9/18 adult patients [7], and 16 of 17 postpubertal patients [17]. In a recent paper with 50 adult patients (18–49 years), Reisch et al. found a prevalence of 61.3% in salt-wasting patients and 47.5% in SV patients [23].

Furthermore, there is a clear association between the prevalence of TART and the genotype of CAH [24]. TART is mostly described in patients with the most severe types of CAH. This may be explained by strongly elevated ACTH levels beginning in utero. The presence of TART in patients with non-classic CAH has only been described anecdotally [12].

Prevalence of TART in Children and Adolescents with CAH

Tumors have also been reported in childhood: Of the 8 patients with testicular tumors described by Avila et al. in a population of 38 patients, 7 were younger than 16 years old [18]. Shanklin et al. reviewed autopsy material of patients with CAH, and noted testicular nodules in 3 of 7 patients less than 8 weeks old, and in all 14 patients older than 14 months [25]. Claahsen et al. found general prevalence of 24% in childhood and adolescence [26]. Other authors found a similar prevalence of 21 and 18.3% [27, 28]. In a recent study, Claahsen-van der Grinten et al. found a high prevalence of TART in CAH children of nearly 100% with a clear increase in prevalence during puberty [29].

Diagnosing TART

The physical examination reveals only a small proportion of all testicular tumors in CAH patients. For the more accurate detection of smaller tumors, imaging techniques such as ultrasound and MR are required [6, 7, 17, 18, 30, 31]. A comparative study in CAH males concluded that ultrasound is the method of choice because it is as sensitive as MR, less costly, and more accessible [32]. Ultrasound features include hypoechoic lesions adjacent to the mediastinum testis, often lobulated and mostly bilateral (Fig. 7.2) [18, 31–38]. In larger tumors, we have observed hyperechoic reflections probably due to fibrotic strands. Tumor margins may be blurred on ultrasound but are always well defined on MR [32, 39]. On MR imaging, most of the masses are isointense on T1-weighted images and hypointense on T2-weighted images, just as are adrenal glands [32].

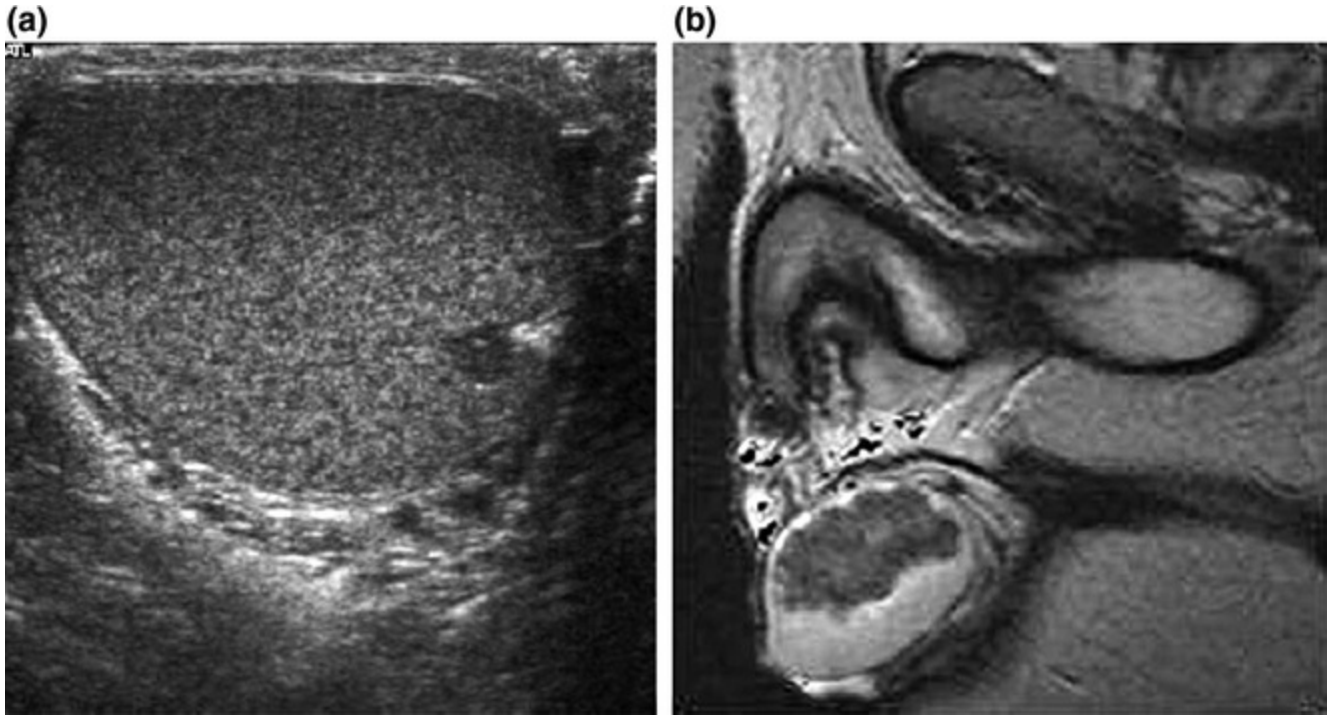

Fig. 7.2 **a** Scrotal ultrasound of a 13-year-old male CAH patient showing a mostly hypoechogenic rounded lesion in the left testis near the rete testis. **b** T2-weighted MR image of long-standing bilateral testicular adrenal rest tumor. Note that heterogeneous low-signal-intensity tumors are displaced surrounding high-signal normal testicular tissue. From Claahsen-van der Grinten et al. [22] (Open Access)

Testicular tumors in CAH may resemble malignant testicular tumors by ultrasonography and MR [38, 40]. However, clinical characteristics such as the bilateral presence of lesions and the location of the lesions adjacent to the mediastinum are helpful in differentiating between a benign testicular adrenal rest tumor and a malignant testicular lesion [33, 39, 41]. On the other hand, the ultrasound characteristics of a testicular tumor can suggest the diagnosis of CAH in males with previously

undiagnosed CAH who present with a testicular mass [41]. In that case, elevated 17-hydroxyprogesterone levels may indicate 21-hydroxylase deficiency. However, 17-hydroxyprogesterone production by a Leydig cell tumor in a non-CAH patient has also been reported [42].

Histopathology

Histologically, the testicular tumors in CAH patients resemble Leydig cell tumors, and differentiation can be difficult (Figs. 7.3 and 7.4) [43]. Some differences may exist; however, TART are bilateral in 83% of cases, whereas Leydig cell tumors are bilateral in only 3% of cases. Malignant degeneration has not been reported in TART, but it occurs in 10% of Leydig cell tumors in adults [43]. In addition, TART are located in the mediastinum testis and may decrease in size when ACTH levels are suppressed by increasing the glucocorticoid dose [20, 41, 43–47]. Rich et al. [43] found that both TART and Leydig cell tumors are circumscribed, lobulated, nodular lesions. TART are typically encapsulated nodules that are dark brown (resulting from a greater concentration of lipochrome pigmentation), whereas Leydig cell tumors are usually yellow intratesticular masses. The color distinction is not always reliable, however, because some Leydig cell tumors are occasionally darker. Reinke crystals, which can be found in 25–40% of Leydig cell tumors, have never been observed in TART. In the surrounding parenchyma, seminiferous tubules show atrophic sclerosis in TART, whereas seminiferous tubules are rarely found in Leydig cell tumors because they are replaced by the tumor [47].

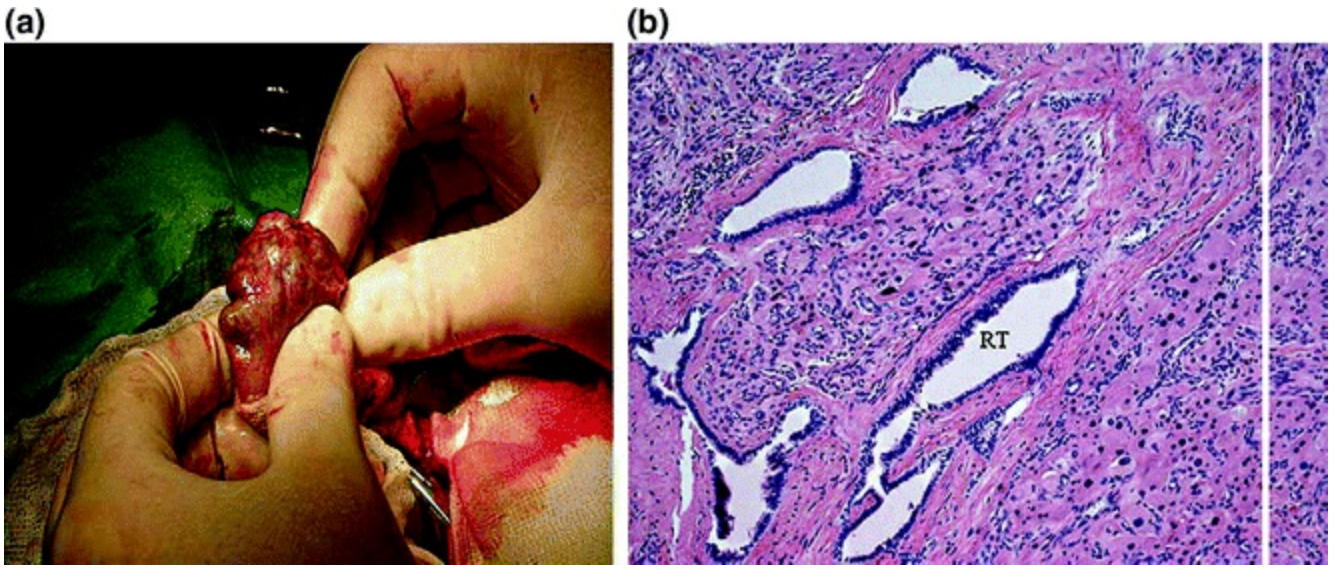

Fig. 7.3 **a** Macroscopic aspect of TART. Note the yellow color and the bands of fibrous tissue. **b** Testicular adrenal rest tumor growing into rete testis (RT) (HE, original magnification × 200). From Claahsen-van der Grinten et al. [22] (Open Access)

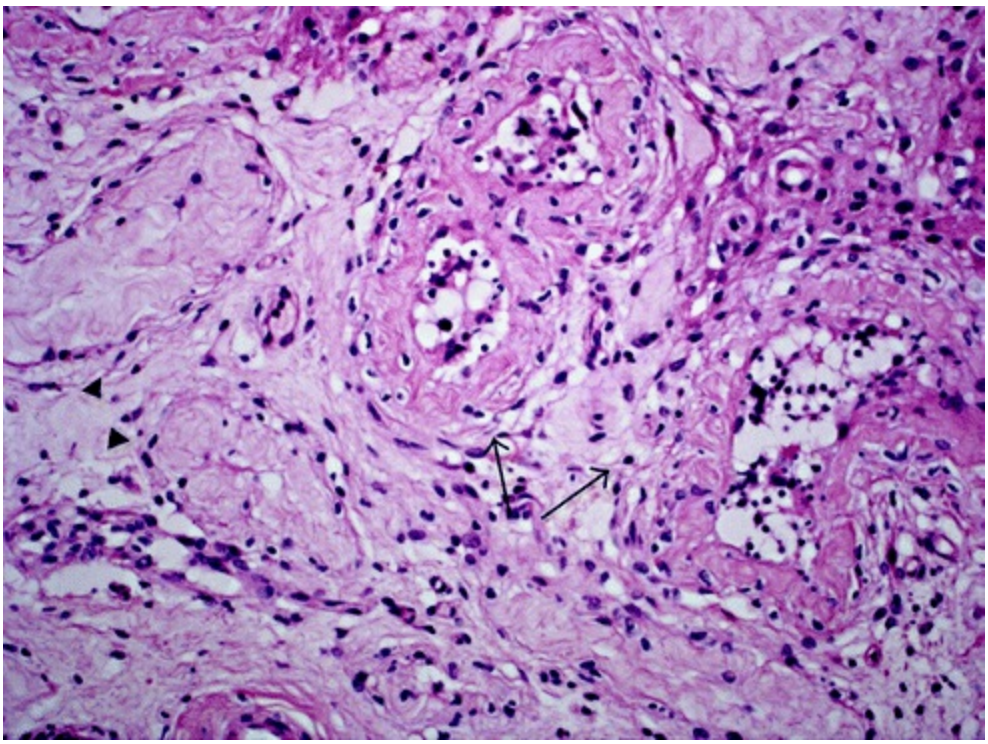

Fig. 7.4 Testicular biopsy of a patient showing seminiferous tubules with hypospermatogenesis and prominent peritubular fibrosis with increased number of peritubular fibroblasts (*arrows*), as well as tubular hyalinisation (*arrowhead*; original magnification × 200). From Claahsen-van der Grinten et al. [22] (Open Access)

Steroid Production

Steroid production by TART has been investigated both in vitro and in vivo.

Cortisol production by TART has been demonstrated in vivo by testicular venous sampling after stimulation with ACTH in non-classic CAH patients [48, 49] and, surprisingly, also in a salt-wasting patient [50].

17-hydroxyprogesterone production by a testicular tumor was demonstrated in vivo by testicular vein sampling after ACTH stimulation [51].

Production of 11-hydroxylated steroids, including 21-deoxycortisol (21-DF), 21-deoxycorticosterone (21-DB), and 11beta-hydroxy-Δ^4-androstenedione (11beta-OHA), has been demonstrated in vitro [51, 52], as well as in vivo [51, 53, 54]. 11-hydroxylase activity is usually restricted to the adrenal cortex. Clark et al. demonstrated 11-hydroxylase activity in vitro in TART from a salt-wasting CAH patient [52]. That study identified abundant angiotensin II receptors, but no LH receptors, in tumor membranes (ACTH receptors were not investigated). In vivo, 11-hydroxylase activity was demonstrated by testicular vein sampling by Blumberg-Tick et al. [53]. Combes-Moukhovsky et al. showed that stimulation with hCG increased the secretion of 11-hydroxylated steroids [54]. LH/hCG receptors have been found in the normal human adrenal cortex [55]. In a study of 7 male CAH patients with bilateral TART undergoing testis-sparing surgery, Claahsen-van der Grinten et al. measured the concentrations of

the adrenal-specific steroid 21DF, and of 17-hydroxyprogesterone (17OHP) and androstenedione (A) in blood taken from the spermatic veins during surgery [56]. In addition, mRNA expression of the adrenal-specific enzymes CYP11B1 and CYP11B2, as well as of ACTH and angiotensin II (AII) receptors, was shown by PCR in 16 testicular tumors from 8 patients [56]. The results show that TART contain adrenal-specific enzymes and produce adrenal-specific steroids suggesting that these tumors arise from adrenal-like cells. The presence at the mRNA level of ACTH and angiotensin II (AII) receptors in the adrenal rest tumors supports this hypothesis.

Testosterone production was shown in vitro by Franco-Saenz et al. [50], but only after stimulation with ACTH or hCG. Interestingly, testosterone production was more responsive to stimulation by ACTH than by hCG. The authors hypothesized the presence of specific receptors for ACTH and hCG in the same cells, a defective receptor with loss of specificity, or the presence of two different cell lines. In vivo, Kirkland et al. showed increased testosterone production after ACTH infusion into the testicular artery [57].

Benvenga et al. hypothesized from longitudinal observations in a single patient that at least three distinct types of testicular tumors in CAH could be discriminated based on their response to dexamethasone-induced ACTH suppression [58]. The first type is ACTH-dependent, both in terms of growth and steroidogenesis. The second type is only partially responsive to ACTH, since suppression of growth lagged behind the prompt suppression of steroidogenesis. In addition, this type seemed to be responsive to LH/hCG. In the third type, growth is unresponsive to dexamethasone-induced ACTH suppression, unresponsive to gonadotropins, and has lost the ability to synthesize cortisol.

The Origin of TART

Based on histopathology, steroid-producing properties, and ACTH responsiveness, these testicular tumors are considered to consist of steroidogenic cells. It is known that cells of the adrenal cortex and gonads share a common precursor called the adreno-gonadal primordium, and develop in close relationship to each other. It was hypothesized that, due to incorrect splitting of cells, ectopic adrenal cortex cells persist and descend within the testes, and are stimulated by ACTH. Aberrant adrenal tissue has been identified in 7.5% of normal testes by Dahl et al. [59]. The aberrant adrenal cells are thought to retain their potential for glucocorticoid production, and to respond with hyperplasia to increased ACTH stimulation. Val et al. suggest that adrenal cells originate from a different cell population of adrenal-like cells migrating from the mesonephros into the developing gonad. Other studies propose that TART may be derived from pluripotent cells within the testes [60].

A more recent study by Smeets et al. [61] described the molecular characteristics of

TART in more detail using qPCR of all relevant genes encoding enzymes important for steroid production. Nearly all genes were highly expressed in TART tissue, including all genes that encode the key steroidogenic enzymes. TART expression levels are generally identical to those found in adrenal tissue. The expression of adrenal cortex-specific genes (*CYP21A2, CYP11B1, CYP11B2,* and *MC2R*) in both TART and adrenal tissue is approximately 1000–10,000 times higher than in testes. In addition, the Leydig cell-specific markers *INSL3* and *HSD17B3* were not only found in testes, but also in TART, both at significantly higher levels than in the adrenal. The authors concluded that TART consist of a more totipotent cell with adrenal and Leydig cell-specific features [61].

Based on clinical, histological, and radiological observations, Claahsen-van der Grinten et al. [22] proposed that the development and growth of TART can be divided into five stages based on histological criteria of TART and the surrounding testicular parenchyma (Fig. 7.5). **Stage 1**: This stage can be defined as the presence of adrenal-like cells within the rete testis not detectable by scrotal ultrasound. **Stage 2**: In CAH patients, the adrenal rest cells may proliferate in the presence of increased concentrations of growth-promoting factors such as ACTH (and possibly AII). In this stage, the adrenal rest cells may become visible by ultrasound as hypoechogenic lesions. **Stage 3**: Further growth of the adrenal rest cells will compress the rete testis. **Stage 4**: Further hypertrophy and hyperplasia of the adrenal rest cells with progressive obstruction of the rete testis may lead to the induction of fibrosis within the tumor and focal lymphocytic infiltration. Several small tumors within the rete testis become confluent, forming a single lobulated structure separated from testicular tissue by fibrous strands. **Stage 5**: Chronic obstruction subsequently leads to the destruction of the surrounding testicular parenchyma causing irreversible damage [62]. Further studies are necessary to validate the proposed classification of TART.

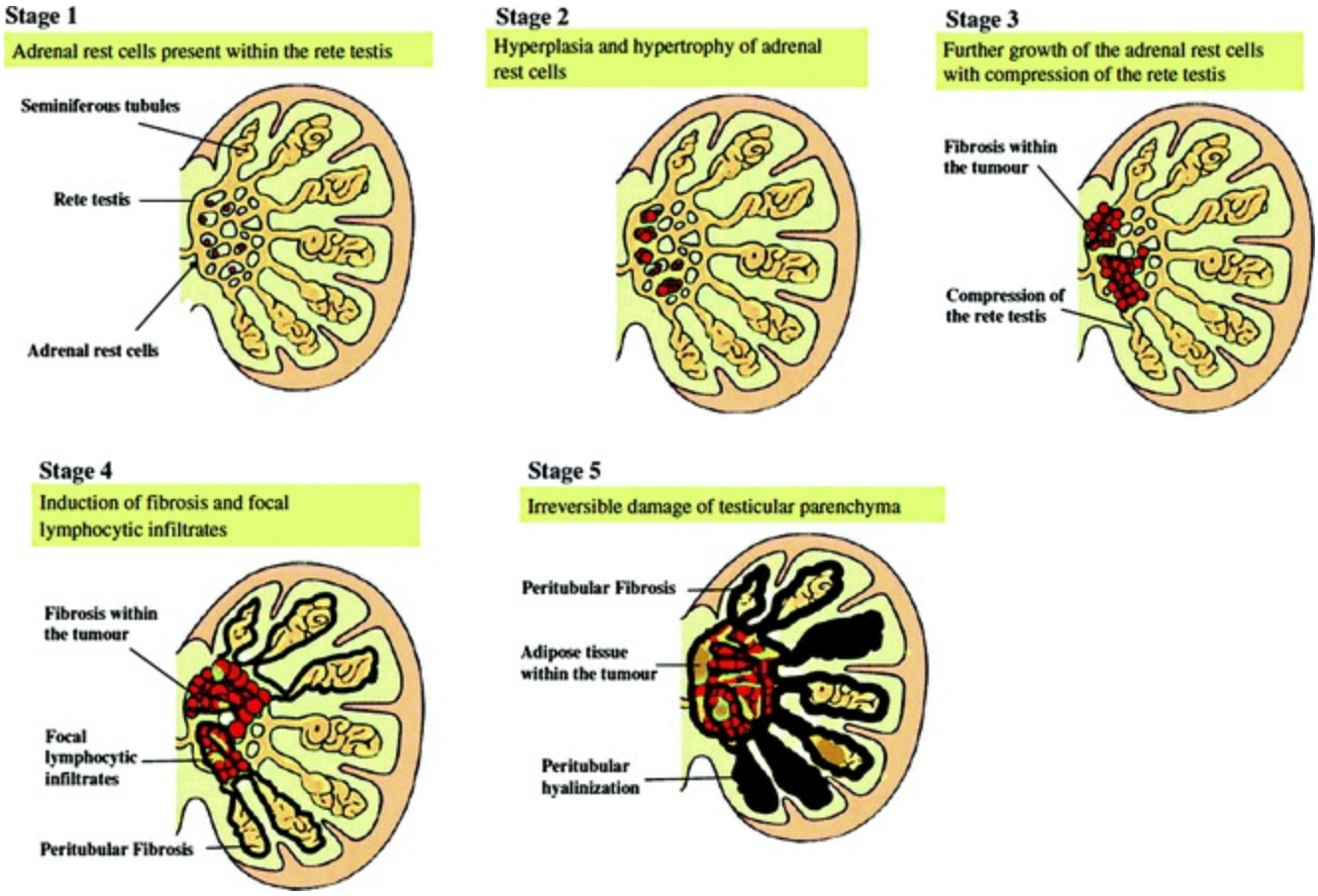

Fig. 7.5 Schematic view of the proposed classification of testicular adrenal rests. From Claahsen-van der Grinten et al. [22] (Open Access)

Relation Between the Presence of TART and Therapeutic Control

Early reports of TART were predominantly in poorly controlled patients [53, 54, 63]. Those observations led to the hypothesis that poor hormonal control and inadequate suppression of ACTH secretion is a dominant etiological factor in the development of TART. This hypothesis is supported by the development of TART in patients without CAH who have elevated ACTH levels, such as Nelson's syndrome [64, 65] or Addison's disease [66]. Furthermore, shrinkage of the tumor after intensifying glucocorticoïd therapy has been described in many case reports.

In more recent studies, however, no correlation was found between the presence of TART and the degree of hormonal control [7, 17, 23, 30, 31, 36]. Still, as stated before, there is a clear association between the prevalence of TART and the genotype of CAH [24]. TART is mostly described in patients with the most severe types of CAH, which may be explained by strongly elevated ACTH levels beginning in utero.

Influence of TART on Fertility

TART may interfere directly (mechanically or paracrine) or indirectly (endocrine) with testicular function. The mechanical effect of the tumors can be considerable as a result of their central location adjacent to the mediastinum testis. It can lead to the obstruction of the vascular supply, and compression and atrophy of the seminiferous tubules. Pathological examination revealed that in the cases of large tumors, the residual testicular parenchyma was abnormal and showed atrophy, sclerosis or immaturity, and decreased or absent spermatogenesis [41]. Around small nodules, however, the testicular parenchyma was generally normal [62]. Murphy et al. showed in one patient that, close to the mediastinum and the tumor, germ cells were reduced and spermatogenesis was impaired. Testicular aspiration in other areas, however, showed normal spermatogenesis. In addition to direct mechanical effects (obstruction or destruction), tumors may have a paracrine effect on the surrounding tissue, i.e., steroids produced by the tumors could be toxic to Sertoli cells or germ cells [67].

Indirect (endocrine) effects of testicular tumors in CAH on fertility could result from steroid secretion by the tumor with suppression of the hypothalamic–pituitary–gonadal axis. The association between infertility, testicular adrenal rest tumors, and depressed serum LH and FSH levels in CAH males has been described [7, 17]. However, the effect of steroid secretion by the tumor cannot be readily separated from the effect of excess steroids secreted by the adrenal glands.

Other Testicular Tumors in CAH Males

There are a few reports of other types of testicular tumors in CAH males. Adesokan et al. presented a case of a testicular adrenal rest tumor in a patient with CAH in combination with a testicular myelolipoma (a tumor of adrenal origin) and a seminoma in a cryptorchid testis [68]. Davis described a Leydig cell tumor with metastases in a CAH patient [69].

Hypogonadotropic Hypogonadism in CAH Males

In male patients with 21-hydroxylase deficiency, as in affected females, adrenal androgens may suppress the hypothalamic–pituitary–gonadal axis both directly and after conversion to estrogens, and thereby lead to hypogonadotropic hypogonadism [70–72].

Augarten et al. described a patient who was referred for secondary infertility and was diagnosed with non-classic 21-hydroxylase deficiency, as was his daughter [73]. Serum FSH and LH levels were decreased and unresponsive to GnRH stimulation. Treatment with prednisone resulted in a decrease in the serum levels of 17-hydroxyprogesterone and androgens, and normalized the response to GnRH. After 4 months of treatment, successful conception took place [73].

Wischusen et al. described a patient with non-classic 21-hydroxylase deficiency, azoospermia, small testes, normal to high serum testosterone levels, and suppressed serum levels of gonadotropins. After treatment with glucocorticoids for several months, the semen quality improved, and he fathered a child [74].

Bonaccorsi et al. presented three cases of male infertility due to 21-hydroxylase deficiency with very low serum gonadotropin levels, two of whom were unresponsive to GnRH stimulation [70]. Unresponsiveness to GnRH was proposed to be caused by increased hypothalamic estrogen production from local aromatization of androstenedione, leading to suppression of GnRH.

Cabrera et al. described 5 patients (out of a group of 30 patients) with poor adrenal control and suppressed gonadotropin levels; azoospermia was documented in 3 [7].

Recent larger series showed hypogonadotropic hypogonadism in 26 of 50 patients (26%) (defined as decreased LH at least once) [8], in approximately 30% of 164 patients [10] (decreased LH, data derived from figure), and in 3 of 22 patients (14%) (defined as decreased FSH, and in all these cases also decreased LH) [13].

The cited cases illustrate that hypogonadotropic hypogonadism is reversible when glucocorticoid therapy is initiated or intensified, or when compliance is improved. More systematically, this was evaluated retrospectively by King et al. who showed the gonadotropin response to intensification of treatment in 9 patients desiring fertility. Recovery of hypogonadotropic hypogonadism was achieved in the majority of cases [8].

It should be emphasized that other causes of hypogonadotropic hypogonadism should be considered in these men, such as hyperprolactinemia, drug abuse (e.g., opioids), and obesity.

Hypergonadotropic Hypogonadism in CAH Males

Hypergonadotropic hypogonadism is mostly seen in patients who have TART. Bouvattier et al. found that FSH levels were significantly higher in patients who also had TART [10], while King et al. [8] found high FSH levels almost exclusively in those men with CAH due to 21-hydroxylase deficiency who also had TART.

It is possible that primary testicular dysfunction causing high FSH and LH levels will be masked by pituitary suppression, as described in the previous section. Conversely, when elevated adrenal androgens are decreased by raising the glucocorticoïd dose, a hypergonadotropic pattern may become clear.

The mechanism by which the testicular damage develops is thought to involve the effects of local steroid production on germ cells and Leydig cells. Also, when TART progresses, local fibrosis may occur, which worsens the testicular damage [62].

Other Causes of Subfertility in CAH Males

Testicular Volume in CAH Patients

Small testes are incidentally reported in males with CAH, especially in poorly controlled patients with low FSH levels [70, 72, 74]. In 101 patients, described in 4 studies, 6 patients had small testes as measured by ultrasonography [5, 7, 17, 18]. More recently, this percentage was confirmed by Bouvattier et al. [10] in a series of 164 men of whom 8% had testicular hypotrophy. Accordingly, small testes have been observed in CAH patients, but not frequently. Testicular volume is relevant with respect to fertility, since it is positively correlated with semen quality [75, 76].

Other Testicular Abnormalities

Varicocele and hydrocele are also known to interfere with testicular function and have been documented by ultrasonography in 4 and 3 cases, respectively, in a group of 18 CAH patients [7]. Semen quality was normal, however, in 3 of the 4 men with varicocele.

Psychological Factors, Compliance, and Transition

Suboptimal psychosocial adaptation to chronic disease has been suggested as a cause for impaired fertility by Jääskeläinen et al. who found a lower child rate in CAH males compared to the healthy male population despite normal gonadal and pituitary function [5].

Arlt et al. showed that subjective health status was significantly impaired, and anxiety and depression scores were increased in both male and female CAH patients [9]. In this multicenter study across the UK, it was explicitly mentioned that the "capture rate" was low, suggesting that a relevant proportion of patients might not be under endocrine control. Male CAH patients may not notice adverse effects from poor adrenal control, and this may contribute to poor attendance and/or poor compliance. To prevent loss to follow-up, the need for dedicated transition from pediatric to adult endocrine care has been emphasized [77].

Treatment of Subfertility in CAH Males

Treatment of Hypogonadism and Subfertility

The optimal treatment is directed by the cause of hypogonadism, but in general, it requires intensification of glucocorticoïd treatment. Both with TART or hypogonadotropic hypogonadism, the glucocorticoïd dose can be raised or (partially)

changed to a more potent glucocorticoïd formulation, such as prednisolone or dexamethasone [8, 78–80]. The adverse effects of treatment intensification are weight gain, striae, and other Cushingoid effects. Theoretically, severe overtreatment could (again) lead to the suppression of the gonadal axis. In practice, the necessary dose adjustments and duration of intensified glucocorticoïd therapy do not lead to the suppression of the axis. In adolescence, high dosages of glucocorticoids may reduce final height. Patients need to be informed about these adverse effects. In general, their motivation for fertility leads to tolerance of some adverse effects for a restricted period of time [78].

It needs to be mentioned that, for optimal adrenal control, mineralocorticoid treatment should be considered. Patients with classic 21OHD almost always benefit from fludrocortisone at doses of 0.05–0.2 mg daily, regardless of their tendency for salt wasting. The dose should be adjusted to avoid both hypertension and orthostatic hypotension, to normalize serum potassium, and to suppress plasma renin activity to the low end of the reference range. It is known that optimizing mineralocorticoid treatment (e.g., fludrocortisone) can have a glucocorticoid-sparing effect, since the maintenance of sodium balance reduces vasopressin and ACTH levels [81].

In patients with TART, if tumor size does not decrease after adjusting medical therapy, surgical intervention may be considered. Walker et al. reviewed the surgical management of these tumors and showed that the predominant intervention was orchiectomy in 19 of 28 patients (68%) [39]. In these patients, the tumors were steroid-resistant or were (initially) misdiagnosed as Leydig cell tumors. Walker performed a testis-sparing enucleation in 3 boys with CAH with steroid unresponsive testicular tumors. Postoperative MR and US of the testis in 2 of the 3 patients showed good vascular flow in the remaining tissue and no evidence of recurrent tumor. No semen analysis or gonadal function was reported. Claahsen-van der Grinten et al. studied pituitary-gonadal function before and after testes-sparing surgery in 8 adult CAH patients with long-standing TART and azoospermia [80]; neither gonadal function nor semen quality improved.

Recently, Bry-Gauillard et al. were the first to describe the use of mitotane in a 29-year-old patient with bilateral TART and azoospermia. Mitotane induces a “chemical adrenalectomy” by inhibiting steroid synthesis. Within 2 years of treatment, tumor shrinkage and an increase in sperm count were observed [82].

In the case of long-standing hypogonadotropic hypogonadism that persists in spite of intensification therapy, gonadotropin therapy has been used [8, 83], but not always successfully. Assisted reproduction techniques, such as intracytoplasmic sperm injection (ICSI) (with preceding testicular sperm extraction (TESE)) and in vitro fertilization (IVF), represent an alternative approach [8, 67].

Prevention of subfertility should ideally begin in (pre)puberty since TART has been described to develop at this age. Screening ultrasonography, adjusting glucocorticoïd

doses (taking into account the possible adverse effects), and semen analysis (possibly around the age of 20 years) may help predict future fertility problems. Since fertility prognosis is uncertain, we advise cryopreservation of semen at a young adult age.

Genetic Counseling

Genetic counseling of the male CAH patient and his partner is an essential part of preconception care. CAH is autosomal recessive. Since the CAH male patient has two homozygous or compound heterozygous CYP21 mutations, the child will be, by definition, a carrier of one CYP21-mutated allele. When the female partner is a (asymptomatic) carrier (incidence of carriers of about 1:50), there is a 50% risk of having an affected child and a 25% risk of having an affected girl (with possibly some level of congenital virilization, depending on the severity of the combined mutations). Couples planning for children should be informed and counseled about these risks, and genetic testing of the asymptomatic partner is recommended [77].

Conclusion

Fertility in men with congenital adrenal hyperplasia due to 21-hydroxylase deficiency can be impaired. Testicular adrenal rest tumors are the most frequent cause of impaired fertility. Their location adjacent to the testicular mediastinum, and their steroid-producing properties, may interfere with spermatogenesis and Leydig cell function. It is sometimes accompanied by hypergonadotropic hypogonadism. Hypogonadotropic hypogonadotropism is a second major cause of impaired fertility, resulting from suppression of the hypothalamic–pituitary–gonadal axis by adrenal androgen excess. Other testicular abnormalities and psychological factors, such as poor adherence, compliance, and transition problems, may also contribute to impaired fertility. Treatment consists of intensification of glucocorticoid treatment and assisted reproduction techniques. Genetic testing of the asymptomatic female partner is recommended for couples planning to have children.

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8. Male Hypogonadism Due to Disorders of the Pituitary and Suprasellar Region

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Introduction

The primary regulator of testicular function is gonadotropin-releasing hormone (GnRH) that is secreted episodically by hypothalamic neurons into the portal vascular system. GnRH stimulates the synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which are produced by gonadotrophs of the anterior

pituitary. LH stimulates the synthesis of testosterone after binding to specific receptors on Leydig cells, whereas Sertoli cells within the seminiferous tubules are the target of FSH ([1, 2], and Chaps. 2 and 3). Accordingly, mass lesions and other disorders of the suprasellar region or pituitary that disrupt GnRH or LH/FSH production result in impaired testicular function that is designated “hypogonadotropic hypogonadism” because LH and FSH production are deficient. These disorders are the subject of this chapter.

Prolactinomas

Physiopathology of Hypogonadism

Prolactinoma is the most common pituitary tumor, with an estimated prevalence in the adult population of 100 cases per million [3]. The frequency varies with age and sex, occurring most often in females between ages 20 and 50 years at which time the ratio between the sexes is estimated to be 4–10:1. After the fifth decade of life, by contrast, the frequency of prolactinomas is similar in both sexes [4, 5]. Men with prolactinomas usually present with macroadenomas (tumors ≥10 mm in diameter), and with very high prolactin levels, erectile dysfunction, loss of libido and/or infertility. These symptoms are related to the hypogonadism that occurs secondary to hyperprolactinemia [6]. They may also have headache and a visual disturbance.

Hyperprolactinemia disrupts male gonadal function at several levels (Fig. 8.1). High levels of prolactin suppress GnRH secretion and consequently, decrease gonadotropin and testosterone production [7, 8]. Experiments in rats revealed that prolactin suppresses GnRH secretion by a direct action on the arcuate nucleus to inhibit kisspeptin expression [9]. In some cases, hyperprolactinemia induces clinical hypogonadism notwithstanding normal serum FSH, LH and testosterone levels [10]. There appears to be an exaggerated diurnal variation in testosterone secretion in men with prolactin-producing tumors, so that normal morning values do not guarantee normal values throughout the day [11]; however, the mechanism for this effect is not known. Others have suggested that hyperprolactinemia reduces the conversion of testosterone to dihydrotestosterone [12, 13]. If so, the symptoms of hypogonadism associated with hyperprolactinemia may not be solely due to the decrease in serum testosterone levels [14]. In fact, testosterone replacement therapy may not reverse the loss of libido typical of men with prolactinoma until the prolactin level is normalized by the administration of a dopamine agonist [15], suggesting the existence of a CNS libido center that is prolactin-regulated. Experiments in rats suggest that hyperprolactinemia impairs sexual function by modulating dopaminergic signaling and by directly arresting penile erection by affecting the penile corpus cavernosum [16].

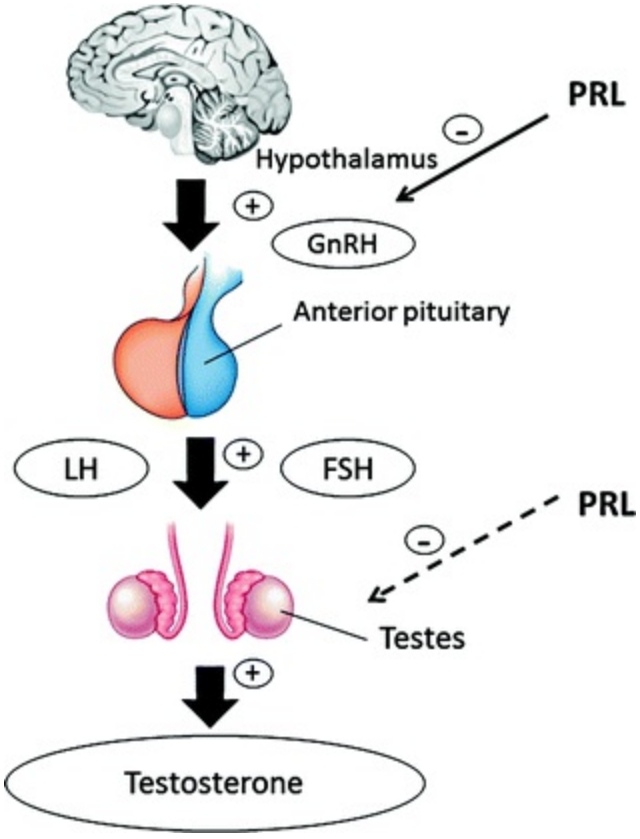

Fig. 8.1 Effects of hyperprolactinemia on the hypothalamic–pituitary–testicular axis

Another mechanism to explain the suppression of gonadal function in men with prolactinoma is a direct tumor mass effect on gonadotrophs. Large adenomas often cause deficiency of multiple anterior pituitary hormones, including the gonadotropins. However, a tumor mass effect appears to be less important than is hyperprolactinemia in the development of hypogonadism in most men with prolactinomas. In fact, there is no significant difference in the prevalence of testosterone deficiency between men with micro- or macroprolactinomas, either before or after medical treatment. Among patients with macroadenomas, however, testosterone levels are inversely correlated with tumor size, perhaps reflecting the increasing likelihood of panhypopituitarism as well as effects of high prolactin levels with larger tumors [17, 18].

Studies in male rats provide evidence that prolactin also affects the testes directly. Prolactin receptors are present in all stages of the cycle of the seminiferous epithelium, on spermatogonia and spermatocytes, and on rodent Sertoli and Leydig cells [19]. PRL modulates testosterone production by regulating the bioconversion of precursor steroids to testosterone under the influence of LH, and by upregulating LH receptor expression. However, there is no evidence so far for direct prolactin effects on the human testis.

The semen analysis in hyperprolactinemic men is characterized by fewer sperm implying spermiogenic arrest, and by reduced sperm motility and/or quality. In addition to the gonadotropin disturbance, hyperprolactinemia may produce structural changes in

testicular cells expressing the androgen receptor [20]. On the other hand, male reproductive functions are normal in prolactin-KO and prolactin receptor-KO mice [21–23]. Thus, the functions of PRL in the testis remain uncertain.

Diagnosis

Young adult women with prolactinomas came to medical attention because of galactorrhea and irregular menses, but in men this tumor can be difficult to recognize. The symptoms and signs of hypogonadism (Table 8.1) may not become obvious until the tumor becomes large, or hyperprolactinemia and gonadotropin deficiency become severe. Therefore, the diagnosis of prolactinoma in men is often delayed until a visual field defect or other symptoms secondary to tumor pressure on surrounding structures occur (e.g., headaches, seizures, altered personality, cranial nerve palsies, symptoms related to the deficiencies of other pituitary hormones, hydrocephalus). With long-standing hypogonadism, the testes are usually soft but of normal size, and the semen analysis frequently reveals low semen volume, oligozoospermia or azoospermia [24]. Most men with hyperprolactinemia have low-normal serum levels of LH, testosterone and dihydrotestosterone, and low frequency and amplitude spontaneous LH secretory episodes [25] together with a normal increase in serum LH levels following GnRH administration [8]. The identification of a high prolactin level is the beginning of the diagnostic process; however, other causes of hyperprolactinemia should be excluded. Prolactin levels in patients with prolactin-secreting adenomas are usually higher than 100 ng/ml and tend to be proportional to the size of the tumor. Adenoma size is also correlated with hormonal dysfunction prevalence at presentation and following treatment [17].

Table 8.1 Clinical manifestations secondary to hypogonadism in men with prolactinoma

Infertility	Loss of pubic hair
Decreased libido	Osteoporosis
Impotence	Apathy
Gynecomastia	Loss of muscle mass
Galactorrhea	Decreased sense of well-being

Lower PRL values are generally found with medication use, pituitary stalk compression, renal failure, cirrhosis, hypothyroidism and polycystic ovary disease [26]. Macroprolactin is a large molecular weight variant in which serum prolactin levels are increased because of binding to an IgG producing a large macromolecule with reduced hormone clearance; it is usually unassociated with clinical problems [27]. Computerized tomography (CT) and magnetic resonance (MR) allow for detailed noninvasive imaging of the pituitary gland. Both methods are effective in identifying

large pituitary tumors; CT is better for defining bone erosions and calcified structures, but MR with gadolinium enhancement provides superior anatomical detail to detect prolactinomas and their relationship to neighboring structures. MR is more sensitive than CT in the identification of microadenomas [28].

Therapy

Current therapeutic options for patients with prolactinomas include observation, medical therapy with dopamine agonists (e.g., bromocriptine, cabergoline), transsphenoidal or transcranial surgery, and conventional or stereotactic radiotherapy.

The first-line treatment of prolactinoma is generally pharmacotherapy with a dopamine agonist [29–32]. These drugs were developed based on the finding that prolactin synthesis and secretion are regulated by the tonic inhibitory effects of hypothalamic dopamine via dopamine D2 receptors on lactotrophs. Bromocriptine mesylate is a semisynthetic ergot alkaloid and long-acting dopamine receptor agonist. It normalizes prolactin levels in 80–90% and 60–75% of patients with micro- and macroprolactinomas, respectively [31]. Generally, the therapeutic doses are in the range of 2.5–15 mg/day, and most patients are successfully treated with 7.5 mg or less. However, doses as high as 20–30 mg/day may be necessary for patients who demonstrate resistance. Headache, nasal congestion, nausea, vomiting and dizziness are common side effects. Quinagolide is a more selective D2 agonist with only mild antagonistic effects at α_1-adrenoceptors. It is better tolerated, and 100 times more potent than bromocriptine and readily crosses the blood–brain barrier. This drug induces an excellent response both in normalizing prolactin levels and in restoring gonadal function, with normalization of libido and improvement in the ejaculation and in nocturnal penile tumescence and rigidity parameters. Usual doses are 0.15–0.6 mg/day. However, at least 6–12 months of treatment is needed to obtain a significant improvement in seminal fluid parameters and circulating testosterone levels. Quinagolide is not available in the USA.

The most commonly used dopamine agonist is cabergoline because of its prolonged duration of action and lower incidence of side effects. Tumor shrinkage and/or disappearance were observed in 60–88% of cases [33]. Cabergoline appears to be more efficacious than bromocriptine in normalizing prolactin levels and in restoring gonadal function. Normalization of serum prolactin levels was followed by restoration of gonadal function in all patients with microprolactinomas, and in most patients with macroadenomas [34]. Moreover, the beneficial effects occurred more rapidly than with bromocriptine or quinagolide [35]. In cabergoline-treated patients, the percentage of immature germ cells was lowered and sperm viability, swollen tails and penetration into bovine cervical mucus were increased significantly during the first 3 months of treatment [36]. Generally, the median starting dose of cabergoline was 1 mg/week in

patients with macroprolactinomas and 0.5 mg/week in those with idiopathic hyperprolactinemia or microprolactinomas. The dosage may be increased up to 1–3 mg twice weekly with a goal of PRL <50 ng/ml. Dosage increases should occur every 4 weeks. In addition, cabergoline may be effective in patients resistant to bromocriptine or quinagolide [31]. Furthermore, treatment with cabergoline and androgen replacement therapy may improve the metabolic profile and reduce the prevalence of metabolic syndrome in male hyperprolactinemic patients with concomitant testosterone deficiency [37].

Approximately 24 and 11% of patients demonstrate resistance to bromocriptine and cabergoline, respectively [31]. So far, there is no evidence that somatostatin receptor agonists are effective in patients with prolactinomas resistant to dopamine agonists.

Surgery may be necessary in men who have not tolerated or have not responded to medical therapy, with invasive macroadenomas and visual field impairment with no immediate response to dopamine agonists, or in patients with complications, such as intra-tumoral hemorrhage or cerebrospinal fluid fistula [38, 39]. A transsphenoidal or endoscopic transnasal approach is the preferred technique for microadenomas and most macroadenomas, while craniotomy is rarely necessary. Surgical success rates are highly dependent on the size of the tumor and the experience and skill of the surgeon. Pituitary tumors are almost always benign adenomas, and the preservation or restoration of anterior pituitary function, including gonadal function, is an important goal of surgery.

Because of the excellent therapeutic responses to medical therapy and surgery, radiotherapy is generally not considered a primary treatment for prolactinomas, but is reserved for patients who are refractory to medical and surgical therapy. Hypopituitarism, including hypogonadism, is a frequent side effect following radiotherapy.

In the uncommon case of hyperprolactinemia and hypogonadism induced by prolactinoma that is resistant or intolerant to medical therapy, and with contraindications to surgery, gonadotropin therapy can be used to induce virilization and advance spermatogenesis sufficiently to achieve fertility [40, 41].

It is noteworthy that, in patients treated with dopamine agonists for hyperprolactinemia, especially those with large tumors and panhypopituitarism, hypogonadism can persist despite normalization of prolactin levels, so that testosterone replacement therapy becomes necessary in addition to dopamine agonist therapy [15].

Acromegaly

Physiopathology of Hypogonadism

Acromegaly is characterized by the excessive production of growth hormone (GH). More than 99% of cases of acromegaly result from a tumor of the pituitary gland

secreting either GH alone, or both GH and prolactin. Gangliocytomas of the hypothalamus or pituitary, and ectopic neuroendocrine tumors secreting GH-releasing hormone (GH-RH), account for less than 1% of cases. Patients with acromegaly have a considerable burden of complications and coexisting morbidities. In particular, acromegaly is associated with cardiovascular and metabolic diseases such as hypertension, glucose intolerance or diabetes, cardiomyopathy and sleep apnea which all increase the risk of morbidities and mortality [42].

Hypogonadism has been reported in 49–70% of acromegalic patients [43]. Secretion of prolactin by the tumor together with GH, stalk disruption by suprasellar growth, and destruction of gonadotrophs by tumor have been implicated in the development of hypogonadism. However, Katznelson et al. [44] described low testosterone levels in 39% of men with somatotroph microadenomas, most of whom had normal prolactin levels. Therefore, other factors may contribute to the pathogenesis of hypogonadism in acromegaly. GH excess is often associated with a low level of sex hormone-binding globulin (SHBG), so that free testosterone levels should be measured to accurately diagnose testosterone deficiency in men with acromegaly. Hypogonadism occurs more often in patients with macroadenomas than in those with microadenomas because of the tumor mass effect on surrounding normal tissue.

GH appears to regulate testicular function by stimulating the local production of IGF-1 which modulates steroidogenesis and spermatogenesis. GH receptors are located in both the male and female gonads and genitalia [45]. Furthermore, GH is important in the development of the male and female reproductive systems, as demonstrated by the expression of GH receptors not only on the testis and ovary but also on Wolffian and Müllerian duct-derived structures [46]. GH administration to rats increases Leydig and Sertoli cell responses to FSH and LH [47]. However, high levels of GH and IGF-1 induce severe testicular alterations and disrupt the hypothalamic–pituitary–testicular axis. Repeated administration of very high doses of GH in dogs reduced LH and testosterone levels as well as testicular weight with germ cell degeneration and epithelial atrophy [48]. In a human study, administration of GH (0.01–0.03 mg/day/kg) for 3 weeks to eight healthy male volunteers increased estradiol levels and decreased anti-Mullerian hormone, inhibin B and LH levels, whereas the GH antagonist pegvisomant reduced only estradiol [49]. The extent of testicular damage appears to be related to the severity of acromegaly. In fact, serum GH and IGF-1 levels correlated negatively with serum dihydrotestosterone levels (Fig. 8.2). Several mechanisms have been proposed to explain these findings, including a change in androgen metabolism [50], FSH and LH suppression by increased somatostatin tone, a decrease in the serum concentration of SHBG, and lactogenic effects of GH separate from those of prolactin [51]. In addition, prostatic dysfunction, leading to alterations of the seminal fluid, may occur in men with acromegaly. In fact, GH/IGF-1 excess causes prostate enlargement with a high prevalence of prostatic abnormalities [52].

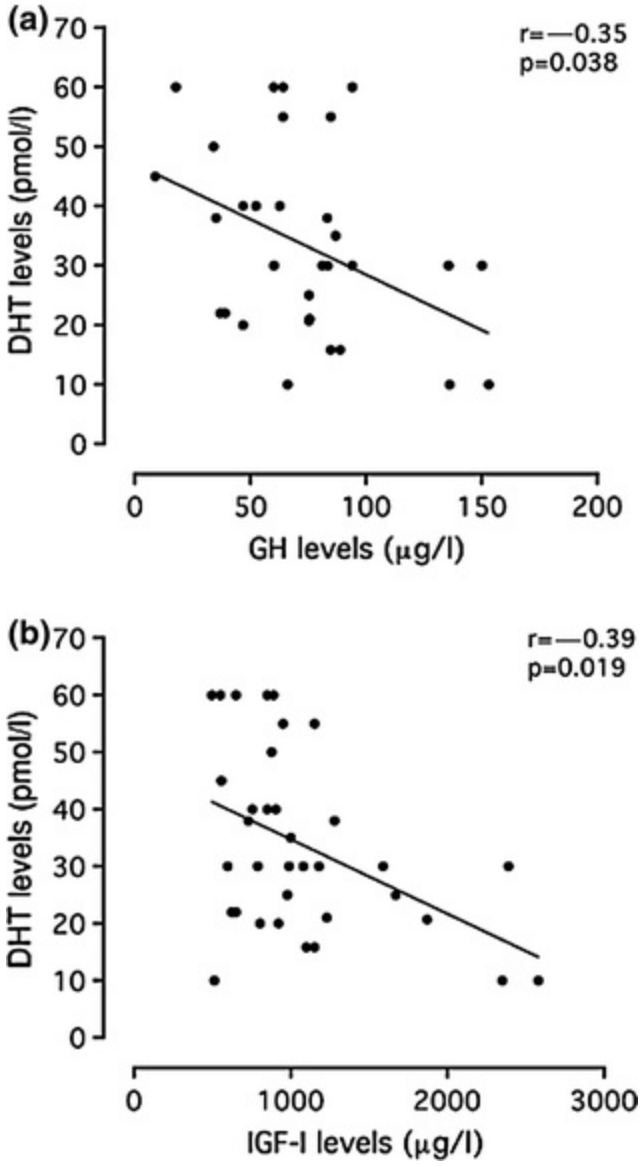

Fig. 8.2 Relationship between serum GH and IGF-1 levels to serum dihydrotestosterone (DHT). Reprinted from Colao et al. [57]

Diagnosis

The symptoms of hypogonadism in men with acromegaly are similar to those of men with prolactinoma and include oligozoospermia and reduced sperm motility. These manifestations, like acromegalic-specific clinical features (Table 8.2), progress slowly. Therefore, the diagnosis of acromegaly is often delayed for as many as 5–15 years.

Table 8.2 Specific clinical features of acromegaly

Recent acral growth	Osteoarthrosis
Prognathism and malocclusion	Kyphosis
Frontal bossing	Carpal tunnel syndrome

Widened spaces between the teeth	Proximal muscle weakness
Increased breadth of the nose	Fatigue
Generalized visceromegaly	Deep and resonant voice
Cardiomyopathy	Hyperhidrosis
Hypertension	Psychological disorders

The specific clinical manifestations of acromegaly are caused by excessive GH/IGF-1 secretion. In addition, pituitary tumor mass effects (headaches, hypopituitarism and visual disturbances) are common, because of the high prevalence of macroadenomas (about 75% of somatotroph adenomas).

It is now known that an elevated random serum GH level is not absolutely necessary for the diagnosis of acromegaly. By contrast, serum IGF-1 levels are invariably high in acromegaly. If the IGF-1 level is increased, and a random GH level is normal, an oral glucose tolerance test (75 g) is used to establish the diagnosis. A normal response is a decrease in the serum GH level to <1 µg/l, but acromegalic patients do not suppress normally, and 20% of patients have a paradoxical GH rise [53].

After the clinical and endocrine diagnosis of acromegaly is made, radiological evaluation of the pituitary gland should be performed, preferably by MR with gadolinium enhancement to localize and delineate the tumor from the surrounding tissues. GH-secreting tumors tend to be larger than prolactinomas at the time of the radiographic evaluation, perhaps due to a later clinical presentation.

Therapy

The treatment goals for men with hypogonadism secondary to acromegaly are the normalization of serum GH and IGF-1, and prolactin levels when hyperprolactinemia is present, and the preservation and restoration of gonadotropin secretion and testicular function.

Trans-sphenoidal adenomectomy is the preferred treatment for GH-secreting tumors, inducing an endocrine remission in 80% of patients with microadenomas, and 50% of those with macroadenomas in experienced centers [54]. It is likely that many centers do not achieve these success rates, however, which is a matter of concern. Surgery relieves the compression of adjacent structures, including the pituitary stalk and surrounding pituitary cells. If gonadotropin secretion is normal prior to surgery, gonadal function can be restored if GH secretion normalizes. Tominaga et al. [55] reported serum testosterone concentrations of less than 300 ng/dl (10.5 nmol/L) in 14/20 men (70%) with active acromegaly. Postoperatively, the serum GH level normalized in 14 patients, while the testosterone concentration returned to normal in 11 patients (79%), restoring gonadal function. This increase in testosterone levels may result from an increase of SHBG production [56], or an increase in LH secretion [57].

The three classes of medical therapy for acromegaly are based on receptor targets of the GH/IGF-1 axis: pituitary somatostatin receptor subtypes, pituitary dopamine D_2 receptors and peripheral GH receptors [58].

Somatostatin analogues normalize IGF-1 levels in 60–70% of patients and achieve tumor shrinkage in about 40% of patients. Long-acting analogues of somatostatin are available and improve compliance. Octreotide-LAR consists of octreotide incorporated into microspheres of a biodegradable polymer. It is injected intramuscularly at a dose of 10, 20 or 30 mg (every 4 weeks). Slow-release lanreotide is a similar preparation and is administered intramuscularly at a dose of 30 mg (every 1–2 weeks) or 60 mg (every 4 weeks). Lanreotide Autogel achieves the same effect as slow-release lanreotide, but is longer acting. It is administered by deep subcutaneous injection at a dose of 120 mg (every 6–8 weeks). Pasireotide is being evaluated in patients with acromegaly. Based on differences in binding affinity and functional activity, pasireotide may have a stronger inhibitory effect on GH secretion with tumors that express somatostatin (sst) receptors other than sst_2 [59]. Important side effects of somatostatin analogues are gallstone formation, which is generally silent, abdominal cramps, and decreased glucose tolerance, especially with pasireotide [60–66]. Suppression of GH and IGF-1 levels by somatostatin analogue therapy for 6 months was associated with a significant increase in LH and total testosterone and dihydrotestosterone levels; in most hypogonadal men, there was also improvement in sperm number and motility [61].

Dopamine agonists are less effective than are somatostatin analogues in normalizing IGF-1 levels (10–30% of patients) and reducing the tumor mass (<20% of cases). However, dopamine agonists are effective in those GH-secreting tumors that also secrete prolactin, or immunostain positively for prolactin. In addition, combined treatment with somatostatin analogues and dopamine agonists induces a greater suppression of IGF-1 levels compared with drug alone and improves testicular function [62].

Pegvisomant is the only GH receptor antagonist available for clinical use [63]. It has been shown in clinical trials to normalize IGF-1 levels in up to 97% of patients and can improve comorbidities such as insulin resistance. Furthermore, because the efficacy of pegvisomant is not dependent on tumor somatostatin receptor expression, pegvisomant effectively inhibits IGF-1 secretion in patients who are non-responders or partial responders to somatostatin analogues. GH secretion is not inhibited during pegvisomant therapy, and pegvisomant does not treat the tumor, necessitating regular monitoring for pituitary tumor growth.

Radiation therapy should be considered for patients with contraindications to pituitary surgery, or those whose operation and/or medical treatment has failed. Radiotherapy induces a slow response and may damage normal pituitary tissues, leading to hypopituitarism, and worsening hypogonadism in 50% of patients 10 years after irradiation. Stereotactic radiation surgery (SRS), either in single or hypofractionated

protocols, has undergone rapid technical improvement. With this approach, the temporal lobe, the optical system and the normal pituitary are exposed to less radiation. SRS may be associated with better biochemical remission, and estimates of hypopituitarism range from 6 to 30% [64].

Cushing's Disease

Physiopathology of Hypogonadism

Cushing's disease is characterized by chronic glucocorticoid excess secondary to hypersecretion of ACTH and other proopiomelanocortin peptides. It is commonly caused by a pituitary corticotroph adenoma.

Gonadal and sexual dysfunctions are common in men with Cushing's syndrome [65–69]. High levels of glucocorticoids decrease serum testosterone levels through several mechanisms (Fig. 8.3). Excessive production of glucocorticoids may produce gonadotropin deficiency by acting at the pituitary and hypothalamic levels. Two regions of the mouse GnRH promoter (distal and proximal negative glucocorticoid response elements) regulate transcriptional repression by glucocorticoids. Glucocorticoid receptors appear to induce glucocorticoid repression of GnRH gene transcription by virtue of their association within a multiprotein complex at the negative glucocorticoid response element [70]. In addition, elevated levels of glucocorticoids directly suppress testicular function, inducing apoptosis in Leydig cells [71], inhibiting LH receptor signal transduction, decreasing the oxidative activity of 11beta-hydroxysteroid dehydrogenase and impairing Leydig cell steroidogenesis [72].

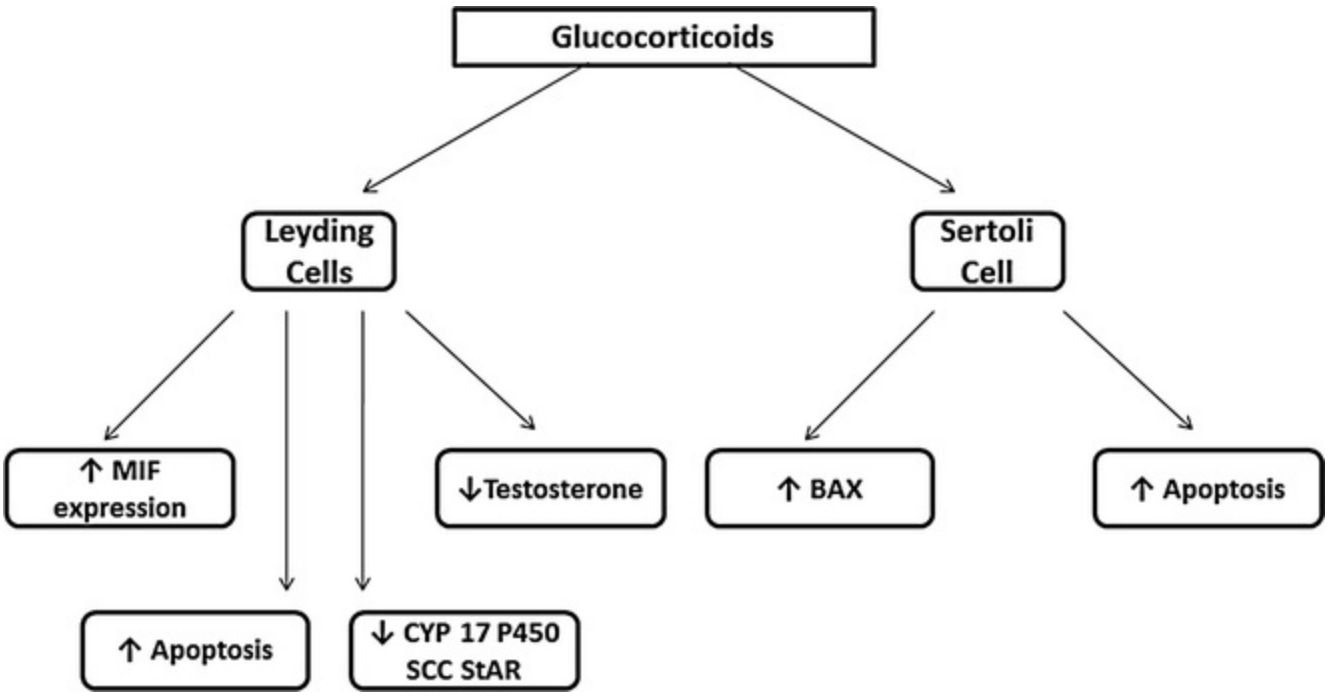

Fig. 8.3 Mechanisms involved in the induction of hypogonadism in men with Cushing's disease

Diagnosis

Since the description by Dr. Harvey Cushing at the beginning of the twentieth century, it is well known that hypogonadism is common in active Cushing's disease [73] (Table 8.3).

Table 8.3 Clinical features of Cushing's syndrome

Centripetal obesity	Proximal myopathy
Buffalo hump	Abdominal pain
Moon face	Psychiatric disturbances
Facial plethora	Hypertension
Purple striae	Backache, vertebral collapse, fracture
Skin thinning	Polydipsia, polyuria
Easy bruising	Renal calculi
Acne, oily skin	Hyperpigmentation
Skin infections	Male hypogonadism
Lethargy	Hirsutism, oligomenorrhea

Males have loss of libido, impotence, oligozoospermia and histological damage to the testes. Basal LH and FSH levels are commonly decreased, the response to GnRH is impaired, and testosterone levels are low. The hypogonadotropic hypogonadism in male patients is reversible and improves after hypercortisolism is suppressed or blocked [65, 68].

When Cushing's syndrome is suspected, initial laboratory testing to detect hypercortisolism includes measurement of 24-hour urinary free cortisol excretion, and/or the 1 mg overnight dexamethasone suppression test [74]. The sensitivity and specificity of the first test in detecting cortisol excess are 95 and 98%, respectively. Because of the difficulty in obtaining 24-hour urine collections in many outpatients, some physicians use the 1 mg overnight dexamethasone suppression test with a diagnostic cutoff of 1.8 μg/dL in the morning cortisol level (sensitivity 98% and specificity 80%). Another diagnostic approach is the late-night (11 PM) salivary cortisol determination [75]. Cortisol is highly stable in saliva, and the level is positively correlated with free serum or plasma cortisol levels and is independent of the rate of salivary flow.

Once the diagnosis of Cushing's syndrome is established, the source of the excess cortisol production needs to be determined. The plasma level of ACTH in the late afternoon is useful in identifying the ACTH-dependent pathologic state. At this time of day, plasma ACTH levels exceed 10 ng/L in Cushing's disease, while plasma ACTH

levels are generally suppressed in Cushing's syndrome secondary to adrenal disease. The high-dose dexamethasone test (8 mg) and the CRH test can be performed to obtain a clearer differential diagnosis. In Cushing's disease, serum cortisol levels and 24-hour urinary free cortisol excretion are generally suppressed after the 8-mg dexamethasone test, and ACTH levels rise following CRH stimulation.

Pituitary ACTH-secreting adenomas are most often microadenomas with a diameter <5 mm in 45% of cases, and only 10–20% of patients with Cushing's disease have a macroadenoma [76]. While MR imaging is required in all patients with ACTH-dependent Cushing's syndrome, pituitary MR imaging is often normal in patients with corticotropinomas. MR should be performed with thin overlapping sections and high field strength (1.5 T) magnets. In the absence of a detectable pituitary tumor, bilateral inferior petrosal venous sinus and peripheral vein catheterization with simultaneous collection of samples for measurement of ACTH should be performed to localize ACTH production to the pituitary and exclude the ectopic production of ACTH [77]. Stimulation with CRH, together with bilateral and simultaneous sampling, increases the sensitivity of this procedure.

Therapy

The hypogonadotropic hypogonadism characteristic of male Cushing's disease is a reversible phenomenon [65, 68]. In fact, cure of hypercortisolism restores the decreased plasma testosterone, FSH and LH levels to normal and improves sexual and gonadal dysfunctions.

Trans-sphenoidal adenomectomy, after accurate preoperative localization of the corticotroph adenoma, represents the treatment of choice for Cushing's disease patients, but can be technically challenging [78]. The remission rate for microadenomas has ranged from 48.7 to 100%, whereas for those with macroadenomas, it has ranged from 30.8 to 100%. A trans-sphenoidal approach was also successfully used in ACTH-producing adenomas located in the pituitary stalk. A recent meta-analysis reported that 11.5–47.4% of patient will experience recurrence of their disease as long as 10 years after surgery, so that follow-up must be life-long [79].

Transcranial surgery (TCS) has been used in the past and is occasionally used today.

Patients unresponsive to surgical treatment, or those in whom surgery is deemed inappropriate (large invasive tumors, or high surgical risk), are referred for pituitary irradiation with adjunctive medical therapy [80]. There is some evidence that stereotactic radiation may lead to biochemical remission faster than with conventional radiation therapy, while estimates of new hypopituitarism at 4–5 years range from 5 to 35%.

The development of novel medical therapies may limit the use of radiotherapy to patients who are unresponsive to or intolerant of medical treatment, or it may result in

the deferral of radiotherapy until after a period of control of cortisol secretion by medical therapy.

Current medical therapies include drugs to decrease ACTH secretion, inhibit corticosteroid synthesis or block their peripheral actions [78]. The first class of drugs (serotonin antagonists, dopamine agonists, GABA agonists and somatostatin analogues) inhibit ACTH release. However, few cases so treated have achieved permanent remission. The second group of drugs includes inhibitors of steroidogenesis (ketoconazole, metyrapone, aminoglutethimide and mitotane). These drugs have the advantage of being rapidly effective in most cases. However, the mechanism(s) of action has little selectivity, and extra-adrenal effects are likely to occur. Ketoconazole inhibits various cytochrome P450 enzymes, including C17-20 lyase involved in the synthesis of sex steroids. Thus, the drug not only lowers cortisol levels, but also decreases testosterone levels such that gynecomastia, impotence, loss of libido and oligozoospermia could occur [81]. The third class of drugs is represented by the glucocorticoid antagonist (mifepristone). Although a potentially important therapy that has been approved by the FDA to control hyperglycemia in adults with Cushing's syndrome, few patients have been treated with mifepristone, and further clinical studies are needed to assess long-term effectiveness and safety, and the impact on gonadal function. Few studies have reported the efficacy of cabergoline treatment in patients with Cushing's disease and Nelson's syndrome [82]. With each of these drugs, improvement in hypercortisolemia and insulin sensitivity may result in a rise in SHBG, LH and testosterone levels.

Finally, bilateral adrenalectomy is reserved for cases in which other treatment modalities have failed. However, the risk of Nelson's syndrome is high with this approach. Adrenal surgery is followed by a rapid and definitive control of cortisol excess in nearly all patients, but it induces adrenal insufficiency.

Non-functioning/Gonadotroph Adenomas

Physiopathology of Hypogonadism

Clinically non-functioning pituitary adenomas (NFPA) represent about 30% of pituitary tumors and are the most commonly encountered macroadenomas [83]. The prevalence of NFPA is 1.5 per 100.000 people, and among them, gonadotroph adenomas account for 43–64% of silent adenomas. Hypogonadism is present in about half of the patients with NFPA, of whom about 50% have mild hyperprolactinemia [83], reflecting stalk compression that might contribute to impaired gonadal function.

The majority of "non-functional" tumors are inefficiently secreting rather than non-secreting. They usually synthesize and immunostain for FSH and/or LH, and/or free α- and β-subunits [84]. Gonadal dysfunction in men with NFPA is primarily related to

intra-sellar expansion of the tumor with compression of residual glandular tissue and may cause hypopituitarism both by direct compression of the portal vessels that limits delivery of hypothalamic releasing factors and by focal ischemic necrosis of the normal pituitary tissue [85]. LH secretion is impaired in about 53% of NFPA [103–105]. However, disruption of ACTH, TSH and GH secretion may further inhibit gonadal function.

Diagnosis

The majority of NFPA are diagnosed incidentally or as a result of headache, visual disturbances or neurological symptoms. Less often, deficient pituitary hormone secretion, including gonadotropin deficiency with hypogonadism, brings the patient to medical attention. These tumors can attain considerable size before manifesting clinically. A mild or moderate increase in the prolactin level is common. A complete endocrine evaluation, including measurement of FSH, LH, α-subunit, testosterone, TSH, FT4, ACTH, cortisol, GH and IGF-1, as well as prolactin should be performed in order to recognize functional lesions and diagnose hypopituitarism. Magnetic resonance imaging with gadolinium enhancement represents the best way to detect a pituitary tumor.

Therapy

Trans-sphenoidal resection of the adenoma is the preferred and most effective treatment for symptomatic NFPA since it may improve visual field defects and permit recovery of pituitary function [86]. In NFPA microadenoma <5 mm, neither radiological nor hormonal surveillance is recommended, while in NFPA microadenoma >5 mm, most authors propose close clinical and MRI surveillance. Some patients with NFPA less than 40 mm in diameter may experience a restoration of gonadal function if the normal pituitary is not destroyed and its ability to secrete gonadotropins is regained after decompression. In NFPA larger than 40 mm, on the other hand, recovery is much less likely, and surgery may in fact worsen testosterone deficiency [87]. This fact reflects the difficulty in preserving or restoring anterior pituitary function in patients with very large tumors. In a recent study that analyzed patients undergoing trans- sphenoidal surgery using intra-operative high-field magnetic resonance imaging, the recovery rate for the gonadotroph axis within 2–3 months after surgery was 32% [88].

Because of their large size, however, surgery alone is often not curative, and radiotherapy is often performed as adjuvant therapy after surgery [89] especially in those with subtotal removal [90] although the risk of hypopituitarism is increased. In one study, the risk of new or worsening hypopituitarism was 21% with hypogonadism in at least 10 of patients [91].

Somatostatin and dopamine receptors have been identified in NFPA. This suggests

that somatostatin analogues or dopamine agonists could be useful in the treatment of refractory NFPA. In a recent study, 36 NFPA macroadenomas patients were found to be positive to DR2. Among them, 9 patients were treated with cabergoline, followed by tumor volume reduction ≥25% in 67%; hypogonadotropic hypogonadism was observed in 2 patients [92]. Furthermore, Greenman et al. found a reduction in tumor volume in 38% of 69 NFPA macroadenomas treated with cabergoline [93]. However, available drugs benefit only a minority of patients, and additional studies are needed to investigate whether subgroups of patients with NFPA will respond to medical therapy.

Gonadotropin therapy is indicated if the patient desires spermatogenesis and fertility (Chap. 20). If fertility is not the requested outcome, testosterone is administered to stimulate virilization, improve sexual function and maintain bone and muscle mass (Chap. 18). In patients with multiple pituitary hormone deficiencies, replacement therapy of all deficient hormones is required for complete improvement of gonadal function.

Non-pituitary Sellar and Parasellar Tumors

Physiopathology of Hypogonadism

Non-pituitary intra-sellar and parasellar tumors include craniopharyngioma, Rathke's cleft cyst, epidermoid, infundibuloma, chordoma, lipoma, colloid cyst, germinoma, dermoid, teratoma, dysgerminoma, ectopic pinealoma, glioma, oligodendroglioma, ependymoma, astrocytoma, meningioma, enchondroma, metastatic tumors and lymphoma. In a recent study of over 2500 patients having a pituitary MRI, non-adenomatous sellar masses accounted for 18% of lesions most notably Rathke's cleft cysts (19%), craniopharyngiomas (15%) and meningiomas (15%) [94, 95]. These masses may interfere with GnRH synthesis and secretion, or directly impair FSH and LH production, inducing hypogonadotropic hypogonadism. In addition, the modest hyperprolactinemia that occurs secondary to hypothalamic stalk compression could impair gonadal function.

Diagnosis

Non-pituitary sellar and suprasellar tumors may present with symptoms of anterior pituitary hormone failure, including gonadotropin deficiency [96]. In one study of patients with craniopharyngioma, 60–70% was diagnosed with hypogonadotropic hypogonadism preoperatively and the risk increased further after surgery [97]. On the other hand, most patients with Rathke's cleft cysts are asymptomatic, and <10% exhibit hypogonadotropic hypogonadism [98]. Mild hyperprolactinemia is common in patients with craniopharyngiomas, meningiomas or cystic lesions [96].

Impairment of visual fields and decreased visual acuity occur because of the

proximity of the lesion to the optic nerves, chiasm and optic tracts. The tendency of these tumors to infiltrate parasellar structures causes cranial neuropathy. Tumors obstructing cerebrospinal fluid flow (craniopharyngioma, meningioma, germinoma) increase intracranial pressure. This event is characterized by visual impairment and headaches. Masses compressing the deep subfrontal region of the brain may induce personality changes and dementia. In addition, these lesions may impair hypothalamic function and disrupt appetite control, causing polyphagia and massive obesity, or starvation.

Although it is difficult to reach a specific preoperative diagnosis on MR imaging, many lesions have characteristic findings that are useful in differential diagnosis [94, 95]. For example, calcifications are suggestive of craniopharyngiomas, meningiomas, chordomas, teratomas and gliomas. Erosion of the floor of the sella is frequently observed in meningiomas of the middle fossa and in Rathke's cleft cysts.

Therapy

Gonadal impairment secondary to non-pituitary intra-sellar and parasellar masses may recover following treatment of the lesion. The choice of treatment depends on the nature of the disease. However, surgery represents the first option in most cases. Surgical treatment generally involves trans-sphenoidal decompression of the mass, when possible, attempting to limit damage and restore normal function of the pituitary gland.

Radiotherapy is recommended for residual tumor after surgery, and for brain metastases. Medical therapy is not generally performed with dysgerminomas or metastatic lesions that require radiotherapy and chemotherapy. Children with precocious puberty due to hamartomas that produce GnRH can be treated with GnRH analogues to suppress gonadotropin secretion since surgery may not be curative or may result in extensive complications, including fatality [99]. These agents do not affect the tumor itself.

When hypogonadism persists after surgical treatment, or remains after conventional treatment, androgen replacement therapy is used to restore physiologic serum concentrations of testosterone (Chap. 18). If fertility is desired, Leydig cell function is stimulated by the administration of human chorionic gonadotropin (Chap. 20). Spermatogenesis may be restored by hCG alone; however, hFSH is sometimes necessary as well. In patients with hypopituitarism, adequate replacement therapy further improves gonadal function.

Non-tumorous Causes of Acquired Hypogonadotropic Hypogonadism

Physiopathology of Hypogonadism

Non-neoplastic diseases of the hypothalamus and pituitary (Table 8.4) may also induce hypogonadotropic hypogonadism [100]. Among them, the most frequent are cystic lesions (53%) and inflammatory lesions (9%). In these disorders, hypogonadism is primarily due to gonadotropin deficiency secondary to hypothalamic–pituitary damage, and it is present in about 23–29% of patients. In addition, GH/IGF-1, thyroid hormone and CRH/ACTH/cortisol play a permissive role in the regulation of spermatogenesis and testosterone production. In this way, deficiency of all or some anterior pituitary hormones, together with the increase in prolactin, may induce hypogonadism.

Table 8.4 Non-tumorous causes of acquired hypogonadotropic hypogonadism

Pituitary apoplexy
Empty sella syndrome
Aneurysm
Pituitary abscess
Tuberculosis
Sarcoidosis
Giant cell granuloma
Lymphocytic hypophysitis
Histiocytosis X
Hemochromatosis
Arachnoid cyst
Mucocele
Post-surgery/irradiation
Head trauma

Hypogonadotropic hypogonadism is also common in patients with lesions of the pituitary stalk [101]. These lesions may be neoplastic or inflammatory, or are sometimes congenital. The abnormality may be limited to the stalk or may extend into the pituitary.

Pituitary apoplexy results in anterior pituitary failure secondary to hemorrhagic or ischemic infarction [102]. Hemorrhage usually occurs into a pituitary tumor, whereas ischemia may involve a normal pituitary gland in the setting of increased intracranial pressure, during anticoagulation therapy, and in women in the postpartum period. Hormone deficiencies following apoplexy include GH (88%), gonadotropins (58–76%), ACTH (66%), TSH (42–53%) and prolactin (67–100%).

Empty sella is defined as a sella that is completely or partially filled by cerebrospinal fluid, owing to arachnoid herniation into the pituitary fossa. This herniation remodels and enlarges the bony sella, and flattens the pituitary gland against

the sella floor. Empty sella, especially a partially empty sella, is usually an incidental anatomical finding, and functional pituitary damage is infrequent. The fact that patients are usually asymptomatic derives from the large functional reserve of the adenohypophysis. However, hypogonadism is not rare and occurs in approximately 15% of cases [103, 104]. Empty sella can also occur following pituitary surgery, radiotherapy, infection, or trauma, and during the evolution of autoimmune hypophysitis, Sheehan's syndrome and neurosarcoidosis. With "secondary" empty sella, flattening of the pituitary is more pronounced, and hypogonadism is more common.

Aneurysms of the cavernous sinus, as well as infectious (bacterial or fungal abscesses, tubercolosis, toxoplasmosis) and inflammatory processes (sarcoidosis, giant cell granuloma, lymphocytic hypophysitis, histiocytosis X) can mimic a pituitary adenoma, causing mass effect and hypothalamic-hypophyseal endocrine dysfunction secondary to infiltration and replacement of normal tissue [94, 95].

An arachnoid cyst is a diverticulum of the arachnoidal membrane of Liliequist. Fifteen percent of these cysts occur in the sellar and parasellar region. This mass may compress the hypothalamic–pituitary unit inducing endocrine disorders, including hypogonadism, producing impotence or low libido.

Mucoceles are slowly expanding, mucous-filled cystic lesions that arise in the paranasal sinuses. These cysts result from the accumulation and retention of mucous secretions in a sinus secondary to the occlusion of the ostium draining the sinus. Sphenoid mucocele with suprasellar extension can also increase prolactin levels by compressing the pituitary stalk.

Radiation of the head for treatment of primary central nervous system tumors, nasopharyngeal tumors or brain metastases may cause the gradual onset of hypothalamic–pituitary failure [106]. The most frequent endocrine abnormalities following cranial radiotherapy are growth hormone deficiency (up to 90%), hypothyroidism (65%), hypogonadism (61%) and mild hyperprolactinemia. Although the mechanism of radiation damage to the hypothalamus and pituitary gland is not known, it presumably involves direct injury to the secreting cells, to the stroma or its microvasculature, or damage to the vascular channels that transfer the hypothalamic hormones to the pituitary.

Head trauma may cause hypogonadism [106–108]. Hypotestosteronemia is found in 60–70% of patients within 1 week following severe traumatic brain injury. GnRH–LH secretion most often recovers, however, with hypotestosteronemia in only 10% of patients 6–9 months after injury [108]. Stalk trauma may increase prolactin levels further worsening gonadal function. Sexual dysfunction, depression and lack of motivation with a history of head trauma may indicate post-traumatic stress disorder [109].

Diagnosis

These diseases are characterized by extensive damage to pituitary function. Therefore, hypogonadism is rarely reported as an isolated manifestation, but instead is frequently associated with hypopituitarism and diabetes insipidus. In addition, headache, vomiting, ophthalmoplegia and visual loss occur with acute onset in pituitary apoplexy or head trauma and may occur progressively in the empty sella syndrome, infectious and inflammatory processes, mucocele, arachnoid cyst, giant cell granuloma and echinococcal cysts.

Considering the importance of hypopituitarism in the induction of hypogonadism, anterior pituitary function testing (in some cases including GnRH stimulation to assess gonadotroph function by measuring FSH and LH; insulin tolerance testing to assess corticotroph and somatotroph secretion by measuring serum cortisol and GH levels; and TRH stimulation to assess TSH levels), prolactin and testosterone levels, and a semen analysis are mandatory in this group of patients.

Nowadays, MR is an essential tool in the differential diagnosis of sellar masses and in the evaluation of patients with head trauma. However, biopsy and histology are often necessary to confirm the diagnosis.

Therapy

The treatment goals in this group of patients are: (1) to reduce or eliminate the mass effects of the lesion, (2) to correct hormonal deficiencies including those that impair gonadal function, and (3) to preserve residual pituitary function. The specific treatments for the various diseases discussed are beyond the scope of this chapter. However, most lesions are initially treated surgically to eliminate the mass effects. Androgen replacement therapy is used to correct hypogonadism (Chap. 18). When fertility is desired, treatment with exogenous gonadotropins (hCG ± hFSH) is generally successful (Chap. 20). Finally, when panhypopituitarism is present, hormonal replacement therapy is mandatory.

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9. Klinefelter Syndrome and Other Forms of Primary Testicular Failure

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Overview

Klinefelter syndrome (KS) is the most common sex chromosome disorder in men affecting roughly 1 in 400–600 males across all ethnic groups [1, 2]. It is also the most common cause of primary testicular failure, resulting in impairments in both spermatogenesis and testosterone production. The hypogonadism associated with KS can range from mild to severe, leading to a marked variation in clinical presentation. Men with KS possess at least one additional or "supernumerary" X chromosome resulting in a 47, XXY genotype. The clinical phenotype in the adult is that of absent spermatogenesis and varying degrees of hypogonadism, manifested by a tall body habitus with sparse body and facial hair, gynecomastia, diminished libido and very small (<6 cc) testes. In childhood, common presenting features can include delayed speech development, learning difficulties at school, unusually rapid growth in mid-childhood and truncal obesity. Laboratory analysis reveals low or low-normal serum

testosterone and elevated serum gonadotropin levels, with FSH elevated to a greater degree than LH. The clinical diagnosis is confirmed using chromosomal analysis (karyotyping) of either peripheral blood leukocytes or tissue, which usually reveals a 47, XXY genotype, although infrequently additional X chromosomes may be present, or an individual may be mosaic (47, XXY/46, XY). Treatment consists of testosterone therapy for improved virilization, sexual function, bone density and quality of life. Gynecomastia is treated with cosmetic surgery after androgen replacement has begun. Infertility can be addressed by testicular sperm extraction "TESE" coupled with intracytoplasmic sperm injection "ICSI" if sperm are recovered during TESE, although these procedures are expensive and effective in only some men. Alternative options, including artificial insemination with donor sperm or adoption are reasonable alternatives. This chapter will summarize the initial historical description of KS as well as our current understanding of the epidemiology, pathophysiology, clinical manifestations and treatment of individuals with KS. In addition, we will discuss the outcomes of assistive reproductive techniques in couples in which than man has KS.

Historical Description of Klinefelter Syndrome

In 1940, Harry F. Klinefelter Jr., a native of Baltimore and a graduate of the Johns Hopkins medical school, spent a year working as a "traveling fellow" with Dr. Fuller Albright in the Metabolic Ward of the Massachusetts General Hospital in Boston [3]. At that time, Dr. Albright was at the height of his formidable academic career and among many other subjects was interested in the endocrinology of sexual development and the therapeutic applications of the recently available steroid hormones. Dr. Albright had identified a group of adult men with a previously uncharacterized syndrome typified by gynecomastia, small testes and varying degrees of eunuchoidism. Dr. Albright assigned Dr. Klinefelter the task of further describing this syndrome for publication.

Klinefelter, Albright and their colleague Dr. Edward Reifenstein Jr. published their description of the syndrome in a classic paper in *The Journal of Clinical Endocrinology* in 1942 [4]. In this paper, they described a cohort of 9 men "characterized by gynecomastia, aspermatogenesis without a-Leydigism and increased excretion of follicle-stimulating hormone." The authors noted the presence of testosterone-producing Leydig cells in testicular biopsy specimens, as well as a marked increase in FSH production, comparable to that seen in castrated men. The authors suggested that the markedly elevated FSH levels were secondary to the absence of a testicular hormone other than testosterone. This postulated hormone, called inhibin by prior researchers, is in fact lacking in individuals with KS, but its identity and role in endocrine feedback in the male would not be fully elucidated for over 50 years [5].

As to the cause of the syndrome, Klinefelter and his colleagues were uncertain. They ruled out testicular inflammation, infection or obstruction of the vas deferens, and noted

on testicular biopsy specimens that the lesion involved the seminiferous tubules without dramatically affecting the histology of the Leydig cells, testicular interstitium or epididymis. They recommended testosterone therapy (available since the late 1930s) for those with signs or symptoms of hypogonadism, but noted that this therapy did not improve the gynecomastia or infertility.

Although Drs. Klinefelter, Reifenstein and Albright first described the clinical and endocrine manifestation of KS in 1942, it was not until 1956 following the ascertainment of DNA as the main instrument of heredity that the additional X chromosomes or "Barr bodies" were identified in tissue from patients with KS [6, 7]. Three years later Jacobs and Strong confirmed the association between the extra X chromosome and KS by cytogenetic analysis of metaphase chromosomes, establishing KS as a sex chromosome disorder with a 47, XXY karyotype [8]. Further work has demonstrated that some individuals may be mosaic (47, XXY/46, XY) with the extra X chromosome detectable only in peripheral blood or the testes; rare individuals with 48, XXXY and 49, XXXXY karyotypes have also been described [9].

Pathophysiology of Klinefelter Syndrome

Most cases of KS appear to occur through sporadic chromosomal non-disjunction during parental gametogenesis in either the sperm or the egg. A seminal study using DNA probes to X chromosome restriction site polymorphisms demonstrated that in one series of 32 individuals with KS the additional X chromosome was of paternal origin in 17 (53%) of cases, of maternal origin in 14 (44%) of cases and due to a post-zygotic mitotic error in one (3%) case (Table 9.1) [10]. Since these are sporadic errors in gametogenesis, there is no evidence that KS is familial, or is likely to recur within a given family after one child with KS is born. Chromosomal analysis of sperm has demonstrated that normal older men produce higher frequencies of XY sperm which could increase their chances of fathering a child with KS [11].

Table 9.1 Mechanism of origin of additional X chromosome in 47, XXY males

Sex chromosomes	Responsible cell division	X-linked loci	Frequency (%)
$X^{M}X^{P}Y$	Paternal meiosis	Identical to father	53
$X^{M1}X^{M2}Y$	Maternal meiosis I	Identical to mother	34
$X^{M1}X^{M1}Y$	Maternal meiosis II	Single maternal chromosome with heterozygosity in distal loci	9
$X^{M1}X^{M1}Y$	Mitotic (post-zygotic)	Single homozygous maternal chromosome	3

In an individual with KS, the extra X chromosome forms a dense chromatin mass, or

"Barr body" within the nuclei of somatic cells, but exactly how the presence of this extra chromosome leads to testicular failure remains a mystery. Microarray analysis reveals over- and underexpression of various genes on the X chromosome and autosomes that presumably contribute to the features of KS [12]. Testicular biopsy specimens from infants with KS may reveal only a reduced number of germ cells [13]. After puberty, fibrosis of the seminiferous tubules begins, eventually leading to small, firm testes and azoospermia [4]. Testicular biopsy specimens from adults with KS reveal marked seminiferous tubule hyalinization with focal areas of immature seminiferous tubule formation and fibrosis with a resulting increase in the amount of interstitium (Fig. 9.1). An absence of functional Sertoli cells in the seminiferous tubules is accompanied by low-level inhibin B in serum [5], the hormone whose existence Klinefelter and colleagues had hypothesized in their original paper.

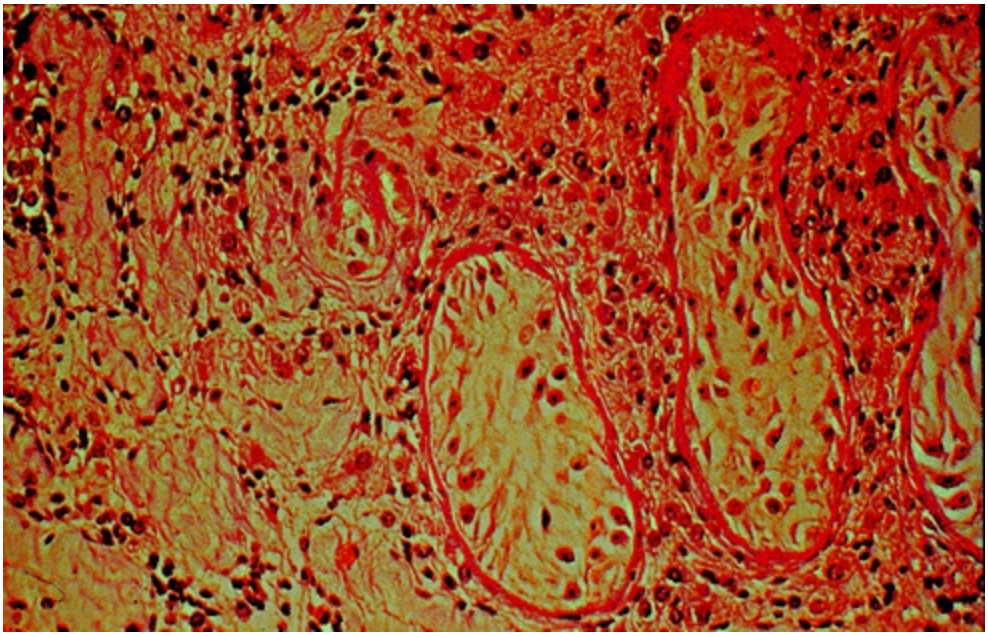

Fig. 9.1 Testicular biopsy specimens an individual with KS. Note focal areas of immature seminiferous tubules interspersed with completely hyalinized tubules and an increase in interstitial tissues (photograph courtesy of Dr. C. Alvin Paulsen)

The hormonal axis in boys with KS can appear normal until puberty, with no apparent differences in levels of testosterone (T), luteinizing hormone (LH) or follicle-stimulating hormone (FSH) compared to controls [14–16]. In addition, gonadotropin production in response to GnRH stimulation is normal. However, at the time of puberty, elevated basal FSH and LH levels and an exaggerated FSH and LH response to GnRH stimulation, and low or low-normal testosterone levels are seen, presaging the hormonal abnormalities seen in adults with KS. Testosterone production in a man with KS declines with aging in a fashion similar to controls [17, 18].

The cause of the language difficulties often encountered by individuals with KS is

not well understood; however, high-resolution MRI scans of the brains of a small number of men with KS have demonstrated a relative reduction in left temporal lobe gray matter compared with control subjects [19]. Interestingly, individuals with KS who had been treated with testosterone had larger left temporal lobe volumes than individuals with KS who had not received testosterone therapy. Moreover, verbal fluency scores were significantly improved in the KS-testosterone groups as compared with the KS-no testosterone group, demonstrating that relatively low levels of testosterone during development may play a major role in the verbal and language problems related to KS.

The pathophysiology of KS-related gynecomastia is also unclear. Individuals with KS do exhibit mildly elevated levels of estradiol, probably from increases in peripheral aromatization and decreased clearance. However, histologic analysis of breast tissue from individuals with KS demonstrates *intra*ductal hyperplasia rather than the ductal hyperplasia seen in high estrogen states in the male such as cirrhosis [20].

Clinical Features of Klinefelter Syndrome

The major clinical manifestations of KS are those of pre-pubertal androgen deficiency and infertility. Because testicular failure occurs before puberty, some of the normal developmental changes in puberty do not occur. This leads to the classic phenotype of KS: long legs, with an arm span frequently greater than height, gynecomastia, decreased muscle mass and increased abdominal adiposity, decreased facial and body hair, and small testes–which often measure less than 5 cc in volume. Long legs may be partly due to overexpression of the short stature homeobox (SHOX) gene on the X chromosome [21]. The penis may be decreased in size but is often of normal length. Gynecomastia is variable, but is often prominent and may require surgical correction. Mosaicism may present more subtly, as affected individuals can appear anywhere along a phenotypic spectrum between classical KS and "normal." Infertility is almost universal; however, children have been fathered by men mosaic for KS [22] and via assisted reproductive techniques (see below). The frequency of various symptoms and signs of KS is summarized in Table 9.2.

Table 9.2 Clinical and physical characteristics of men with Klinefelter syndrome

Feature	Percent
Infertility	99–100
Small testes	99–100
Gynecomastia	50–75
Decreased facial and pubic hair	50–80
Decreased penis size	10–25

Hormonally, roughly one-half of affected men have decreased total testosterone levels, almost all have elevated gonadotropins, particularly FSH, but estradiol levels are usually fairly normal (Table 9.3) [18]. More than 90% of men have increased levels of serum gonadotropins, particularly FSH, as inhibin B levels are very low [5]. Because of low levels of androgens, individuals with KS can have low bone mineral density and are at increased risk of osteoporosis, even despite seemingly adequate androgen therapy [23]. This is likely due to failure to achieve peak bone mass early in life, as testosterone replacement before the age of 20 can improve bone mass to normal levels, whereas testosterone therapy started later in life is less effective [24]. As a result, calcium and vitamin D supplementation as well as baseline densitometry are probably prudent in individuals with KS, especially those who began androgen therapy later in life.

Table 9.3 Serum hormones in men with Klinefelter Syndrome

Hormone	Mean ± SD	Reference range	% Abnormal
Testosterone (ng/mL)	2.7 ± 1.6	2.8–8.8	45
FSH (IU/L)	35.4 ± 16.2	1–8	100
LH (IU/L)	22.3 ± 11.6	2–12	83
Estradiol (pg/mL)	26.5 ± 13.2	18–44	6
Prolactin (ng/mL)	16.5 ± 11.3	5–20	33

At one time, individuals with KS were thought to be frequently retarded and/or at increased risk of criminal behavior [25, 26]. These studies were based on pre-selected (usually committed or incarcerated) populations, however, and were not representative of the syndrome as a whole. Subsequent population-based studies have demonstrated a non-significant, but slightly increased rate of incarceration among individuals with KS. This increased rate of imprisonment is most likely accounted for by lower average scores on IQ tests compared with controls [27].

Individuals with KS can exhibit significant deficits in language processing skills including: reading and spelling, verbal processing speed, verbal and non-verbal executive abilities and motor dexterity [28, 29]. In general, this leads to lower average school performance and an increased frequency of psychological consultation. In addition, adolescent males with KS exhibit lowered sexual activity compared with their peers [30]. Expression of genes on the supernumerary X chromosome in the central nervous system may contribute to these behaviors [31]. It is important to remember that individual variation in mental function is marked, and some individuals with KS perform well above average on intelligence tests. Moreover, it seems likely that early developmental interventions and language tutoring in boys can assist in preventing disabling difficulties with language skills later in adult life [25, 32].

Morbidity Associated with Klinefelter Syndrome

Data from large population-based studies of chromosome abnormalities at birth have demonstrated that the KS occurs in about 1 in 400–600 male births [33–35]. However, only 25% of men with KS appear to receive a diagnosis [32], suggesting that many men with KS do not receive appropriate medical care. The failure to diagnose most men with KS, could likely be addressed by routine testicular examination in clinical settings, as the testes of most men with KS are much smaller than normal (usually less than 5 cc in KS vs. greater than 15 cc in normal men). However, testicular examinations are frequently not performed by clinicians seeing young men for reasons other than infertility or testicular discomfort. Alternatively, greater awareness of KS in the general population could improve rates of diagnosis.

In adults, overall age-matched mortality appears to be roughly doubled [36, 37]. Most of this increase is from non-neoplastic diseases of the cardiovascular, respiratory and digestive systems, and diabetes; however, the incidence of breast cancer and autoimmune disease is somewhat increased [38, 39].

In particular, the risk of breast cancer is 20-fold higher in men with KS, who account for 4% of all breast cancer cases in men. Fortunately, breast cancer in men is rare, so the absolute risk of breast cancer in a given man with KS is well under 1%. As a result, screening mammography is not generally recommended [40]. Men with KS also have an increased risk of extra-gonadal germ cell tumors [41]. Such patients are young and frequently present with advanced disease, often of the mediastinum. It is thought that these neoplasms arise from primordial germ cells that failed to complete migration to the fetal testes during embryogenesis. These germ cell precursors may then undergo malignant transformation partly in response to the elevated gonadotropin levels seen in individuals with KS. Fortunately, these tumors are rare even in individuals with KS, and most of these tumors respond well to chemotherapy.

There may be an increased risk of autoimmune disease in individuals with KS. Systemic lupus erythematosus, rheumatoid arthritis, ankylosing spondylitis and even rare forms of vasculitis such as Takaysu's arteritis have been reported in KS patients, although the true relative increase in risk is difficult to calculate due to the small number of individuals affected [42–44]. It has been speculated that this possible increased risk of autoimmune disease is due to an increase in the estrogen to testosterone ratio, as it is believed that elevated levels of circulating estrogen may place premenopausal women at increased risk of autoimmune disease [45]. Alternative explanations for this increased risk of autoimmune disease in individuals with KS include abnormalities in "regulatory" T-lymphocyte subsets, as is observed in women with autoimmune disease. It is interesting to note that androgen therapy has been observed to improve clinical symptoms of autoimmune disease as well as correcting some of the laboratory features of autoimmunity [46].

Diabetes mellitus, usually type 2, is more common in individuals with KS than in the general population [47, 48] and may be present even before puberty [49]. The mechanism of diabetes in men with KS is probably due to a post-receptor defect in insulin action [50].

In addition to diabetes, men with KS may be at increased risk of other elements of the metabolic syndrome [51], especially when KS occurs in combination with obesity [52, 53]. As a result, screening for hypertension and hyperlipidemia should occur early in men with KS for the purposes of preventing premature atherosclerosis [51].

Taurodontism (an enlargement of the pulp in molar teeth) is seen in almost half of men with KS [54]. Taurodontism is diagnosed by dental X-rays and predisposes to premature dental caries and tooth loss. Good dental hygiene and frequent cleaning may be protective.

Early varicose veins are a feature of KS with 20% of men having severe varicosities at a relatively early age [55]. In addition to lower-extremity venous ulcers and an increased risk of deep-vein thrombosis have been noted [56, 57]. There is speculation that a defect in venous basement membrane connective tissue is present in some individuals with KS.

Diagnosis of Klinefelter Syndrome

KS is unique in that it can be diagnosed at almost any age: in utero, in a pre-pubertal boy, in an adolescent or even an adult male presenting to either a general practice or to an infertility clinic. Chromosomal karyotyping on amniotic fluid obtained from amniocentesis for an abnormal triple-screen or for advanced maternal age will reveal KS with great accuracy. KS can be suggested in pre-pubertal children by a testicular size of less than 2 cm after the age of six years [58]. Additional clues to the diagnosis of KS in a pre-pubertal male are verbal learning disabilities or taurodontism. After puberty, men with KS will often exhibit tall stature (usually greater than 184 cm.) with disproportionally long legs and frequently manifest an arm span that is greater than their height [59]. In adults, the diagnosis of KS should be considered in men with gynecomastia, primary hypogonadism, infertility or osteoporosis. Since serum testosterone levels may be normal, serum gonadotropins should also be measured. Peripheral blood karyotyping can be used to confirm the diagnosis, although this test can be negative in mosaic individuals, and tissue karyotype may be necessary [9].

Treatment of Klinefelter Syndrome

Testosterone therapy results in a more “male” phenotype, with increases in facial and pubic hair, muscle size and strength, increased libido and bone mineral density and improved mood [24, 49, 60, 61]. Optimally, testosterone therapy is begun at puberty

allowing boys with KS to experience pubertal changes in tandem with their peers. In addition, this allows for optimal enhancement of bone mineral density. Even if testosterone therapy is not initiated until adulthood, it is still associated with beneficial improvements in mood, behavior and sense of well-being [61]. Testosterone therapy has no beneficial impact on infertility or on gynecomastia, which requires surgical resection if bothersome.

Testosterone therapy is safely and easily accomplished by either periodic intramuscular injection of testosterone esters, or transdermal gels described in detail elsewhere in this volume. The replacement dose for intramuscular esters of testosterone is 200 mg IM every other week in adults. Adolescents are usually started at lower doses (e.g., 50–100 mg every 2–4 weeks) and are gradually increased to adult doses over 2–3 months [14]. Side effects of testosterone therapy include weight gain, mostly due to increases in lean body mass, and acne. In older men, erythrocytosis is seen with some frequency. Induction or a worsening of sleep apnea may occur [60]. In general, testosterone therapy is both beneficial and well tolerated by individuals with KS, most of whom continue therapy long term [62]. Twenty-year follow-up of cohorts of men with KS treated with testosterone have demonstrated marked improvements in mental health, working capacity social adjustment and inter-personal relationships that are similar to improvements seen in hypogonadal control cohorts [63]. As a result, most physicians feel that early recognition and treatment with testosterone therapy is of great benefit to the long-term health and welfare of men with KS.

Klinefelter Syndrome and Infertility

Why men with KS are infertile is unknown. Mouse models of KS suggest that common genes that escape X inactivation may be responsible for the infertility and other manifestations of KS [64]. Before 1992, donated sperm or adoptions were the only options for fertility for individuals with KS. Since then, however, dramatic advances in the treatment of infertility in individuals with KS have occurred. Numerous births resulting from intracytoplasmic sperm injection (ICSI) of sperm obtained from surgical testicular extractions “TESE” in patients with KS have been published [65–67]. While some patients with non-mosaic KS do not have sperm on testicular biopsy [68], this technique offers a chance at fertility for those who do, roughly 50% of men [69]. Early analysis of spermatozoa obtained by testicular biopsy in individuals with KS has revealed an increased prevalence of sperm with additional X chromosomes [70], higher rates of aneuploidy [71] and trisomy 21 [72]. Roughly 5% of testicular spermatozoa from men with KS are aneuploid, compared with less than 1% from men with normal karyotypes, likely due to meiotic errors in euploid spermatocytes [73]. The good news, however, is that follow-up of babies born from men with KS by TESE/ICSI has not demonstrated an increased risk of genetic abnormalities [74, 75]. As a result, pre-

implantation genetic diagnosis of embryos from couples with KS is not currently recommended to prevent the transmission of genetic abnormalities.

Recently, two groups have explored whether performing "early" TESE in adolescents or young adults will result in a finding sperm in a greater proportion of procedures. These studies enrolled young men between 12 and 25 of age [76, 77]. Unfortunately, the sperm retrieval rate in the young men in these studies was still only 50%. As a result, it is probably reasonable to wait until a man with KS desires fertility to perform TESE, as early TESE does not appear to increase the chance of finding sperm, allowing for fertility treatments.

Other Forms of Primary Hypogonadism

KS is the most common form of primary hypogonadism. Other causes of primary hypogonadism (i.e., low serum testosterone and elevated gonadotropins) include: toxic exposures (especially radiation and/or chemotherapy as part of treatment for cancer), history of testicular torsion, trauma or congenital cryptorchidism, history of post-pubertal mumps orchitis, autoimmune orchitis, HIV and rare congenital deficiencies in testosterone biosynthetic enzymes, gonadotropin receptors or other uncommon conditions such as myotonic dystrophy. Frequently, the diagnosis is clear by history and physical examination and routine laboratory testing. Referral to an endocrinologist skilled in distinguishing among these uncommon forms of primary hypogonadism is appropriate if the diagnosis remains unclear.

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10. The Undescended Testis

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Introduction

An undescended testis, the most appropriate term, is one that is not fully descended into the scrotum by 3 months of age. Undescended testes may be palpable or truly cryptorchid (hidden or non-palpable). It is usually unclear whether an innate defect of the testis caused non-descent or a normal testis has not descended because of a defect or obstruction. Regardless, of course, appropriate therapy involves surgical repositioning the cryptorchid testis into the scrotum because a non-scrotal position is associated with

impaired spermatogenesis and malignant predisposition. When congenital, surgical correction is optimally done as soon as possible after 6 months of age. When a previously normally positioned testis is found to be non-descended (hence, an ascended testis), corrective surgery can be performed as soon as the diagnosis is verified. The major concerns relate to degeneration of germ cells, resulting in diminished spermatogenesis, and malignant transformation.

Definitions

Cryptorchidism

The undescended testis, a physical finding, may or may not be visible or palpable. The testis may be palpated at various locations along the usual path of descent, or at ectopic locations that deviate from that path (Fig. 10.1). Etiologies are genetic, hormonal, anatomic, or metabolic, as occurring with galactosemia [1]. Unilateral non-descent accounts for more than 75% of cases, the remainder are bilateral [2–4]. It is important to verify lack of descent, determine whether the maldescended testis was congenital, ascended, or acquired to exclude a retractile testis, particularly during the mid-childhood years when the cremasteric reflex is most pronounced [5].

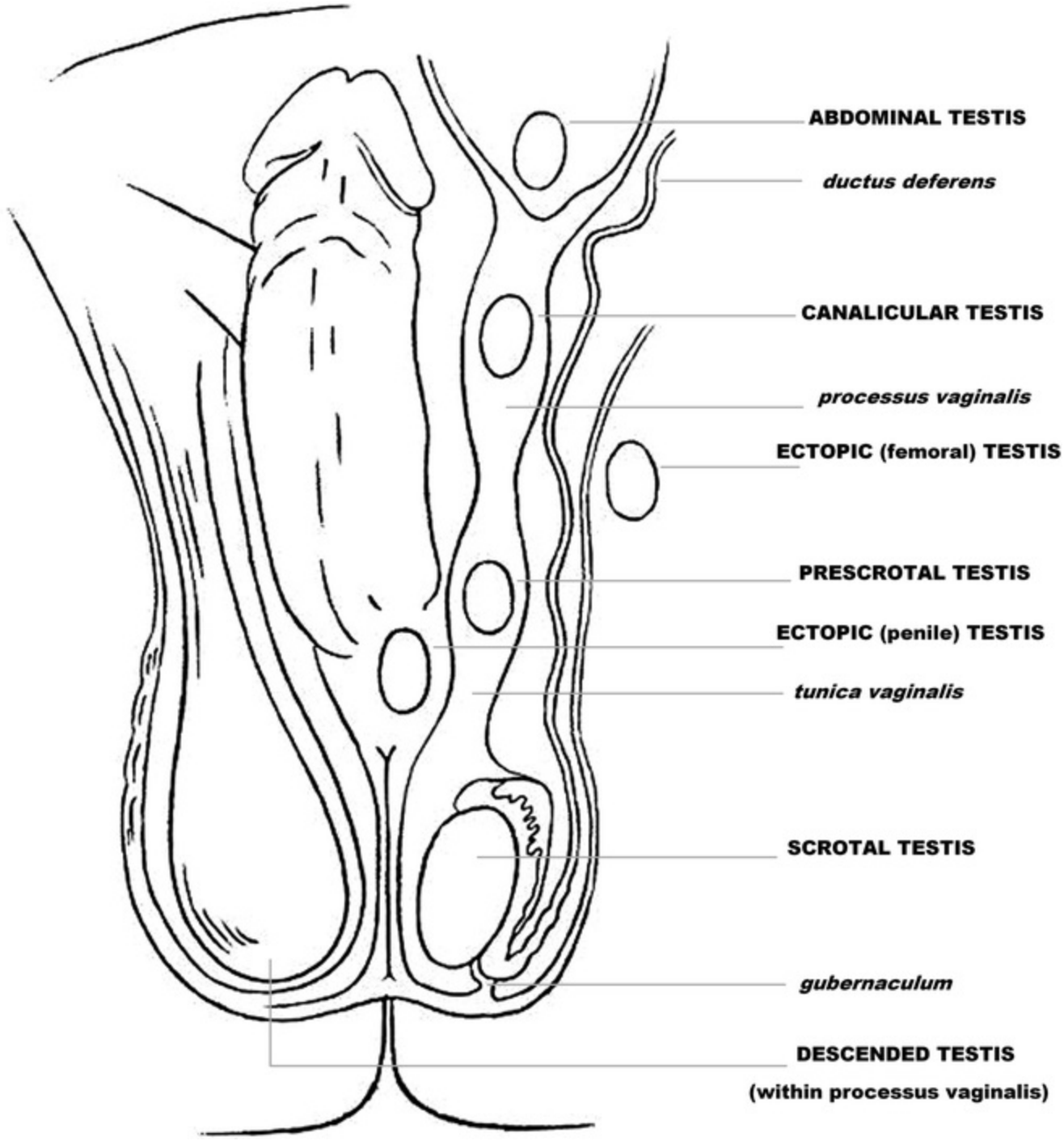

Fig. 10.1 Undescended testes along the pathway of normal testicular descent into the scrotum, and ectopic locations. Illustration by Dr. James Grubola, Professor of Fine Arts, University of Louisville

Congenital cryptorchidism is present since birth. An **ascended testis**, documented to be descended at birth and during infancy, has subsequently ascended above the scrotal position during childhood. Commonly occurring from about 5 years of age until pubertal onset, ascent may be a consequence of atypically low activity of the hypothalamus–pituitary–testicular axis during childhood. A **retractile testis** that readily retracts into a supra-scrotal position by the cremasteric reflex is considered a normal

testis and usually resides in the scrotum. A continuum may exist so that retractile testis is the less-affected end of the spectrum of the ascended testis. In this review, cryptorchidism that occurs as a complication of inguinal area surgery or trauma is referred to as **acquired cryptorchidism** [6], although many reports have used the term "acquired" to refer to ascended testes in general, occurring without a previous insult.

A non-palpable testis may also indicate an **absent testis,** which is felt to be a consequence of agenesis, dysgenesis or an intrauterine accident such as occluded blood supply or testicular torsion (vanishing testis syndrome). Bilateral absence of the testes is referred to **anorchia**.

An **ectopic testis** is undescended and located elsewhere than along the normal pathway of descent (Fig. 10.1). The mechanism may be an abnormal primary attachment of the gubernaculum to structures other than the base of the scrotum, such as laterally or medially in the femoral, penile, or perineal areas.

Orchidopexy is the surgical relocation of the testis into the scrotum. It is the most successful therapy for cryptorchidism, while hormonal therapy is not recommended.

Epidemiology

The prevalence of cryptorchidism is 2–9% in full-term infants [7–14]. Among premature infants, the prevalence is as high as 21%, but diminishes with advancing gestational age and increasing birthweight. It is also noteworthy that there is an increased prevalence of both cryptorchidism and hypospadias among those born early and with low birthweights, although these findings may be more likely to be associated with a more pervasive development defect [15]. As a consequence of spontaneous descent after birth in both the term and premature infant, the prevalence of cryptorchidism decreases to approximately 1–2% by 3 months of age (Table 10.1) [14, 16]. Spontaneous descent is more likely among boys who weigh <2500 g at birth or are gestational age <37 weeks [8]. The likelihood of descent in the 3 months after birth is greater for bilateral maldescent and if the scrotum is large and well developed at birth [7, 9].

Table 10.1 Prevalence of cryptorchidism in relation to age and gestational age [7, 8, 10, 12]

At birth	
Overall	2.7–6.7%
Bilateral	3.0%
Unilateral	1.9%
Related to birth and gestational age	
>2500 grams	2.7–5.5%
<2499 grams	21.0–28.9%

>37 weeks	2.1%
At 3 months of age	
Overall	0.9–1.6%
3 months from EDC	1.3%
Based upon birthweight	
<2000 gm	7.7%
2000–2500 gm	1.7–4.6%
>2500 gm	0.9–1.4%

After the age of one year, the prevalence of non-descent ranges from 0.7 to 2.0% of males with unilateral and bilateral cryptorchid testes [3, 7, 9, 14, 17, 18], similar to the 0.82% found among untreated Nigerian schoolboys aged 5–13 years [19].

The incidence of cryptorchidism and other urogenital malformations has been reported to have increased in recent decades. The estimated prevalence varies considerably in different regions [20, 21], however, perhaps from different levels of exposure to anti-androgenic chemicals, with hormone-disrupting actions, such as pesticides and phthalates [22]. Increasing incidence trends have been reported from Denmark and the United Kingdom [9, 10, 14, 23, 24]. These studies used rather similar diagnostic criteria for the ascertainment of cryptorchidism, the children having been prospectively examined by trained researchers.

Normal Development and Descent of the Testis

Testicular development involves a cascade of sequential steps under the control of numerous genes [25]. Several transcription factors are known to contribute to the initial development of the adrenogenital primordium [26, 27] including EMX2 (*Empty Spiracles 2*), LHX1 (*Lim Homeobox Gene 1*), WT1 (*Wilms Tumor 1*), and WNT4 (*Wingless-type MMTV Integration Family Member 4*). Mutations in these genes cause agenesis or dysgenesis of the gonads, adrenal glands, and the urogenital system. Further development to the bipotential gonad requires LHX9 (*Lim Homeobox Gene 9*), NR5A (*Nuclear Receptor Subfamily 5 Group A Member*; previously called *SF1*, *Steroidogenic Factor 1*), GATA4 (*GATA Binding Protein 4*), DMRT1/2 (*Doublesex- and Mab3-related transcription factor*), and CBX2 (*Chromobox Homolog 2*).

The primordial germ cells (PGCS) migrate from the epiblast to the developing gonadal ridge under the guidance of Kit ligand and c-Kit receptor interaction [28]. Soon thereafter, testicular differentiation of the gonad is initiated by the SRY gene product (*Sex determining region of the Y-chromosome*) [29]. Expression starts in the somatic cells that differentiate into Sertoli cells forming the seminiferous cords [30]. Peritubular myoid cells surround the cords, and Leydig cells develop in the interstitial space. The Sertoli cells regulate testicular development. SRY regulates SOX9 (*SRY-box*

Containing Gene 9) in the Sertoli cells which determines the gonadal structure [31]. If SRY is missing (as in 46, XX), WNT4 and RSPO1 (*R-spondin 1*) lead the gonadal development toward the ovary [32]. FOXL2 (*Forkhead box L2*) is necessary for the maintenance of the ovary. SOX9 regulates the production of fibroblast growth factor 9 (*FGF9*) and prostaglandin D2 (*PGD2*) in Sertoli cells, and these reciprocally stimulate SOX9 expression. SOX9 and FGF9 together inhibit the ovarian genes WNT4, RSPO1 and FOXL2 that, in turn, suppress SOX9. Thus, there is a complex gene interaction network in the gonads securing normal development, and if this fails, gonads may become dysgenetic [32].

Sertoli cells secrete growth factors that influence Leydig and peritubular myoid cell differentiation, such as Desert hedgehog (*DHH*) and platelet-derived growth factors (*PDGF*). After differentiation, Leydig cells start to secrete testosterone and insulin-like peptide 3 (*INSL3*) which masculinize the genital structures and regulate testicular descent into the scrotum. Placental human chorionic gonadotropin and pituitary luteinizing hormone stimulate Leydig cell differentiation, proliferation, and hormone production.

Sertoli cells also secrete anti-Müllerian hormone (AMH) from the 7th week of gestation [33], which leads to the involution of Müllerian ducts. AMH is an excellent marker of the presence of testicular tissue in an infant.

Other developmental events that relate to testicular descent involve the formation of associated ducts, the gubernaculum, and the tunica vaginalis (the serous membrane covering a portion of the testes and epididymis). The gubernaculum develops from mesenchyme, becoming a ligamentous band of tissue that connects the lower end of the epididymis and the base of the scrotum. The processus vaginalis, a peritoneal out-pocketing next to the internal inguinal ring adjacent to the gubernaculum, elongates progressively through the inguinal canal. The testicular ductal system develops from the paired Wolffian ducts under testosterone stimulation, with the portion adjacent to the testes becoming rete testes with ejaculatory ducts at the distal end. Concomitantly, dihydrotestosterone produced in target tissues, such as fetal genital skin, by 5-alpha reductase type 2, *SRD5A2*, stimulates male external genital differentiation.

Testicular descent [34, 35] occurs after a morphologic reorientation repositions the testis caudally at the level of the lower pole of the metanephric kidney. Then, trans-abdominal migration relocates the testis from the posterior abdominal wall to the inguinal region, accomplished by 15 weeks of fetal life, followed by trans-scrotal migration. Cranial suspensory ligament regression allows both relative intra-abdominal testicular repositioning and trans-inguinal migration. Androgen control of this process is implied by the persistence of intra-abdominal testes in patients with androgen insensitivity syndrome [36], failure of regression in animals treated with an anti-androgen [37], and cranial ligament regression in female animals treated in utero with androgens [38].

Trans-inguinal migration through the inguinal canal to the base of the scrotum occurs after 26 weeks of fetal life [39]. Descent is usually accomplished by the 32nd week but may not be fully complete until post-natal life. Concomitantly, the testis carries the processus vaginalis into the scrotum. Leydig cell-derived insulin-like peptide 3 (*INSL3*) regulates gubernacular development and regression together with androgens [40]. This androgen-dependent phase involves the gubernaculum, cremaster muscles, the genitofemoral nerve, neurotransmitters, cytokines, intra-abdominal pressure, and epididymal maturation. Some patients with maldescent fail to have attachment of the epididymis to the testes [41]. The gubernaculum, which is intertwined with cremaster muscle, plays a role [42, 43], regressing, perhaps creating a relative negative pressure toward the inguinal canal, concomitant with trans-inguinal migration [42].

It is recognized that the factors causing testicular descent are complex and incompletely understood. Androgen stimulation involves paracrine factors and catabolic enzymes such as acid phosphatase that stimulate gubernacular regression [44]. Animal studies suggest a role for the genitofemoral nerve [45]. In spite of these potential genetic regulators, identifiable genetic causes of cryptorchidism are rare. While INSL3 plays a role in establishing gonadal position in animals [46], mutations in the coding region of the INSL3 gene do not appear to commonly cause human cryptorchidism [47]. It has been shown that RXFP2 (relaxin family peptide receptor 2), a receptor of INSL3, is required for gubernacular development and is necessary for testicular descent [48]. Polymorphisms in the RXFP2 gene may play a role in undescent [49]. Systemic factors may be more frequent with bilateral cryptorchidism while paracrine regulators may be disrupted when only one testis is undescended. Anatomic problems precluding descent could include a detached epididymis [40], testicular-splenic, or testicular hepatic fusion [50]. Inguinal hernia is a common finding with cryptorchidism and is more likely a result than a cause of maldescent.

A **hormonal role** (gonadotropin-stimulated testosterone production) in testicular descent has been postulated for many years, [51] although factors controlling testicular descent are clearly more complicated. Animal data are consistent with time-specific androgen stimulation of both the abdominal and inguinal phases of descent [52, 53]. Orchidectomy will halt gubernacular regression, and estrogen treatment prevents testicular descent in animals, perhaps via down-regulation of INSL3 expression [54–57].

The testis secretes AMH by the 8–9th fetal week, followed within a week by Leydig cell testosterone production, and by week 12, the hypothalamic-pituitary-testicular (HPT) axis is functional to play a crucial role in final testicular descent [58]. After a decline from about week 17 to term, the HPT axis is reactivated in the first few months after birth, with near adult circulating levels of testosterone at one to three months of age although much of the circulating testosterone is bound to sex hormone binding globulin, with values approaching those seen in men, at this stage of development [59].

This may stimulate spontaneous descent of undescended testes occurring at this age. Failure of spontaneous descent after 6 months of age may be related to diminished hormone levels.

There are contradictory reports of hormonal levels in boys with cryptorchid testes. Early studies reported decreased testosterone levels in cryptorchid infants [60] or preterm cryptorchid infants [61] compared with babies without cryptorchidism, possibly a result of diminished LH secretion [62, 63]. However, more recent research using more sensitive assays has not confirmed that result [64]. A prospective study that compared hormone levels at 3 months of age during "minipuberty" in cryptorchid and control boys reported different results between Danish and Finnish boys. The Finnish group of cryptorchid boys had slightly elevated LH and FSH levels but lower inhibin B levels as compared with non-cryptorchid boys, while among the Danish neonates with cryptorchidism, only FSH levels were significantly higher than in non-cryptorchid boys. Inhibin B levels were lower in both Danish cryptorchid and control newborns than in boys in Finland [65] while testosterone levels did not differ between the groups. When androgen bioactivity was measured in a recombinant cell bioassay, however, all 3-month-old boys with measurable bioactivity had fully descended testes, while all 16 boys with testicular position in the inguinal canal or higher had unmeasurable androgen bioactivity [66]. The explanation for these provocative findings is unclear but may relate to the sensitivity of the testosterone radioimminoassay or differences between populations, and deserves further study. AMH levels have also been reported to be diminished among boys with cryptorchidism [67], suggesting a role for AMH in testicular descent or a consequence of dysfunctional testis. During childhood after the completion of minipuberty, physiologically low LH, FSH, and testosterone levels among cryptorchid boys are not different from values in boys without cryptorchidism [64].

An older report of diminished LH responses to GnRH stimulation among prepubertal boys with undescended testes [68] has led to the unsubstantiated suggestion of mild hypogonadotropic hypogonadism (HH) among cryptorchid boys during childhood. Although patients with congenital HH often do have cryptorchidism, HH is rare among adults with a history of cryptorchidism [69]. Elevated FSH levels during the peripubertal period in some cryptorchid boys suggest instead partial gonadal failure [70], perhaps a consequence of maldescent during childhood. Inhibin B levels vary considerably among boys, and those with cryptorchidism generally have levels within the reference range [71].

Germ Cell Development and the Congenitally Cryptorchid Testis

At birth, the numbers of germ cells in cryptorchid testes has been reported to be similar to those in descended testes [72, 73]. However, after the transformation of gonocytes into adult dark (AD) spermatogonia which begins between 3 and 9 months of age, the undescended testis has fewer AD spermatogonia. **This apparent diminished transformation resulting in diminished germ cells provides the primary rationale for early surgical intervention** [74]. Further, regarding the risk of tumor development, intra-tubular germ cell neoplasia in situ (GNIS) in the second and third decades contain enzymatic markers similar to neonatal gonocytes, suggesting that failure of transformation into AD spermatogonia may result in testicular tumors. Testes that remain cryptorchid beyond infancy acquire a progressively more abnormal microscopic appearance [75–79] with progression with increasing age [80]. Comparison of findings at 9 months and 3 years (see below) confirm these differences and document that orchidopexy at the earlier date reverses the process [81]. The poorer growth of the cryptorchid testis that results in diminished volume compared with descended testes is also reversed by spontaneous or surgical descent [82].

The study comparing germ cells in testes with orchidopexy at 9 versus 36 months [81, 82] is the most compelling evidence for the age of recommended surgery for the congenital cryptorchid testis, even though this study could not be fully controlled since cryptorchidism is an outcome of a set of heterogenous disorders [83]. The recommendation for orchidopexy is between 6 and 12 months of age because spontaneous descent is unlikely to occur after 6 months of age while there is progressive loss of germ cells after 12 months of age. The dramatic reduction in germ cell number between 9 and 36 months of age (Fig. 10.2) provides a clear indication for earlier surgery that is beneficial for testicular growth and germ cell development [84, 85]. Other studies have verified these results [86–88]. An assessment of hormones and semen in adult men born with cryptorchidism who had bilateral biopsies at orchidopexy found that more severe histopathology tended to be associated with lower sperm density and higher FSH levels [89], although the association was not statistically significant perhaps because of the small sample size and wide age range at surgery. In clinical practice, biopsy at orchidopexy has not been shown to have value in predicting future fertility.

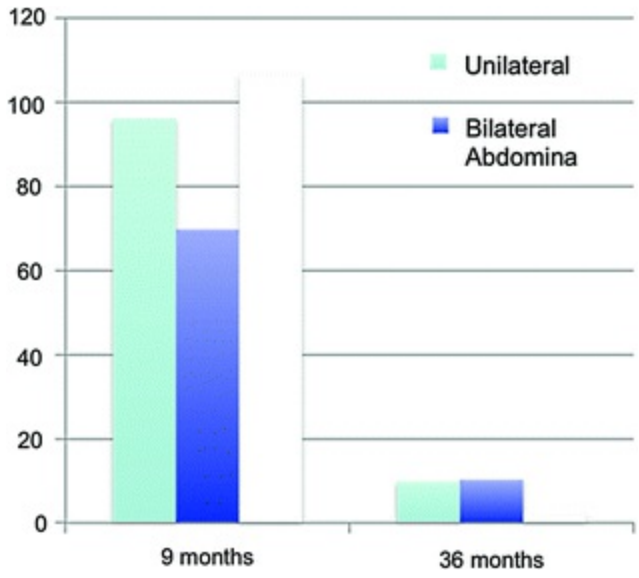

Fig. 10.2 Mean germ cell numbers in testicular biopsy specimens among boys having unilateral, bilateral, and intra-abdominal testes at surgery at 9 months and 3 years. Data (mean + SD at 9 months respectively) 96.1 + 68.7, 69.7 + 49.2, 106.6 + 79.0 and at 3 years 9.8 + 21.3, 10.2 + 13.1, 2.2 + 3.4

It has been theorized for some time that diminished germ cells of the undescended testis might be a consequence of relative gonadotropin deficiency, and hence, GnRH might be a beneficial adjunct therapy after orchidopexy. Pooled effect estimates in a meta-analysis of comparative clinical trials indicated that cryptorchid children treated with GnRH had a higher number of germ cells per seminiferous tubule at various prepubertal ages after biopsy when compared with controls [90]. This suggests that some boys with cryptorchidism may benefit from GnRH treatment as adjunctive to orchidopexy in improving the fertility index. However, longer outcome follow-up is necessary to determine if fertility is increased.

Ascended Testis

Ascent during childhood of a previously normally descended testis (acquired undescended testis) occurs when the testis becomes tethered above the scrotum in an unacceptable position. Acquired UDT is seen in 1–3% of boys during childhood. This may occur among some boys with a retractile testis due to spasticity of the cremaster muscle, or as a consequence of improper elongation of the spermatic cord due to fibrous remnant of the processus vaginalis [23, 91–96]. It is now widely accepted that testes may ascend from the full scrotal position during early- or mid-childhood years inasmuch as boys with undescended testes have been reported whose medical records clearly documented descended or retractile testes that become persistently above the scrotal location [97–100]. The age distribution at orchidopexy is bimodal, with an initial and larger peak within the first five years of life, and a second peak between 8 and 11 years

of age. This distribution could be a consequence of acquired cryptorchidism or ascending testes [101]. The pathogenesis of the acquired undescended testis is unclear. It has been suggested that ascending testis may occur more frequently among boys born with undescended testes that undergo spontaneous descent in early infancy by 3 months of age [102]. There is also a suggestion that altered feeding patterns (decreased breast feeding and use of soy formulas) may increase the incidence of ascending cryptorchidism [103], although this has not been confirmed.

Previously, it was felt that ascending testes had the potential for normal function. Since most boys with ascending cryptorchidism have spontaneous testicular descent during puberty [104, 105], surgery was traditionally deferred and performed only in those boys in whom spontaneous descent failed to occur at puberty [106]. One retrospective study involved 2 groups of adult men with a history of ascending testes: group 1 had orchidopexy for lack of spontaneous descent at ages 4.75–17.8 years ($n = 26$) while group 2 represented men with spontaneous testicular descent during puberty ($n = 32$) [107]. Overall, men who had unilateral ascent had smaller testes, lower sperm concentration and progressive sperm motility, and softer testicular consistency than control men. No significant differences in fertility parameters were found between men with spontaneous descent versus those with orchidopexy. Those who had bilateral ascent also had smaller mean testicular volume and softer testicular consistency, but also a lower paternity rates, higher LH and FSH and lower inhibin B levels, and reduced sperm concentrations and progressive motility than control men. This latter group was not different from men who had bilateral congenital cryptorchidism. Testicular volume, reproductive hormones, semen quality (better for unilateral than bilateral), and successful paternity rates did not differ between the groups. Small testicular size for age of ascending testes was noted at surgery [108]. Testicular volumes smaller than normal for age by ultrasonography were observed at follow-up after operation in boys and men who had previously been treated with orchidopexy at diagnosis of uni- or bilateral ascended cryptorchidism ($n = 155$) [109]. Testicular size of ascended undescended testes estimated by orchidometer after spontaneous descent at puberty ($n = 494$) or after pubertal orchidopexy ($n = 85$) found that >90% had volumes within normal limits, while in over 50% of unilateral cases the affected testis was smaller at follow-up than the other testis that had always been descended [110]. An evaluation of men with unilateral cryptorchidism who delayed surgery until age 20–30 years found severe maturation arrest (22%), germinal cell aplasia (70%), or tubular atrophy (8%) in the orchidectomy specimens, and no differences in semen parameters or testosterone levels before or after surgery suggesting that testicular function by this age was essentially from the descended testis [111].

A reduced number of germ cells per tubule have been reported in unilaterally ascended testis and also in the normally descended contralateral testis [112], which is interpreted as an innate defect in testicular development in both testes in unilateral as

well as bilateral cryptorchidism. By contrast, when a testis is found to be absent, the contralateral descended testis often has an increased volume, increased germ cell proliferation, and dissimilar maturation patterns compared with the contralateral descended testis of patients with unilateral cryptorchidism. These findings do not suggest an underlying endocrinopathy or other testicular defect, or risk of infertility for patients with a solitary testes [113]. In a systematic follow-up of testicular growth in puberty, testes that had been cryptorchid (either unilateral or bilateral) grew slower and remained smaller than descended testes, and there was no compensatory growth in the contralateral testis as compared with control [114]. In contrast, testes of monorchid boys were larger than control.

The Clinical Evaluation

Physical Examination

If the scrotum is empty, it is fundamental to decide whether the testis is present or not. Repeated physical examinations may be necessary if it is unclear whether a testis is present, or to determine the location of a palpable testis. Incompletely descended testes are not usually fixed in position. A testis may at one examination be just palpable at the internal inguinal ring, and on another occasion be impalpable, or the location may be high scrotal on one occasion and within the inguinal canal on another. Also, the location upon physical examination may differ from that when the patient is under general anesthesia in preparation for surgery. If a testis is not palpable, laparoscopy may be used to determine not only its presence, but also its location [115, 116].

In a prepubertal male, confirmation of an undescended testis often requires two physical examinations because of intra- and inter-observer bias in diagnosing undescended testes [115]. After 6 months of age, the cremasteric reflex is active, and the testes readily retract into the upper scrotum, or even to the external inguinal ring if a boy is anxious, nervous, or cold, all of which are common occurrences during a medical examination. A careful physical examination is essential to determine whether a testis is cryptorchid or retractile [117]. A retractile testis is diagnosed when a testis can be pulled into the scrotum and held there for a brief period, after which it does not immediately retract.

Laboratory Testing

LH, FSH, and T levels are measured in the newborn, during infancy from 6 weeks to 4 months, and at puberty to exclude hypogonadotropic hypogonadism. Inhibin B levels may also be useful [118]. When neither testis is palpable, hormone stimulation, usually with hCG, is also useful since a rise in the serum testosterone level indicates the presence of functional Leydig cells. AMH levels can also be used as an index of Sertoli

cell mass [119].

Treatment

An evaluation and treatment guideline for cryptorchidism was recently published by the American Urological Association [120]. Imaging for cryptorchidism is not recommended prior to referral, which should occur by 6 months of age. Orchidopexy is the most successful therapy to relocate the testis into the scrotum, while hormonal therapy is not recommended. Successful scrotal repositioning of the testis may reduce but does not prevent the potential long-term issues of infertility and testis malignancy. Appropriate counseling and follow-up of the patient is essential.

A Nordic consensus on treatment of undescended testes was published in 2007 summarizing current information and making treatment recommendations [119]. These include a recommendation against hormonal treatment based upon poor results and the possibility of long-term adverse effects upon spermatogenesis, and recommend orchidopexy between 6 and 12 months of age, or at diagnosis, if later, to be performed by pediatric urologist/surgeons with pediatric anesthesiologists.

The rationale for treatment of cryptorchidism includes the increased risk of infertility and testicular cancer and the need to correct hernias and decrease the risk of torsion. While bilateral cryptorchidism is associated with infertility if the testes remain undescended beyond puberty, the beneficial effect on fertility of orchidopexy during childhood has not been definitively shown [121]. However, the benefit of reducing the risk of malignancy is assumed. For boys with unilateral cryptorchidism, it has been questioned whether treatment to bring the testis into a scrotal location influences fertility since the percentages of men with azoospermia or oligospermia have been reported to be similar for men with surgical treatments or no treatment [121]. However, the question of whether early surgery is beneficial has not been verified since outcome data are for those whose surgery was generally at ages considerably older than is recommended currently. While semen analysis may be abnormal, evidence (see below) suggests that paternity rates have not been shown to differ from control men [122].

Surgical Treatment

Diagnostic laparoscopy can be used to determine the location of a non-palpable testis and, if needed, to complete orchidopexy [116]. The diagnostic laparoscopy is used to plan the surgical approach, and when no evidence for a testis or associated vasculature is found, it will prevent unnecessary open surgical exploration [116]. Surgical therapy for the palpable undescended testis is commonly orchidopexy using a transverse groin incision over the internal ring, incising the external oblique aponeurosis lateral to the external ring. The testis is located and together with the spermatic cord is freed and

placed into the scrotum. Success rates are related to whether the testis was palpable or not, the age at surgery, and the choice of surgical procedure. Success rates are generally greater than 90%, being somewhat lower for abdominal testes [119].

Hormonal Treatment

While human chorionic gonadotropin (hCG) testing is used in bilateral cryptorchid patients with non-palpable testes for diagnostic purposes [123], it is not currently recommended as a treatment modality. When descent occurred with such hormonal therapy, it was seldom permanent [124, 125]. Furthermore, hCG treatment has been associated with inflammatory changes in the testes and long-term adverse effects on germ cell development [126–129]. Hormonal treatment can be considered for patients in whom the testis is in the upper scrotum, or with a high likelihood of retractile testes that cannot be confirmed by routine physical examination or those with a high anesthesia risk. Infants with hypogonadotropic hypogonadism can also be treated with gonadotropins during minipuberty, which may induce testicular descent.

Consequences of Cryptorchidism

Hormone Production and Sexual Development

Testosterone levels are generally normal in adult men who were cryptorchid [130, 131, 133]. A minority of formerly unilateral cryptorchid men has elevated FSH and inhibin B levels, however, while inhibin B levels are lower among formerly bilaterally cryptorchid men than controls, and correlate inversely with other markers of fertility, including FSH, LH, total and free testosterone, sperm density, and sperm motility/morphology. FSH levels for men with bilateral and unilateral cryptorchidism are compared with results in control men in Fig. 10.3. Few formerly unilateral cryptorchid men had elevated FSH and low inhibin B levels, while the majority of men who had orchiopexy for bilateral cryptorchidism had elevated FSH and low inhibin B levels. Figure 10.4 shows that FSH levels are inversely related to sperm density, a relationship that has also been reported elsewhere [132]. There is also a positive relationship between inhibin B levels and sperm density for formerly unilaterally cryptorchid men (Fig. 10.5).

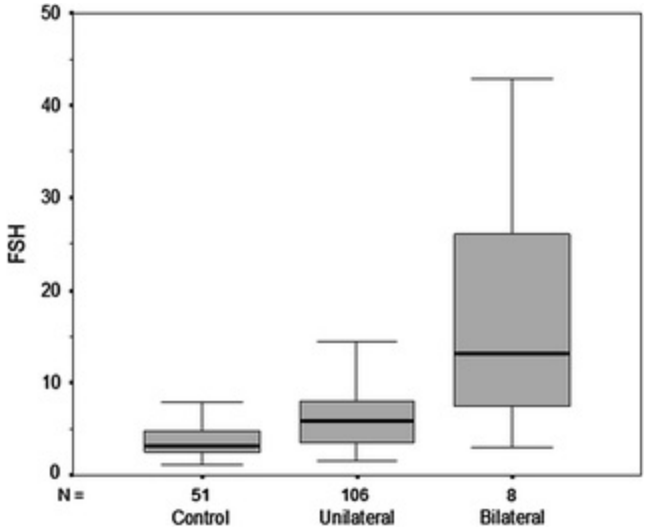

Fig. 10.3 Circulating FSH levels among formerly cryptorchid men compared with control men. The majority of men in the unilateral group have FSH levels within the normal range while almost half of men who had bilateral cryptorchidism had elevated FSH levels

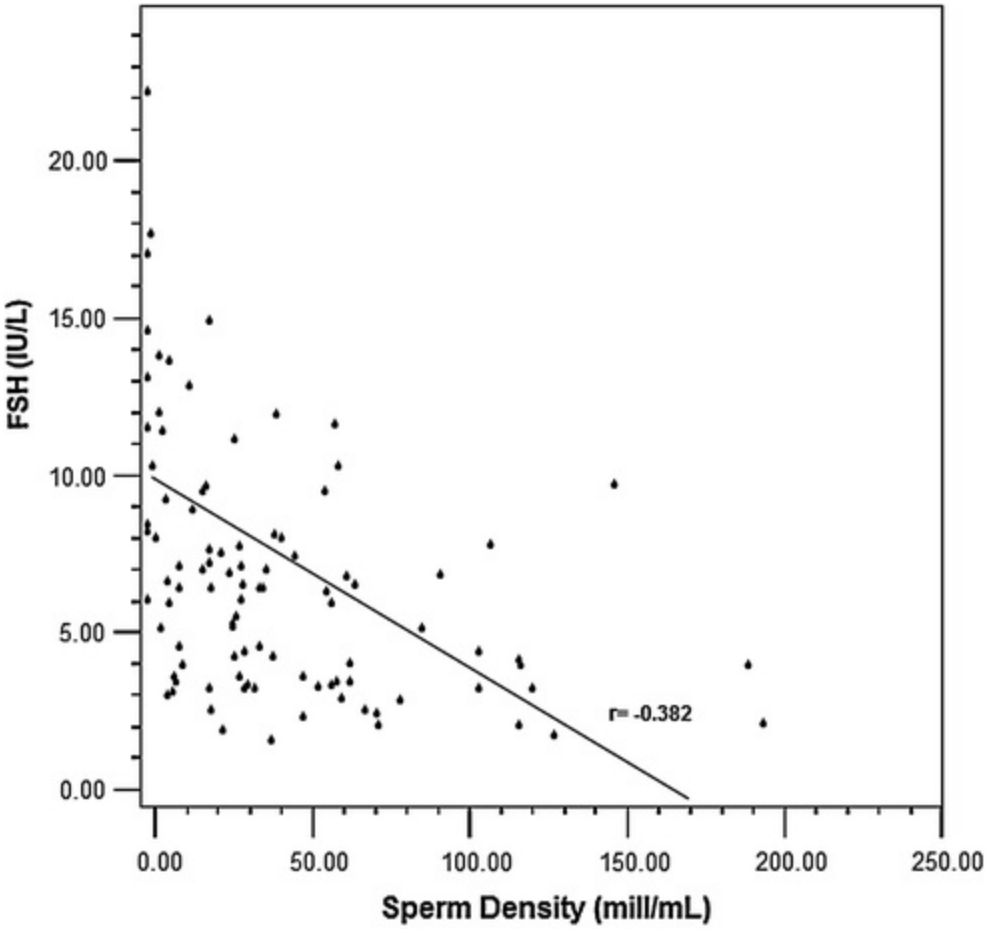

Fig. 10.4 Inverse relationship between FSH levels and sperm density among formerly cryptorchid men

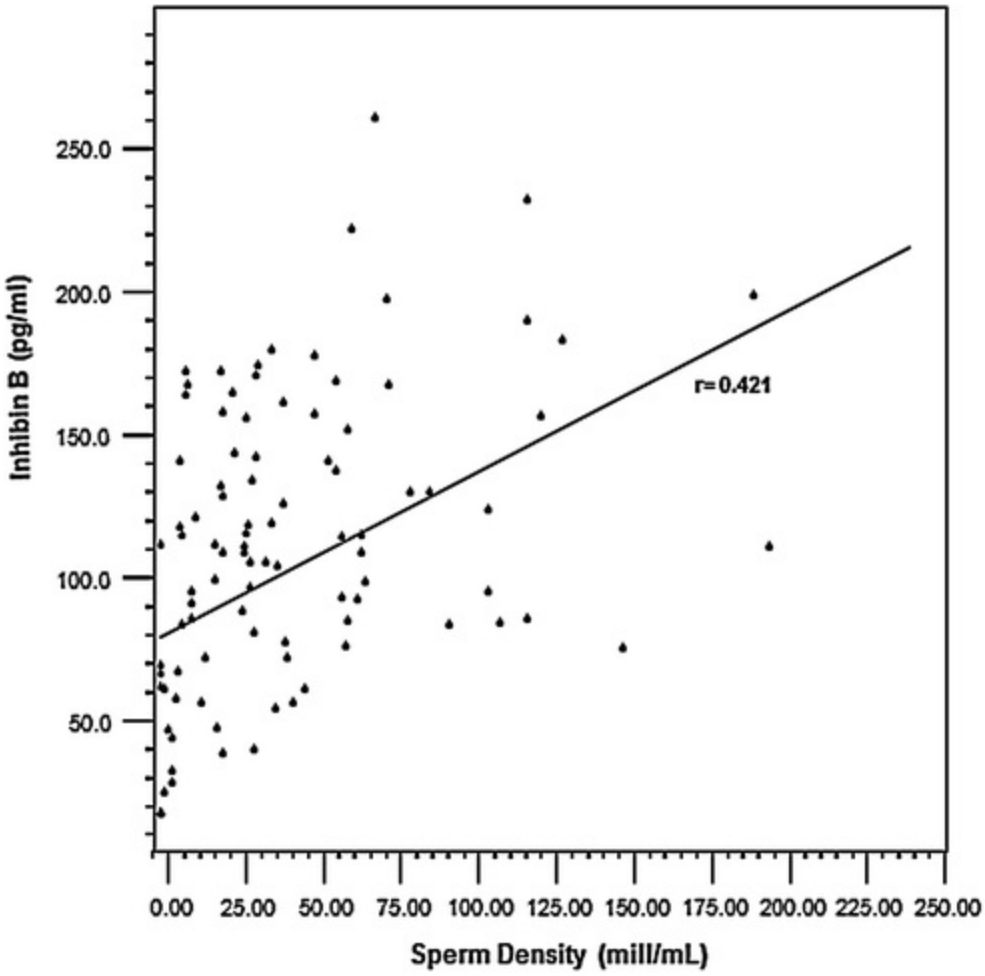

Fig. 10.5 A direct relationship is present between Inhibin B levels and sperm density among formerly unilateral cryptorchid men

Testosterone production in cryptorchid patients results in full stimulation of growth and pubertal maturation of androgen-responsive tissues [133]. Compensatory hypertrophy of the non-affected testis in unilateral cryptorchidism, or in the less-affected testis in bilateral cryptorchidism, may or may not occur, either before or after correction of the cryptorchidism [115, 134]. Catch-up testicular growth may occur after orchidopexy, and mean testicular volume for men with corrected unilateral maldescent is usually within the normal adult range [135] although the undescended testis in unilateral cryptorchid boys remains smaller than their descended testis [115].

Among men with idiopathic infertility, there is a significant positive correlation between testicular volume and indices of spermatogenesis (sperm density and motility), and an inverse relationship with serum FSH levels [137]. On the other hand, no correlation has been found between testicular volume at orchidopexy and paternity, hormone levels, sperm counts, and testicular volume in adulthood in either unilaterally or bilaterally cryptorchid men [89].

Infertility

While the mechanisms remain incompletely understood, the abnormalities in germ cell development in cryptorchidism lead to diminished sperm count and poor sperm motility that increase the risk for infertility.

Germ Cell Counts (Unilateral and Bilateral)

In men with bilateral orchidopexy between 10 and 16 years of age [136, 137], germ cell counts at biopsy at the time of orchidopexy were positively correlated with adult sperm density and combined testicular volume although the percentage of tubular sections containing spermatogonia did not predict sperm density in adulthood [138]. Assessment after orchidopexy when unilateral or bilateral cryptorchidism was performed before 2 years of age [139, 140] found that two-thirds of men, the portion having biopsy evidence for the second stage of maturation to adult dark (Ad) spermatogonia, had normal sperm density. An age relationship was found: all with orchidopexy and biopsy before age 2 years had germ cells, while there was an increased risk for germ cell absence with orchidopexy at older ages [141]. Among boys with good fertility potential (normal numbers of tubular germ cells and normal gonadotropin and inhibin B levels), the mean number of adult dark spermatogonia was significantly greater than in those with low fertility potential [142]. The relationship between sperm density (million/ml) after bilateral cryptorchidism for men who had testicular biopsy at orchidopexy during the first 2 decades of life [83] demonstrating germ cell loss and normal or abnormal adult dark spermatogonia is shown in Table 10.2.

Table 10.2 Median and range of sperm density (million/ml) after bilateral cryptorchidism for men who had testicular biopsy at orchidopexy during the first 2 decades of life [83]

Category	Median	Range
Mild	37	0–83
Severe	1	0–58
Normal adult dark	58	0–127
Abnormal adult dark	1	0–58

Germ cell loss was categorized as mild, moderate, severe, and normal or abnormal adult dark spermatogonia. No significant differences were noted for any group

Age of Treatment and Fertility

Because of progressive damage to germ cells over time, earlier treatment is believed to improve fertility [143]. Age at orchidopexy and sperm concentration is inversely related; however, published data do not consistently suggest a better prognosis for

fertility with earlier surgery although few subjects have had surgery at a very young age [144]. Surgery before age 8 years in one series showed a better prognosis [145], while a second study found no difference among men with unilateral or bilateral cryptorchidism treated before 7 years [146]. A comparison of cases in which surgery was performed between 2 and 7 years of age versus 10 and 12 years of age also found no difference in fertility potential [147]. However, in one report [148] of formerly cryptorchid men who had surgery in the first 2 years of life, the percentage with normal sperm counts and motility exceeded 95%, with an even better fertility prognosis if orchidopexy was performed in the first year of life, with no significant difference between the patients with bilateral or unilateral cryptorchidism.

Spermatogenesis

Using semen analyses as an index of fertility, variable results among men who had cryptorchidism suggest considerable differences in fertility potential. Sperm density was found to be normal in only 4% of men with bilateral maldescent, and in 23% with unilateral cryptorchidism [131], consistent with other reports [130, 149]. Sperm density for formerly bilateral or unilateral cryptorchid men compared with control men is shown in Fig. 10.6. After unilateral cryptorchidism, it appears that the formerly undescended testis may have little sperm production however, since a novel study of men after unilateral vasectomy of the unaffected normally descended testis revealed that most had azoospermia or very low sperm density [149, 150].

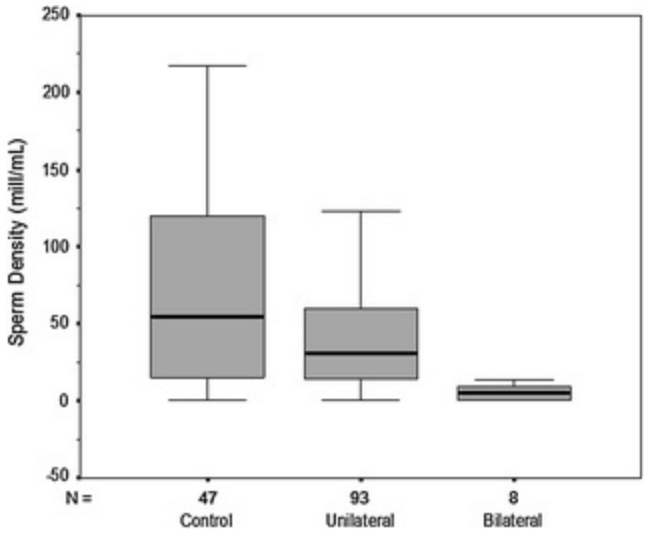

Fig. 10.6 Sperm density among formerly cryptorchid men shows diminished levels among formerly bilaterally cryptorchid men while the majority from the unilateral group is normal. Note that control men are men who did not have cryptorchidism but may have reproductive abnormalities

Paternity and Related Factors

Paternity rates (attempted at least 12 months, with and without success) among men with orchidopexy for uni- or bilateral cryptorchidism were compared with a control group of men matched for age at operation for non-reproductive system [2, 3, 151]. Differences of sperm density related to successful paternity are shown for these groups (Fig. 10.7), together with percentages of men from the Pittsburgh cohort together with composite literature reports (Fig. 10.8).

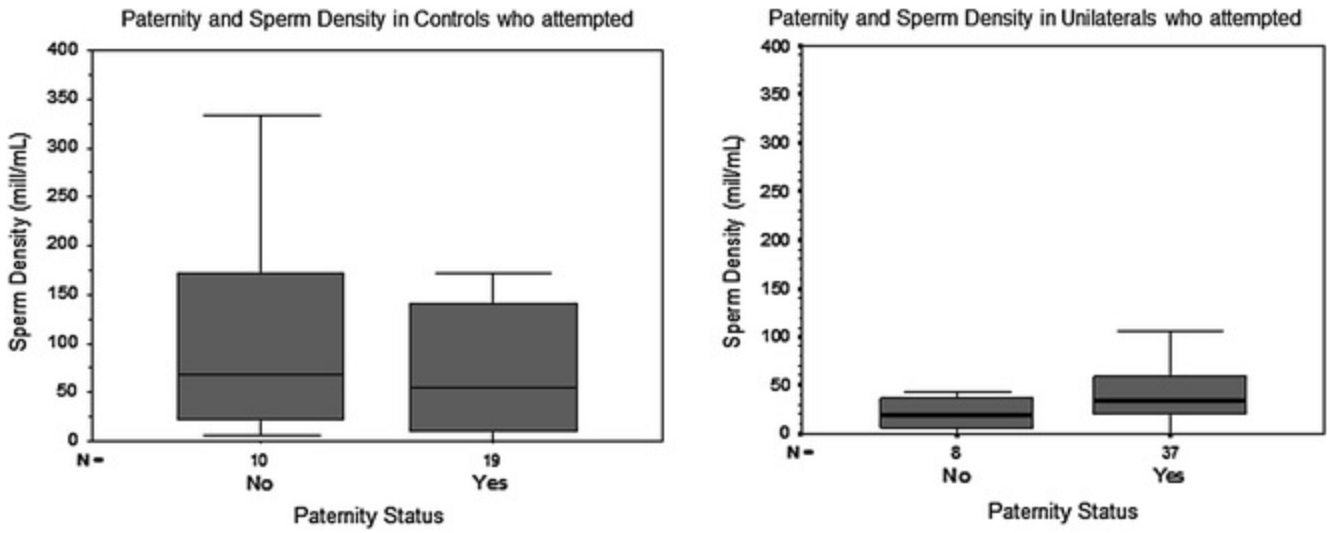

Fig. 10.7 Sperm density compared with success at attempted paternity for formerly unilateral cryptorchid men and control men

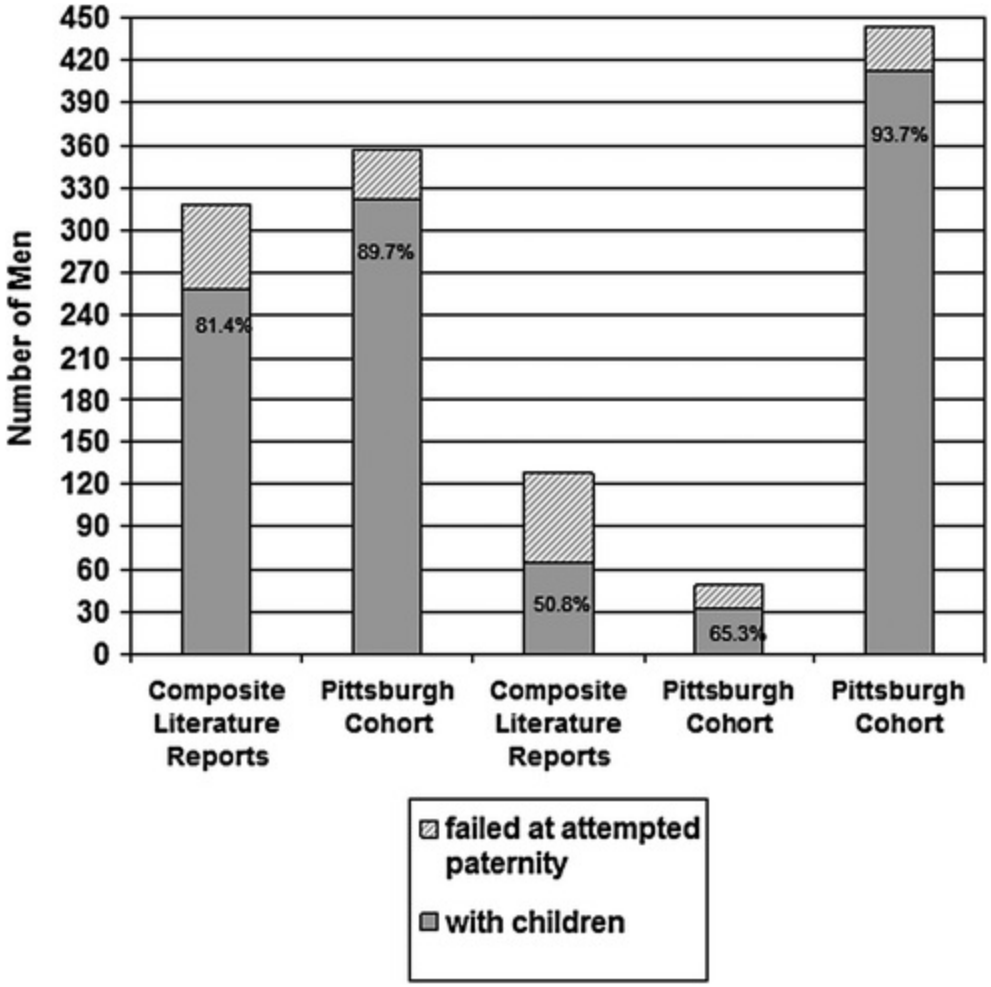

Fig. 10.8 Paternity data from a meta-analysis of reported cases and patients from the Pittsburgh cohort. The first 2 columns are from formerly unilaterally cryptorchid men, the next two from formerly bilateral cryptorchid men, and the final column the control group from the Pittsburgh study

Rates were significantly reduced among formerly bilaterally cryptorchid men (success rate 65% of 49 men). However, no significant difference was found between formerly unilaterally cryptorchid men and controls (success rates 90% of 359 men and 93% of 443 men, respectively). The men in these studies were operated on between 1955 and 1975, and their age ranged from one month to adolescence at the time of operation. Hence, cases with both congenital and ascended cryptorchidism were likely included. Among the formerly unilaterally cryptorchid men, no significant association was observed between the age at orchidopexy, successful paternity or sperm concentration [2, 152]. Inhibin B levels correlated negatively and FSH levels correlated positively with age at operation ($n = 84$), with men operated by the age of two years ($n = 10$) having higher inhibin B levels, which suggests a beneficial effect of earlier orchidopexy. Inhibin B levels correlated positively with sperm concentration ($n = 117$, with no association among control men ($n = 58$) [153]. Furthermore, early orchidopexy may benefit adult-age Leydig cell function insofar as a weak negative association

between age at orchidopexy and testosterone levels among adult men with a history of operated unilateral cryptorchidism was found (n = 105) [154]. Men with a single testis (due to absence or orchiectomy) have similar success rates in attempted paternity as men who have been treated surgically for unilateral cryptorchidism during childhood or control men [155].

Another survey of attempted paternity among 94 men with unilateral or bilateral cryptorchidism who had surgery during childhood [156] found 34 of 53 men (64%) had succeeded within 12 months of attempted pregnancy, with subsequent success after longer attempts in 19%, and no success for 17%. Because uni- and bilateral groups were assessed together, these data do not allow a comparison between these groups.

Testicular Cancer

An increased risk for testicular cancer is a second major potential consequence of cryptorchidism. The origin of malignant cells appears to be germ cells that remain in the early stages of differentiation. This may be a consequence of excessive proliferation of precursor germ cells with associated loss of intercellular communications [157] or a failure of maturation of these precursors. Testicular germ cell neoplasia in situ (GNIS; previously called carcinoma-in situ, CIS) precedes germ cell tumor [158], although it is not clear that all instances of GNIS ultimately develop into tumors. Placenta-like alkaline phosphatase antibody-positive cells (a marker of both undifferentiated germ cells and unclassified germ cell neoplasia) have been identified at biopsy during orchidopexy at young ages [159].

A recent meta-analysis found an overall cancer risk of 2.90-fold (95% CI-2.21-3.82), with heterogeneity related to laterality, surgery, age of surgery, and unilateral or bilateral [160]. Reports of the increased risk of tumors in males with cryptorchidism vary from 4-fold to tenfold [161] and suggest a lifetime risk that is 4–5 times greater than in the general population [162, 163], with odds ratios of 9.3 with bilateral cryptorchidism, and 2.4 for unilateral. A much greater cancer risk is present among men with uncorrected cryptorchidism (RR 15.9 or 11/69) [164].

The rate of development of testicular cancer has typically been assessed as the proportion of testicular cancer patients with a history of cryptorchidism, rather than the percentage of formerly cryptorchid men who develop testicular tumors. In either situation, the risk may differ depending upon the inclusion of patients with unilateral or bilateral cryptorchidism, age and mode of therapy, occurrence of spontaneous descent or ascent, location of the undescended testis, and the presence of other developmental anomalies.

A case-control study of men with testicular germ cell tumors found a significant association with cryptorchidism, with an odds ratio of 3.82 [165]. The odds ratio was 5.86 for bilateral, and 2.71 for unilateral cryptorchidism. When the men in the unilateral

group who developed a tumor in the contralateral testis were assessed separately, the odds ratio for the affected previously undescended testis was 4.02, and for the unaffected testis was 1.42. Positive associations with testicular cancer for the entire population, not just those who had cryptorchidism, were also noted for early onset of puberty (voice change, shaving), infertility, and a sedentary lifestyle. A protective effect of exercise was found, but no association with vasectomy was identified.

An increased cancer risk is likely among boys with intra-abdominal testes, an abnormal karyotype, abnormal external genitalia, and the presence of malignant precursor cells [166].

Tumors and Age at Orchidopexy

Testicular cancer may have its origin in the fetus when germ cell development is abnormal and GNIS—cells form inside seminiferous cords and begin to proliferate during puberty to appear as cancer in young men [167]. If so, surgical correction of cryptorchidism may not influence the risk of testicular cancer dramatically. While there are reports suggesting that treatment of cryptorchidism before puberty is beneficial compared with post-pubertal surgery [168, 169], even the small benefit was challenged in another large registry study [170]. Thus, it is not clear whether bringing the testis into a scrotal location reduces the risk of testicular cancer. When age at correction was considered for patients with previous unilateral cryptorchidism, no increased risk of cancer was found for those who had successful surgery before the age of 10 years (odds ratio 0.60), whereas the risk was increased with surgery at age 10 years or older (odds ratio 6.75). It is noteworthy that the risk associated with undescended testes was eliminated in that study by orchidopexy before the age of 10 years. In addition, there was a significant association between age at orchidopexy (age 3 to >14 years) and risk of cancer. Data are not sufficient to assess whether there is a risk for the contralateral testis in unilateral cryptorchidism.

Although the benefits of orchidopexy in reducing the risk of neoplasia may be uncertain, the general view is that bringing the testis into a scrotal or near scrotal position is indicated. A scrotal position would allow for earlier detection of physical changes that might indicate malignancy. For this reason, orchidopexy has been recommended even for post-pubertal males who are healthy except for cryptorchidism since the risk of malignancy is considered to exceed the perioperative morbidity and mortality [171]. Most recommend biopsy of the testis at the time of orchidopexy in pubertal and post-pubertal boys.

Summary

Cryptorchidism, the condition in which one or both testes have not descended fully into

the scrotum, is one of the most frequent developmental anomalies of the human male. Although there is spontaneous descent in the first few months of life in many males born with this condition, the prevalence thereafter is about 1%. Genetic direction of testicular descent remains poorly understood. There are multiple etiologies of cryptorchidism, both anatomic and hormonal, with some testes having the potential for normal function.

The life-long consequences of cryptorchidism are poor semen quality and increased risk for testicular cancer. Germ cells in the undescended testis fail to undergo normal differentiation from early infancy, hence the recommendation for surgical treatment between 6 and 12 months of age. Men born with bilaterally undescended testes frequently have decreased inhibin B and elevated FSH levels and have a substantially reduced paternity rate. By contrast, only a small portion of men who had unilateral cryptorchidism remain infertile. The risk of developing a testicular tumor, primarily of germ cell origin, is increased substantially particularly among the bilateral group and those not corrected before puberty.

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11. Male Hypogonadism and Liver Disease

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Introduction

Chronic liver disease (CLD) and cirrhosis are common chronic medical illnesses, often from preventable causes, and remain the 12th leading cause of mortality in the United States [1]. New cases of CLD are diagnosed by persistently elevated liver enzymes, radiological evidence of cirrhosis, histopathology, or a clinical consequence of CLD such as variceal bleeding or ascites [1]. The progression of CLD from hepatocyte injury to cirrhosis is characterized by a diffuse, fibrosing, and generally irreversible condition

that disrupts the normal architecture of the liver, particularly in the sinusoids [1]. The major etiologies of CLD include alcohol, hepatitis B or C infection, hemochromatosis, and nonalcoholic fatty liver disease (NAFLD) (Table 11.1). Data from National Health and Nutrition Examination Survey (NHANES) demonstrate that the prevalence of chronic liver disease ranged from 12 to 15% from 1988 to 2008 in the United States [2]. The overall prevalence rate of hepatitis B infection was approximately 0.3%, hepatitis C virus 1.6%, and alcoholic liver disease 2%. These prevalence rates have stayed relatively stable in the past two decades while that of NAFLD has increased from 5.5 to 11% in the last 20 years. NAFLD represented more than 75% of liver disease cases from 2005 to 2008. Hepatitis C virus (26%) remained the most common cause of cirrhosis with the highest number of liver transplants, while alcohol (20–25%) was a close second [2].

Table 11.1 Causes of chronic liver disease in the United States with reported prevalence and associated level of hypogonadism

Causes of chronic liver disease	Prevalence in United States	Associated type of hypogonadism
Nonalcoholic fatty liver disease (NAFLD)	11%	Unclear, but likely more secondary
Alcoholic liver disease (ALD)	2%	Both primary and secondary
Hepatitis B/C	0.3% Hep B, 1.6% Hep C	Unclear, potentially more primary
Hereditary hemochromatosis	0.4%	Predominantly secondary, but likely a component of primary as well

CLD or cirrhosis is associated with multiple systemic processes, and one of the underappreciated comorbidities is testosterone deficiency [3, 4]. Men with advanced liver disease present with phenotypic manifestations typical of hypogonadism. The exact prevalence of low testosterone in liver disease patients has not been carefully studied, but limited literature suggests that hypogonadism, defined by low free testosterone, occurs in 70–80% of patients with CLD, with evidence of both primary testicular failure and disruption of the hypothalamic-pituitary-gonadal (HPG) regulation (Table 11.2) [4–6]. Among men with cirrhosis, low serum testosterone has been reported in up to 90% [7–10] with worsening hypogonadism corresponding to worsening liver failure [11].

Table 11.2 Total testosterone, free testosterone, and SHBG in various chronic liver diseases

	Total T	Free T	SHBG
Nonalcoholic fatty liver disease (NAFLD)[a]	Decreased	Decreased	Decreased
Alcoholic liver disease (ALD)[a]	Decreased	Decreased	Increased

Hepatitis B/C[a]	Decreased	Decreased	Increased
Hereditary hemochromatosis[a]	Decreased	Decreased	Increased
End-stage liver disease (decompensated cirrhosis)	Decreased	Decreased	Decreased
Post liver transplantation	Increased	Increased	Decreased

[a]Patient with stable chronic liver disease without evidence of decompensate cirrhosis including signs of hepatic encelopathy, ascites, or variceal bleeding

Pathophysiology

The pathogenesis of hypogonadism in liver disease is complex, differs by diagnosis, and is hypothesized to be mediated in part by toxic metabolites affecting the hypothalamic-pituitary-gonadal (HPG) axis, hyperestrogenemia, and altered protein synthesis [12].

Cirrhosis, particularly end-stage cirrhosis, is most commonly associated with central hypogonadism [11]. Individuals with cirrhosis may lose luteinizing hormone (LH) pulsatility, suggestive of hypothalamic dysfunction [12]. The response to human chorionic gonadotropin (hCG) is blunted, consistent with an impaired gonadotropic response [12–14]. Systemic disease, in general, is associated with central hypogonadism, and is hypothesized to be due to downregulation of GnRH production by elevated inflammatory cytokines including interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α) resulting in functional suppression of the HPG axis [7, 15]. Hyperprolactinemia may also be seen in liver disease, particularly in alcohol-related liver disease, likely due to alterations in the dopaminergic system that contribute to GnRH suppression with suppression of the HPG axis [16–22].

However, hypogonadism in the setting of liver disease may also be due to testicular damage. In particular, ethanol and its metabolites have been shown to exert a toxic effect on Leydig cells and are involved in onset of the fibrogenic processes [12, 23]. In vitro studies of isolated Leydig cells, isolated perfused rat testes, and testicular homogenates have consistently shown reduced testosterone synthesis [13, 24]. Despite damage to Leydig cells, testosterone levels in patients may be normal early in the disease process due to a compensatory increase in LH.

Estrogen levels can be elevated in men with cirrhosis, which is hypothesized to be due, at least in part, to reduced metabolism and clearance of estrogen, as well as portosystemic shunting further resulting in impaired/bypassed estradiol metabolism, and increased peripheral aromatization of testosterone [7, 11, 25–27]. Estrone and estradiol production may also be increased secondary to increased production of androstenedione from the adrenal glands. Increased androstenedione may be due to a combination of a shift in androgenic steroidogenesis from cortisol to androstenedione with worsening

liver disease and to inflammation-mediated inhibition of conversion of DHEA to DHEA-S [12, 28]. Ethanol may also exacerbate hyperestrogenemia by increasing aromatase activity and interfering with sex hormone endocrine homeostasis before the onset of severe liver dysfunction [29, 30].

Elevation in estrogen in conjunction with decreased testosterone levels results in an increased estrogen/androgen ratio, felt to contribute to feminizing features of liver disease, including altered fat distribution, gynecomastia, spider angiomatas, palmar erythema and reduced prostate volume [13].

The liver regulates testosterone bioavailability via production of SHBG and albumin and is also responsible for the production of enzymes important in the aromatization, conversion to dihydrotestosterone (DHT) and breakdown of androgens. Plasma testosterone binds with high affinity predominantly to SHBG (~60%) and albumin (~38%) [31]. SHBG levels are frequently elevated in certain forms liver disease, resulting in increased total testosterone, but lower free or bioavailable testosterone levels. SHBG levels are low in NAFLD and NASH and are often normal in hemochromatosis. Regulation of SHBG is multifactorial with hepatic signaling molecules and increasing levels of estrogen and decreasing levels of testosterone hypothesized to be important driving factors in SHBG elevation. Thus, early in liver disease, total testosterone levels may be elevated, when free testosterone levels are actually low. SHBG levels normalize after clinical decompensation, likely due to severity of impaired synthetic capacity despite hormonal stimuli. Testosterone, in turn, is important in protein synthesis (including albumin), modulation of liver regeneration, inhibition of cytokine release, and anabolic effects related to liver metabolism [12].

Hemochromatosis

Hemochromatosis is characterized by iron overloading leading to systemic illnesses such as diabetes mellitus, hyperpigmentation, arthralgias, cardiomyopathy, cirrhosis and hepatocellular carcinoma, and hypogonadism [32]. The most notable iron overloading syndrome, hereditary hemochromatosis (HH), is one of the more common autosomal genetic disorders in Caucasians with an estimated prevalence of 1 per 220–250 individuals. HH can be divided into the primary HFE gene defect and non-HFE-related disease. The most common HFE gene defect is a missense mutation in which tyrosine is substituted for cysteine at amino acid position 282 of the protein product C282Y. The defective HFE protein signals that the body is incorrectly deficient in iron because it fails to stimulate normally the production of hepcidin, leading to increased iron absorption from the upper intestine. In addition to HH, there are multiple secondary iron overload syndromes such as thalassemia major and parental iron overload from frequent blood transfusions [32].

Liver injury and hypogonadism are two of the more common abnormalities in HH

[33]. Reported prevalence rates of hypogonadism have ranged from 10 to 100% [33–35]. While liver disease is seen earlier in the disease course, hypogonadism is generally seen in its later stages. The exact timeframe for the manifestation of these symptoms is not clear. However, McDermott et al. in 2005 reported the 20 year follow-up of 141 men age 24–77 years who were diagnosed with HH with evidence of iron deposition on liver biopsy at a single center in Ireland. At the time of diagnosis of HH, those with hypogonadism were on average 56 years of age while nonhypogonadal males averaged 52 years of age [33]. They found that only 9 men (6.4%) had abnormally low testosterone levels with low gonadotropin concentrations. This is in contrast to patients with juvenile (non-HFE) HH who have more severe iron overload, and generally present with delayed pubertal development rather than cardiac or liver disease [35].

Liver injury is hypothesized to occur as a consequence of cellular degradation products from hepatocyte injury from iron deposition, activating Kupffer cells which stimulate hepatic stellate cells to synthesize collagen leading to fibrosis and cirrhosis. In HH, C282Y homozygotes with serum ferritin levels >1000 micrograms/L have a higher risk of cirrhosis with prevalence of 20–45% while fewer than 2% of patients with ferritin <1000 micrograms/L have cirrhosis [32].

In HH, testosterone and LH levels are often low [33]. Iron deposition is found in the pituitary with high predilection for gonadotrophic cells. On the other hand, the secretion of ACTH and TSH is usually normal. There is decreased expression of transferrin and transferrin receptor in iron loaded gonadotrophs secondary to iron deposition [36, 37]. The depressed LH and testosterone with pituitary iron deposition is most consistent with secondary hypogonadism, but there may also be a component of primary hypogonadism since iron deposition is also found in testicular cells. Given that many of these studies did not involve a concomitant liver biopsy, it is not clear to what degree liver disease may contribute to hypogonadism in this setting.

SHBG levels are sometimes increased in HH [38]. Yeap et al. [36] showed that heterozygotes with the C282Y polymorphism without evidence of iron overload tend to have higher SHBG levels than do men who do not carry this mutation, but have normal total and free testosterone levels. These data suggest that the C282Y polymorphism may somehow influence SHBG levels, while the hypothalamic-pituitary-gonadal axis remains intact in the absence of significant iron overload [34]. It is nevertheless important to note that patients with HH may have other causes of hypogonadism such as obesity or Klinefelter syndrome [39].

Phlebotomy, with the goal of normalization of ferritin levels, remains a key treatment for hemochromatosis. Limited data suggest that phlebotomy can partially reverse hepatic fibrosis in approximately 30% of cases, and significantly lower the rate of HH-related HCC if iron removal is achieved before cirrhosis develops [40]. However, it is unclear if testicular atrophy or biochemical hypogonadism can be reversed with phlebotomy since it is hypothesized that hypogonadism may be a

manifestation of irreversible iron deposition and damage to the pituitary gland [41].

In conclusion, HH is a genetic iron overloading syndrome with liver disease usually presenting before hypogonadism. Although advanced liver disease may contribute to hypogonadism, HH seems to occur predominantly due to iron deposition and damage to the pituitary gland. Primary hypogonadism due to iron deposition in the testes is less commonly seen [42]. Phlebotomy has been shown to reverse some degree of CLD before progression to cirrhosis, but further studies are needed to understand its potential impact on hypogonadism.

Alcohol-Induced Liver Injury

Alcoholic liver disease (ALD) is classified into three histologic stages: alcoholic fatty liver, alcoholic hepatitis, and cirrhosis. Fatty liver is a result of steatosis in the setting of heavy drinking, occurring in about 90% of individuals who drink more than 60 mLs per day [43]. In general, fatty liver can be arrested or significantly reversed with alcohol cessation [44]. Alcoholic hepatitis is characterized by inflammatory changes and degeneration of hepatocytes. Patients may have clinical signs of nausea, abdominal pain, hepatomegaly, fever, jaundice, and bleeding. Alcoholic hepatitis is associated with a very high short-term mortality rate and chronically, approximately 40–50% of patients are at risk for progression to cirrhosis [45]. Cirrhosis remains the final stage of ALD with about 10–15% of patients with alcoholism generally at risk [46].

While dose relationship is the most important risk factor for ALD, other important risk factors need to be considered. In general, men who ingest more than 2–3 oz (60–90 mL) of alcohol per day, or woman who ingest more than 1 oz (30 mL) per day for 10 or more years, are at higher risk of cirrhosis [43]. Two important categories include individuals with (1) *alcohol dependence* which includes those who drink excessively and develop physical tolerance; and (2) *alcohol abuse* which includes those engaged in negative social and health consequences of drinking [46]. A large study from Denmark also suggested that the type of alcohol was important, with beer or spirits more likely to be associated with liver disease compared to other drinks such as wine [47]. Binge drinking, female gender, obesity, malnutrition, and family history of alcohol dependence were also associated with a higher risk of ALD [48].

The bidirectional nature of ALD and hypogonadism remains an active area of research. ALD is known to contribute to both biochemical and clinical hypogonadism (Fig. 11.1).‘ In fact, alcoholic patients seem to have more profound phenotypic and biochemical evidence of hypogonadism than other forms of liver disease potentially since alcohol has a direct toxic effect at each level of the hypothalamic-pituitary-gonadal axis.

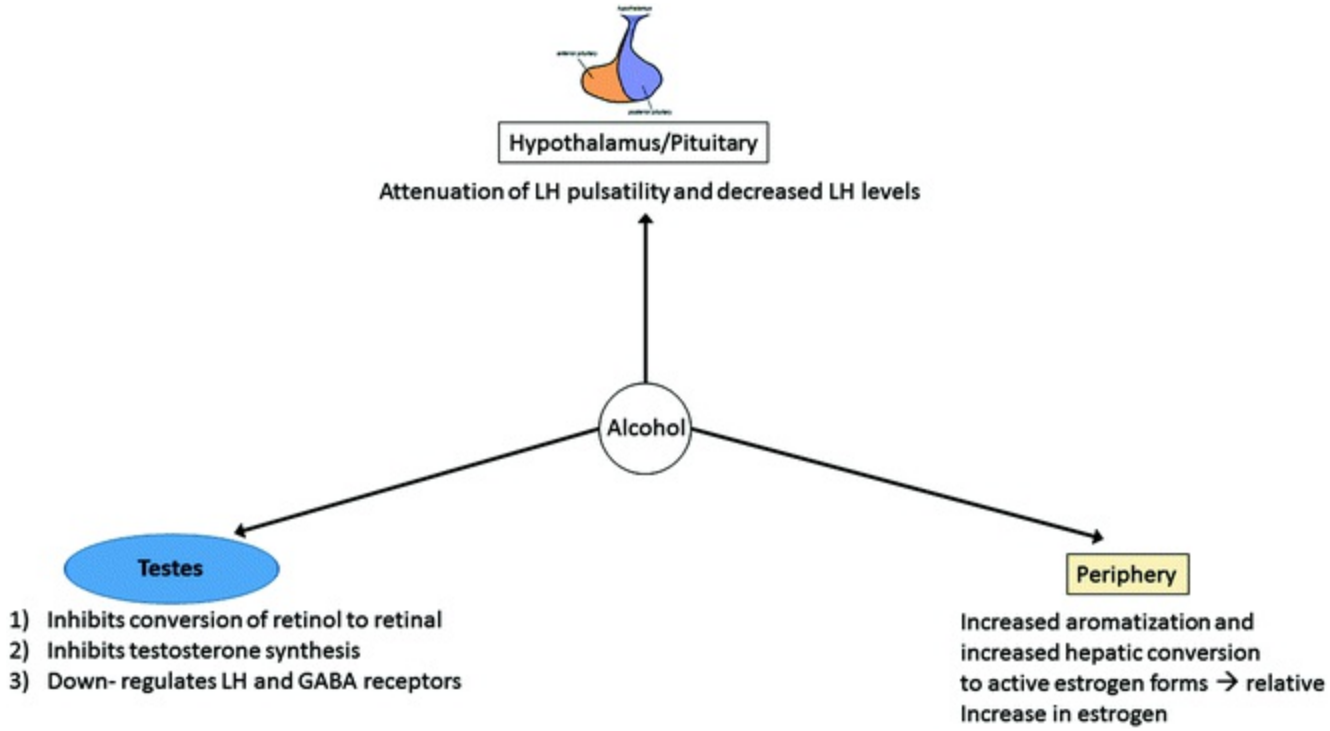

Fig. 11.1 Proposed mechanisms for hypogonadism in the setting of alcoholic liver disease. Alcohol is postulated to affect the *hypothalamus/pituitary* via attenuation of LH pulsatility, though the role of GnRH pulsatility remains unclear at this time. Alcohol also affects the *testes* with inhibition of conversion of retinol to retinal (important in spermatogenesis), inhibition of multiple enzymes necessary for testosterone biosynthesis, and downregulation of LH and GABA receptors. Alcohol additionally acts *peripherally* via increased aromatization of testosterone to estrogen in the setting of portosystemic shunting of androgens and increased conversion to active estrogen forms, overall resulting in higher relative estrogen levels

Most studies suggest that the hypogonadism in alcoholism far precedes, sometimes by years, the development of liver disease. In men with significant history of alcohol abuse, but without histological or biochemical evidence of liver disease, more than 50% showed primary gonadal damage with testicular atrophy and infertility, and notably seminiferous tubular atrophy, marked peritubular fibrosis, and loss of germ cells [28]. Additionally, impairment of conversion of testosterone to its active metabolite dihydrotestosterone (DHT), in the setting of reduced levels of 5-alpha reductase, has been reported [49]. Ylikahri et al. reported in 1974 that short-term alcohol administration (<4 h) to young healthy male volunteers did not alter testosterone levels, but longer administration (weeks) resulted in a reduction in testosterone concentrations [50]. Badr et al. demonstrated that mice treated with high volumes of alcohol (one-third of caloric intake) showed much higher reductions in testosterone and marked testicular changes compared to iso-calorically pair-fed controls without alcohol intake [51].

Ethanol has several direct adverse effects on the testes. Alcohol inhibits the conversion of retinol to its active metabolite retinal, which is essential for normal spermatogenesis [52]. High doses of ethanol impair testosterone synthesis from the precursor cholesterol by inhibiting multiple enzymes including 3-beta-hydroxysteroid

dehydrogenase, 17-oxoreductase and 17–20 desmolase [53]. While short-term alcohol exposure leads to increased expression of LH receptors in Leydig cells, long term exposure downregulates LH receptors as well as gamma amino butyric acid (GABA) receptors, suggesting alcohol may affect LH receptor through cell membrane potential [54].

If alcohol produced solely direct testicular atrophy, a much higher level of gonadotropins, especially LH would be expected, but that is often not the case, suggesting that ethanol also affects hypothalamic-pituitary function. While some older data suggest that ethanol may reduce the pulsatility of LH secretion independent of the liver disease [52], the effect of ethanol on GnRH pulsatility has not been thoroughly studied. Bannister et al. [55] measured LH levels in blood samples drawn every 15 min for 6 or 8 hours in men with chronic alcohol liver disease without clinical signs of gonadal failure compared to those with phenotypic signs of hypogonadism such as testicular atrophy and hair loss. In men without clinical signs of hypogonadism, the pulsatility of LH was preserved, but those with overt signs of gonadal failure showed severely decreased LH hormone levels and an attenuation of pulsatility [52].

In the periphery, alcohol may increase aromatase activity, converting androgens to estrogens even beyond what can be explained by obesity. In alcoholic liver disease, estrogen sulfate may be converted to active estrogens in the liver through NF-kb [55]. Relative estrogen levels may further be increased as a result of portosystemic shunting leading to increased androgen substrate for bioconversion by extra-hepatic peripheral aromatase. It is unclear to what degree the relative excess estrogen may contribute to the inhibition of LH secretion [49]. Estrone and estradiol levels may also be increased secondary to increased production of androstenedione from the adrenal cortex [16].

Many of the studies that examined alcoholism and hypogonadism were performed in the 1970s and 1980s, highlighting the need for new research. In summary, existing data suggest that acute alcohol consumption results in transient primary hypogonadism while longer term alcohol use, even in the absence of liver damage, is associated with both primary and secondary hypogonadism. In those who develop cirrhosis, hypogonadism seems to be more profound than in other causes of liver disease. The reversibility of hypogonadism in those with chronic liver disease who abstain from alcoholism has not been well studied.

Hepatitis B/C

Hepatitis B (HBV) and Hepatitis C (HCV) are the leading cause of hepatocellular carcinoma, cirrhosis, and transplantation. Within the United States, HBV and HCV are responsible for 15 and 26% of cirrhosis cases, respectively. This section will focus on predominantly HCV given HBV rates have been declining with increasing vaccination use [2].

It is estimated that between 2.5 and 4.7 million people in the United States had chronic HCV between 2003 and 2010, with major risk factors including current or prior intravenous drug use, and treatment with blood products before 1987 [56]. About Approximately 20% of patients clear HCV without additional treatment. Of the 75–80% who develop chronic infection, up to 25% will develop cirrhosis over a period of 20–30 years. It also important to note that more than 50% of HIV-infected intravenous drug users have HCV [56].

While HCV is associated with multiple immune-mediated conditions such as mixed cryoglobulinemia, glomerulonephritis, thyroiditis, arthritis, and peripheral neuropathy [57], it remains to be seen if there is a direct relationship between HCV with hypogonadism, especially in the setting of compensated liver disease. Multiple studies have suggested that hypogonadism is not seen with hepatitis C infection until liver architectural damage is present, as evidenced by elevated SHBG and total testosterone levels [58].

Ferri et al. (2002) compared 207 men with HCV (all with compensated liver disease) to age-matched men without liver disease, and demonstrated that plasma levels of total and free testosterone were generally lower in the former group [59]. However, testosterone levels were not significantly correlated with the severity of the liver disease based on ultrasound and laboratory testing.

It is postulated that one mechanism by which HCV induces hepatocyte injury is through the production of reactive oxygen species (ROS) via expression of core protein with direct mitochondrial injury [60]. HCV may also induce ROS-mediated damage to sperm given that somewhere between 30–80% of those with hypospermatogenesis have evidence of ROS damage, including damage to sperm membranes and DNA [61]. Lorusso et al. showed that patients with chronic HCV have impaired sperm quality, particularly the percentage of spermatozoa with normal progressive motility and morphology [62].

The exact prevalence of low testosterone in HCV has not been thoroughly studied, likely due to multiple co-morbidities in HCV patient such as alcohol use, HIV, and HBV infection. Also, many studies looking at the relationship between hypogonadism and HCV were conducted in the setting of interferon treatment. More recently, antiviral drugs such as ledipasvir (inhibitor of viral phosphoprotein) and sofosbuvir (RNA chain terminator) have shown sustained virologic response up to 94–99% in those with genotype 1 HCV infections [63]. Additional studies are needed to examine the impact of these new treatments on testosterone levels.

There have been ongoing concerns that elevated testosterone levels may be a risk factor for the development of HCV-related HCC given that males have a higher risk of HCC than females. One prospective Japanese study of 46 males with HCV-related cirrhosis found that increased total testosterone was associated with HCV–HCC risk, but a case-control study in Australia with 35 HCV patients did not find any significant

association [64]. White et al. (2012) performed one of the largest cross-sectional studies in the U.S. of 308 HCV compensated liver patients with mild to advanced fibrosis. They reported a significant 27% increase in advanced fibrosis risk and 16% inflammatory activity risk for each 1 ng/ml increase in total testosterone [65]. The study examined risk of fibrosis and inflammatory activity based on three groups of total testosterone: low (≤4.11 ng/mL), middle (4.12–5.91 ng/mL), and highest (≥5.91 ng/ml). HBV was reported to augment androgen receptor (AR) signaling using HepG2 carcinoma cells, which may lead to elevated testosterone and subsequent proliferation, apoptosis, and inflammatory response of the hepatocyte, increasing risk of HCC [66]. Increases in total testosterone may be explained by high SHBG levels, and SHBG has been proposed as a marker for HCC [67]. Given somewhat similar mechanism of invasion between HCV and HBV, it may be that HCV could have a similar response.

In summary, whether HCV causes hypogonadism independent of liver disease remains unclear, and should be a focus of further studies. There are data to suggest that HCV may induce ROS-mediated damage in the testes. Additionally, limited data suggest that patients with higher levels of endogenous testosterone and SHBG may be at increased risk for HCC, fibrosis, and inflammatory activity, but the mechanism is not well understood at this time.

Nonalcoholic Fatty Liver Disease

According to the American Association for the Study of Liver Diseases (AASLD), the diagnosis of nonalcoholic fatty liver disease (NAFLD) requires (1) evidence of hepatic steatosis by imaging or histology, and (2) no other causes for secondary hepatic fat accumulation such as alcohol consumption or steatogenic medications. In general, NAFLD is associated with metabolic syndrome including obesity, diabetes mellitus, and dyslipidemia. NAFLD should be differentiated into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). While NAFL is defined by the presence of hepatic steatosis without hepatocellular injury, NASH includes hepatocyte injury. NAFLD is a spectrum of liver injury progressing from lipid accumulation within hepatocytes, to inflammation and ballooning of hepatocytes, and to eventual scarring and cirrhosis (Fig. 11.2). Estimates of the worldwide prevalence of NAFL range from 6.3 to 33%, with a median of 20%, while the prevalence of NASH is estimated to be 3–5% [68].

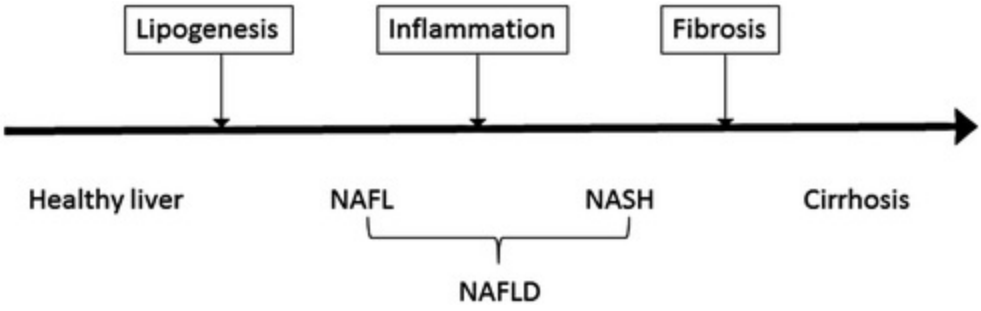

Fig. 11.2 Schematic of progression from fatty liver to cirrhosis. Nonalcoholic fatty liver disease (NAFLD) is subdivided into nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). The former has a prevalence of 6.3–33% and is characterized by hepatic steatosis without hepatocellular injury. The latter has a prevalence of 3–5% and is characterized by hepatocellular injury. Importantly, NASH can progress to cirrhosis, which could potentially lead to decompensated liver disease and/or hepatocellular carcinoma

It is well documented that men with metabolic syndrome have an increased prevalence of hypogonadism [68, 69]. However, the bidirectional nature of the relationship is complex and multifactorial (Fig. 11.3; see Chaps. 15 and 17). It is important to understand these associations in order to determine if these risk factors are independently associated with the development of hepatic steatosis to inform the development of relevant treatment strategies [70]. The actual prevalence of hypogonadism in NAFLD is not established at this time.

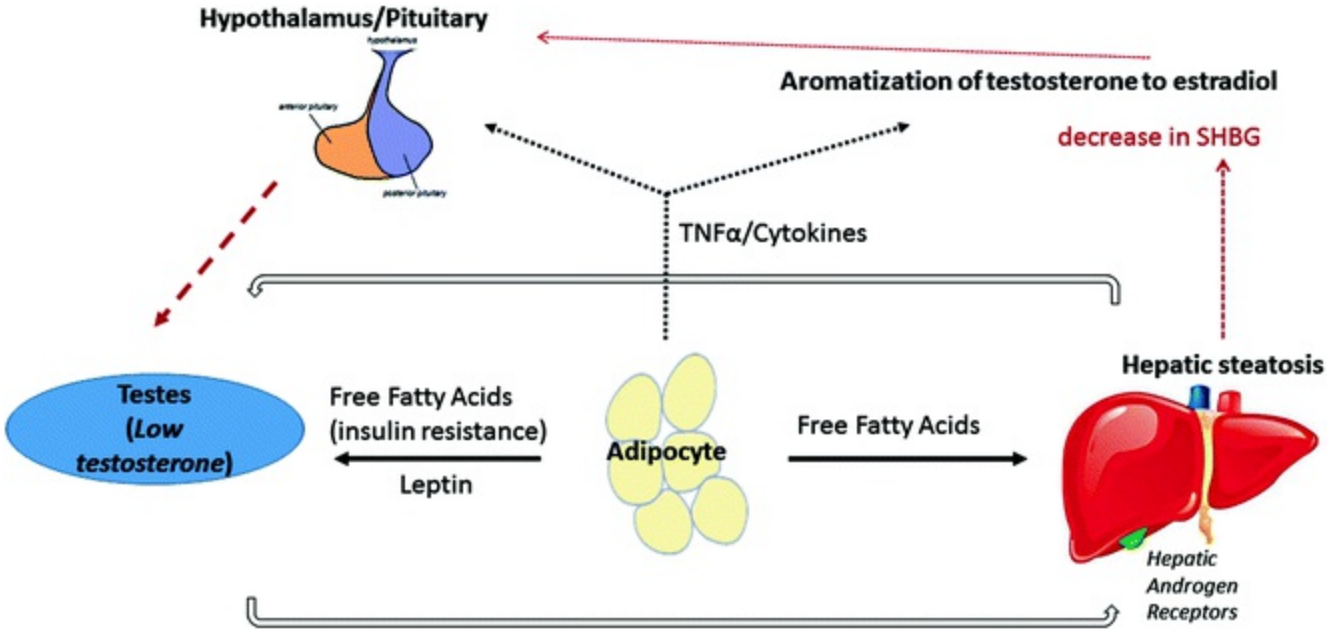

Fig. 11.3 Proposed mechanism of the bidirectional relationship between hypogonadism and nonalcoholic fatty liver disease. Adipose tissue may play a central role in the bidirectional relationship between hypogonadism and nonalcoholic fatty liver disease. Increased free fatty acid secretion results in insulin resistance and subsequently decreased testosterone production. Leptin may also be directly be toxic to the testes. Downregulation of testosterone production may lead to reduced activation of androgen receptors on hepatocytes, further increasing hepatic steatosis. Free fatty acid production from adipocytes can also have a direct steatogenic effect on the liver. Adipose tissues can also

increase cytokine production, leading to central downregulation of the HPG axis and promoting peripheral conversion of testosterone to estrogen [69–72]

One leading hypothesis is that obesity is the predominant driver of hypogonadism in patients with NAFLD/metabolic syndrome. Excess of leptin, an adipokine, in obese men directly inhibits Leydig cell androgenesis. Both subcutaneous and visceral adipose tissue may directly affect gonadotropin release by the pituitary by either increased secretion of cytokines such as TNF-α or IL-2, or by increased conversion of circulating androgens into estrogens, which further contribute to negative feedback. Aromatase is expressed in adipose tissues, and obese patients may have increased expression of the enzyme. Additionally, SHBG, a major testosterone transport protein, is decreased in obese patients, contributing to low total testosterone levels [71] (see Chap. 15).

Another idea is that increased visceral adipose tissue which is partly due to testosterone deficiency increases hepatic exposure to free fatty acids, which can lead to worsening hepatic insulin resistance and systemic insulin resistance [71]. Leydig cell function may also be directly impaired in men with insulin resistance [72]. There is some evidence that testosterone treatment can decrease visceral adiposity and improve insulin sensitivity [73].

There have been a number of studies that suggest that androgen deficiency and low SHBG levels in metabolic syndrome patients may be independently associated with hepatic steatosis. In a large retrospective cohort study of 1912 Korean men, hepatic steatosis was associated with lower serum testosterone levels (14.2 vs. 17.2 nmol/l). When these results were adjusted for confounders such as age, BMI, lipid panel, diabetes, exercise, and high-sensitivity reactive protein (hs-CRP), low serum testosterone still remained associated with hepatic steatosis [70]. Additionally, there have been other studies showing that testosterone replacement in hypogonadal men is followed by reduction in visceral adiposity and circulating TNFα levels [74], and in obese men with obstructive sleep apnea, by reduced liver fat measured by CT scan [75]. Furthermore, low SHBG has been associated with NAFLD, which is likely one of the major reasons for low testosterone. SHBG levels are lower with increasing obesity [76], type 2 diabetes [77], and metabolic syndrome. The TNFα and IL1β increase seen in metabolic syndrome has been shown to reduce SHBG expression in HepG2 cells by decreasing HNF4α (hepatocyte nuclear factor 4-α), which is responsible for activating promoters of multiple genes expressed in liver that function in lipid metabolism [78].

Finally, there is growing research in the relationship between testosterone and microRNAs (miRNAs) in the liver. Some studies suggest that downregulation of miRNAs in liver may ultimately lead to liver diseases, and that testosterone is linked to miRNA regulation in the mouse liver [79]. Specifically, testosterone seems to affect miRNA upregulation of 6 miRNAs: miR-22, miR-690, miR-122, let-7A, miR-30D, and let-7D.

Currently, the treatment for NAFLD is limited with most of the focus on dietary and

lifestyle modification, vitamin E, metformin, and pioglitazone, while testosterone treatment is not routinely used [68]. It is likely that obesity and androgen deficiency are interdependent, and further studies need to address whether treatment of androgen deficiency is helpful in men with hepato-steatosis.

Implications of Hypogonadism in End-Stage Liver Disease

Cirrhosis is defined histologically as a diffuse hepatic process described by fibrosis and conversion of normal liver tissue into abnormal nodules after progression from chronic liver injury. In general, cirrhosis is not a reversible process. Cirrhosis is generally classified into two main stages of compensated and decompensated. Decompensated cirrhosis is characterized by ascites, variceal bleeding, encephalopathy, and hepatorenal syndrome with an estimate of 5–7% yearly progression from the compensated to decompensated stage. Child-Pughs score and MELD-Na which includes INR, bilirubin, creatinine, sodium, and recent dialysis, are the predominant scores used to evaluate for the prognosis of end-stage liver disease in cirrhosis patients [1, 80].

Testosterone levels are generally low in men with advanced liver disease and continue to decline with hepatic decompensation [7]. SHBG levels also decline despite an initial increase in the early course of liver disease [81]. The previous sections focused on the implications of hypogonadism in stable, chronic liver disease, and this section will discuss the relationship between hypogonadism and decompensated liver disease with focus on the implications of hypogonadism in end-stage liver disease. There are data to suggest that low testosterone levels predict mortality in cirrhosis, independent of MELD-Na score, predict prognosis and can be used to prioritize for liver transplantation [82, 83].

In cirrhosis, the phenotypic presentation includes gynecomastia, palmar erythema, hair loss, and poor libido, which is likely due to increase in the estrogen to androgen ratio. Also, the chronic use of spironolactone as a diuretic also functions as an antiandrogen and can increase the level of estradiol [84], further potentiating gynecomastia and impotence. Muscle wasting is common in cirrhosis, which may be partly due to decreased nutrition with decreased appetite in the setting of chronic inflammation. Although muscle wasting is associated with mortality in patients with cirrhosis, it was unrelated to the extent of liver dysfunction by conventional scoring systems, and its relationship with hypogonadism remains unclear. However, low testosterone contributes to muscle wasting through its effects on cell differentiation and myocyte proliferation [85].

Low bone mineral bone density (BMD) is seen in upward of 60% of patients undergoing liver transplant evaluation, with micro-architectural deterioration of bone tissue leading to increased risk of bone fragility and twofold increase in the risk of

fracture [86]. There are multiple hypotheses concerning low BMD including the effect of impaired osteoblast activity from alcohol or iron overload, impaired calcium absorption from hyperbilirubinemia, and low vitamin D levels [87, 88]. The level of liver failure based on Child-Pugh system seems to correlate with the increase in bone loss, while the role of testosterone deficiency needs further investigation.

Up to 80% of patients with cirrhosis have insulin resistance [89]. Since there is a strong relationship between low testosterone levels and insulin resistance in men with diabetes, androgen deficiency may play a role in the development of insulin resistance in cirrhosis.

Testosterone is known to suppress the immune system, reducing macrophage activation and the generation of cytokines such as IL-2 [90], but the data remain controversial in terms of how hypogonadism, liver disease, and the immune system are linked. There has been some interest in the use of testosterone pre-transplant for its immune suppressive effects. Finally, cirrhotic patients report higher levels of psychological distress and general fatigue, which in part may be from androgen deficiency [83].

Thus, low testosterone levels may partly contribute to many of the complications seen in patients with cirrhosis, such as muscle wasting, low BMD, insulin resistance, alterations in immune system, and psychologic changes.

Diagnosis

The diagnosis of hypogonadism in the setting of liver disease can be challenging. Due to elevation in SHBG levels, total testosterone levels may be high despite low free and bioavailable testosterone levels. Alternatively low SHBG levels may result in low total with normal free testosterone levels. Signs and symptoms of hypogonadism, as outlined above, may overlap with those of liver disease and associated comorbidities, further underscoring the need for an accurate biochemical assessment of testosterone.

The 2010 guidelines from the Endocrine Society recommend utilizing total testosterone (TT), sex hormone binding globulin (SHBG), and luteinizing hormone (LH) levels to diagnose hypogonadism. The guidelines further recommend considering free and/or bioavailable testosterone levels in the setting of conditions, associated with elevated SHBG levels, including hepatic cirrhosis and hepatitis [91].

Testosterone Treatment in Liver Disease Patients

The impact of treatment of hypogonadism in liver disease is controversial. The 2010 Endocrine Society guidelines recommend testosterone treatment for symptomatic men with classical androgen deficiency syndromes that is aimed at inducing and maintaining secondary sex characteristics and at improving sexual function, sense of well-being and

bone density [91]. The guidelines do not specifically comment on androgen treatment in the setting of liver disease.

Few trials have examined the effects of testosterone therapy in men with liver disease and are limited by small sample size, study design, and heterogeneous patient groups. In an early study, Wells et al. (1960) randomized men (n = 97) with biopsy-proven cirrhosis on hospital admission, regardless of baseline testosterone levels, to receive prednisolone/corticotropin (n = 27) (20 mg daily of prednisolone for the first 4 weeks followed by 140 mg every 4 weeks beginning week 7, along with long-acting corticotropin every 2 weeks), intramuscular testosterone (n = 26) (100 mg of testosterone propionate on alternate days for the first 4 weeks followed by 300 mg every 2 weeks), or a control group (n = 27) for a total of 14–86 weeks. Both the prednisolone and testosterone groups had an increase in serum albumin levels. Furthermore, mortality occurred in 55% of controls compared to 26% in the prednisone group and 31% in the testosterone group. Overall, these findings were felt at the time to suggest a possible mortality benefit with testosterone therapy. However, interpretation of these results is difficult as treatment duration and patient follow-up were variable and the study was not blinded. Fenster et al. (1966) randomized 32 men with alcoholic cirrhosis to receive intramuscular testosterone versus methenolone versus placebo for 1 month, and found no benefit with treatment [92]. Puliyel et al. in 1978 randomized participants (n = 14 men, n = 17 women) with biopsy-proven cirrhosis of any etiology to receive intramuscular testosterone (n = 12) or standard of care (n = 9) for 4 weeks. At the end of the trial, the testosterone group had a subjective feeling of improvement and an increase in albumin with a decrease in ascites and pedal edema [93]. The size and duration of the study are notable limitations.

A Cochrane review (2006) (n = 499) demonstrated that anabolic steroids (testosterone, oxandrolone, methenolone) got men with alcoholic liver disease (hepatitis and cirrhosis) reduced gynecomastia, but had no effect on sexual function, liver biochemistry, liver histology, complications of liver disease (RR 1.25, 95% CI 0.74–2.10), liver-related mortality (RR 0.83, 95% CI 0.60–1.15), or overall mortality (RR 1.01, 95% CI 0.79–1.29). Testosterone was not associated with an increase in nonserious adverse events (RR 1.14, 95% CI 0.50–2.59) or serious adverse events (RR 4.54, 95% CI 0.57–36.30). There was, however, a trend toward statistical significance with the treatment group reporting three patients with portal or hepatic thrombosis, one with myocardial infarction, and three with polycythemia versus only one with polycythemia in the control group [94]. Overall, however, this study has been criticized in that a high proportion of noncirrhotic subjects with alcoholic hepatitis ceased alcohol consumption during the study, potentially impacting the ability to detect benefits of testosterone treatment.

The Copenhagen Study Group for Liver Disease (1986) performed a double-blind placebo-controlled multicenter trial in which they randomized men to either oral

testosterone 200 mg three times/day (n = 134) or placebo (n = 87) for a median of 28 months. Despite improvement in gynecomastia, no differences were seen in liver biochemistry, histology, or mortality (25%; 95% CI 18–33%) versus placebo (21%; 95% CI, 13–31%). A difference in adverse events could not be excluded. This study has been criticized, as a low testosterone level was not an inclusion criterion, thus potentially impacting the ability to detect benefits of testosterone treatment [95].

Most recently, Yurci et al. (2011) enrolled men (n = 12) with low free testosterone levels with various etiologies of cirrhosis, and treated all participants with 50 mg of testosterone gel for 6 months. Gynecomastia was reduced (subareolar breast tissue 28.83 ± 17.18 mm; 15.00 ± 6.74 mm, $P = 0.007$), while muscle strength, as assessed by hydraulic hand dynamometer, improved (34.03 ± 7.24 kg; 39.18 ± 5.99 kg, $P < 0.001$). Further, although BMD was not significantly improved, the study was not powered for this outcome. No adverse events were seen in this study [96].

Thus, reduction in gynecomastia appears to be the only consistent benefit across existing studies of testosterone therapy in men with liver disease. Although adverse events have not been increased, larger trials are needed to adequately evaluate safety. Concerns regarding testosterone utilization specific to a population with liver disease have focused on the risk of hepatocellular carcinoma. Small cohort and retrospective studies have shown an association between higher endogenous testosterone levels and HCC [64, 97]. In a Taiwanese study of men without cirrhosis, free testosterone was associated with HCC, but in multivariable analysis, independent associations with estradiol and SHBG were not seen [64]. A study in a Japanese population suggested that an elevated estradiol/testosterone ratio was associated with increased risk of HCC [97]. Low endogenous testosterone levels have also been associated with higher rates of hepatocellular carcinoma in a largely Caucasian cohort of men [97], with low testosterone hypothesized to reflect increased severity of liver disease, rather than an involvement in tumorigenesis [7]. Other studies have not demonstrated a link between testosterone levels and hepatocellular carcinoma [67]. In terms of men being treated with testosterone, reports of hepatocellular effects are limited to oral oxymetholone, which is no longer approved for use due to its established hepatotoxicity [98].

Liver transplantation results in changes to the HPG axis (affected by pre-transplant metabolic insults, immunosuppression and graft function) and protein synthesis, which can in turn affect recovery from hypogonadism. Total and free testosterone levels may be reduced beyond pre-transplant levels for up to one month after surgery, felt to be due at least in part to high-dose immunosuppressive therapy [12, 99, 100]. More than one-third of men after liver transplant do not normalize testosterone levels, many with persistent evidence of hypergonadotropic hypogonadism [12, 101]. Increase in gonadotropin output, notably LH, has further been described as a key factor in normalization of free testosterone levels in the first year after surgery. Hyperprolactinemia also resolves in the majority of patients, which is felt to be due to a

combination of changes in endocrine-metabolic function and the suppressive effect of calcineurin inhibitors on prolactin gene expression [12, 102, 103]. Tacrolimus, like cyclosporine, may have a direct toxic effect on Leydig cells [104, 105]. Furthermore, glucocorticoids are known suppress GnRH and inhibit the synthesis of adrenal androgens.

Conclusions

In conclusion, hypogonadism in the setting of liver disease is common, and its etiology is multifactorial. In general, chronic liver disease is associated with central hypogonadism, though exceptions exist, most commonly in alcoholic liver disease because ethanol has a direct toxic effect on the testes. In NAFLD and NASH, low testosterone may be explained by low SHBG. The role of testosterone treatment in hypogonadal men with chronic liver disease remains controversial in the absence of reliable safety data, although adverse events have not been increased in smaller studies. Despite early and limited data on the possible beneficial effect of testosterone in improvement of morbidity and mortality in liver disease, taken in sum, testosterone treatment is associated only with a possible improvement in gynecomastia. Hypogonadism may be reversible after liver transplantation. Well-designed and adequately powered studies are needed to understand the consequences of hypogonadism in liver disease, as well as efficacy and safety of treatment strategies.

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12. Male Hypogonadism Due to Cancer and Cancer Treatments

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Introduction

Survival rates after cancer treatment have increased dramatically in recent decades, resulting in an increasing focus on the harmful effects of cancer treatment for these patients [1]. One of the major long-term effects of cancer and its treatment is compromised reproductive function in both males and females [2]. These effects may occur as a result of direct effects on the gonads or indirect effects via damage to the hypothalamus or pituitary. In males, there may be impairment of testicular function prior to the commencement of treatment while exposure to cytotoxic therapies may result in damage to the seminiferous epithelium leading to oligo- or azoospermia [3]. In addition to effects on the germinal epithelium there may also be damage to the Leydig cells, resulting in impaired testosterone production [4].

The precise nature of the damage, and the potential for recovery, is determined by a number of factors which include intrinsic factors, such as the genetic factors that modulate the sensitivity of an individual's germinal epithelium, and the cancer diagnosis, and extrinsic factors such as the specific treatment regimen in terms of the agent, dosage and frequency [2, 5–11]. The state of testicular development, between prepubertal and adult, may also influence the effects of cancer treatment on subsequent development and function [11]. Age and developmental stage also determine the options available to preserve reproductive potential in these patients [2].

This chapter will describe the effects of cancer and its treatments on male reproductive function in terms of damage to the seminiferous epithelium and testosterone production. We will also discuss the options, both established and experimental, for fertility preservation in these patients.

Pre-treatment Testicular Dysfunction

Alterations in germinal epithelial function have been demonstrated in patients with cancer before any treatment is commenced. This is particularly apparent in men with testicular cancer and Hodgkin's Disease (HD). Because of severe debilitation or impotence, not all men are able to provide semen for cryopreservation prior to treatment. As a result many of the studies relating to germinal epithelial function prior to treatment examined only men who were able to produce a semen sample, and therefore may have selected a slightly healthier cohort leading to an underestimation of the extent of gonadal dysfunction pre-treatment.

Hodgkin's Disease

The literature describes varying degrees of semen quality impairment in HD patients prior to treatment. Some studies found a high degree of impaired spermatogenesis based on semen analysis [12–15] while others found normozoospermia in more than 50% of HD patients [16–21]. Pubertal patients with HD have significantly lower mean inhibin

B levels and higher mean FSH and LH levels than controls, which is indicative of spermatogenetic dysfunction among the paediatric HD population [22]. The pathogenesis of HD-associated dyspermia is not clearly understood. Studies incorporating testicular biopsies have failed to find any evidence for direct tumour involvement in the testis [12, 23]. Poor semen quality has been attributed to hypothalamic dysfunction, increased catabolism, malnutrition and immunological imbalances, affecting the lymphocytic cell and cytokine profiles (interleukin and tumour necrosis factor) [24].

Testicular Cancer

Testicular cancer is also associated with oligozoospermia in over 50% of men before treatment [25, 26]. In patients with unilateral testicular cancer, irreversibly impaired spermatogenesis in the contralateral testis has been demonstrated in up to 24% of patients [27]. This observation supports the concept of a "testicular dysgenesis syndrome" (TDS) comprising low sperm counts, hypospadias, cryptorchidism and testicular cancer, and is thought to be related to foetal development complications [28] (see Chap. 14). Accordingly, the prevalence of testicular cancer among men with an abnormal semen analysis has been calculated to be 20 times higher than in the normal population [29]. Other possible causes of dyspermia include local tumour effects, to which elevation of scrotal temperature, alterations in testicular blood flow, and disruption of the blood–testis barrier may contribute. Furthermore, sperm antibodies have been found in a high proportion of patients with testicular cancer [30]. Finally, testicular cancer may be hormonally active, and local hormone production may impair spermatogenesis.

Pediatric Malignancies

The preliminary histological findings of testicular biopsies obtained from prepubertal males before initiation of gonadotoxic therapy suggest possible abnormal germ cell maturation [31]. Testicular biopsies were cryopreserved for experimental fertility preservation for 29 patients with solid tumours and for 5 patients who underwent hematopoietic stem cell transplantation (HSCT) for benign disease. A total of 27 patients had adequate tissue for histological analysis. In total, 22 children (81% of patients) had normal and 5 (19%) had increased numbers of germ cells per tubule for their age. However, 19% (5/26) of boys older than 6 months had no evidence of adult dark spermatogonia and 56% (9/16) of those older than 6 years had no evidence of primary spermatocytes on biopsy, which would be expected based on age norms [32–34]. These observational studies suggest maturation delay of spermatogenesis. If boys with serious childhood diseases truly have delayed maturation in spermatogenesis, this may affect the fertility potential of testicular tissue cryopreserved for fertility

preservation. Further investigation regarding the impact of these preliminary histological findings is warranted.

Cancer Treatment and Gonadal Function

Cancer treatment may result in damage to the gonad either as a result of direct effects on the gonad, or indirectly from damage to the hypothalamic-pituitary unit with resulting downstream effects on gonadal function and fertility (Fig. 12.1).

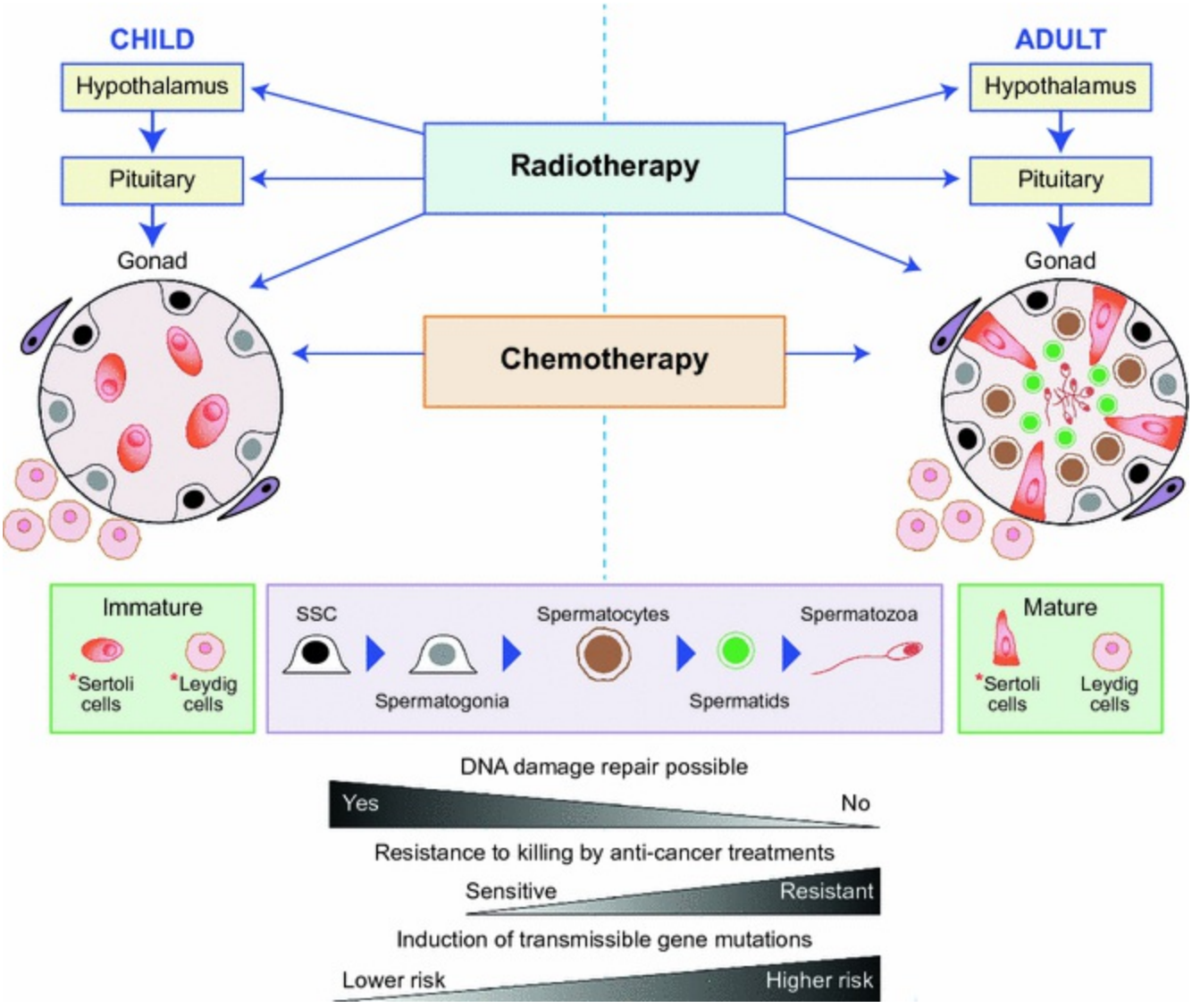

Fig. 12.1 Effect of cytotoxic therapies on the hypothalamic–pituitary–testicular axis in children and adults. Differences in hormonal profile, cellular composition and cellular maturity can result in differential effects of cancer treatment in children compared with adults. SSC—spermatogonial stem cell. * Insufficient evidence to determine effects of cancer treatment

Cancer Treatment and Hypothalamic-Pituitary Function

Following cranial irradiation, gonadotropin deficiency is the second most common pituitary hormone abnormality after growth hormone deficiency. Although in the short term, doses of 30–50 Gy may result in precocious puberty (more frequently in young girls than boys), and doses of at least 30 Gy may result in true gonadotropin deficiency in the longer term [35]. In a study of 45 children treated with cranial radiation, severe gonadotropin deficiency was observed in 11% of cases, resulting in lack of, or slow progression of puberty, with a reduced gonadotropin response to GnRH [36]. Studies in post-pubertal patients have demonstrated that in some the serum levels of testosterone are reduced, and LH and FSH levels may be low. However, demonstration of a preserved pituitary response to GnRH [37] and growth hormone-releasing hormone [38] following cranial irradiation suggests initial hypothalamic hypogonadism, with pituitary insufficiency occurring later.

Cancer Treatment and Spermatogenesis

Chemotherapy-Induced Damage to the Germinal Epithelium

Testicular cells, especially rapidly dividing germ cells, are highly sensitive to cytotoxic treatment (Fig. 12.2). Low doses of these treatments deplete the pool of differentiating spermatogonia, while reserve spermatogonial stem cells (SSCs) survive, and spermatocytes and spermatids continue their maturation into sperm [39] (Fig. 12.2). Testicular involution after such gonadotoxic damage is a slow process that among sexually mature men occurs over several weeks until temporary or permanent azoospermia ensues.

Risk of Infertility	Chemotherapy Agent (cumulative dose for prolonged azoospermia)	Mechanism of action
High or Moderate	**Alkylating agents** Cyclophosphamide (19g/m^2) Busulphan (600g/m^2) Melphalan (140g/m^2) Ifosfamide (60g/m^2) Procarbazine (4g/m^2) BCNU (carmustine) CCNU (lomustine) **Platinum agents** Cis-platinum (600g/m^2) Carboplatin (2g/m^2)	Direct DNA or RNA damage Induction of Apoptosis
Low	**Antimetabolites** Methotrexate Mercaptopurine Cytarabine **Vinca Alkaloids** Vincristine Vinblastine **Antitumour Antibiotics** Bleomycin Dactinomycin Doxirubicin **Podophyllotoxins** Etoposide **Other** Asparaginase	Inhibition of DNA or RNA Synthesis Inhibition of Mitosis Deamination of Proteins Introduction of DNA Strand breaks (antimetabolites)

Fig. 12.2 Risk of infertility based on the prospect of prolonged azoospermia according to cumulative dose of chemotherapeutic agent exposure [40, 41], Busulfan: [42], Ifosfamide: [43], Carbo: [44], Procarbazine: [45], Cis-platinum: [46, 47]

Recovery of sperm production after a cytotoxic insult in puberty or adulthood depends on the ability of mitotically quiescent SSCs to survive and resume mitotic activity and to produce differentiating spermatogonia. If the damage is severe (e.g. as a result of a high cumulative dose of alkylating agents), all SSCs may commit to apoptosis and the patient becomes permanently infertile. Spermatogonia have been shown to be similarly susceptible to such depletion at all stages of life.

Alkylating agents, including cyclophosphamide and the combination of mechlorethamine and procarbazine, have been associated with increased risk of impaired spermatogenesis [48, 49]. Agents with the potential to cause impaired spermatogenesis include also ifosfamide at doses >60 g/m^2 [43, 50–52]. Higher doses have greater risks compared to lower doses [48, 49, 53]. The threshold dose of cyclophosphamide, in relation to infertility, has been shown to be between 7.5 and 10 g/m^2 [10, 54, 55]. Recent observations suggest that slow recovery of spermatogenesis may occur even after these high doses, and permanent sterility may appear after 19–20 g/m^2 of cyclophosphamide [40, 41]. Similar threshold doses for

incipient testicular failure have also been reported for other alkylating agents [41, 45, 55, 56], carboplatin and cisplatin [5, 44]. However, a recent large study of non-irradiated childhood cancer survivors failed to identify any threshold dose for alkylating agent exposure that predicted impaired spermatogenesis or azoospermia after a median follow-up of 21 years [53]. There may be other factors, in addition to absolute doses and regimen, such as genetic variation in drug metabolising pathways, which modulate the impact of alkylating agent exposure on spermatogenesis or its recovery [53].

Spermatogenetic Failure After Treatment of Haematological Malignancy

At diagnosis, between 40 and 70% of children with acute lymphoblastic leukaemia (ALL) and 50% of those with acute myeloid leukaemia (AML) are considered to be standard risk, and as a result will receive treatment that combines chemotherapy agents from a number of different classes [57]. These regimens typically involve the use of antimetabolite or vinca alkaloid chemotherapy which inhibits DNA and RNA synthesis and mitosis. Evaluation of testicular biopsies of ALL patients immediately following termination of therapy has revealed that 40% exhibit severe impairment of the tubular fertility index (with less than 40% of their seminiferous tubules containing spermatogonia; [58]). This index tends to improve for most patients as more time elapses after treatment [58, 59]. Long-term follow-up of childhood leukaemia survivors revealed that treatment with these cell cycle-specific cytotoxic drugs, without a high dose of alkylating agent cyclophosphamide, does not totally deplete SSCs, and that spermatogenesis is reinitiated from the surviving reserve stem cell population [40, 60].

Gonadotoxicity following exposure to the ABVD (doxorubicin, vinblastine, dacarbazine and bleomycin) protocol, without a high dose of alkylating agent for HD, is relatively mild with 90% of adult patients having normal gonadotropin levels and sperm counts 12 months after therapy [19, 61]. However, more than 3 courses of alkylating agent (cyclophosphamide, nitrogen mustard, or procarbazine)-based chemotherapy for HD is known to cause azoospermia or result in inhibin B and FSH levels consistent with oligozoospermia in 70–90% of patients [21, 49, 61–63]. Standard-risk non-Hodgkin lymphoma (NHL) patients who are treated with chemotherapy including antimetabolites, vinca alkaloids and low-dose alkylating agents have a low risk of developing testicular insufficiency similar to that of ALL patients.

Patients initially classified as standard-risk leukaemia or NHL may be re-assigned to a more gonadotoxic treatment regimen in the case of treatment failure/relapse. 30–40% of patients with acute leukaemia carry high-risk features. To be cured, these patients require treatment with allogeneic HSCT. Conditioning for HSCT is associated with a significant risk of germ cell failure with azoospermia in 85% of men and

oligozoospermia in the remainder [64–66]. Germ cell failure with raised serum levels of FSH and decreased testicular growth in puberty is observed among most of the male patients irrespective of the type of conditioning therapy [65, 67]. The probability for recovery of spermatogenesis after HSCT is associated with the type of conditioning therapy, age of the patient, time interval since transplantation and absence of chronic graft versus host disease [65, 66]. One-third of adult HSCT patients receiving high-dose cyclophosphamide treatment had sperm in the ejaculate after a recovery period of 1 year, while those patients receiving cyclophosphamide combined with busulfan or thiotepa showed first recovery after 3 years. Following total body irradiation (TBI), recovery of spermatogenesis never occurred before the 4th year after transplantation [65]. Similar findings have recently been reported in the paediatric population. HSCT conditioning with busulfan or cyclophosphamide was associated with a larger adult testicular volume, lower serum levels of FSH and the more frequent presence of spermatozoa compared to those conditioned with TBI [68].

Spermatogenetic Failure After Testicular Cancer

The other group of patients in whom the effects of chemotherapy on testicular function have been widely investigated is those with testicular cancer [44, 69–71]. To attempt to delineate which abnormalities are a result of cytotoxic chemotherapy, several of these studies also examined pre-treatment testicular function, or compared chemotherapy-treated patients with those who underwent orchidectomy alone [69–71]. All have demonstrated greater testicular dysfunction in the cytotoxic-treated groups, with evidence of germinal epithelial damage indicated by raised FSH levels and/or reduced sperm counts. Lampe and colleagues [44] analysed data from 170 patients with testicular germ cell cancers who underwent treatment with either cisplatin- or carboplatin-based chemotherapy. 40 (24%) were azoospermic pre-treatment, and a further 41 (24%) were oligozoospermic. For a median of 30 months after the completion of chemotherapy, only 64% of those who were normospermic before therapy remained normospermic, whilst 54 (32%) of the total cohort were azoospermic and 43 (25%) were oligozoospermic. The probability of recovery to a normal sperm count was found to be higher for men with a normal pre-treatment sperm count, those who received carboplatin- rather than cisplatin-based therapy, and those treated with less than five cycles of chemotherapy. Brydøy and colleagues [72] reported that the paternity rate, by natural conception or using assisted reproductive techniques (ART), when sperm reappeared in their semen, was approximately 10% at 5 years in the patient group having received high-dose cisplatin (total cisplatin dose >850 mg), and about 35% in the group with low-dose cisplatin (total cisplatin dose ≤850 mg). However, even in the high-dose cisplatin group, the paternity rate increased to 85% after 15 years post-treatment. These data showed that the time necessary for the restoration of

spermatogenesis in order to father children was as long as 5–10 years or more.

Irradiation-Induced Damage to Germinal Epithelium

The germinal epithelium is very susceptible to irradiation-induced damage (Fig. 12.3). This depends on the field of treatment, total dose and fractionation [73, 74]. The progenitor and differentiating spermatogonia are radiosensitive to scattered doses as low as 0.1 Gy leading to short-term cessation of spermatogenesis [9]. Doses of 2–3 Gy also affect SSCs and cause long-term azoospermia [9, 75–77]. Doses in excess of 6 Gy deplete the SSC pool and lead to permanent infertility [9, 78].

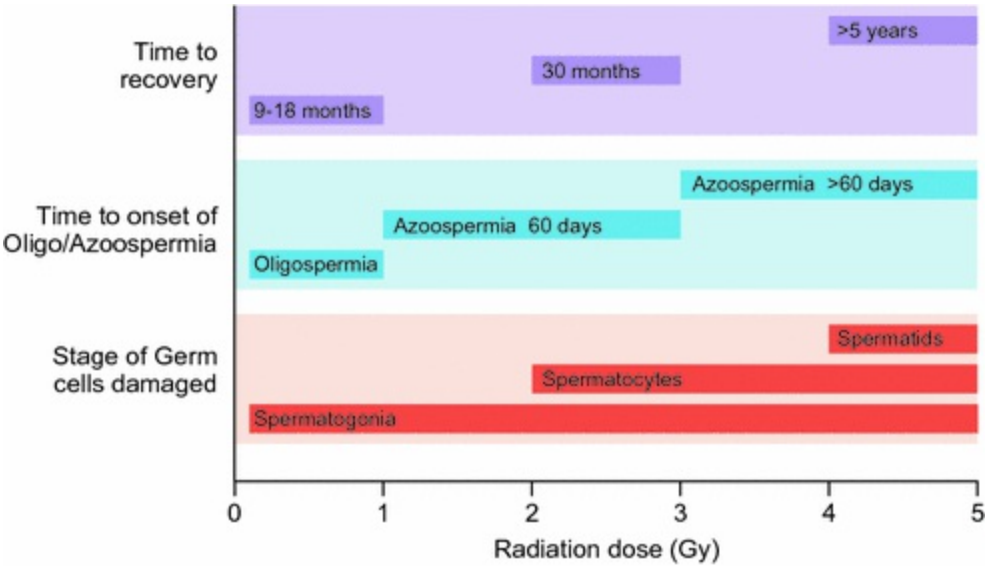

Fig. 12.3 Dose-dependent effects on spermatogenesis following single-dose irradiation in adults

TBI, as conditioning for haematological stem cell transplantation, is associated with significant germ cell failure [64]. Following treatment with TBI (10 or 13 Gy), azoospermia was found in 85% of men, and oligozoospermia occurred in the remainder [65]. Following TBI, recovery of spermatogenesis never occurred before the 4th year after transplantation; therefore, azoospermia after hematopoietic stem cell transplantation may be overestimated if semen samples are evaluated too soon after transplantation [65]. Fractionation of radiotherapy has been shown to increase germ cell toxicity possibly because of repeated hits to activated reserve stem cells [78–80]. However, a long-term follow-up study of 106 male survivors after paediatric allogeneic HSCT does not support this notion [68]. The mean adult testicular volume of 6 mL after single-fraction TBI was significantly smaller than the 11 mL after fractionated TBI. Lower serum levels of FSH after fractionated TBI further suggest that fractionation may slightly decrease the detrimental effect of irradiation on the testis [68].

Whilst most of the literature concerning radiotherapy effects on testicular function concerns the use of traditional radiotherapy techniques, recent technological advances

have provided new methods for delivering radiotherapy which result in more localised treatment with less scatter dose [81]. These improvements will likely reduce damage to the testis for many treatments targeting radiation to the pelvic region without delivering radiotherapy to the gonad.

Cancer Treatment and Leydig Cell Function

While effects on the seminiferous epithelium are manifest by impacts on spermatogenesis and fertility, Leydig cell dysfunction may manifest as raised LH levels with normal or low–normal testosterone levels. Such Leydig cell effects are usually associated with much higher doses of radiotherapy/chemotherapy than those which damage seminiferous epithelium.

Chemotherapy and Leydig Cell Function

Evidence for Leydig cell dysfunction following chemotherapy has been described in several studies [4, 42, 44, 82, 83]. In a study of 209 men with a variety of haematological malignancies (including 160 with HD) treated with either MVPP or ChlVPP/EVA hybrid, LH levels were higher in patients compared with a cohort of age-matched controls (mean LH 7.9 versus 4.1 IU/l) [4]. Increased LH levels suggest pituitary compensation to a reduction in testosterone production causing reduced hypothalamic-pituitary negative feedback. Testosterone may still remain within the reference range as mild Leydig cell dysfunction may be reflected by raised LH together with low–normal to normal testosterone levels. This combination was found in 67 (32%) following chemotherapy, compared with 2 (4%) of healthy controls. A further 12 (6%) of the chemotherapy-treated patients had a raised LH level alone [4]. These results suggest that a significant proportion of men treated with cytotoxic chemotherapy have biochemical abnormalities indicative of compensated Leydig cell dysfunction, although recent evidence suggests that there is probably no increased risk for frankly subnormal levels of testosterone after treatment with alkylating agents, [40, 43, 50, 52, 67] or cisplatin [47].

Testicular function following high-dose chemotherapy used as preparation for bone marrow transplantation has also been studied. In a study of 155 men treated with cyclophosphamide (200 mg/kg) or busulphan and cyclophosphamide (busulphan 16 mg/kg, cyclophosphamide 200 mg/kg) [42], 67 of 109 who received cyclophosphamide (61%), but only 8 of 46 (17%) patients treated with busulphan and cyclophosphamide, had recovery of testicular function defined by normal LH, FSH and testosterone levels with evidence of sperm production, after an average of 2–3 years post-transplantation. The only prospective study to examine testicular function following high-dose treatment reported data in 13 men who received either BEAM

(BCNU, etoposide, Ara-C and melphalan) (n = 11) or melphalan and single-fraction TBI (n = 2) [82]. All had previously received multi-agent chemotherapy and four had abnormal semen parameters before transplantation. All patients were azoospermic 2–3 months post-transplantation with raised FSH levels. LH levels increased and testosterone levels decreased after transplantation indicating Leydig cell dysfunction in addition to germ cell failure. The association between Leydig cell dysfunction and germinal epithelial function was clearly illustrated in a study of 68 patients treated with high-dose chemotherapy (either cyclophosphamide, BCNU and etoposide, busulphan and cyclophosphamide or BCNU, etoposide, doxorubicin and melphalan) as conditioning for bone marrow transplantation. They demonstrated that the majority of those with raised FSH (88% of the patients) also had raised LH level (69% of the patients). In addition, none of the 13 patients with a normal FSH level had an increased LH level. Although this is not surprising given the relative susceptibilities of the Leydig cells and the germinal epithelium to damage, it leaves open the possibility that germinal cell dysfunction plays a role in Leydig cell insufficiency [4]. There was also evidence of recovery of Leydig cell function with a gradual recovery of germinal cell function during the first 10 years after chemotherapy which further indicates that germinal epithelial dysfunction may be etiologically important in the occurrence of Leydig cell insufficiency [4].

In men with testicular cancer, there are several important considerations regarding the effect of treatment on gonadal function. These men are generally young adults in the age range where preservations of reproductive function and fertility are particularly important. In addition to the potential effects of cytotoxic treatment, these men are likely to have significant pre-existing testicular abnormalities (e.g. cryptorchidism, germinal aplasia, contralateral malignant/pre-malignant cells) that may be compounded by treatment. Separating the testicular effects of cancer treatment from pre-existing testicular dysfunction is challenging [44, 69–71, 83]. In an attempt to determine which abnormalities result from cytotoxic chemotherapy, several of these studies also examined pre-treatment testicular function, or compared chemotherapy-treated patients with those who underwent orchidectomy alone. In a study of 170 patients with testicular germ cell cancers treated with cisplatin- or carboplatin-based chemotherapy, compensated Leydig cell dysfunction, as indicated by a raised LH level in the presence of a normal testosterone level, was found in 59–75% of men following chemotherapy, compared with 6–45% in those following unilateral orchidectomy alone [44]. An age-adjusted increase in hypogonadism, defined as LH >12 IU/L or testosterone <8 nmol/L, has also been described in a cohort of 1183 patients with testicular cancer with odds ratios ranging from 3.5 to 7.9 depending on treatment regimen [83].

Radiotherapy and Leydig Cell Function

The testis is one of the most radiosensitive tissues, with very low doses of radiation causing significant impairment of function. Damage may be caused during direct irradiation of the testis or, more commonly, from scattered radiation during treatment directed at adjacent tissues. As previously described, radiotherapy effects on gonadal function depend on the dose, treatment field and fractionation schedule [84]. While relatively low doses may result in damage to the seminiferous epithelium and oligozoospermia [85], much higher doses (>20 Gy) appear to be required to cause Leydig cell dysfunction. However, significant rises in LH have been demonstrated following single-radiation doses above 0.75 Gy [9] and fractionated doses above 2 Gy [77]. No change in testosterone levels was seen at these doses, indicating compensated Leydig cell damage and LH values gradually return to normal levels over 30 months.

Radiotherapy doses delivered directly to the gonad are often in the range of 20–24 Gy which result in eradication of germ cells [86] causing permanent azoospermia [87]. Such radiation doses delivered to the testis have also been shown to result in Leydig cell insufficiency [88], whilst studies involving lower radiation doses have not resulted in testosterone deficiency [9, 75]. In a study of 20 men, previously treated with unilateral orchidectomy for testicular cancer, who received direct testicular irradiation at a dose of 20 Gy in 10 fractions for pre-malignant changes in the remaining testis, there was a significant increase in mean LH levels within the first three months (10.4–15.6 IU/L), with a decrease in mean serum testosterone levels (13.3–10.8 nmol/L) [89]. Similar results were observed in adults treated with high-dose (30 Gy) testicular irradiation following unilateral orchidectomy. Serum testosterone levels were significantly reduced (12.5 versus 16.0 nmol/L), and LH levels were significantly increased (6 versus 16 IU/L) compared with a control group who had undergone unilateral orchidectomy without subsequent radiotherapy [11]. This study also highlighted the potential importance of age in determining the extent of Leydig cell dysfunction. In the five adult men treated with the same testicular dose of irradiation during childhood, the effect on Leydig cell function was more dramatic. The median LH levels was >32 IU/L and the median testosterone level was <2.5 nmol/L, and there was no response to hCG stimulation, suggesting that the prepubertal testis is much more vulnerable to radiation-induced Leydig cell damage [11]. Several other studies have demonstrated Leydig cell dysfunction for male survivors of childhood cancer receiving radiotherapy, including direct radiotherapy to the testis and total body irradiation [40, 67, 68, 90, 91]. Differences in Leydig cell sensitivity between those treated in childhood or in adulthood may be due to important differences in the Leydig cell populations between children and adults. The adult Leydig cell population is not present in prepuberty; however, studies in rats indicate that the stem cell progenitors that give rise to the adult Leydig cell population are present in the prepubertal testis [92]. Sensitivity of these two populations to cytotoxic therapy may decrease from prepuberty to adulthood.

A study using radiotherapy to impair Leydig cell function in 7 men with prostate cancer demonstrated that even with relatively high doses of radiotherapy (17–24 Gy in 2–3 fractions), Leydig cell function is maintained over 3 years of follow-up [93]. However, the results of these studies should be considered in the context of the patient population, namely aged men, with pre-existing pathology/treatment and relatively low pre-radiotherapy testosterone levels.

Clinical Impact of Impaired Leydig Cell Function

While a substantial proportion of men treated for cancer have evidence of mildly impaired Leydig cell function, as indicated by raised LH and low/subnormal testosterone levels, the clinical impact of this effect is not entirely clear. For men with true hypogonadism, there is a clear benefit of androgen replacement on sexual function, bone density, body composition and quality of life [94, 95]; however, there are limited data in patients with cancer. In a cohort of 203 men treated with either MVPP, ChlVPP/EVA hybrid or high-dose chemotherapy for a variety of malignancies, 32% were identified with biochemical evidence of mild Leydig cell insufficiency as defined by a raised LH and a testosterone level in the lower half of the normal range, or frankly subnormal [4]. They had a significantly reduced bone mineral density at the hip compared with a similarly treated cohort with normal hormone levels, with some evidence of altered body composition, reduced sexual activity and alterations in mood [96, 97]. The men were then enrolled into a 12-month randomised, single-blind placebo-controlled trial of testosterone replacement [98]. During the 12-month study period, however, there was no significant improvement in bone density, body composition, sexual function, energy levels or mood in the testosterone-treated group compared with the controls.

Thus, based on the evidence available, the mild biochemical abnormalities (raised LH, low/normal testosterone) observed in many men following cytotoxic chemotherapy are of limited clinical importance in the vast majority of patients, and androgen replacement cannot be routinely recommended for such patients. However, it remains possible that a minority of men with more marked biochemical abnormalities may benefit from androgen therapy. More studies are needed.

Genetic Damage Following Cytotoxic Therapy

In addition to impairment of steroidogenesis and sperm production, there has been concern that cytotoxic chemotherapy may also result in transmissible genetic damage. Animal studies have demonstrated untoward effects in the offspring of animals treated with cytotoxic agents, but no clear evidence for this has been reported in humans. Increased aneuploidy frequency has been observed in human sperm following

chemotherapy for HD [99, 100] and an increase in chromosomal abnormalities has been demonstrated several years after treatment for testicular cancer [101], while a study of men who had been treated for cancer in childhood showed that their sperm carried as much healthy DNA as controls [102]. A study of 25 childhood cancer survivors, their healthy partners and 43 offspring also revealed no increased genomic instability in the latter [103]. The majority of epidemiological studies concerning the outcome of pregnancies have not shown any increase in genetically mediated birth defects, altered sex ratios or birthweight effects in the offspring of cancer survivors [104–106]; however, one study involving 8162 children with a paternal history of cancer from a cohort of almost 2 million children, demonstrated a modest increase (Relative Risk −1.17) in the risk of congenital abnormalities [107]. Given the evidence thus far, it is reasonable to conclude that patients treated with cytotoxic therapy who remain fertile are at no/low increased risk of fathering children with genetic abnormalities.

Fertility Preservation for Males Undergoing Cancer Treatment

Semen Cryopreservation and Assisted Reproduction

At present the only established method for preserving reproductive potential in patients undergoing potentially sterilising therapy is cryopreservation of semen for use in ART such as intracytoplasmic sperm injection (ICSI). This should be offered to all men in whom their treatment has a risk of subsequent infertility, and the British Fertility Society recommends that all post-pubertal patients requiring treatment for cancer should be offered sperm banking. A semen sample is most frequently obtained by masturbation, although penile vibratory stimulation or electrostimulation can also be performed under anaesthetic; however, the motility and sperm count may be lower when the latter method is used [108]. Epididymal aspiration and testicular biopsy have also been used to obtain gametes for cryostorage; however, the risk of compromising testicular function questions the suitability of the latter technique. In the UK, semen cryopreservation is governed by the Human Fertilisation and Embryology Act. This legislation stipulates that the individual must understand the implications of what is proposed and that written, informed consent must be obtained prior to semen cryopreservation [109].

Despite recommendations, the option of sperm banking is not always offered. This may be due to issues such as cost, lack of facilities and a poor prognosis from the underlying condition [110]. Typical rates for storage range between 40 and 70% [111], with lack of information often cited as the most common reason for failing to bank [112]. In a study of 902 men with HD, 40% cryopreserved semen before the start of treatment at the median age of 31 years. Three hundred thirty four men wanted to have a child during the median follow-up of 13 years (range 5–36). Ninety nine of 128 (77%) men with no spontaneous success had cryopreserved semen. Altogether, 48 of 78 (62%)

men who used the cryopreserved sperm had success with ART and had at least one child. Patients treated with alkylating- or second-line chemotherapy were more likely to subsequently use cryopreserved sperm. The availability of cryopreserved semen doubled the odds of post-treatment fatherhood [113].

Despite the success of semen cryopreservation as an option to males undergoing potentially gonadotoxic therapy, there are important limitations to its utility for certain patient groups. Men with malignancies may have impaired testicular function prior to treatment [114]. There may be difficulty producing a semen sample, and even when a sample can be produced, there may be oligozoospermia and/or reduced sperm motility. Oligozoospermia is found in a third to a half of patients with HD, non-Hodgkin's lymphoma and testicular cancer pre-treatment, and also in men with leukaemia or soft tissue cancers [17, 115]. Whilst successful fertilisation may be achieved with only a few viable sperm using ICSI, pregnancy rates using this method are lower with abnormal than with normal semen [116]. Another important patient group is children, who despite the relative quiescence of the hypothalamic–pituitary–gonadal axis, are still at significant risk of infertility as a result of cytotoxic therapies [3]. Therefore, alternative strategies to preserve or restore fertility in young patients are currently under investigation, although they remain experimental.

Hormonal Therapy for Fertility Preservation

Hormonal suppression of testicular activity has been postulated as a potential strategy for the preservation of fertility in patients treated for cancer (for detailed review see [3]). This is based on the hypothesis that suppression of the HPG axis prior to cytotoxic therapy would protect the gonad from cytotoxic damage, a concept based largely on a belief that the prepubertal testis is somewhat protected from permanent chemotherapy-induced gonadal damage [10]. In fact, the prepubertal testis is susceptible to damage from cytotoxic therapy and germ cell proliferation has been shown to occur in the prepubertal human and non-human primate testis [117, 118]. On the other hand, numerous studies in rodents (reviewed in [119]) have demonstrated protection of spermatogenesis when hormonal suppression is commenced using agents such as sex steroids, GnRH antagonists or analogues before or during treatment. It has even been shown that recovery of spermatogenesis can be enhanced when hormonal suppression is induced after treatment. However, differences in hormonal and spermatogenetic regulation in rodents and primates may account for the failure to translate this strategy successfully into primates, including humans (see below).

Treatment of adult rats for 6 weeks with testosterone and estradiol, prior to irradiation or procarbazine treatment, resulted in a higher germ cell repopulation index and sperm head count compared to rats receiving irradiation alone [120–122]. Short-term (2 weeks) treatment of rats with a GnRH analogue prior to procarbazine treatment

also resulted in a strain-dependant increase in stem cell index and subsequent recovery of sperm counts close to normal values and significantly higher than procarbazine-only treated rats at 90 days [123, 124].

GnRH analogues have also been shown to stimulate recovery of spermatogenesis following irradiation or procarbazine treatment in adult rats [125–128]. The use of a depot formulation of the GnRH analogue, goserelin, in irradiated rats receiving 3.5 Gy, resulted in a 91% repopulation index compared to 31% in the controls at 10-week post-irradiation [126]. Similar results were obtained for treatment with GnRH analogues following procarbazine treatment [127]. GnRH analogues administered to rats up to 15–20 weeks after irradiation can also result in increased numbers of tubules containing differentiating germ cells derived from surviving stem cells 6 weeks later [127, 128]. These results, along with studies demonstrating no cytoprotective effect from chemotherapy or irradiation in a mouse strain with congenital complete gonadotropin deficiency [129], suggest that the mechanism for restoring spermatogenesis is not due to suppressing germ cell activity during cytotoxic treatment. Instead, studies have suggested that the mechanism may involve a reduction in the high levels of intra-testicular testosterone caused by cytotoxic treatment [130] or release of germ cells from the block on differentiation [131]. Transplantation studies have indicated that the block in spermatogonia differentiation following irradiation may be due to damage to somatic cells rather than germ cells [132].

Attempts to reproduce the beneficial effects of GnRH analogues/antagonists in rodents on radiation- induced cytotoxicity in non-human primates have been unsuccessful. For example, the GnRH antagonist cetrorelix failed to increase the proportion of seminiferous tubule cross sections containing germ cells in rhesus monkeys subjected to testicular irradiation [133, 134].

Studies using hormonal manipulations in humans have also failed to demonstrate preservation or restoration of fertility in patients treated for cancer [119]. These studies included the use of GnRH analogues [135, 136], GnRH analogue plus testosterone [137], GnRH analogue plus an antiandrogen [138], testosterone [139] or medroxyprogesterone acetate given before or after treatment [140] in men receiving chemotherapy and/or radiotherapy for HD and testicular cancer. One further study in adult patients treated with cyclophosphamide for nephritis demonstrated that testosterone therapy was associated with recovery by six months in 5/5 patients compared with 1/5 who did not receive testosterone [141]. Overall, no consistent effect of treatment was demonstrated in these studies. However, it must be recognised that in one study there was no control group, and the majority of studies involved 20 patients or less, and are likely to be underpowered to demonstrate any significant effect. Several studies demonstrated that the majority of patients in the control group recovered spermatogenesis, which also reduces the chances of demonstrating a protective effect of hormonal manipulation, whilst the study in which no recovery was seen in either group

may indicate that the cytotoxic treatment may result in a complete loss of spermatogonia.

Many of the human studies were carried out before it became clear that hormonal treatments in the rodent studies were demonstrating restoration rather than protection of spermatogenesis, and none of the studies involved the continuation of gonadal-suppressive therapy for a significant period of time after the completion of chemotherapy or radiotherapy. Thus, carefully designed, adequately powered studies are warranted to determine whether suppression of gonadal function with a GnRH agonist or testosterone for a fixed time during and after the completion of irradiation or chemotherapy may prove successful in reducing the impact of these treatments on fertility.

Ex Vivo Techniques for Fertility Preservation

As discussed above, cryopreservation of semen is considered routine clinical practice in many countries. Pubertal boys and men who are able to produce a semen sample should always be offered the possibility to cryopreserve their sperm if they are at risk of infertility due to a gonadotoxic treatment. Unfortunately, prepubertal boys, who are not able to produce sperm at the time their gonadotoxic treatments start, do not have this possibility [142]. In some adults, there may also be other factors, such as ethical or religious beliefs, that exclude cryopreservation of semen as an option for fertility preservation. For these boys and men, testicular biopsy, cryopreservation of the testicular tissue and ex vivo differentiation of male germ cells might be an option, although this approach remains experimental, and currently there are no functional techniques available to generate mature human gametes ex vivo (Fig. 12.4).

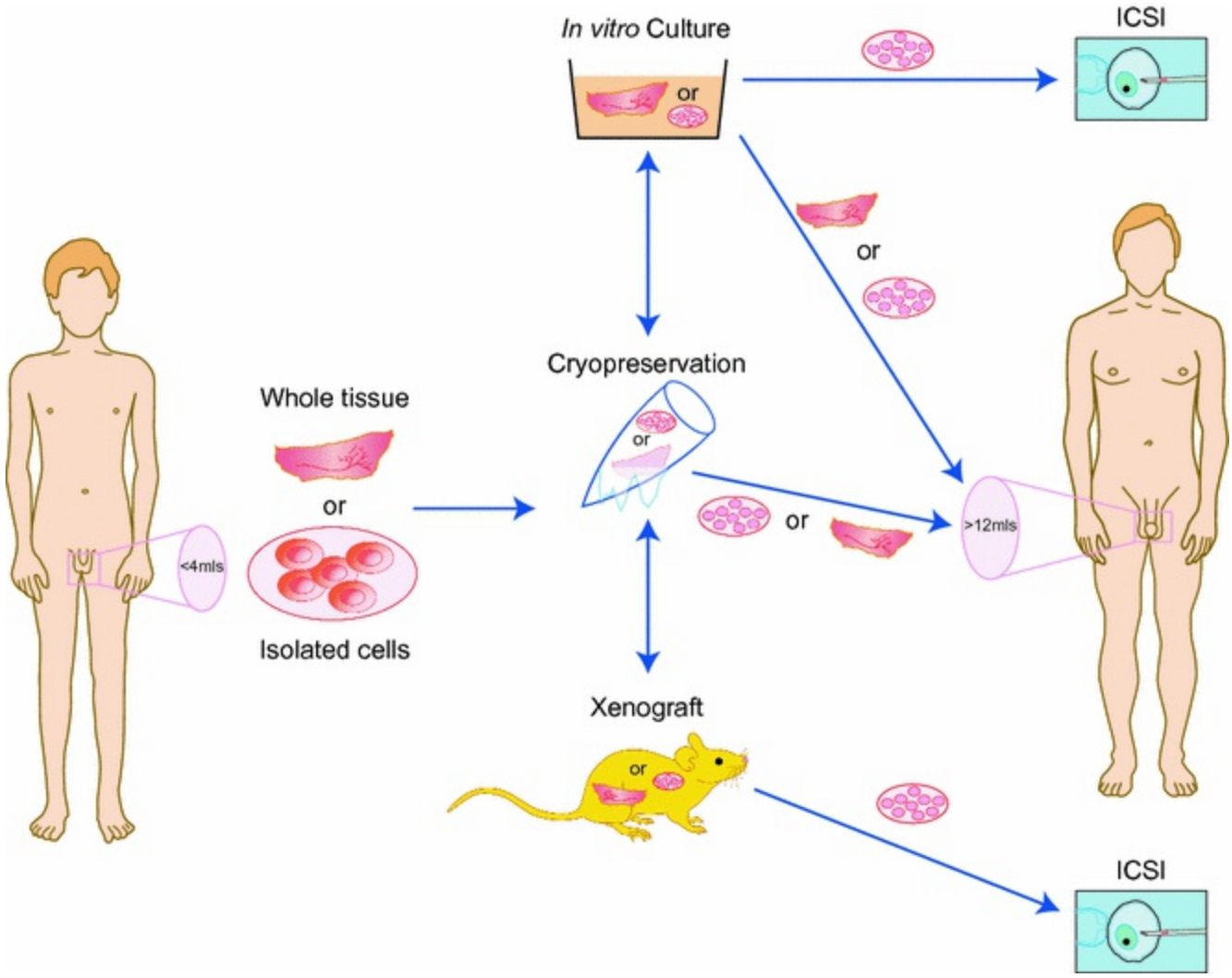

Fig. 12.4 Experimental approaches for fertility preservation in young men prior to cytotoxic therapy. Testicular tissue is removed from the testis prior to cancer treatment. Tissue or isolated cells may be cryopreserved, cultured or xenografted. The material may be replaced in the host testis following the completion of treatment or alternatively differentiated germ cells may be recovered and used for assisted reproduction, e.g. intracytoplasmic sperm injection (ICSI)

Although our understanding of human spermatogenesis has increased dramatically in recent decades, details of the highly complex process of controlling human spermatogenesis are still inadequately understood (see Chap. 3). The limited access to normal human testicular tissue, accounting also for the lack of functional in vitro models, might be one of the main reasons behind this. The use of animal models to elucidate the process of human spermatogenesis is limited as a result of species differences [143, 144]. However, in recent decades, different strategies, including animal studies with a focus on male germ cell or tissue transplantation [145–149], or the use of different types of in vitro cultures [150–156], including various pluripotent stem cells types [157, 158] and the recently described very small embryonic-like stem cells (VSELs) [159, 160], have explored the spermatogenic progress in detail.

Prominent experimental conditions for ex vivo differentiation of mammalian male germ cells include (i) in vitro culture systems; (ii) testicular tissue (xeno) grafting; and (iii) SSC transplantation into the seminiferous tubules of the testis [155, 161, 162]. Among these experimental strategies, germ cell or testicular tissue transplantation have demonstrated possibilities to mature SSCs and more advanced germ cells in testicular microenvironments similar to those present in vivo.

Among the three ex vivo differentiation strategies, auto-transplantation of cryopreserved SSCs or testicular tissue into the patient´s testis represents a potential clinical tool for preservation of fertility following gonadotoxic treatment. However, the risk of reintroducing the disease due to re-transplantation of malignant cells back into the patient, particularly in patients with ALL, is one of the major limiting factors for future clinical application of this technique. The establishment of "decontamination" strategies to eliminate malignant cells from testicular tissue fragments is extremely challenging. So far, a specific marker for SSCs has not been identified [163]. Decontamination protocols involving enrichment and isolation of SSCs or the depletion of malignant cells have become a focus for research during recent years [164–166]. However, transplantation of cryopreserved testicular tissue or germ cells back into the patient after treatment is currently unsafe for patients with haematological cancer [142].

100 Years in Search of the Conditions Required for Male Germ Cell Differentiation in Vitro

At the beginning of the last century, organ culture conditions focusing on the differentiation of male germ cells from a variety of different species revealed several important factors necessary for male germ cell differentiation, including in vitro maintenance of the microenvironment provided by the seminiferous epithelium and thereby the intact cell-to-cell communication pathways of somatic and germ cells [155]. These early experiments, followed by many others in the following decades, also demonstrated the importance of maintaining temperature below that of the body [167–169], the need for cell–cell contacts [170–172], the supportive effect on viability of media components including pyruvate, glutamine, vitamins A, C and E, the non-supportive effect of gonadotropins for the success of germ cell cultures [167], as well as the negative influence of high oxygen levels on the viability of cells in vitro [162]. Studies focusing on in vitro differentiation of male germ cells combined with somatic cells, and studies involving enriched germ cell types such as spermatogonia and spermatocytes, clearly demonstrated that a microenvironment resembling the three-dimensional organisation of the seminiferous epithelium in situ (thereby providing cell–cell contact) should be provided [150, 155, 173–175].

The first successful differentiation of human male germ cells with completion of meiosis and the spermiogenic process in vitro was reported in the late 1990s. Tesarik

and colleagues obtained human sperm in vitro via the use of seminiferous tubule cultures at 30 °C [153, 176]. The same group also reported the birth of healthy infants as a result of ICSI using in vitro-differentiated sperm from azoospermic patients [153]. In these reports, the differentiation of primary spermatocytes into haploid spermatids, which in vivo takes up to 32 days [176, 177], was reported to occur within 48 h [176] in vitro. Although earlier investigators reported a similar speed for germ cell differentiation in vivo and in vitro, another group reported four years later a similar feature of in vitro maturation. Primary spermatocytes obtained from azoospermic patients with meiotic arrest exhibited features of haploid round spermatids, when cultured for 2–5 days on Vero cells [178].

So far, differentiation of undifferentiated spermatogonia to elongated spermatids has been reported in connection with organ culture and three-dimensional cell culture methodologies [150, 151, 155, 179–182]. In 2011, Sato et al. reported the first successful differentiation of murine SSCs into functional sperm in vitro using organ culture [150, 179, 183] in combination with 10% Knockout Serum Replacement or AlbuMAX (40 mg/ml) instead of conventional foetal bovine serum. Recently, a study using testicular material from 16 patients with cryptorchidism (mean age ± SEM 29 ± 2 years) demonstrated the differentiation of human SSCs up to functional haploid spermatids [184]. Germ cell differentiation in that study was achieved using DMEM/F12 cell culture medium, 10% foetal bovine serum, retinoic acid and stem cell factor in a conventional single cell culture for 7–10 days [184].

In parallel with the successful establishment of organ cultures for murine spermatogenesis, the establishment of three-dimensional culture systems has recently provided novel insights into the process of male germ cell differentiation/proliferation and the formation of testicular somatic microenvironments in vitro [154, 155, 174, 175, 185]. Three-dimensional culture systems, using agar, methylcellulose or extracellular matrixes, were first established as clonogenic assays for multipotent hematopoietic cells [186], and were subsequently developed and adapted to the requirements needed for clonal expansion of male germ cells [154, 155, 174, 181, 182]. Regardless of the matrix used to generate the three-dimensional environment, differentiation of male germ cells through meiosis could be demonstrated, pointing out the important effect of physical contact between the cells, in accordance with results from previous organ culture experiments [155].

Methods for establishing a decellularized testicular matrix (DTM) from cadaveric human testicular tissue in order to support spermatogenesis in vitro for fertility preservation have recently been reviewed [156]. In a recent study, Baert and colleagues reported important insights into the components of the basal membrane. Interestingly, proteomic analysis of the DTM used revealed, in addition to well-known components such as laminin, collagens I and IV, glycosaminoglycans and fibronectin, more than 100 unique proteins belonging to, or associated with testicular extracellular matrix. These

new findings indicate a need for further experiments to elucidate the role and potential use of scaffolds made from human DTM for fertility preservation.

In addition to experimental definitions of appropriate culture conditions, current studies are also focusing on optimal cryopreservation techniques for immature testicular tissue and their effects on SSCs in murine and human cells/tissue. These studies suggest that the cryopreservation process itself does not impact on survival or differentiation capacity of the germ cells [152, 187–190]. Consideration must be given to potential abnormalities such as genetic/epigenetic instabilities of in vitro-generated gametes, which have been described, for example, in connection with bone marrow or human embryonic and foetal mesenchymal stem cells [191, 192]. However, initial experiments focusing on the effects of long-term culture of SSCs are promising and suggest genomic stability of these cells [193]. However, additional studies are mandatory to address efficiency and safety concerns in order for these techniques to be used clinically.

Summary

Testicular dysfunction may be present in males with cancer prior to commencing treatment often in relation to the underlying malignancy. Treatment with chemotherapy and radiotherapy may also result in significant testicular damage, primarily as a result of damage to the seminiferous epithelium resulting in infertility, whilst Leydig cell impairment may also occur, albeit generally at higher exposures.

For chemotherapy, alkylating agents, such as cyclophosphamide and procarbazine, are most commonly implicated in damage to the seminiferous epithelium. A large proportion of men receiving procarbazine-containing regimens for the treatment of lymphomas are rendered permanently infertile, whilst alternative regimens such as ABVD appear to have a significant advantage in terms of testicular function, with a return to normal fertility in the vast majority of patients. Leydig cell dysfunction can occur in men receiving chemotherapy, although this may be of little/no clinical significance in the majority of patients. Radiotherapy-induced damage to the germinal epithelium is common with changes to spermatogonia following as little as 0.1 Gy, and permanent infertility after fractionated doses of 6 Gy and above, whereas clinically significant Leydig cell impairment occurs rarely with doses of less than 20 Gy.

The management of males about to commence treatment for cancer should always include counselling regarding the possible effects of treatment on testicular function, and sperm banking should be offered to all post-pubertal patients undergoing potentially sterilising therapy. Regular semen analyses should be offered to men following cytotoxic treatment to allow appropriate family planning. Measurement of testosterone and LH are appropriate for men with symptoms consistent with testosterone deficiency who have received significant doses of irradiation to the testes, procarbazine-containing chemotherapy or high-dose chemotherapy. Mild elevations of LH accompanied by

testosterone levels within the normal range (the most common abnormality) do not generally require treatment, but patients with subnormal testosterone levels and markedly elevated LH levels may benefit from androgen replacement. For prepubertal patients, there are currently no established options to preserve fertility; however, experimental strategies to cryopreserve testicular tissue biopsies and generate mature games ex vivo are currently being developed, and may be an option for these patients in the future.

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13. Exercise and Male Hypogonadism: Testosterone, the Hypothalamic-Pituitary-Testicular Axis, and Exercise Training

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Introduction

Athletes who compete in endurance-based sports events are swimming, running, cycling, and skating faster than ever before, and world records in many events are being

broken on a nearly annual basis [1]. There are several factors contributing to this improvement in human exercise performance. First, coaches working with athletes have improved scientific knowledge because of advances in the fields of Sports Medicine—Exercise Physiology. Second, sporting equipment changes have allowed some improvements in events [1, 2]. Third and perhaps most important, is the greater level of exercise training that athletes are performing in our modern era [1–4]. For example, it is not uncommon for marathon runners to complete 150–250 km of intensive running per week, or for tri-athletes to spend 3–4 h per day in swim, run and cycle training. This large volume of exercise training results in physiological changes and adaptations that are highly beneficial to the human organism, such as enhanced cardiac output, an enhanced arterial-venous oxygen difference, increased erythrocyte number, decreased body adiposity, and increased mitochondrial density [3]. However, this great volume of exercise training can also place a tremendous amount of stress on the human organism, and can result in unwanted physiological responses and medical problems which can compromise to some extent the ability of an athlete to perform.

A physiological system that is extremely sensitive to the stress of exercise training is the endocrine reproductive system. A growing body of research over the last 35 years reveals how chronic exposure to endurance exercise training results in the development of endocrine reproductive dysfunction [5–9]. The vast majority of the research on exercise and endocrine reproductive dysfunction has focused upon sporting women [10–12]. A growing number of studies, however, have begun to address the question of how exercise training affects the endocrine reproductive system in men. Comparatively, however, published research reports in this area remain relatively few in number. Nevertheless, many researchers hypothesize that the effect of endurance exercise training on the male endocrine reproductive system may be comparable to that found in sporting women; i.e., endurance-exercise-trained athletes of both sexes can develop hypogonadism-like characteristics. Specifically, some men chronically exposed to endurance exercise training develop low basal, resting levels of total and free testosterone. Most of these men display clinically "normal" levels of the hormone testosterone, but the levels are at the extreme low end of normal (<15 nmol/L) [13].

A general label applied to these men has been "endurance trained men with low resting testosterone." While this is an accurate descriptive phrase for their condition, it is cumbersome to use. According to Taber's Medical Dictionary, "hypogonadism" can be defined as "defective internal secretion of the gonads" [14]. This is a simplistic definition not giving an indication of the level of "defect" [clinical versus subclinical] or the root of the "defect" [e.g., hypergonadotropic versus hypogonadotrophic]. Nonetheless, we chose to use this definition, and will refer to "endurance trained men with low resting testosterone" as having the "exercise-hypogonadal male condition" (EHMC) [15, 16]. This term serves as an operational definition for this special case of exercise men discussed in this chapter. These men have several common characteristics;

- Their low testosterone levels do not appear to be a transient phenomenon related to the stress–strain of an acute exercise bout, or prior drug ("doping") usage.
- In many cases, it appears that an adjustment in the regulatory axis [to allow a new lower set point for circulating testosterone] has occurred.
- They typically have a history of early involvement in organized sport and exercise training resulting in many years of almost daily exposure to varying intensities of physical activity.
- The type of exercise training history most prevalent in these men is prolonged, endurance-based activities such as: distance running [10 km or marathons], cycling, race walking, cross-country skiing, or triathlons.

A second endocrine reproductive disturbance also occurs in male athletic populations. This problem is a pseudo-hypergonadism brought about by the use of anabolic-androgenic steroid pharmaceutical agents. The term "pseudo-hypergonadism" is used because in reality, the usage of exogenous anabolic-androgenic steroids results in an atrophy of the gonads and reduced endogenous sex-steroid hormone production because of hypothalamic-pituitary suppression. The extremely high circulating levels of anabolic-androgenic pharmaceutical agents actually mask the endogenous hypogonadism which is occurring.

Evidence points to large numbers of athletes taking anabolic-androgenic steroid agents in an attempt to stimulate muscular growth, development, and strength. The usage of these agents has been a persistent occurrence in sports for approximately 70 years. Unfortunately, athletes and coaches continue to ignore the reports of serious medical side effects associated with such usage [17].

The intent of this review is to present an overview of select endocrine reproductive problems that occur in men involved in exercise training. Specifically discussed are: (a) how endurance exercise training affects the male reproductive endocrine system to induce hypogonadal-like conditions resulting in suppressed circulating testosterone levels [denoted here as the EHMC], and (b) anabolic-androgenic steroid abuse and the reproductive dysfunction that is associated with the use of these pharmaceutical agents.

The following discussion is delimited to the relationship between exercise endocrinology and reproduction in men. Research in this area in women is the subject of several authoritative reviews [12, 18].

Physiological Actions of Testosterone

Testosterone is the most important sex hormone in males, and has multiple physiological functions. These roles can be dichotomized into two major categories:

- Androgenic effects, which are related to reproductive function and to the

development of male secondary sex characteristics.

- Anabolic effects, which are related more generally to the stimulation of tissue growth and development.

A complete discussion of these categories is beyond the scope of this chapter. What follows, instead, is a brief overview of the major androgenic and anabolic effects of testosterone.

Androgenic Effects

The foremost reproductive role of testosterone is to stimulate the production of sperm. Activation of androgen receptors in Sertoli cells, peritubular myoid cells and Leydig cells by testosterone stimulates and catalyzes the maturation and development of sperm through down-stream target mechanisms that are incompletely understood [19–22].

Testosterone also stimulates the differentiation, development, and function of the male accessory sex glands (prostate, seminal vesicles, and epididymis) that aid in sperm development and function as well as in copulation. Finally, testosterone stimulates the development of male secondary sex characteristics, such as the deeper male voice, increased body hair, penile growth, sex drive (libido), and male behavior patterns [20–23].

Anabolic Effects

Testosterone and 5-α dihydrotestosterone (DHT) are powerful anabolic hormones that stimulate nitrogen retention and protein synthesis [19]. During puberty, testosterone acts in concert with other hormones to increase bone mass, and to initiate the adolescent growth spurt. In the adult, testosterone is needed to maintain protein anabolism and thereby structural proteins. Athletes who use anabolic steroids (i.e., pharmacologically derived testosterone-like compounds) take advantage of this effect to increase skeletal muscle mass [3, 6, 19, 20, 22]. This latter action by testosterone is due to androgen receptor activation of muscle gene transcription including that of insulin-like growth factor (IGF)-1 [24]. In a related fashion, testosterone also decreases myostatin expression (TGF-β family member and inhibitor of muscle development) [24]. Testosterone also stimulates hematopoiesis and increases sodium reabsorption in the kidney. Finally, studies in animals reveal that testosterone also plays a role in increasing skeletal muscle glycogen synthesis and storage [20].

Factors Affecting Circulating Testosterone

Hypothalamic-Pituitary-Testicular Axis

Testosterone production by the testis is under control of the hypothalamic-pituitary unit

[25]. The operation and components of this axis are discussed in detail elsewhere in this volume (see Chap. 1) and will not be repeated here. The hypothalamic-pituitary-testicular axis hormones addressed within this discussion are testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), inhibin, and prolactin (PRL).

Cardiovascular Factors

Testosterone is freely diffusible through tissues, and thus its secretion from the testis is directly affected by testicular blood flow. Testicular blood flow is a function of cardiac output, vascular vasoconstriction and vasodilatation. Therefore, factors that influence vascular tone can also affect testosterone secretion (e.g., increased sympathetic nervous system activity) [26].

The metabolic clearance rate (MCR) of testosterone varies among normal men, but is approximately 100 L per day [19, 20]. MCR involves target tissue uptake as well as degradation by the liver, and is influenced by the portion of the hormone bound to carrier proteins (principally sex hormone-binding globulin [SHBG]). The degradation process involves the conversion of testosterone into functional metabolites, such as estradiol and dihydrotestosterone, and degradation products, such as 17-ketosteroids, sulfates, and glucuronides, which are excreted into the urine [19, 20, 27]. Hepatic clearance is primarily a function of hepatic blood flow, such that changes in the hepatic blood flow influence the removal rate of testosterone [19]. This latter phenomenon is influenced by exercise, as hepatic blood flow is reduced during exercise as blood is shunted toward the active muscles [3, 28–30].

During exercise, there is movement of plasma from the vascular space. Since testosterone is bound to carrier proteins, an increase or decrease in the plasma volume leads to dilution or concentration of the circulating testosterone level. These changes, however, are not indicative of changes in the normal hormonal turnover rate [28]. For example, it is not uncommon during prolonged exercise (≥1 h) for transient plasma volume decreases of 10–20% to occur [28] leading to a compensatory increase in testosterone (total) concentration of a similar magnitude. Whether these highly transient changes in the concentration of testosterone due to changes in plasma volume have a persistent physiological impact is a point of debate, and remains to be determined.

Other Factors

In addressing factors affecting changes in blood levels of testosterone, it is important to consider the other physiological and non-physiological factors that could account for variations in testosterone response among studies. Examples include the blood-sampling method, diurnal variations in hormone concentrations, hormone detection methodology, experimental research protocol or design, or age, emotional stress, diet, and sleep patterns of the subject-patient. These factors are discussed in detail elsewhere [6, 27,

28]. Collectively, all of the aforementioned considerations must be carefully examined when comparing the hormonal results of research studies if a valid interpretation of the endocrine system's responses to exercise is to be made. Figure 13.1 is a summary that illustrates some of these physiological and non-physiological factors that impact upon circulating testosterone levels in men [31].

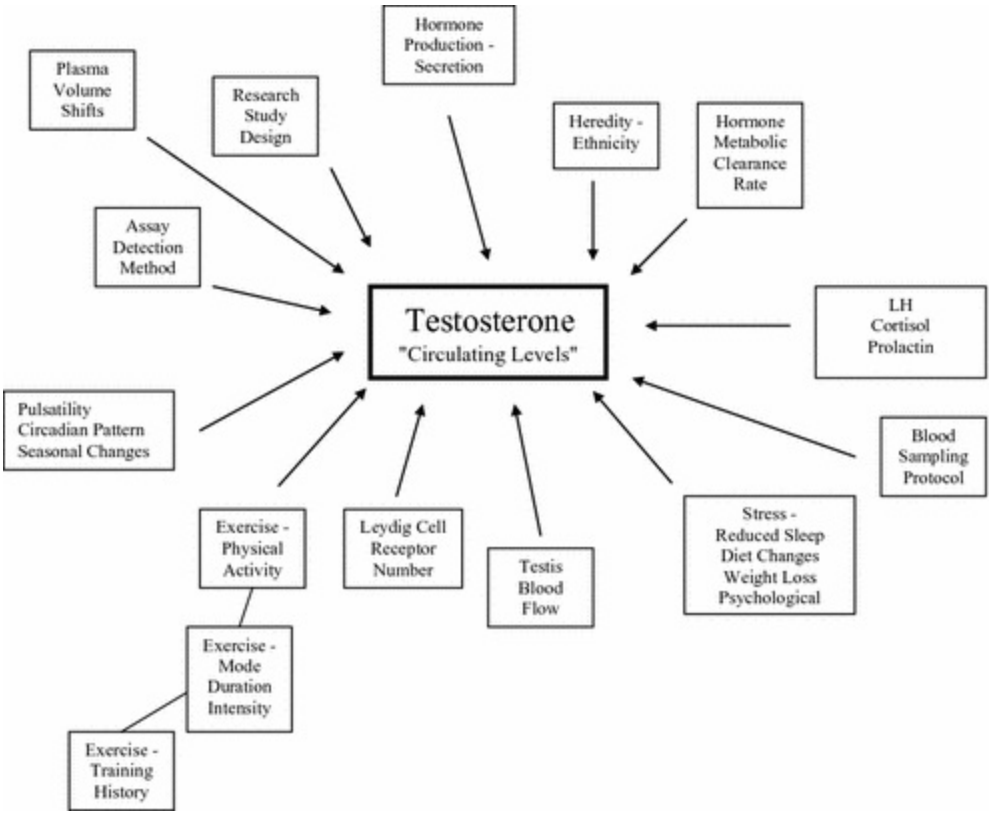

Fig. 13.1 Illustrated are the factors that influence circulating testosterone levels in normal men at rest and in response to a physical exercise bout. This figure is based upon the information from references [22, 25, 27, 28]

Exercise and Pituitary—Testicular Function

Normal Men—Exercise Responses

Testosterone

It is generally accepted that short-term, maximal, and anaerobic-type exercise results in an elevation of the circulating testosterone level (see Table 13.1 for definitions of exercise-types terminology) [28, 32–34]. It is still an issue of some debate whether this effect is due solely to hemoconcentration and reduced metabolic clearance, or whether there is also an increase in testosterone production [6, 27, 35].

Table 13.1 Categorization and definition of exercise intensive and exercise-type relative to exercise studies of physically fit men

Category term	Relative intensity (%VO_{2max})	Energy pathway predominating	Typical duration	Other terminology
Light or easy exercise	<35	Aerobic	>30 min	Submaximal
Moderate exercise	>35 < 70	Aerobic	>30 min < 180 min	Submaximal
Heavy exercise	>70	Aerobic—anaerobic	<120 min	Submaximal
Maximal exercise	100	Aerobic—anaerobic	<15 min	Maximal or max
Supra-maximal exercise	>100	Anaerobic	<1 min	Sprints, power

In quantifying exercise intensity it is typical to express it as a relative percentage of an individual's maximal oxygen uptake (VO_{2max}; i.e., = maximal aerobic capacity)
Based upon; Bouchard et al. [169]

Unlike changes during maximal exercise, testosterone responses to submaximal exercise are more variable, and seem dependent on the duration and intensity of exercise. Progressive increases in testosterone levels during moderate intensity exercise lasting 45–90 min have been found [28, 36, 37]. However, 90 min of submaximal moderate intensity exercise has also been reported to produce no change or a slight decrease in testosterone concentrations [28, 36]. Exercise of a moderate or hard intensity until exhaustion of greater than ~2 h duration typically lowers testosterone concentrations [28, 36, 37].

Several explanations for these dissimilar changes during submaximal exercise have been proposed. Initially, hemoconcentration may increase the testosterone concentration; but as exercise continues, testicular testosterone production declines. The latter change may be due partly to reduced testicular blood flow [29, 30]. Hepatic blood flow may also decline reducing hepatic clearance [29, 30]. This latter change may be offset by an increased uptake of testosterone by peripheral target tissue (i.e., skeletal muscle) as exercise duration continues, gradually reducing circulating testosterone levels.

Interestingly, these acute effects of a bout of maximal and submaximal exercise on testosterone levels are transient. That is, the changes are typically short-lived and normalize during the recovery period (1–24 h) [28, 35, 38–40].

Gonadotropins

Research results examining the gonadotropin response to maximal and anaerobic-type exercise are somewhat contradictory [35, 41, 42]; however, the majority of the evidence indicates there are small transient increases in the circulating gonadotropins [40, 43–49]. In addition, the outcomes of these studies, and their interpretations, are subject to methodological limitations. In many cases, researchers did not obtain blood samples

frequently enough to assess the highly pulsatile gonadotropin secretory profile [12, 19, 27]. Resistance-type exercise induces small increases in serum gonadotropin levels in both men and women [46, 49–51].

Likewise, the prolonged (~1–2 h) submaximal exercise responses of the gonadotropins vary considerably for both men and women [28, 46, 47]. Evidence of significant increase, decrease, and no change has been reported [36, 40, 43, 44, 46, 47]. If submaximal exercise is extended for extremely long periods, or until exhaustion occurs, gonadotropin levels are suppressed [46, 51–54].

These acute exercise-induced gonadotropin disturbances are transient and last for relatively short periods into the recovery from exercise [35]. The exception to this point is that submaximal exercise of many hours duration can produce disturbances that last several days [53, 54]. Interestingly, scant research exist on the influence of exercise on the pulsatile release of the gonadotropins in men, and these findings are inconclusive [36, 37, 41, 43, 44].

Other Pituitary Hormones—Prolactin

PRL presents an interesting paradox in the reproductive physiology of exercise. At physiological concentrations, PRL may be necessary for normal testicular function, while high PRL levels disrupt both central and peripheral aspects of the hypothalamic-pituitary-testicular axis [19, 55]. Circulating PRL concentrations increase during most exercise. The magnitude of the increase appears approximately proportional to the intensity of the activity [28, 46, 56]. Short-term, maximal, and anaerobic-type exercise increases PRL levels [28, 46, 56–60]. Submaximal exercise of 30 to 60 min duration, provided it is intense enough, increases circulating PRL levels as well [28, 47, 57–60]. Extending the duration of a submaximal exercise bout augments the magnitude of the PRL response [27, 47, 56, 57].

There are several factors that further increase the PRL response to prolonged exercise: (a) consumption of a diet rich in fat, (b) fasting for an extended period of time prior to exercise, (c) performing exercise in conditions promoting an increase in body temperature, and (d) administration of β-adrenergic blocking agents prior to exercise [28, 47, 57, 60]. On the other hand, PRL release is inhibited by α-adrenergic blockade [28, 47]. Exercise training history (i.e., training type or mode) also seems to have a positive or negative influence on this response to submaximal exercise [28].

PRL changes following maximal exercise or submaximal exercise of 1–2 h duration appear transient in nature [57–59]. Interestingly, Hackney and associates have reported nocturnal PRL levels are 2–3 times greater following 1.5 h of intensive endurance exercise than when no daytime exercise was performed [43, 61].

Exercise-Hypogonadal Males

Basal Hormonal Responses

Retrospective comparative studies examining isolated, single blood samples have found lower testosterone levels in chronically endurance-trained males. The subjects in those studies were typically endurance athletes (e.g., distance runners) who had been involved with the physical training aspects of their sport for 1–15 years. In those studies, total and free testosterone levels were only 60–85% of the levels of matched sedentary controls [7, 40, 59, 62–64]. Many of the early studies reporting this finding suffered from small sample sizes. However, recent work with larger numbers of subjects has substantiated those findings [65, 66]. These low resting testosterone levels seem highly reproducible, and are not just an aberration of the athletes' seasonal training regime [66]. Furthermore, SHBG levels have not been reported to differ from sedentary, age-matched control men [65].

Prospective studies have also been conducted in which blood samples have been collected over weeks or months of endurance training regimens. Thus far, findings from such studies have been inconsistent. Some reports reveal significant reductions (decreases of 20–40%) in resting testosterone levels following 1–6 months of intensive training [66–70], whereas other studies have found no significant change in resting testosterone after 2–9 months of training [71–74]. Differences in the initial training status of the subjects, or the training dosage administered within these studies, may explain the discrepant findings. It is also possible that some of these prospective studies were too brief compared to the retrospective studies in which the men with low testosterone levels had been training for many years. To this point, landmark work by Safarinejad and colleagues [75] showed that prolonged training exposure (60 weeks) resulted in significant reductions in free and total testosterone, gonadotropins, and stimulated gonadotropin production.

Exercise-hypogonadal men also display other reproductive hormonal abnormalities in addition to low basal testosterone levels. The most frequently reported finding involves no significant elevation in resting LH to correspond with the decrease in testosterone (i.e., hypogonadotropic-hypogonadism) [40, 64, 76]. Additionally, resting levels of PRL may be decreased [40, 64]. Altered LH and PRL levels at rest in EHMC men have been interpreted by some researchers to indicate dysfunction of the hypothalamic-pituitary-testicular axis. These findings for LH and PRL have been reported in several retrospective and prospective studies [7, 40, 62, 64, 76, 77]. Interestingly, the long-term prospective study of Safarinejad and colleagues [75], noted earlier, reported a significant decline in basal LH and FSH with training exposure by 12 weeks.

There are a number of retrospective investigations in which basal, resting blood samples were collected (i.e., serially every 20 or 30 min for 4–8 h periods) in EHMC men and in sedentary control men. Results are similar to those of the isolated-sampling

studies: resting total and free testosterone concentrations of the trained subjects were typically only 60–80% of those found in control men [62, 78]. As in isolated single blood-sampling studies, resting LH levels were not significantly elevated. Again, these findings have been interpreted to represent dysfunction in the hypothalamic-pituitary function.

As noted, the hormonal findings described above have primarily been found in endurance athletes (i.e., distance runners and tri-athletes). It is highly unlikely, however, that these hormonal alterations are limited to this athletic—exercise group. The prevalence of these phenomena in endurance athletes most likely represents the tendency of researchers to focus upon this group, following the initial lead of early studies conducted in the 1980s. As scientists studying exercise expand their endocrinological studies to include other athletic groups involved with some components of endurance training, it is highly likely that comparable data will come forward as is beginning to be seen in preliminary findings [79].

Exercise Responses—Testosterone, Gonadotropins, and Other Hormones

Few studies have compared whether EHMC men and normal men respond differently to exercise. Evidence suggests that in response to a single exercise bout (maximal or submaximal in nature) the direction of the hormonal changes is similar. Testosterone and PRL are increased while the gonadotropin responses are variable in both groups of men [40, 77]. In the recovery from exercise, however, the two groups differ. In normal men, the reproductive hormones display some degree of negative feedback "rebound" inhibition during the hours of recovery following exercise [40, 58]. In EHMC men, this rebound effect is diminished or eliminated. Currently, it is unclear whether this represents an adjustment in regulatory aspects of the controlling axis, or whether a temporal displacement shift has occurred in the axis of the EHMC men. Obviously, the resting, basal levels of the hormones in each of these groups are vastly different; therefore, relative changes in response to exercise must be compared. Figure 13.2 displays recovery hormonal responses of exercise-hypogonadal men and normal men as relative change following an identical exercise bout [40].

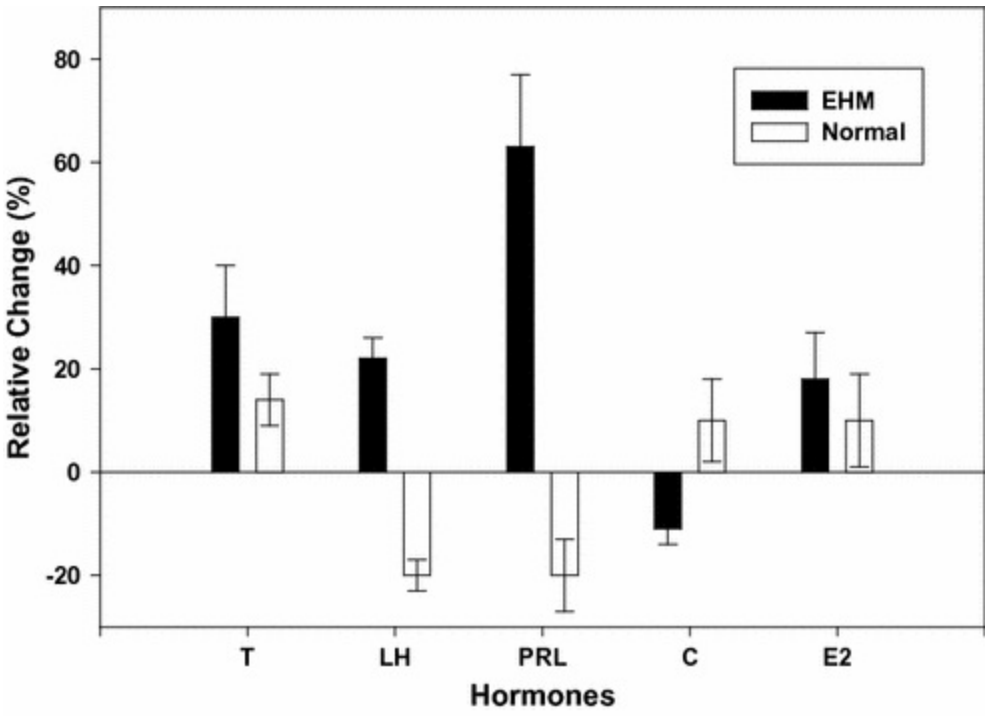

Fig. 13.2 Comparison of the percent change in hormone levels of exercise-hypogonadal (EHM) condition men versus normal, untrained men. Comparison is between a 4 h(overall mean response) period following a maximal exercise bout and that of a four period of rest in both groups of men. The plotted mean (± SE) responses of each group represent a delta value (i.e., exercise session 4 h overall response –control session 4 h overall response). Abbreviations *T* testosterone*, *LH* luteinizing hormone*, *PRL* prolactin*, *C* cortisol, *E*2 estradiol. The * denotes significance differences between the groups ($P < 0.05$). The data are based upon the findings in reference [40]

Mechanistic Studies

Some studies have attempted to elucidate the mechanism for the proposed hypothalamic-pituitary-testicular axis dysfunction in exercise-hypogonadal men. These studies have focused on examining whether the dysfunction is central (hypothalamic or pituitary) or peripheral (testicular) in nature.

Several investigators have reported that EHMC men have altered PRL and LH release when either an exogenous stimulus and, or an exercise bout are used to provoke hormone secretion [40, 52, 75, 76, 80]. Figures 13.3 and 13.4 illustrate an augmented PRL response and a reduced LH response to metoclopramide and GnRH stimulation, respectively, in EHMC men when compared to the matched sedentary controls [80]. Hyperprolactinemia is associated with decreased circulating testosterone levels [19, 81]. However, while the PRL response to metoclopramide was augmented, there is no evidence that EHMC men are chronically hyperprolactinemic. The converse has been actually been demonstrated (see earlier discussion). The reduction in LH release could also reduce testosterone levels. Reports indicate that testicular sensitivity and response to exogenous stimuli are comparable in exercise-hypogonadal men and sedentary males [40, 80, 82]. In contrast, Kujala et al. reported that the testicular response to a stimulus

(human chorionic gonadotropin [hCG]) is attenuated following 4 h of exhaustive exercise [52].

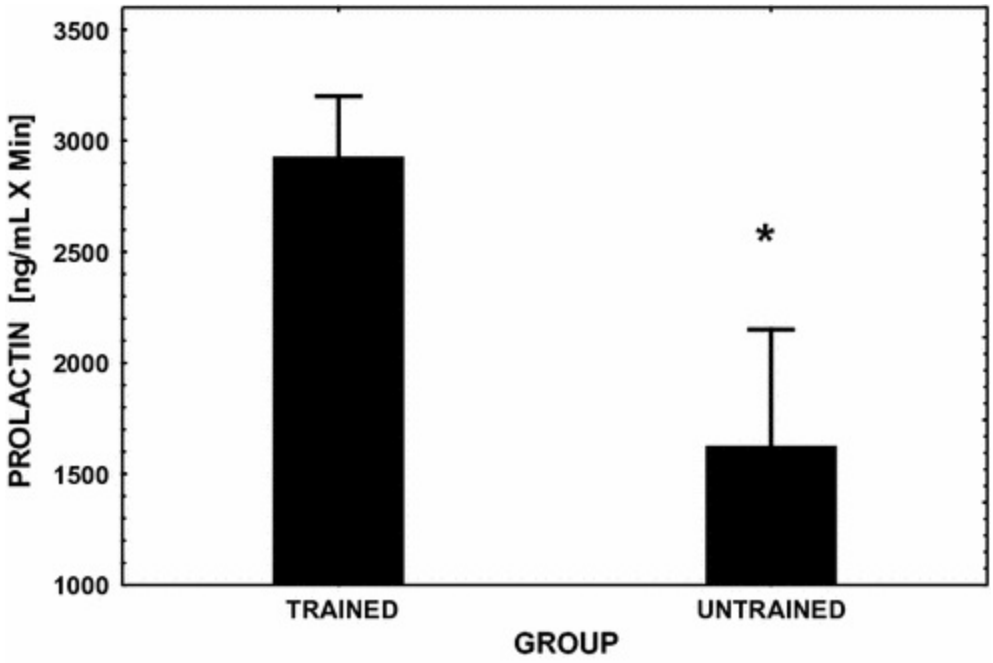

Fig. 13.3 Mean (± SE) integrated area under the PRL response curve over 3 hfollowing the injection of the dopamine antagonist metoclopramide (see reference [80] for dosage). This challenge to the pituitary was performed in endurance exercise-trained male runners and in age-matched sedentary controls. Significant between group differences ($P < 0.05$) are denoted with an asterisk

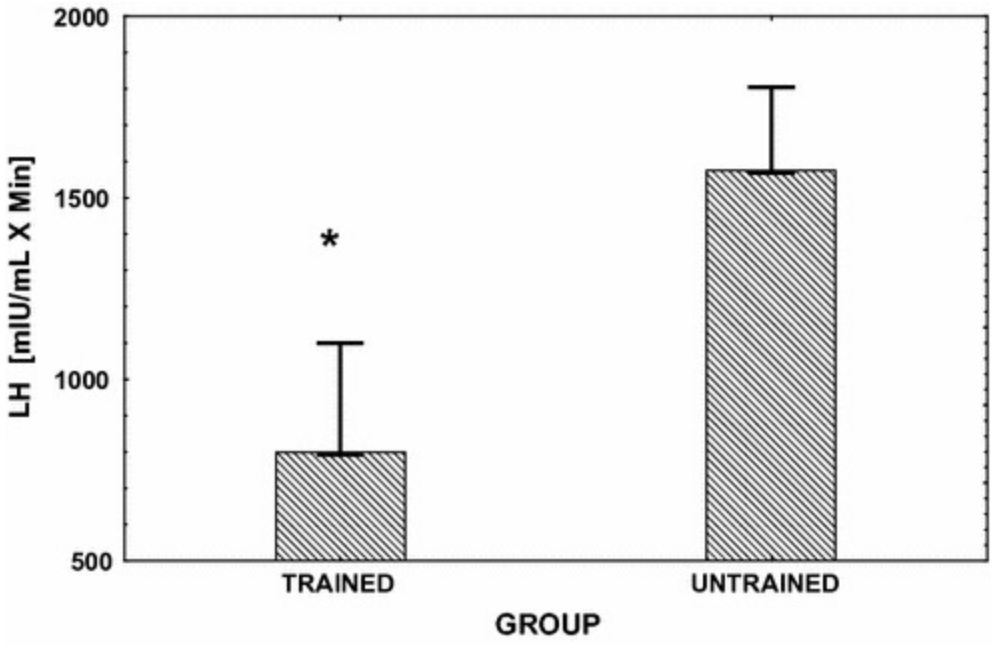

Fig. 13.4 Mean (± SE) integrated area under the LH response curve over 3h following the injection of GnRH (gonadorelin hydrochloride) (see reference [80] for dosage). This challenge to the pituitary was performed in endurance exercise-trained male runners and in age-matched sedentary controls. Significant between group

differences ($P < 0.05$) are denoted with an asterisk

Collectively, these findings suggest the development of both a central and a peripheral problem in the hypothalamic-pituitary-testicular axis; however, data in this area are limited and not yet definitive.

Cortisol

Researchers have demonstrated that acute pharmacological or pathological increases in cortisol secretion are associated with a decrease in circulating testosterone levels [38, 83]. Several investigators have alluded to these hormonal changes as a potential mechanism for the low testosterone levels in EHMC men [6, 27, 38, 83]. A single, acute exercise bout at high intensity (>60% of maximal aerobic capacity) could transiently increase circulating cortisol, which could bring about the observed reductions in testosterone via inhibitory effects on GnRH, LH or Leydig cell function [19]. Current evidence suggests, however, that hypercortisolemia is an unlikely mechanism for exercise-hypogonadism, since there are relatively small, transient changes in cortisol levels in response to exercise [43, 49, 50, 55, 58, 84–87], and exercise-induced changes in cortisol are well within the physiological range, regardless of whether the exercise is of moderate or high intensity. This point is illustrated in Fig. 13.5 [88]. Nevertheless, systematic research examining the role of exercise-induced cortisol changes upon testosterone production remains inadequate.

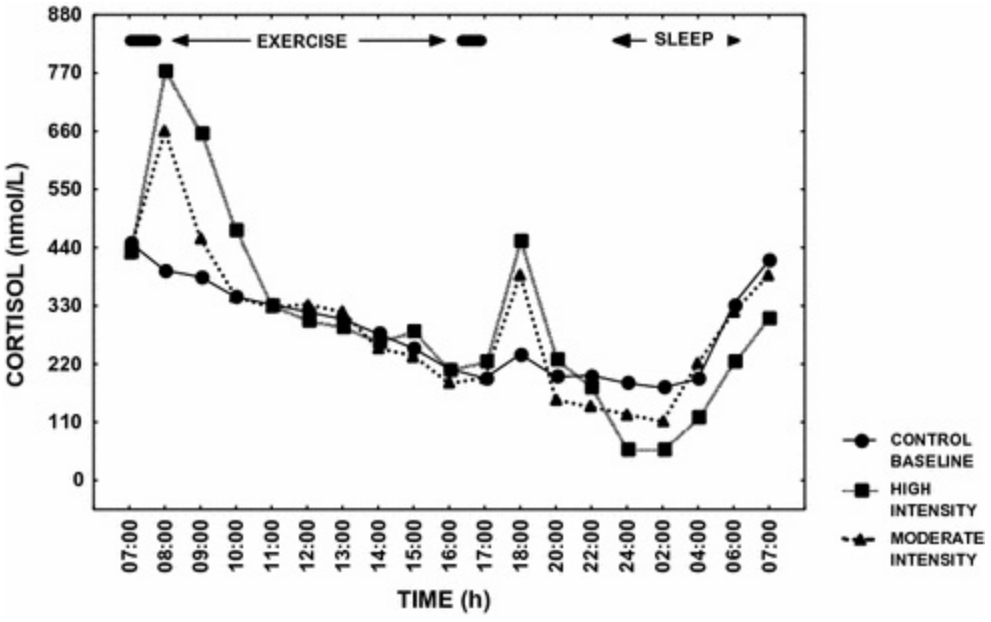

Fig. 13.5 Mean cortisol levels over 24 h in endurance athletes on three separate occasions; *Filled circle* control—baseline day with no exercise, *Filled triangle* exercise day involving two moderate intensity exercise sessions morning and afternoon, and *Filled square* exercise day involving two high intensity exercise sessions morning and afternoon. Significant increases ($P < 0.05$) in cortisol occurred on the exercise days; however, the changes were transient in nature. The data are based upon the results in reference [88]

Physiological Consequences of Low Testosterone Levels in Athletes

There is evidence that the low resting testosterone level in men doing endurance training has detrimental effects on testosterone-dependent physiological processes. However, the extent of the evidence is somewhat limited. Several reports of decreased spermatogenesis or oligospermia in EHMC men have been published [21, 75, 89–93]. One of the most well-controlled studies was by Arce et al. [90]. Some key findings from that study are displayed in Table 13.2. Other investigators have reported that endurance-trained men may have a lowered sex drive—libido, but a direct cause-and-effect linkage between a lower sex drive and circulating testosterone levels was not proven in those reports [82, 91, 94–97]. Accordingly, other factors may have affected the libido of those athletes (e.g., overall fatigue and psychological stress), but this area has not been well studied in exercising men [22, 94]. Additionally, there is some evidence that endurance training has no effect upon sperm characteristics and the spermatogenesis process [74].

Table 13.2 Semen characteristics of endurance-trained runners, resistance trained weight lifters, and sedentary controls (values are means ± SE)

	Runners	**Weight lifters**	**Controls**	**P value**
	(*N* = 10)	(*N* = 8)	(*N* = 10)	
Totals				
Volume (ml)	4.2 ± 0.5	3.0 ± 0.5	2.5 ± 0.5	0.9
Sperm density ($x10^6 \cdot ml^{-1}$)	78 ± 12*	122 ± 15	176 ± 25	<0.01
Total sperm count ($x10^6$)	332 ± 74	342 ± 36	376 ± 59	1.86
Normal motile count ($x10^6$)	55 ± 11	104 ± 20	107 ± 22	0.09
Motility				
Forward progressive (%)	40.8 ± 4.7**	58.0 ± 4.6	58.7 ± 2.4	<0.01
Nonprogressive (%)	5.0 ± 1.0	2.8 ± 1.5	2.0 ± 1.0	0.17
Nonmotile (%)	54.2 ± 4.9	39.2 ± 3.9	39.3 ± 1.9	1.01
Morphology				
Normal (%)	40.2 ± 2.1†	54.8 ± 2.9	47.0 ± 3.3	<0.01
Large (%)	2.7 ± 0.8	1.7 ± 0.9	2.3 ± 1.0	0.76
Small (%)	4.3 ± 0.8	2.6 ± 0.7	2.4 ± 0.5	0.10
Amorphous (%)	34.1 ± 2.5	30.1 ± 2.6	37.4 ± 2.8	0.19
Immature (%)	17.2 ± 2.4**	10.5 ± 2.1	10.9 ± 1.2	0.03
Round cells ($x10^6$)	8.3 ± 1.7**	0.6 ± 0.4	2.5 ± 0.9	<0.01
In vitro sperm penetration of CM—Penetrak (mm)	22 ± 5*	–	43 ± 7	0.04

These data are reproduced from: Arce et al. [90]
*$P < 0.05$ for runners versus controls. ** $P < 0.05$ for runners versus weight lifters and controls
† $P < 0.05$ for runners versus weight lifters

Relative to the androgenic-anabolic actions of testosterone, there are no documented detrimental effects of the lower testosterone levels (i.e., decreased protein synthesis and muscle mass development). However, this area needs more thorough examination. An additional area in need of research concerns the impact of the exercise-associated decline in testosterone levels on bone demineralization in trained men. Currently, there are no conclusive findings that endurance training results in male osteoporosis [98]. In fact, the bone mineral density of the lower extremities was increased in high mileage runners [99–101]. On the other hand, several compelling case reports have described male athletes with extremely low testosterone levels and reduced bone mineral density [102, 103].

Thus the question arises, is it necessary for endurance-trained males to supplement with testosterone-like substances in order to safeguard against loss of androgenic-anabolic processes? This question has not been addressed thoroughly in the literature. In one case study, Burge et al. [82] described a male runner with hypogonadotrophic hypogonadism who responded favorably to clomiphene citrate treatment over a 5-month period. Testosterone and gonadotrophin concentrations increased into the normal range, and the subject's sexual function improved. Whether treatment was necessary is an issue of debate. There is little evidence that disruptions in testosterone-dependent processes are sufficient to warrant action, but physicians must use their own medical judgment on a case-by-case basis. In extreme cases, especially among men with a very low body mass index, such steps may be necessary, and a well-developed pharmacological course of therapy could be highly advantageous and efficacious (see section "Anabolic Steroid—A Banned Substance").

Conversely, there may be beneficial physiologic adaptations from lowered testosterone levels. Some research indicates that lowering testosterone levels (i.e., but, not to hypogonadal levels) may have cardiovascular protective effects and decrease the risk of coronary heart disease [104]. A study from Germany demonstrated that a pharmacologically induced reduction in endogenous testosterone levels resulted in significant increases in high-density lipoprotein in men [105]. Whether the lowering of testosterone directly contributed to the exercise-related increase in HDL remains to be determined [106]. Nonetheless, it is important to recognize that increased physical activity promotes a healthy cardiovascular risk profile, including increased circulating HDL [107].

The Overtraining Syndrome

Several researchers have suggested that reproductive hormones in exercise-hypogonadal men are suppressed because they are "overtraining" and/or are developing the "Overtraining Syndrome." This terminology can be confusing to non-Exercise Physiology researchers; hence some explanation is warranted. The terms "Overtraining Syndrome" and "overtraining" are frequently used interchangeably. The term "overtraining" actually refers to the *process* of heavier than usual exercise training, while the term "Overtraining Syndrome" refers to the *product* of too much of the overtraining process [2, 4, 7–9, 108–112]. The Overtraining Syndrome is a pathological condition in which an athlete experiences consistent and persistent exercise performance incompetence that does not reverse itself after a few days of rest and recovery. Furthermore, there is no underlying medical reason or explanation for the declining performance. This exercise performance impairment can manifest itself within athletic competition as well as during exercise training. Concurrent with the declining physical performance is a host of other psycho-physiological consequences that are adverse and negative in nature. Some of the most general and commonly reported consequences and symptoms are listed in Table 13.3.

Table 13.3 Consequences and symptoms of the overtraining syndrome

↓ Physical performance
↑ Severe constant fatigue
↑ Persistent muscle soreness
↑ Overuse muscular-skeletal injuries
↓ Appetite
↑ Lethargy
↑ Depression
↑ Disturbed sleep patterns
↑ Overall mood disturbances—shifts
↑ Immune system deficits
↑ Mental concentration difficulties
Δ Submaximal—maximal heart rate responses to exercise (↑, ↓)
↓ Maximal oxygen uptake
Δ Submaximal—maximal lactate response to exercise (↑, ↓)

See references: [2, 4, 113]
↑ Increase
↓ Decrease
Δ Change; decrease and, or increase

One commonly reported endocrine change among overtrained athletes is suppressed circulating levels of testosterone [2, 4, 7, 9, 110–112]. Furthermore, in some cases, suppressed levels of LH, FSH, and inhibin have been reported as well as a reduced testosterone to cortisol ratio [27, 108–113]. While these changes appear nearly identical to those reported for EHMC men, it does not seem to represent the same phenomena. The hormone changes that are found in overtraining athletes appear to be only temporary. When the exercise training load of these overtraining athletes is reduced, their diets are adjusted to allow macro-nutrient—caloric balance, and increased periods of rest are incorporated into the training program, their hormonal profiles return to normal [113, 114]. Incorporation of more rest into the training of EHMC men seems to have no substantial impact upon their resting hormone levels. Neither do EHMC men display Overtraining Syndrome symptoms (see Table 13.3). Furthermore, most researchers studying the exercise-hypogonadal issue have been careful to insure that their subjects under investigation are not going through periods of intensive training; and that when they are evaluated they are well rested.

Thus, the hormonal changes found in EHMC men appear to be a more stable accommodation within the hypothalamic-pituitary-testicular axis while those in overtraining athletes are transient (i.e., returning to normal after adequate rest-recovery) and are associated with a declining physical performance.

Athletes and Anabolic-Androgenic Steroids

Anabolic-androgenic steroids are pharmacological agents that are similar to testosterone in structure [115, 116]. They have anabolic-androgenic actions like testosterone; however, these agents have been chemically modified in an effort to enhance their anabolic actions. Athletes use anabolic-androgenic steroids in an attempt to facilitate physiological development beyond that achieved with exercise training alone (e.g., increased body weight, muscular strength-power-speed or endurance) [115, 116]. These agents are used by athletes involved in many sports. However, sports such as track and field (mostly in the throwing events), weight-lifting, bodybuilding, and football report the highest prevalence of usage [103]. Regrettably, evidence points to nonathlete use being on the rise with the intent being for altering the body image [117, 118]. For the remainder of the discussion, anabolic-androgenic steroids will be referred to as AAS.

Physiologic Adaptation and Exercise Performance

It is now clearly established that testosterone and AAS supplementation increase muscle mass and maximal voluntary muscle strength [119–122]. Early studies suggested equivocal effects of AAS usage on athletic or exercise performance [119]. It is

important to recognize, however, that because of ethical reasons most of those sanctioned studies did not administer AASs at the high dosages used by athletes. This prompted researchers to report no effects whereas anecdotal reports by individual users touted large, substantial effects. Those anecdotal reports were tainted by their lack of scientific control for confounding factors such as extent of training and "the placebo effect." Nonetheless, the findings in those "non-experimental" studies suggested body mass gains of 10 or 20 kg, as well as up to 30% increases in muscle strength [120]. Several well-controlled short-term studies have found smaller, but significant, increases in body mass, lean body mass, muscular strength, and concurrent reductions in fat mass [119, 120].

AAS usage is not limited to "weight-lifting–power" athletes. Endurance athletes (e.g., distance runners and tri-athletes) have also been found to use AAS. Interestingly, relative to endurance athletes, most studies have failed to demonstrate a substantial, beneficial effect of AAS on maximal oxygen consumption (i.e., key determinate to aerobic—endurance capacity) [115–119]. It has been proposed that endurance athletes who use AAS choose to do so because they recover from exercise training bouts more rapidly. This idea seems logical since performing more exercise training is a major stimulus to improve the cardiovascular-respiratory system, and thereby aerobic performance, and androgen treatment should negate the decline in testosterone that normally follows heavy exercise.

In addition to the myogenic effects of exogenous testosterone use, androgens have also promote erythropoiesis (i.e., hence potentially increasing oxygen carrying capacity of the blood and cardiovascular function; see later section, Erythropoietic Effects) [123]. The impetus for this line of research stemmed from early studies that found following puberty (and subsequent increases in testosterone), men developed a higher red blood cell and hemoglobin mass [124]. Since then, the use of exogenous synthetic androgens has been shown to affect hematocrit by stimulating erythropoietin production, altering iron metabolism by suppressing hepcidin, and by direct effect on bone marrow cells [123, 125]. Hypogonadal men have a lowered hematocrit and hemoglobin [126], and androgen treatment can return those parameters to normal levels [127], although it is unclear whether the same effect would occur in exercise-induced hypogonadism.

Mechanisms of Action

AAS are thought to affect athletes through mechanisms that are both physiological and psychological in nature [120]. These proposed mechanisms include: (a) activating steroid hormone receptors and androgen-responsive genes in skeletal muscle cells, (b) producing an anti-catabolic effect within the skeletal muscle, and (c) inducing motivational psychological effects. It is presently unclear to what extent each of these mechanisms contributes to the overall physiological adaptation and performance

changes found in AAS users. Most likely, these elements combine synergistically to bring about the changes noted.

Steroid Hormone Receptors

AAS activate androgen receptors in skeletal muscle cells, which stimulate the promoters of specific genes and induce protein synthesis [119, 120]. Of these proteins, one that appears to be important in increased muscle mass and strength is insulin-like growth factor-1 (IGF-1) [128]. IGF-1 mRNA in skeletal muscle is increased by testosterone treatment of older men [129], and is reduced in testosterone deficiency [130]. In certain cells, most notably prostate epithelium, the action of testosterone is amplified by its irreversible bioconversion to 5-α dihydrotesterone by the enzyme 5-α reductase. In skeletal muscle cells, however, the activity of this enzyme is weak [131]. The number of muscle androgen receptors is increased by androgens in certain experimental models [132, 133]. A number of transcription factors have been shown to interact with androgen receptors and to up-regulate androgen receptor function [134]. Of these factors, supervillin, a 205-kDa actin-binding protein, has been shown to be androgen regulated [135].

Importantly, exercise training seems to be necessary for AAS to exert any beneficial effect on performance. As an illustration, research has shown a greater exercise performance improvement with AAS usage by experienced weight lifters than by novice subjects [115, 119, 120]. The experienced weight lifters were capable of training with heavier weights, and of producing relatively greater muscle tension during exercise. AAS effectiveness also appears dependent upon the number of unbound steroid hormone receptor sites in skeletal muscle, which may be increased by resistance exercise training (i.e., intensive, powerful exercise activities [anaerobic]) [48, 51, 119, 120].

Anti-catabolic Actions

There is evidence that a portion of the anabolic effect of testosterone and AAS is via an antiglucocorticoid action. Cortisol is catabolic because one of its actions is to induce protein degradation [28, 36, 55]. Cortisol is secreted in large amounts in response to exercise, which may provide proteins for energy metabolism, and deplete the free amino acid pool of proteogenesis precursors [28, 36, 55]. Glucocorticoid receptors are found in skeletal muscle cells, and AAS can bind these receptors [136, 137] to block the binding of cortisol. This prevents cortisol from inducing muscle protein breakdown and depleting the free amino acid pool [34, 115, 120]. For example, in men with severe burn injury, testosterone treatment markedly reduced protein breakdown [129]. A similar effect could enhance recovery from exercise training by allowing amino acid precursors to be available for tissue repair and regeneration. Interestingly, athletes have indicated that AAS help them train more intensely and recover faster [115, 120].

Erythropoietic Effects

In healthy individuals, Medlinsky et al. [138] demonstrated in 1969 that testosterone administration increased erythropoietin production, the major regulatory hormonal mechanism for red blood cell production leading to increased hemoglobin and hematocrit. Furthermore, androgens directly affect bone marrow, iron uptake into erythrocytes, and enhance hemoglobin synthesis partly by suppressing hepcidin production [139, 140]. Moreover, androgens can increase the concentration of 2,3-DPG, facilitating a rightward shift in the oxyhemoglobin disassociation curve and hence promote oxygen delivery at the tissues; however, this affect was shown only in anemic patients or older adults and may not occur to a great extent in healthy younger athletic populations [139, 141, 142].

Psychological Effects

An important aspect of the AAS effect may be enhancement of the "motivational psychological state" of the athlete [120, 143]. This thought is supported by the notion that athletes indicate their emotional sense of well-being; euphoria, aggressiveness, and tolerance to stress, are all enhanced when they are using these agents. These emotions are positive motivators, and allow the athlete to train harder and to perform more intensive exercise training. This in turn would allow a greater exercise stimulus to be presented to muscle cells, and thus result in improved adaptation.

Side Effects of Usage

There are a large number of side effects associated with AAS usage [144]. Some of these effects are transient in nature; however, other effects seem more permanent and persist long after usage has stopped. Table 13.4 lists those side effects that have been reported in the literature. Brooks and associates [120] have categorized and subdivided the principal side effects into those attributable to:

Table 13.4 Major side effects associated with anabolic steroid usage in athletes

Acne
Aggressiveness
Altered electrolyte balance
Alterations in clotting factors
Altered thyroid function tests
Alterations in libido
Clitoral enlargement (women)
Depressed spermatogenesis
Decreased endogenous testosterone production

Decreased gonadotropin production
Dizziness
Depressed immune function
Decreased high-density lipoprotein
Edema
Elevated creatine kinase and lactate dehydrogenase
Elevated total cholesterol
Elevated triglycerides
Elevated blood glucose
Elevated blood pressure
Gastrointestinal distress
Gynecomastia and breast tenderness
Increase apocrine sweat gland activity
Increased nervous tension
Liver toxicity
Lower voice (women)
Masculinization (women)
Muscle cramps—spasms
Nosebleeds
Polyuria
Prostatic hypertrophy (and perhaps cancer)
Premature closure of epiphyses (children)
Psychosis
Wilm's tumor

See references: [120, 145]

- normal physiological actions of male sex-steroid hormones that are inappropriate in the recipient, and
- toxic effects caused by the chemical structure of the drug (principally "C-17 α alkylated" oral AAS).

One extremely important area concerning side effects involves the greater risk of cardiovascular disease associated with AAS use. For example, during AAS use, especially when taken orally, total cholesterol tends to increase, while HDL-cholesterol demonstrates a marked decline [145]. It appears that AAS influence hepatic triglyceride lipase (HTL) and lipoprotein lipase (LPL). HTL is primarily responsible for the clearance of HDL-cholesterol, while LPL facilitates the cellular uptake of free fatty acids and glycerol. Androgens and AASs stimulate HTL, presumably resulting in

decreased serum levels of HDL-cholesterol [145, 146]. AAS use may also provoke a transient hypertensive state. Data suggest that high dose usage significantly increases diastolic blood pressure. The increases in diastolic blood pressure typically subside after abstinence from AAS [145–148].

There is evidence that AAS use elicits structural changes in cardiomyocytes, and that myocardial ischemic tolerance is decreased after usage [145, 149, 150]. Echocardiographic studies in bodybuilders using AAS reported mild hypertrophy of the left ventricle with decreased diastolic relaxation, resulting in decreased diastolic filling [145, 148]. Some investigators have associated cardiomyopathy, myocardial infarction, and cerebrovascular accidents with abuse of AASs [130, 131]. However, a possible causal relationship has not been directly proved [151, 152]. Such physiological effects have negative influence on cardiovascular health risk factors; unfortunately, no rigorous scientific study-based data appear ethically available due to the risks associated with the long-term use, but case studies with self-administered AAS users have pointed to such associations [144, 151–153].

Many additional serious side-effects of AAS use exist which can compromise the overall health, and impact upon the morbidity and mortality of a user [153]. It is especially noteworthy to mention the serious liver disorders that can occur; such as hepatotoxicity, fatty liver and liver neoplasm, as well as anecdotal reports of permanent hypothalamic-pituitary-testicular suppression (see Table 13.4 for complete listing) [153].

Prevalence of AAS Usage

Usage of AAS by athletes is illegal, and often has negative ramifications on athletic eligibility (see Discussion below). As a consequence, the number of athletes using these agents is difficult to determine (i.e., most individuals are unwilling to admit use). Mistakenly, body builders are often cited for prevalent use of AASs. In self-report studies, estimate usage rates range from 5 to 30% of the professional and amateur athletic populations [143, 154, 155]. This even includes high school athletes and expectant athletes. For example, Buckley and associates [155] studied a North American sample of 3400 high school seniors, and found that over 6% used AAS periodically. In that study, the majority of the users were sports athletes. Interestingly, 35% were nonathletes who cited improved appearance as their main reason for taking the drugs. Regrettably, 20% of AAS users in that study had obtained their drugs from a health care, medical professional. Research by Yesalis et al. [156] in the 1990s put the total number of users within the United States at over 1 million. There is no evidence that number has declined in spite of the warnings issued by healthcare providers of the dangers of usage [143, 154–157]. In fact, recent work by Albertson et al. [158] suggests that usage, especially recreational, may be increasing.

Other Anabolic Substances

There are many other "anabolic agents" an athlete can use for supplementation. One that has received a large amount of press coverage is androstenedione, a precursor for both androgens and estrogens (testosterone and estradiol). Androstenedione is a weak androgen, and is poorly converted to more potent androgen forms. Instead, most orally administered androstenedione is metabolized to testosterone glucuronide and other metabolites before release into the general circulation [159]. Several well-controlled studies have been conducted to examine the effects of androstenedione usage [119, 160, 161]. Collectively, based upon evaluations of circulating androgen levels, muscle strength or morphology, these studies suggest that no credible evidence exists for an enhanced anabolic effect.

Dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) are the most abundant circulating steroid hormones in humans. DHEA, as with androstenedione, may serve as a precursor for androgens and estrogens; although, direct actions though nuclear and G-protein receptors have also been reported [162]. Theoretically, use of DHEA or DHEA-S as a supplement could have beneficial anabolic effects. In older adults, its use increased circulating androgen levels and subjective ratings of physical and psychological well-being [163]. It is perhaps most beneficial to those with adrenal insufficiency and those on high dosage glucocorticoid therapy [119]. Its effects on muscle strength and performance in athletes remain questionable [117, 119].

Recombinant growth hormone is used by some athletes (e.g., weight lifters) to increase muscle mass and strength. Growth hormone is a powerful endogenous anabolic hormone. It facilitates amino acid uptake and protein synthesis within many body tissues of which skeletal muscle is strongly influenced. Growth hormone also stimulates production of IGF-1, which is also anabolic. In addition, growth hormone has metabolic effects such as stimulating glucose uptake, free fatty acid mobilization, and lipolysis [28, 36, 55]. Human studies examining the efficacy of growth hormone usage in exercising men are limited [164, 165]. Muscle hypertrophy has been demonstrated, but the effects upon muscular strength are equivocal. Conversely, there are negative consequences to supplementation with growth hormone. Large dosages can result in edema, cardiomegaly, arthralgias, elevated blood glucose, peripheral neuropathy, atherosclerosis, and heart disease [120]. The risk for developing some of these side effects is enhanced when growth hormone is used in conjunction with AAS.

Anabolic Steroids—A Banned Substance

The International Olympic Committee (IOC) and all major sports governing organizations throughout the world have banned the use of substances taken for the purpose of unfairly and artificially improving performance in competition. Included on the banned substance list are varieties of AAS and similar agents.

In an attempt to deter usage by athletes, it is typical for major sporting events (Olympics, International—National Championship competitions) to have drug-testing programs ("doping control") to determine if any banned substance has been used to enhance performance. Furthermore, sport governing organizations perform random testing of national level athletes annually. Unfortunately, the logistical and financial considerations of such banned substance-testing make frequent, large-scale examination of all athletic competitions impossible. Therefore, many violators go undetected. Furthermore, the allure of sport fame and fortune are so strong in many societies that athletes are willing to risk being detected in order to gain a competitive edge. For example, AAS have been on the IOC list of banned substances since 1975 [143]. Since that time, 15 total winter and summer Olympic Games have taken place. Every one of these Olympic Games has involved a "doping scandal" of some type in which athletes were caught using banned substances. Many of these cases involved AAS usage.

It is important to note, however, that although many athletes abuse AAS as performance-enhancing drugs, there are legitimate uses of these substances. In cases of medical need, the World Anti-Doping Association (WADA) permits athletes to apply for a therapeutic use exemption (TUE) for a banned substance. In 2014, 897 TUE were granted, representing a 41% increase from 2013 (2014, WADA Annual Report). Of the TUE in 2013, only a small number were for anabolic agents (~3%) as noted at the WADA Paris Symposium that year [166].

WADA has strict guidelines for the control of therapeutic use exemptions (TUE), particularly with those substances otherwise considered to be performance enhancing. Specifically, WADA requires that the use of testosterone replacement therapy (TRT) be granted to only those male athletes who have what are considered to be an "organic androgen deficiency" [166]. This deficiency category is divided into primary (e.g., Klinefelter Syndrome, cryptorchidism, direct testicular trauma and torsion, and radiation or chemotherapy treatments) and secondary (e.g., pituitary disorders, sickle-cell disease, and anatomical issues) androgen deficiencies [166]. These are separate and distinct from disorders considered to be "functional androgen deficiencies" most commonly associated with athletes, resulting from severe stress, overtraining, or chronic systemic illnesses. According to the TUE Physician Guidelines on androgen deficiency:

> TUE should only be approved for androgen deficiency that has an organic etiology.
> TUE should not be approved for androgen deficiency due to functional disorder.
> TUE for androgen deficiency should not be approved for females.
> WADA—TUE Physician Guidelines [166]

In order for athletes to be granted a TUE, an application to WADA must be submitted, with information regarding the history, physical examination and

testing/laboratory evaluation by the primary physician, most commonly an endocrinologist. Even then, there are strict requirements for the application and dosage for TRT, since testosterone has a dose-proportional response [122], thereby opening the possibility for unfair advantages in athletes with TUE [167]. Of course, there will always be athletes and coaches who may try to take advantage of this system, but it is important to understand that physicians still have a legitimate recourse for TRT use when practicing medicine with elite athletes. Please note that many sports also impose their regulations, dictated by individual governing bodies, which may not align with WADA guidelines.

Conclusions

Endurance exercise training does have significant effects upon the major male reproductive hormone, testosterone, and the hypothalamic-pituitary axis that regulates testicular function. A growing body of evidence suggests that testosterone is chronically lowered in endurance exercise-trained men, and we have referred to this condition as "exercise-hypogonadism." While the mechanism of this lowering of testosterone is currently unclear, it may be related to a dysfunction or a readjustment within the hypothalamic-pituitary-testicular regulatory axis brought about by years of endurance training. Currently, the time course of these changes, including their reversibility, remains unresolved, and is in need of further scientific investigation [168]. The lowered testosterone levels of the men with EHMC could potentially disrupt anabolic or androgenic testosterone-dependent processes. Conversely, the alterations in testosterone levels brought about by endurance training could have cardiovascular protective effects, and may thus be beneficial to these men.

Similar reproductive hormonal profiles exist in EHMC men and in men undergoing overtraining who develop the Overtraining Syndrome. However, the hormonal changes with the Overtraining Syndrome are transient, and appear to reflect the stress of excessive physical training. An increase in the rest and recovery portions of training regimens seems to eliminate any hormonal abnormalities in overtraining men, but not so in EHMC men.

Some athletes are using anabolic-androgenic steroid agents in an attempt to induce muscular growth, development, and strength gains. Current evidence suggests that they are effective in producing the outcomes that athlete's desire. Unfortunately, athletes seem to be ignorant of, or are willing to ignore, the serious medical side effects and health consequences associated with usage of these agents. Unfortunately, their usage has been a persistent occurrence and problem in sports for the last 60 + years, even though there are legitimate medical usages that are permitted.

While there is a large volume of literature concerning reproductive endocrine dysfunction in exercising women, the number of studies in men is relatively small. Thus,

many questions regarding the male reproductive adaptive process to exercise training remain unanswered. Consequently, this area of exercise endocrinology is in need of continued study and investigation.

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14. Environmental Causes of Testicular Dysfunction

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Introduction

The notion that our environment can adversely impact male reproductive development and/or function is not an idea at the top of the list for most clinicians. There are probably very good practical reasons for this stance—for example, few will have encountered patients in whom reproductive dysfunction was diagnosed as being altered by some environmental factor. Does this mean that such causes are rare or does it mean that they go unrecognized? Unfortunately, we probably still lack sufficient understanding to definitively answer this question, but hopefully this chapter will go some way toward addressing it.

When viewed from an evolutionary perspective, it is clear that the environment and reproductive function are inextricably linked, as evidenced by the fact that the majority of mammals ('seasonal breeders') exhibit seasonal hypogonadism and infertility under the influence of daylight length and other environmental cues [1]. Humans are not seasonal breeders, but as we undoubtedly have seasonally breeding ancestors in our

evolutionary past, residues of such effects are likely, as discussed later in this chapter. But perhaps the most important lesson to be learned is that seasonal breeding evolved so as to ensure that offspring are born at the optimum time of year for survival, which usually equates to the availability of a good food supply. Thus, environmental factors, of which daylight is the most invariable, were adopted as cues via which to regulate function of the reproductive system—this seems to work primarily via central regulation of the hypothalamic–pituitary axis [2]. However, effects of the environment on reproduction are far more pervasive than this. The well-established relationship between food intake/energy balance and onset of puberty/maintenance of menstrual cycling in females is another example, and the mechanisms via which these effects occur are now being established (e.g., leptin) [3]. These examples remind us that we have evolved so as to be 'in tune' with our environment and, if we accept this premise, we must also recognize that when our environment or lifestyle changes dramatically, then there may be health consequences. The major changes in Western diet (increased intake of refined carbohydrates, dairy products/fats) and lifestyle (e.g., widespread artificial light, increased sedentary habits, increased recreational and pharmaceutical drug use) over the last century are therefore certain to have had some impact, but the extent to which these changes may have affected our general or reproductive health is unclear, with the notable exception of obesity and obesity-related disorders.

In the past 20 years the specter of more widespread environmental adverse effects on male reproductive function has arisen, fuelled by the possibility of 'falling sperm counts' and increase in other male reproductive disorders [4, 5]. Though this remains a somewhat controversial topic, some aspects, such as the increased incidence of testicular germ cell cancer over the past 50+ years, and the high prevalence of young men with low sperm counts (<20 million/ml) are beyond dispute [4]. Such changes must have a lifestyle/environmental cause, and the various possibilities are outlined below. There are strong beliefs that testis germ cell cancer may be the tip of an iceberg, signaling to us about a more fundamental underlying syndrome of disorders ('testicular dysgenesis syndrome') that may have a common origin in fetal life during the masculinization process [4–6]. Much of this is still hypothesis, but the evidence in its favor has grown substantially, based on clinical and experimental animal studies [4, 7, 8]. True or not, this hypothesized syndrome has played an important part in opening our eyes to the fundamental importance of fetal and neonatal life in 'setting up the male reproductive system for adulthood.' This reality is very much in step with thinking in other areas of medicine in which the fetal origins of adult disease are recognized increasingly as being of fundamental importance. Such developments have turned the spotlight on the pregnant woman and, with the dramatic changes that have been occurring in women's lifestyles in the past two decades or so, and the later age for first pregnancies, it seems inevitable that this must have lifelong consequences for the fetus. These may be good or bad, but if they adversely affect the reproductive health of the

offspring [4–6], such effects can remain 'hidden' for 30 or more years, with the result that recognition of the effect and its 'cause' is extremely difficult to investigate in a definitive manner.

In parallel with the developments outlined above, there has been a veritable explosion of studies aimed at establishing that the reproductive health of humans and wildlife is being adversely impacted by so-called endocrine disruptors. This is a broad and complex area with much controversy and uncertainty, and whether or not endocrine disruptors really do exert significant effects on human male reproductive health still remains an open question [4, 5, 9]. However, we must recognize that man-made products such as DDT and PCBs, which undoubtedly exert a range of biological effects in wildlife, persist in us all and are to some extent passed from one generation to the next via breast milk and the food chain. Although these products have been banned/restricted in use for decades, their persistence makes them a continuing issue, and indeed, they have been added to by more recent brominated and fluorinated compounds which also bioaccumulate to a significant degree, and evidence for their potential adverse adult-onset reproductive effects following maternal exposure during pregnancy has begun to emerge [4, 10, 11]. These and numerous other man-made chemicals are part of our everyday environment, and it is more likely than not that some of these compounds will have a biological effect upon some of us. Such effects may be benign, beneficial or perhaps most likely adverse, but proving cause and effect in human studies is fraught with problems; hence, evidence for this is sought from animal experimental studies. However, as detailed later in this chapter, emerging evidence for animal–human differences in susceptibility to the adverse effects of certain key contaminants questions how reliable it is to use animal models to estimate human risk.

Environmental/Lifestyle Effects on the Adult Male

These fall into four different categories, namely the effects of season, occupation, lifestyle/diet and chemical exposures (Fig. 14.1). These are dealt with separately, but it is emphasized that they are not independent factors, and that combinations of circumstances, rather than individual factors, may be more important in causing reproductive health changes. In a similar vein, some individuals may be predisposed to an adverse effect of a chemical or lifestyle factor, for example, because of their genetic background.

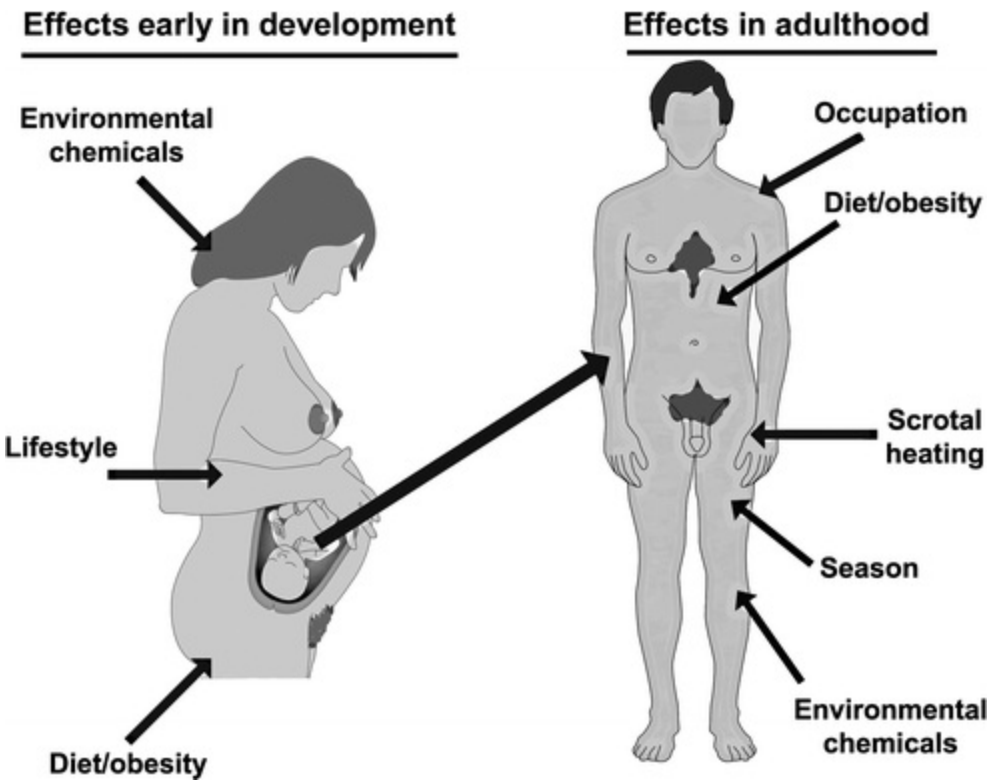

Fig. 14.1 Main environmental and lifestyle factors affecting testicular development and/or function in adulthood

Seasonal Effects on Testicular Function

Though humans are considered to be non-seasonal mammals, we are undoubtedly sensitive to photoperiod [12], as exemplified by 'seasonal affective disorder' and by seasonal trends in the frequency of births and in the incidence of twins. Such effects are most obvious in Northern Europe where photoperiodic changes are most extreme [13]. Furthermore, both longitudinal and cross-sectional studies have demonstrated that sperm counts in men are consistently ~30% lower in summer than in spring and/or winter [14–16], though not all studies have reported such effects, and they may be less apparent or absent in tropical countries [17]. An alternative explanation is that exposure to the higher summer temperature is responsible for lowering sperm production (see below), though temperature changes do not account for all of the seasonal trends in births, especially in Northern Europe [12]. If the reported 'seasonal' changes in sperm counts/semen quality are an echo from our seasonally breeding ancestors, then this may be rather variable in its effect and, in most men, is unlikely to exert a major effect on their fertility. It is likely that any effects of season in humans/individuals will be driven by seasonal changes in the duration of melatonin secretion, as in seasonally breeding animals (below).

Studies in seasonally breeding animals have established the key pathways via which daylight length is able to regulate the reproductive axis, and melatonin secreted by the pineal gland plays a pivotal role [1, 2]. It is therefore curious that young men with

hypogonadotropic hypogonadism (HH) show abnormal blood levels of melatonin, being increased in males with idiopathic HH and decreased in males with Kallmann syndrome, when compared with controls [18, 19]. However, as melatonin levels may be normalized by testosterone replacement therapy in HH men, it implies that the abnormal melatonin level may be a consequence rather than a cause of the HH [18], although this reasoning may not extend to men with hypergonadotropic hypogonadism [19]. More generally, the evidence for seasonal fluctuations in testosterone levels in men is equivocal [20] and is likely to be highly confounded by the influence of other factors such as obesity, activity levels and age.

Occupational Effects on Testicular Function: General Aspects

Numerous published studies have retrospectively surveyed the occupations of men attending infertility clinics and/or compared occupations of fertile and infertile groups. There is some consensus in showing, for example, that farmers/agricultural workers or lorry drivers, painters or welders may be over-represented among infertile men, but overall the findings of such studies are inconsistent and have failed to identify common occupational causes of male infertility [21–24]. Occupation is only one of a range of factors that may cause male infertility, and therefore searching for such factors among patients at the infertility clinic may not be the most sensitive approach. Unfortunately, alternative approaches such as the direct investigation of particular working groups also have various problems [25]. Low participation rates are common and may be biased toward those who have experienced, or suspect, a fertility problem [26]. This makes interpretation of any findings difficult. Another common problem is the low numbers of workers who may eventually compose the ‘exposed’ or control groups, as many published studies involve 30–50 men or less per group [23, 25, 27]. As sperm counts and other semen parameters show great variation between subjects, detection of a workplace/occupational effect against such a background requires considerable numbers of subjects; otherwise, the study will lack sufficient power to detect anything other than a major change in semen parameters [25]. For example, ~80 control and ‘exposed’ men would be required to detect a 20% fall in sperm count. Moreover, because within-subject variation in semen parameters can also be substantial, a rigorous study should involve two or more semen samples per subject, adding considerably to study complexity and cost.

Finally, there are likely to be many confounding factors in any occupational study. These may include age, ejaculatory frequency/abstinence, smoking and alcohol consumption, recreational drug use, time spent seated (see below), recent infection or febrile period, past history of cryptorchidism and sexually transmitted disease [28]. Attempts can be made to control for some of these factors, but with the generally small numbers of subjects involved, this is inevitably less than satisfactory. Against this

background, it is therefore unsurprising that relatively few occupations or workplace exposures have been shown **consistently** to impact significantly on male reproductive health (usually on sperm counts/fertility). However, there is reasonable evidence to suggest that exposure to some solvents [29], older glycol ethers [23, 30] and inorganic lead [31, 32] can impact one or more aspects of semen quality in exposed workers and in some instances affect fertility or miscarriage rates (though effects on the latter two parameters are small). Nevertheless, other studies that showed no significant effect of similar exposures can also be found in the literature [e.g., 33, 34]. This would support the view that such workplace exposures have relatively minor effects on semen quality and male fertility, though in some individuals, such as those with a low sperm count for other reasons, such effects might significantly impact fertility.

Information on occupational impacts on testosterone levels in men is even more equivocal, in part for reasons of study group sizes as just outlined for semen quality aspects. Moreover, any adverse effect on testosterone levels should automatically trigger a compensatory increase in LH secretion that will return testosterone levels toward normal, unless the factor(s) in question exerts its adverse effect via altering secretion of LH. While any such compensatory change should theoretically be detectable via LH measurement and calculation of the LH/testosterone ratio, the inter-individual variation in LH and its pulsatile secretion pattern as well as the circadian rhythm in testosterone levels can make differences difficult to detect unless changes are extreme, which would be unusual. Reasons such as these probably explain why the literature on this topic is small with highly equivocal, and usually very minor, findings. These will not be reviewed in any detail here, but an example is a Chinese study of 154 electrical power-plant workers, half of whom were exposed to electromagnetic fields, which was associated with a 3% average drop ($p = 0.015$) in total testosterone levels [35]; such a change is likely to be incidental. Where occupation has been associated with altered testosterone levels, it has been mainly in connection with pesticide/fungicide exposure, which is outlined below. A more convincing study involved Italian policemen exposed to traffic fumes [36], compared with police office workers. Those exposed to traffic fumes exhibited ~20% reduction in free testosterone levels, a difference that was even more marked (~29%) when those in the age range 30–40 years were compared [36]. Unfortunately, LH levels and the LH/testosterone ratio were not reported in that particular study, although a similar earlier study by the same authors ($N = 166$ traffic-exposed vs. 166 non-exposed) reported a mean 50% increase in LH levels (from 1.8 to 2.7 mIU/ml; $p < 0.001$), although no testosterone levels were reported in that study! [37] Thus, it *appears* that high exposure to traffic pollutants may induce compensated Leydig cell failure (i.e., decreased or normal testosterone in the face of elevated LH), but this requires direct confirmation in a properly designed study in which both LH and testosterone are measured in the same men. As to mechanism, there is some evidence that diesel exhaust might suppress expression of steroidogenic

factor-1 in developmental animal studies [38].

Occupational/Environmental Exposure to Pesticides/Fungicides/PCBs

The occupational exposure associated with the greatest change in sperm count and fertility was workplace exposure to the nematicide, dibromochloropropane (DBCP; Table 14.1). This toxin induced azoospermia or oligospermia in a high percentage of exposed workers, both those involved in its manufacture and those involved in its application on crops [39–41]. In a substantial proportion of DBCP-exposed workers who became azoospermic, no recovery of sperm counts occurred following removal from exposure [39, 40]. This *lone* example is frequently cited as reassuring evidence of the rarity of such workplace effects on sperm counts and male fertility. In reality this reassurance is a veil. The effects of DBCP were revealed, even though the affected workforces comprised fairly low numbers, because it had **catastrophic** effects on sperm counts/fertility. We can be reassured that similar catastrophes will also reveal themselves, but more modest effects of occupation on sperm counts/fertility will remain difficult or impossible to identify in all but very large, well-controlled studies. The latter are hugely expensive and laborious, both of which act as strong deterrents to their application.

Table 14.1 Main examples of environmental chemicals that have been studied in relation to potential impacts on spermatogenesis or steroidogenesis in adult men

Chemical type	Uses	Nature of exposure	Possible effects
Dibromochloropropane (DBCP)	Nematicide	Occupational	Irreversible azoospermia
DDT/DDE and related pesticides	Pesticides	Food chain and environmental	?Changes in semen quality and/or LH and testosterone
Polychlorinated Biphenyls (PCBs)	Electrical equipment + others	Food chain and environmental	?Changes in semen quality and/or LH and testosterone
Organophosphates	Pesticide	Occupational	?Changes in semen quality and/or LH and testosterone
Pyrethroids	Pesticide	Environmental	?Changes in semen quality and/or LH and testosterone

The DBCP experience has had several important repercussions, the most scientifically important being the impetus that it gave to studies of wider pesticide exposure and male reproductive function, especially changes in semen quality and male fertility. Conversely, it has also helped trigger the default view that pesticide exposure results in lowered sperm counts/infertility, which has become more or less accepted as dogma in some circles. There have also been numerous studies of pesticide exposure

and hormone levels in men, which is the primary focus of this chapter. However, as there is clear evidence that men with low sperm counts are at increased risk of having compensated Leydig cell failure [42, 43], it makes sense to consider the evidence for pesticide effects on both aspects of male reproductive function together.

There are numerous studies that show significant associations between exposure to various pesticides/fungicides and reduced semen quality (Table 14.1), but there are nearly as many studies showing no significant association. As this is not the primary focus of this chapter, only a brief overview is given here, and readers are referred to a 2008 review [44]. This paper reviewed all studies spanning a 15-year period and reported that only 13 of 20 studies showed a significant negative association between environmental or occupational pesticide exposure and semen quality with similar disparity for other parameters (e.g., sperm DNA damage). When considering individual pesticides or classes of pesticides, the results are in general equally inconsistent, for example, for persistent organochlorine pesticides such as DDT/DDE [45–48]. Recent studies that have focused on other pesticides such as organophosphates [47, 49, 50] or pyrethroids [51] have perhaps shown a more consistent association between exposure and reduced semen quality.

In terms of pesticide exposure and sex hormone levels in men, the perspective is quite similar to that for semen quality, although not as many studies have investigated hormone levels. Thus, reduced blood testosterone levels with or without altered (up/down) LH levels have been reported in men exposed to persistent organochlorine pesticides such as DDT/DDE [52], to organophosphates [53] or pyrethroids [51, 54]. However, for each of these classes of compounds there are comparable studies (mostly larger in size) showing no significant alteration of testosterone and/or LH levels in association with exposure to DDT/DDE or other persistent organochlorine pesticides [55–59], to organophosphates [60] or pyrethroids [61].

The inconsistencies in studies that have assessed the association between pesticide exposure and male reproductive function may be partly attributed to inter-study variation in a range of factors, such as methods, country of study, exposure assessments and outcomes [44]. Perhaps more important may be that many of the studies are based on either male partners of infertile couples or farmers/occupationally exposed men, neither of which is representative of the general male population. It is perhaps noteworthy that the majority of studies unaffected by such limitations have mostly found no association between pesticide exposure and male reproductive dysfunction [46, 48, 58, 61], although there are exceptions [53, 62], bearing in mind also that there will be significant under-reporting of negative data in the published literature. Arguably, the most informative study was the US LIFE study which was prospective and involved 501 male partners of couples who were just stopping contraceptive use, and in whom exposure to 36 PCBs, 9 organochlorine pesticides and 10 polybrominated diethyl ethers was measured [48]. Although a range of statistically significant associations was found

between exposure to individual chemicals or classes of chemicals and semen quality, most of the associations were positive not negative. In this study, as well as in all of the others cited, it should be emphasized that where significant associations have been found between exposure(s) and semen quality or testosterone levels, the 'effect' sizes, whether negative or positive, have been generally small and could be incidental. The tentative conclusion, based on present data, is that for the general population at current levels of pesticide exposure, there is unlikely to be any biologically meaningful effect on adult male reproductive function although there may be some risk for those who are more highly exposed via their occupations, such as farmers.

Occupational/Lifestyle Effects on Scrotal Temperature

Another situation in which occupational effects on male reproductive function might become apparent is if the 'occupational effect' applies to a large proportion of men; 'scrotal heating' may be one such example. Scrotal, and therefore testicular, temperature has to be maintained some 3–4 C lower than core body temperature if normal spermatogenesis is to occur, and this is achieved via a vascular heat exchanger system, the pampiniform plexus (Fig. 14.2). Interference with the normal scrotal cooling process can profoundly affect sperm production, quality and fertility [63–65], although there is no clear evidence for effects on testosterone levels. A straightforward example of this is the effects of repeated Finnish sauna exposure over 3 months, which lowered sperm counts and motility and induced adverse effects to sperm chromatin packaging but did not measurably alter testosterone levels; all of the adverse effects of the sauna were reversible within 6 months [66]. In theory, occupational or lifestyle factors that interfere with scrotal cooling/thermoregulation could be significant factors in lowering sperm counts [66, 67]. Examples of such potential factors are exposure to radiant heat, the wearing of tight trousers/underwear, time spent having hot baths and time spent seated (Fig. 14.2). Most of the older studies that sought to investigate such factors could rely only on establishing an association between such factors and poor semen quality, and perhaps not surprisingly the evidence proved equivocal or only marginally convincing in most cases [reviewed in ref. 25]. However, several epidemiological studies have shown that occupations involving prolonged sitting, and thus reduction in airflow around the scrotum [63, 66], are associated with significant reductions in sperm counts. An example is car or taxi driving [68, 69]. Away from occupational situations, the worst case might be paraplegic men who are confined to wheelchairs [70], although the poor semen quality in such men may have other contributory causes such as impaired accessory sex gland function, infection and increased proinflammatory cytokines [71].

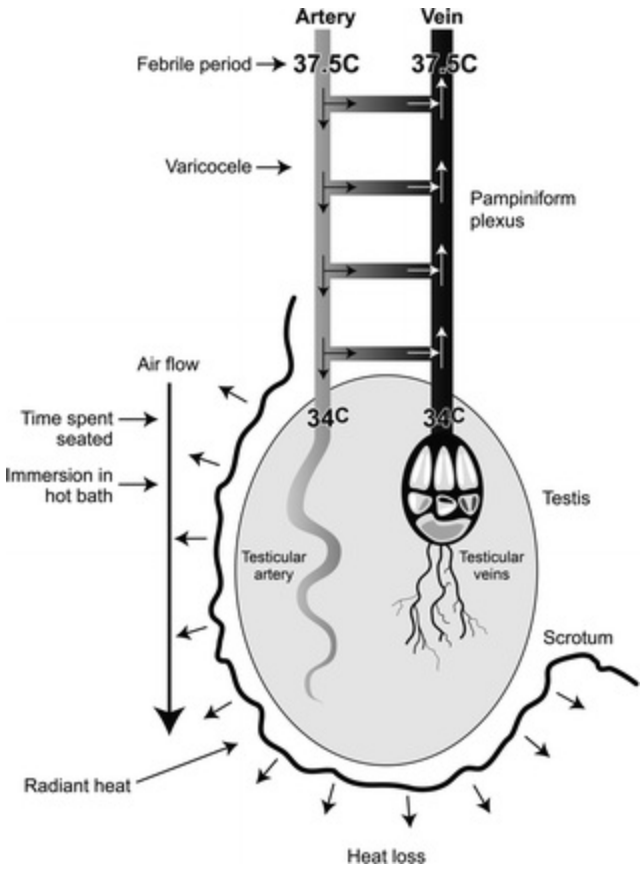

Fig. 14.2 Schematic diagram to illustrate the central mechanisms involved in cooling of the testis, and the various occupational or lifestyle factors that can interfere with their function. The primary mechanism of cooling the testis is by heat loss from the scrotum, and this necessitates air flow around it. The resulting cooling of the testis means that testicular venous blood is also cool when it leaves the testis and this blood is used to cool incoming warm arterial blood within the pampiniform plexus via heat exchange and via the diversion of ~40% of incoming arterial blood into the testicular vein via numerous arterio-venous anastomoses; the presence of a varicocele may interfere with heat exchange within the pampiniform plexus

More objective studies became possible once the technical problems relating to measurement of changes in scrotal temperature had been overcome, as this enabled *quantification* of scrotal heating. Scrotal temperature was measured continuously in normal young men or in men from couples planning their first pregnancy, and a significant relationship between average daily scrotal temperature and sperm counts was established [72]. Moreover, the same studies linked scrotal temperature to the amount of time spent seated, i.e., the more sedentary the lifestyle/occupation, the higher the average scrotal temperature, and this has been confirmed in more extensive recent studies [73]. Application of continuous scrotal temperature monitoring in reasonably large numbers of healthy men has demonstrated unequivocally that increase in average scrotal temperature is clearly linked to lower sperm counts as well as to associated changes in blood levels of FSH and inhibin-B [72–74]. Such findings raised the expectation that our increasingly sedentary lifestyles in the West might induce negative effects on semen quality in a high proportion of men [25], but this expectation is proving

to be overly simplistic. For example, although time spent seated is an important determinant of scrotal temperature, the former has only a weak relationship with semen quality [74] and fertility [75], and other factors such as obesity [76], night-time scrotal temperature [73] and genetic determinants of scrotal temperature [77] may prove to be more important. There is reasonably strong evidence that physical activity is associated with better semen quality [75, 78] and marginally higher testosterone levels [78], when compared to men who have sedentary lifestyles. While this may be related to lower scrotal temperature, as physical activity is likely to improve scrotal cooling, there may be other effects of physical activity that also impact testis function.

From a clinical perspective, the number of men in whom infertility is induced partly or totally by elevated scrotal temperature is likely to be low. However, there are three other points to be kept in mind. First, even quite mild elevations in scrotal temperature in animal studies can increase DNA damage and miscarriage rates [64, 79], so its effects on sperm can be qualitative as well as quantitative. Second, 'treatment' to lower overall scrotal temperature in patients who have poor semen quality, though being a 'shot in the dark,' is relatively noninvasive and can be quite effective in some cases [80]—and there are no side effects! The discovery that there are genetic determinants of scrotal temperature [77] also reinforces the possibility that some individuals may be predisposed to adverse effects of prolonged seating or radiant heat on semen quality. While such individuals cannot yet be recognized, careful attention to lifestyle factors to minimize scrotal heating in all men with fertility problems is therapeutically benign and can have only beneficial effects. Third, the well-established, if inconsistent, effect of a varicocele on semen quality in men, may itself induce such effects by interfering with normal cooling of incoming arterial blood in the pampiniform plexus [76] (Fig. 14.2), and it is possible that such an effect might be exacerbated by the time spent seated or make such individuals more susceptible to effects of radiant heat than men without a varicocele.

Other Lifestyle/Dietary Factors Affecting the Adult Male

The preceding section dealing with scrotal temperature obviously applies as much to sedentary lifestyles outside, as well as inside, of the workplace. Probably, the most important other factors are dietary habits, in particular obesity and alcohol intake, smoking, stress and recreational/sporting drug use.

Unlike the well-established relationship between calorific intake and maintenance of menstrual cycles in women, there is no such straightforward relationship in men regarding sperm counts. Underweight and overweight men both have a slightly increased risk of having low sperm counts, though there is considerable variation between studies, especially with regard to obesity [81, 82]. Moreover, there is only limited evidence that it is obesity per se that causes any semen quality changes

(Fig. 14.3). Thus, although a 14-week residential weight loss program in 43 severely obese men was shown to increase semen volume and total sperm count [83], similar studies with small numbers of obese men undergoing bariatric surgery-induced weight loss have so far failed to find consistent benefits, and some studies even point to deleterious effects [84]. Nevertheless, the evidence that male obesity can negatively impact fertility is convincing. For example, obesity is over-represented among infertile men [80, 81], and in the setting of assisted reproduction, male obesity is associated with reduced embryo quality and pregnancy rate in the absence of any significant changes in semen quality [85]. Indeed, a recent meta-analysis of 30 studies (n = 115, 158 men) reported that male obesity was associated with a 66% increase in risk of infertility and a 35% reduction in live birth rate per cycle of assisted reproduction in the absence of significant differences in conventional semen parameters [86]. The underlying cause of obesity-related male infertility remains unclear and is likely to be multi-factorial [87]. However, there is compelling evidence emerging that points to changes in fatty acid composition of semen and sperm as being important [88, 89], and both human [90] and animal [91] studies provide evidence that the fat composition of our diets may be important in determining this and affecting semen quality (Fig. 14.3). Thus, it may be specific dietary factors in obese men that is important in relation to changes in semen quality and/or fertility, and that therefore not all obese men will be affected.

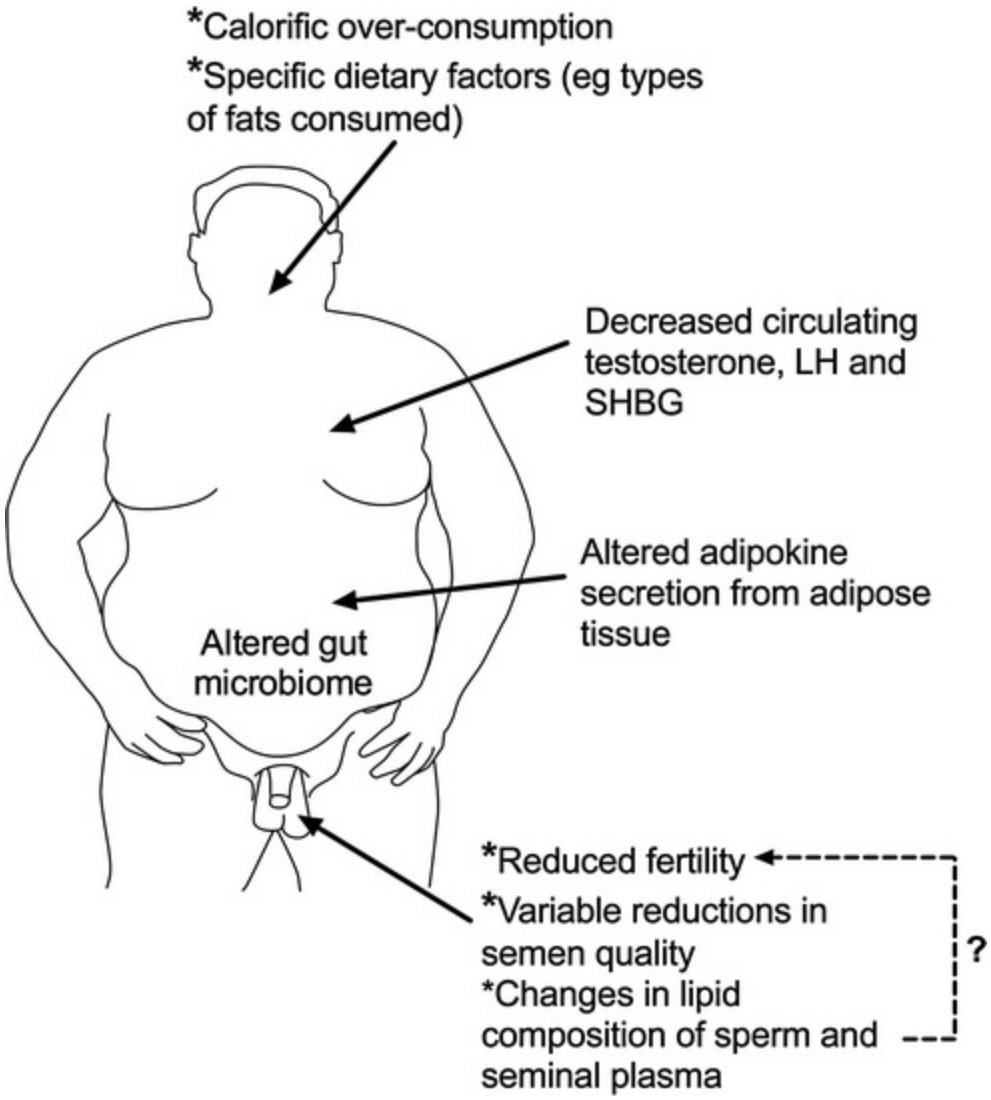

Fig. 14.3 Schematic representation of the major reproductive changes in men that are associated with obesity and current thinking as to what may be the key causal factors and pathways

In contrast to the equivocal data on semen quality, the evidence that visceral obesity in men is associated with a fall in total testosterone levels in both young [92] and older [93] men is much more compelling (Fig. 14.3), and it is now widely accepted that visceral obesity and its associated metabolic changes invariably impact testosterone levels, encapsulated in the metabolic syndrome [94]; however, metabolic syndrome-associated changes in testosterone levels are dissociated from any change in semen parameters [95]. The primary mechanism via which obesity/the metabolic syndrome reduces testosterone levels is via suppression of pituitary LH secretion, but the mechanisms that underpin this are complex and incompletely known, but certainly involve changes in estradiol and sex hormone-binding globulin levels [96, 97] (Fig. 14.3). These interplay with the effects of adipokines, such as leptin and adiponectin, which may act both centrally [97, 98] or directly on the testis [99], although a recent systematic review suggests that direct testicular effects are probably

of minor importance in comparison with central effects [99]. As other chapters in this book deal with these aspects, in particular the relationship of hypogonadism to the metabolic syndrome, they will not be discussed further here. However, there is one emerging aspect that has been hitherto overlooked, and which may turn out in the long term to be an important player, the gut microbiome.

There are various lines of evidence that the gut microbiome can affect male reproductive function, although the number of studies is presently too few to enable assessment of how important this might be [100]. However, its potential importance is highlighted by a series of intervention studies in mice, which have shown that feeding them from 8 weeks of age (young adulthood) with the probiotic species *Lactobacillus reuteri* was able to protect the mice against diet-induced, age-related obesity [101]. However, far more interesting was that this intervention completely prevented the age-related development of impaired spermatogenesis, reduced testis weight and hypogonadism [102], although until such studies are repeated, it is too early to conclude that similar effects could apply to man. The protective effects of the probiotic treatment were equally apparent in mice maintained on either a standard (control) diet or maintained on a 'Western style diet.' A series of studies by these authors have shown that the beneficial effects of the probiotic feeding regime appear to result from modulation of specific proinflammatory cytokines, such as interleukin-10 and interleukin-17A [102, 103]. As obesity/metabolic syndrome and hypogonadism are linked intrinsically to a more proinflammatory cytokine profile in blood in men [94, 104], it raises the interesting possibility that such health-relevant changes could be ameliorated either via probiotic supplementation or by dietary changes that alter the gut microbiome, an area richly deserving of further study.

Smoking by men sometimes emerges from epidemiology studies as a risk factor for low sperm counts or altered sperm morphology, but this is an inconsistent finding and 'effect' sizes are small [105]. Conversely, smoking is consistently associated with increased rather than decreased total and free testosterone levels, the mechanism for which is uncertain [106, 107]. As outlined below, smoking by mothers during pregnancy is associated with a consistent and substantial (20–40%) reduction in sperm counts in resulting male offspring in adulthood [25], whereas testosterone levels are unaffected [108]. In a large population-based cross-sectional study of >8000 young men, moderate alcohol intake was not associated with any change in semen quality, but was positively associated with free testosterone levels [109], whereas heavy alcohol intake is associated with lowered total testosterone levels [110].

Psychological stress arising for various reasons is usually associated with reduced semen quality with effects both on sperm count and motility, and testosterone levels are also usually lowered [111, 112]. The mechanisms behind such changes involve a complex interplay at various levels between the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes; among these, direct inhibitory effects of adrenal

cortisol on testicular testosterone production and indirect effects via cortisol-induced decrease in SHBG levels are probably important [111]. It should be noted, however, that much of the available data stem from experimental animal studies, as studies in men are much more difficult to control for confounding factors [111].

Though Western males are generally less physically active today than 30 or so years ago, for those who do participate in sports, it is far more competitive and this has led to usage of performance-enhancing drugs, the most common being anabolic androgenic steroids [113]. Outside of sports, there has been an explosion in use of the same drugs in pursuit of improving physique and body image [113]. Such use is now considered to be perhaps the single most important determinant of hypogonadism in young men [114], although this will be dependent on factors such as type of steroid, dose administered and perhaps duration of administration [115]. Although much of the anabolic steroid use is clandestine, clinicians faced with athletic young men with hypogonadism should always bear this possibility in mind [113, 115]. Similar awareness should be practiced for other non-steroidal drugs, as we live in an age when use of a range of 'recreational' drugs is widespread. For example, 25% of young American men report using marijuana within the last month [116], and 45% of Danish young men within the past 3 months [117]. In the latter study, which was a population-based study ($N = 1215$), regular weekly use of marijuana was associated with ~30% decrease in sperm count, and if marijuana use was combined with other recreational drugs, the decrease was >50% [117]. Considering the scale of marijuana/other recreational drug use, these statistics suggest that this may be a very important determinant of low sperm counts in young men. As has been found with tobacco smoking, marijuana use is associated with a significant increase in total (but not free) testosterone levels [117]. In contrast, use of opiate-based drugs by young men is associated with a uniform and quite substantial decrease in testosterone levels, based on a meta-analysis of 17 studies [118].

Finally, vigorous exercise such as long-distance running may result in temporary suppression of testosterone levels in men and minor decrements in semen quality, though the changes are by no means as pronounced as the anti-reproductive effects that can occur in female athletes [119, 120]. In contrast, less severe exercise generally has no effect on testosterone levels [120] (see Chap. 13).

Environmental/Lifestyle Effects on the Adult Testis that Arise During Fetal Development

Testicular Dysgenesis Syndrome (TDS) and the Masculinization Programming Window

There is growing evidence that a syndrome of inter-connected disorders affecting the

human male, so-called TDS, may have a common origin in fetal life during the period of masculinization [4, 6–8]. Manifestations of this syndrome in adulthood can include low sperm counts/reduced fertility and/or testicular germ cell cancer as well as a history of cryptorchidism and/or hypospadias (Fig. 14.4)—recent evidence suggests that lowered testosterone levels or compensated Leydig cell failure could also be a part of TDS [43, 121]. Some of these disorders appear to be increasing in incidence, with environmental/lifestyle causes implicated in this increase [4, 6]. An integral part of this syndrome of disorders is evidence for impaired testosterone production/action [7, 8], during a specific fetal period termed the 'masculinization programming window' (MPW; Fig. 14.4) [7]. This period has been shown in animal studies to be the critical period when sufficient fetal androgen action is required to program later development of all male reproductive organs, including their ultimate size [7, 8, 122, 123]. The available evidence suggests that an MPW also exists in humans and probably lies within the period 8- to 12-weeks gestation [7, 8], and based on the animal studies and emerging human data, the level of androgen exposure during the MPW could be deduced at any age by measurement of the anogenital distance (AGD) [7, 8].

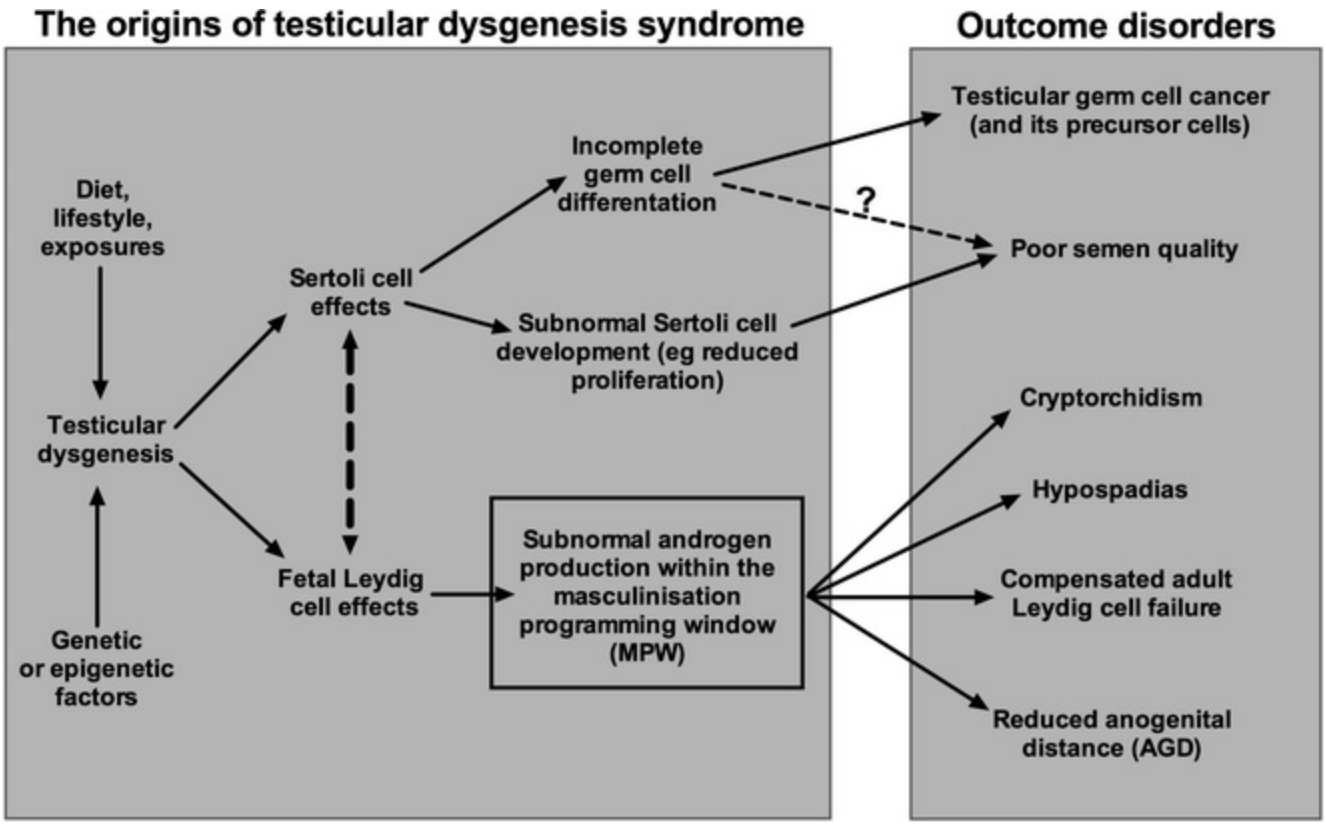

Fig. 14.4 Schematic diagram to illustrate the cellular basis and general pathways via which the disorders that comprise 'testicular dysgenesis syndrome' (TDS) are thought to arise in the human. Abnormal testicular cell differentiation/function (=dysgenesis) appears to be an integral part of this syndrome of disorders, but several causal pathways might lead to this occurrence, including genetic, environmental and/or lifestyle factors. Note also that the resulting disorders that arise because of TDS occur with differing frequency, varying from quite common (poor semen quality, cryptorchidism) to rare (testicular germ cell cancer). Note also that some of the disorders may occur for

reasons other than TDS (e.g., cryptorchidism, hypospadias) or because of effects after birth/in adulthood (e.g., poor semen quality). TDS disorders are thought to arise primarily as a consequence of, or associated with, subnormal testosterone production and/or action within a discrete fetal time window, termed the 'masculinization programming window (MPW),' which is reckoned to occur somewhere within the time period of 8- to 12-weeks gestation. In theory, androgen exposure within the MPW can be determined retrospectively by measurement of anogenital distance (AGD), though in practice such use may have limitations (see text)

As a result of the discovery of the MPW and the emerging credibility of the TDS hypothesis, the focus has shifted toward trying to identify what factors, acting via the pregnant mother, might adversely impact fetal testis testosterone production, a task that is severely constrained because of the inaccessibility of the human fetus (it is only a few cm in length during the MPW) and because fetal androgen production cannot be monitored directly [8]. Therefore, most of the available evidence derives from epidemiological studies that have looked for associations between occurrence of 'TDS disorders' (e.g., cryptorchidism, hypospadias) and specific maternal exposures or lifestyle factors. This literature is too extensive to discuss in detail here, so just the aspects that are most directly relevant to this chapter are presented. Further details and reference trails can be found elsewhere [4–8].

It is first worth making some general points, in particular to remind readers of just how common are the disorders that may comprise the TDS. For example, the two most common congenital malformations in children of either sex are both TDS disorders, namely cryptorchidism and hypospadias, which affect 2–9 and 0.3–1.0% of boys at birth, respectively [4, 6, 8]. This is not to imply that all cases of cryptorchidism and hypospadias are part of TDS, because they may arise as a consequence of other deficiencies/syndromes which might not be directly connected to TDS. One way in which some potential discrimination might be applied is via measurement of AGD, because if AGD is abnormally small, it would imply that there had been androgen deficiency/resistance during the period of the MPW, so that the case in question could be classified as likely to be TDS. Where this has been done for cases of cryptorchidism and hypospadias and reference made to population-based normal data for AGD in one particular center, it is clear that the majority (but not all) of cases of cryptorchidism and hypospadias have AGD measurements below the normal 50th centile [124]. Therefore, based on AGD it may be possible to at least putatively identify possible TDS and non-TDS cases, at least for cryptorchidism and hypospadias. Reduced AGD has also been associated with androgen receptor polymorphisms such as longer CAG repeat length, which impairs androgen signaling [125].

Extending the same line of thinking to adult-onset cases of suspected TDS, namely those with low sperm counts, low testosterone/compensated Leydig cell failure or testicular germ cell cancer, has only so far been done in a superficial way, and only in relation to sperm count [8]. The available studies support the view that men with lower sperm counts and/or infertility have an associated shorter AGD [126–129], although a

recent Chinese study did not find this [130]. It seems unlikely, however, that AGD could be used clinically in any diagnostic or predictive way, because of considerable inter-individual variation and confounding by body size and adiposity [8, 131, 132].

Of more direct relevance to the present chapter is the potential association of AGD with blood testosterone levels and Leydig cell function. There is a single human [127] and a single experimental rat [122] study showing a similar magnitude, positive association between AGD and adult blood testosterone levels. However, several other pieces of data support a role for prenatal events, including fetal androgen exposure, as a determining factor for adult Leydig cell function. First, birth weight has been shown to be positively associated with blood testosterone levels in adult men across the full birth weight spectrum [133], and this is independent of adult obesity/BMI, which can itself be a determinant as discussed earlier. Second, a population-based study of >8000 European young men showed that low sperm counts, a TDS disorder that occurs in ~20% of young men [134], are not associated with any change in blood testosterone levels but are associated with compensated Leydig cell failure [43]. Evidence that such a change could emanate from fetal life as a consequence of subnormal fetal androgen action on adult Leydig stem cells, comes from an experimental animal study showing that compensated adult Leydig cell failure is induced in rats and mice when fetal androgen production/action is reduced [121]. The human health significance of compensated Leydig cell failure is not clear at present, although one line of thinking is that it might identify a subset of men whose Leydig cells may 'wear out' during aging due to their compensated 'over-functioning,' which might then lead to frank hypogonadism and its attendant morbidities. However, frank hypogonadism is not a very prevalent feature among aging men, whereas ~10% exhibit compensated Leydig cell failure [135].

An indirect but powerful argument in favor of environmental factors impacting testosterone levels in men is the evidence from three independent studies showing that the more recently a man has been born, the lower will be his testosterone level at any age [136–138]. This implies that something new in our lifestyle or exposures is exerting an adverse effect on testosterone levels in men, the most likely explanation being the increase in adiposity and its associated lowering of testosterone levels [92–94]. However, specific dietary factors and/or chemical exposures could be contributory factors, including those occurring in fetal life (via the mother). Unfortunately, it seems unlikely that it is feasible to design studies that could address the latter possibility in a meaningful way.

What Maternal Lifestyle Factors or Exposures Might Impact Reproductive Development/Androgen Exposure of the Male Fetus?

Of necessity, all studies that have addressed this question in human pregnancy are association studies. By far the largest and most consistent finding is that smoking by mothers during pregnancy is associated with reduced testis size and sperm counts in resulting male offspring [reviewed in ref 25]; the reductions reported are substantial, ranging from 20 to 40%. The mechanism underlying the presumed effect is unknown but is presumed to reflect a smoking-induced reduction in Sertoli cell number [25]. Maternal smoking has not been associated with any obvious change in adult male testosterone levels [108], although this has not been studied in as much depth as sperm counts. More recently, experimental evidence has emerged to suggest that protracted use of acetaminophen and/or other painkillers in early pregnancy might reduce testosterone production by the fetal human testis [139, 140]. This finding is supported by the fact that 4 independent epidemiological studies have shown a significantly increased risk (~twofold) of cryptorchidism in sons of mothers who reported protracted use of acetaminophen during pregnancy [139, 141–143]. Whether acetaminophen use in pregnancy is associated with adult-onset TDS disorders, and whether it might predispose to development of compensated Leydig cell failure have not been studied yet and will prove challenging to investigate given the time gap between exposure and sought for 'effects.' Nevertheless, this possibility is in urgent need of investigation as the majority of women report use of acetaminophen and/or other painkillers in pregnancy [144].

The issue of environmental 'endocrine-active' chemicals has received enormous attention over the past few decades, and there is widespread belief that such compounds do impact human health, and in particular male reproductive health; such effects are presumed to be mainly during fetal or early postnatal life and thus to affect the developing testis. The initial focus was on 'environmental estrogens,' because if pregnant rats or mice are exposed in utero to high levels of potent exogenous estrogens, such as diethylstilbestrol (DES) or ethinyl estradiol, testosterone production by the fetal testis is reduced by 80–90%, a change that will lead to irreversible impairment of masculinization and development of the male reproductive system [145–147]. However, there have been two important research developments which strongly suggest that, at least as far as the developing fetal human testis is concerned, environmental estrogenic chemicals are unlikely to pose any significant risk, irrespective of their estrogenic potency or the level of exposure. First, evidence from both in vitro cultures [148] and ex vivo xenografts [149] of human fetal testes into nude mice demonstrates that even after exposure to very high doses of DES, no inhibition of testosterone secretion is evident, whereas exposure of rat and mouse fetal testes to the same estrogen doses profoundly suppresses testosterone production [146, 147]. This dramatic difference in effect is presumably explained by the absence of ESR1 (estrogen receptor-α) from human fetal Leydig cells, whereas it is present in fetal rodent Leydig cells [146, 149]. Second, detailed studies by Fowler and colleagues of the human fetal testis have shown

that it has enormously high levels of bioactive estrogens, far higher than in the fetal human ovary and ~1000 times higher than the already extremely high levels in maternal blood [150]. Why there should be such high bioactive estrogen levels in the fetal human testis is not known, but in their presence it makes sense to not have any endogenous system that would allow estrogens to suppress fetal Leydig cell steroidogenesis. Thus, rodents are not a good model for the human when considering whether or not estrogenic chemicals pose any threat to the steroidogenic function of the human fetal testis.

Partly because of the findings above, the focus of concern regarding environmental chemicals and their potential contribution to TDS disorders, has switched to those that can potentially alter production of endogenous testosterone by the fetal testis or interfere with the downstream actions of androgens via androgen receptor (AR) antagonism and thus interfere with masculinization. An important driver of such studies has been the finding that exposure of pregnant rats to high doses of certain phthalate esters, which are widely used plasticizers to which we are all exposed, can induce a spectrum of reproductive disorders in the male offspring that is remarkably similar to human TDS [147, 151–154]. However, in a rather remarkable replay of the estrogen story described above, in vitro and xenograft studies involving human fetal testes have shown categorically that exposure to the same phthalates at comparable doses that cause TDS disorders in exposed male rats has no effect on testosterone production by the fetal human testis [155–157]. These negative findings are supported by similar negative findings after exposure of pregnant marmosets to high doses of phthalates during the presumptive MPW [158]. However, the latter study also assessed the effects of the same dose of phthalates on the newborn marmoset testis and found that it acutely caused ~50% suppression of blood testosterone levels [158, 159], indicating a fundamental difference between the fetal and newborn primate testis in its susceptibility to the adverse effects of certain phthalates. However, it needs to be kept in mind that the doses of phthalates that are needed to induce suppression of fetal testosterone in rats or in newborn marmosets are far in excess of human exposure levels. Having said this, epidemiological studies have provided evidence for a negative association between exposure to certain phthalates and blood testosterone levels in men [160, 161] although the ‘effects’ are small and are not found consistently in every study [162].

Other than exposure to phthalates, most human studies have focused on whether exposure to pesticides, most of which have varying degrees of weak endocrine-disrupting activity, might be associated with TDS disorders or altered AGD. Of necessity, most of the studies have focused on the association with cryptorchidism, because of its high prevalence, and/or hypospadias, and have investigated boys born to mothers who were either occupationally exposed (e.g., greenhouse workers) or who lived in an area where relatively high pesticide exposure is likely to have occurred (e.g., farmer’s wives, or recent use of DDT for malaria control). Some of the studies determined pesticide exposure indirectly, for example via a detailed questionnaire

[163], or based on the nature of their job (e.g., greenhouse workers) [164, 165], or where they lived (e.g., farming area) [166], while other studies have measured pesticide exposure directly in breast milk [167] or in maternal blood [168, 169]. The results have proved to be very mixed, and overall fewer studies have found a significant association between cryptorchidism and maternal pesticide exposure than studies that have found no significant association [166]. Moreover, even in the positive studies, the 'effect' sizes have been small. It is not possible to discuss all of the findings and inconsistencies here, so readers are referred to a recent review [166]. It is worth emphasizing that the inconsistency of findings may simply be a reflection of the inherent difficulties in doing such studies in populations in which other confounding factors may be present (e.g., maternal age or parity, maternal diet/obesity, ethnic or geographic differences), although similar arguments could be voiced to dismiss studies in which positive associations have been found. On the other hand, it does seem reasonable to conclude that, when viewed together, the various studies support the view that maternal pesticide exposure is unlikely to be a major cause of cryptorchidism in boys from the general population. Nevertheless, it would seem prudent for pregnant women to minimize exposure to pesticides, especially if their exposure is likely to be high in comparison with the general population (e.g., greenhouse workers), as these tend to show a little stronger evidence for an association between exposure and occurrence of cryptorchidism [166].

Whether exposure to pesticides during pregnancy might increase risk of adult-onset TDS disorders or hypogonadism is unknown as such studies are extremely difficult to undertake.

Future Prospects

Arguably, the most important development with regard to male reproductive dysfunction in recent years has been the recognition that disorders presenting in adulthood might have their origin in fetal or neonatal life, in particular during the presumptive MPW. Such thinking is very much in line with the evolution in thinking about the origins of other common medical disorders such as cardiovascular disease and type 2 diabetes. However, even on the assumption that the fetal period is the most important in terms of shaping lifelong reproductive health, we do not appear to have made great progress in identifying what factors acting via the mother might be responsible for predisposing toward the development of a reproductive disorder.

This is not to imply that **ALL** male reproductive dysfunction has its origins in fetal life but rather to indicate that susceptibilities are put in place that may then predispose to effects by other genetic, environmental or lifestyle factors—these may exert their effects at various stages in life or throughout long periods of life. There is nothing remotely controversial in such thinking as all human health disorders ultimately result from an interaction between genetic and environmental/lifestyle factors.

An important (and overlooked) aspect of the environmental/lifestyle components is that they are intrinsically preventable. Before this possibility can be realized, however, the causative factor(s) have to be identified and their mechanisms of effect established. As this chapter will have demonstrated, we are a long way from achieving this for most environmental and lifestyle factors that are recognized as potentially impacting on male reproductive health, whether during development or in adulthood. The exception is time spent seated and scrotal heating, as methods and studies are now in place that are able to more precisely assess their risk to sperm counts and whether this impacts on fertility, sperm DNA damage and so on.

Perhaps the other important development has been the recognition that diet and obesity can profoundly affect both the spermatogenic and steroidogenic functions of the adult testis, even if the pathways of effect are still to be fully resolved. It seems remarkable that we have previously overlooked or underestimated the importance and degree of potential impact of such dietary factors, especially when considering the extent of the evidence that links such factors to the most prevalent (and increasing) obesity-related disorders in modern societies. A reasonable extrapolation of this thinking is that we may also have overlooked the effects of the changing nature of dietary factors during pregnancy and their potential impact on the developing fetal testis. Indeed, there is now a substantial body of evidence which indicates that, for pregnancies, the diet of either mother or father may exert lifelong health-changing effects both on the developing fetus and on the children which that fetus then fathers or mothers, so-called intergenerational effects [170, 171]; the fact that this includes reproductive effects and Leydig cell steroidogenic effects in grandsons as the result of grand-paternal exposure to a high fat diet is a sobering thought [170, 172].

Such intergenerational effects result from epigenetic changes (?to the germ cells) in the parents that are then transmitted to the offspring, though the precise mechanisms underlying this are only just beginning to emerge. Remarkably, it appears that paternally mediated effects (of diet) seem to involve changes (e.g., in non-coding small RNAs) to seminal plasma and epididymal sperm rather than to testicular germ cells [172–175], but we can expect rapid expansion of understanding in this area in the coming years. Although the importance of these primarily experimental findings to human health remains to be determined, such findings bring me back neatly to where I started this chapter. Because, from an evolutionary perspective, intergenerational effects of parental diet are likely to represent yet another mechanism via which we are attuned to our environment, but in this case the effect is to attune the future offspring to their anticipated (dietary) environment.

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15. Sex Hormone-Binding Globulin and the Metabolic Syndrome

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Introduction

The metabolic syndrome (MetS) incorporates a cluster of clinical signs and symptoms including elevated blood glucose, central obesity, increased blood pressure, and abnormal HDL cholesterol or triglyceride levels that are collectively associated with increased risk for type 2 diabetes mellitus (T2DM) and cardiovascular disease. The MetS was originally considered to be an age-related condition but is now known to be associated with insulin resistance. Remarkable worldwide increases in the incidence of T2DM have been attributed to changes in lifestyles and nutrition choices that promote the MetS in young adults and children. The prevalence of the MetS is similar in men and women, but sex differences in the risk for cardiovascular disease and T2DM have been linked to differences in androgen exposures. Interest in the relationship between androgens and the MetS has been stimulated further by the emerging use of testosterone treatment for middle-aged and older men because of concerns that it may increase risk for cardiovascular disease. However, recent studies suggest that these risks are either

uncertain [1] or unfounded [2], and may be outweighed by the beneficial effects of testosterone replacement in terms of obesity, T2DM, sexual function and osteoporosis [3], especially when testosterone replacement is managed conservatively [1]. It is now well accepted that the plasma concentrations of testosterone, and its access to target tissues and cells, are regulated primarily by sex hormone-binding globulin (SHBG). There is also a substantial body of information that plasma SHBG levels represent a biomarker of the MetS and the risk associated with developing cardiovascular disease or T2DM, and emerging evidence suggests that SHBG may even participate in the predisposition to these diseases. This chapter provides a contemporary overview of how SHBG is produced and functions in the context of the MetS, and how it may be utilized as a biomarker of its associated diseases.

Sex Steroids, SHBG and Diseases Associated with the Metabolic Syndrome

Clinical and epidemiological studies over several decades have implicated circulating testosterone, as a major risk factor for the MetS and its associated diseases in men [4, 5] and women [6]. It is now generally acknowledged that there is an inverse relationship between total testosterone levels and the number of MetS risk factors [5] and men with low circulating testosterone levels are at increased risk for developing the MetS [4, 7], while the opposite is true in men with high testosterone levels [8], irrespective of their body mass index. By contrast, as summarized recently [6], elevated circulating testosterone levels in women are associated with increased risk for the MetS and T2DM. Estrogens have also been implicated in the genesis of the MetS [9], and it has recently been reported that a low estradiol: testosterone ratio is protective against the MetS, at least in men [7].

It is also now well accepted that SHBG is the major determinant of plasma testosterone levels in healthy men [10], and that SHBG regulates the access of both testosterone and estradiol to target tissues [11, 12]. In recognition of this, most studies of sex steroids and the MetS have examined how SHBG might be co-implicated as a risk factor, and there is substantial evidence that SHBG levels are low in MetS patients [5], and that low plasma SHBG levels predict the development of the MetS in both men [13, 14] and women [15, 16]. Several studies also indicate that low plasma SHBG levels are an independent risk factor beyond traditional risk factors such as BMI, blood pressure, glucose and triglycerides in men [13, 14], but evidence for this in women is less compelling.

While reduced insulin sensitivity is a consistent finding in T2DM, diabetes is uncommon in insulin resistant (IR) patients unless there is also β cell dysfunction [17]. As such, the relationship between SHBG, sex steroids and T2DM differs from that

found in patients with MetS. In the Tromso study [18], men with higher estradiol levels had an increased risk of incipient diabetes independent of obesity while men with lower total testosterone and SHBG levels had an increased risk of diabetes that was no longer significant after adjusting for waist circumference. Moreover, a meta-analysis of 23 cross-sectional and 10 prospective studies found that a low concentration of SHBG was associated with a higher risk of developing T2DM, but this relationship was much stronger for postmenopausal women than for men [19], and a study of adults at high risk for developing T2DM found that obesity and glycemia were far more important determinants of incident diabetes than was SHBG for either sex [20]. Other disorders associated with the MetS in which SHBG levels may be low include nonalcoholic fatty liver disease (NAFLD), obstructive sleep apnea (OSA), psoriasis, and gout.

SHBG is found in the fetal circulation and in umbilical cord blood. Although the significance of SHBG, and the factors which regulate its expression at this stage of development are unknown, cord blood SHBG levels were lower among babies born to overweight mothers, most of whom had gestational diabetes [21]. SHBG levels are low in children and adolescents diagnosed with MetS [22], and in the children of parents with MetS [23]. Detailed metabolic profiling of young adults revealed a strong association between SHBG and circulating lipids and metabolites reflecting the degree of adiposity and IR, and low SHBG levels in early adulthood predicted the development of IR even after adjustment for adiposity, insulin and testosterone levels [24].

Many studies have shown that weight loss through caloric restriction, metformin treatment in combination with lifestyle changes, or following bariatric surgery increases serum SHBG levels, and a few studies have also shown the associated improvement in IR (Table 15.1). Improvement in IR with resistance training is also associated with a rise in SHBG even though BMI was unchanged [25]. In adult men, the rise in SHBG following bariatric surgery is associated with a rise in testosterone that is greater in younger men [26], whereas there is a decline in testosterone levels when women with PCOS lose weight [27].

Table 15.1 Hormone changes following bariatric surgery in men

Ref.	n	BMI (kg/m^2)		Testosterone ng/dL (nmol/L)		SHBG nmo/L		MetS		HOMA-IR	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
[148]	22	46.2 ± 0.9	−16.6%	340 ± 21	636 ± 186		+21.6				
[149]	33	50.3 ± 6.1	31.5 ± 4.7	248 ± 91	546 ± 165	18.3 ± 11.8	42.7 ± 18.1				
[26]	20	43.6	34.8	8.1	13.2	19	39				
[150]		46.9 ± 7.8	30.9 ± 5.0	256 ± 121	508 ± 161	25.0 ± 12.3	48.5 ± 16.1			11.8 ± 7.8	3.5 ± 4.7
[151]	20	50.4 ± 8.7		302 ± 102							
[152]	28	50.1 ± 11.2	35.9 ± 7.02	(8.31 ± 3.24)	(12.7 ± 3.8)	23.4 ± 17.4	37.8 ± 18.5	75%	25%		

Post measurements were made 6–12 months after surgery

Overall, why SHBG is associated with the MetS and to a lesser extent with T2DM, and the role SHBG plays in determining how androgens or estrogens influence MetS and T2DM risk are only partly understood, and may differ by sex, as discussed below.

SHBG: Regulator of Androgen and Estrogen Action

Plasma SHBG is produced as a glycoprotein by hepatocytes, and is released into the blood circulation as a homodimer of identical subunits, each of which contains a single steroid-binding site for active androgens and estrogens [12]. Human SHBG binds 5α-dihydrotestosterone (DHT) with about 5 times greater affinity than testosterone and about 20 times higher affinity than estradiol [28]. There are sex differences in human SHBG production by the liver at different stages of the life cycle, and the occupancy of SHBG steroid-binding sites in men and women differs greatly [11]. Plasma levels of SHBG are usually about twofold higher in women than in men but far fewer SHBG steroid-binding sites are occupied in women than in men, in whom SHBG is largely occupied by testosterone [11, 28]. The obvious corollary of this sex difference is that SHBG functions differently in women than in men.

In women, SHBG appears to play a primary role in limiting the access of both testosterone and estradiol to their target cells. This has been evident ever since the early findings that low plasma SHBG levels are directly associated with increased bioavailable testosterone and symptoms of hyperandrogenism [29]. To illustrate this further, marked 5–10 fold increases in plasma SHBG levels after administration of some oral contraceptives accentuate reductions in free testosterone levels, and are often used to reduce hyperandrogenic symptoms, including hirsutism [30]. In addition, low plasma SHBG levels in obese postmenopausal women are linked to increase risk for endometrial cancer because they increase free estradiol levels [31]. Plasma levels of SHBG also increase by 5–10 fold in women during pregnancy, and it is assumed that this is caused by estrogen-dependent increases in liver SHBG production [29]. The functional importance of elevated plasma SHBG levels during pregnancy is unclear but it may limit the exposure of pregnant women to fetal adrenal androgens that escape metabolism by the placenta, as illustrated by the transient manifestations of severe androgen excess in a pregnant woman with a rare case of SHBG deficiency due to mutations with both *SHBG* alleles [32]. Women with gestational diabetes have lower plasma SHBG levels [33], and recent reports indicate that low pre-pregnancy levels of SHBG are associated with increased risk for gestational diabetes even in women who were of normal weight with no previous history of this condition [34].

Measurements of plasma SHBG and total testosterone levels are used together to calculate the plasma concentrations of free testosterone, which are generally considered

to be the most reliable measure of androgenic activity in both sexes [35]. However, the mathematical algorithms used for this purpose are less than optimal, and the recent identification of human SHBG variants with abnormal steroid-binding activities or immunological properties highlights the pressing need for more direct methods to measure free testosterone levels based on sensitive and specific mass spectrometric methods [36]. The following sections summarize recent advances in our understanding of the functional properties of SHBG, as well as its production by the liver in health and disease, and highlight gaps in our knowledge that remain to be filled.

Structure and Function of Human SHBG

The evolutionary origins of SHBG remain obscure, but its monomeric form comprises two laminin G-like (LG) domains found in the C-terminal regions of several extracellular proteins (Fig. 15.1a), including protein S and gas6 [37–39]. Protein S and gas6 are potential ligands for the Axl/Sky subfamily of receptor tyrosine kinases and they interact with these receptors through their “SHBG-like domains” [40]. Protein S and gas6 do not bind steroids, and SHBG appears to have acquired its function as a steroid-binding protein early in the evolution of vertebrates because it is present in the fishes [41, 42].

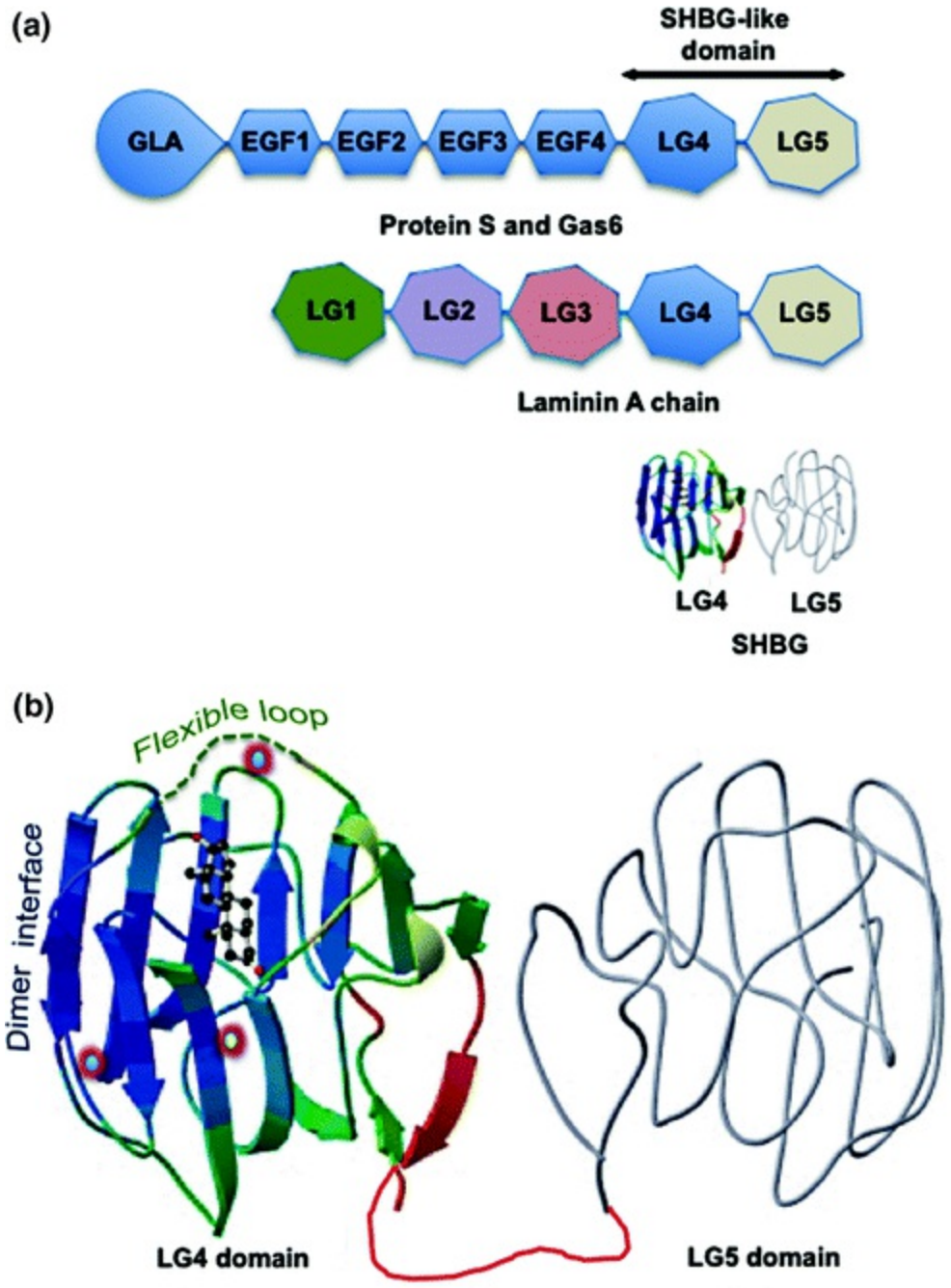

Fig. 15.1 Human SHBG and its structural and functional properties. **a** Domain structures of human SHBG and other proteins with tandem Laminin G-like (LG) domains. Human SHBG resembles the LG4 and LG5 domain structure found in the C-terminus of the laminin A chain. Protein S and Gas6 are large extracellular proteins that comprise a GLA (multiple γ-carboxyglutamic acid) domain followed by several EGF-like domains and similar tandem LG4 and LG5 "SHBG-like domain" that interacts with the Axl/Sky subfamily of receptor tyrosine kinases. **b** The crystal structure of the SHBG LG4 domain in complex with 5α-dihydrotestosterone (DHT) together with the predicted structure of the LG4 domain (*gray*). The unstructured flexible loop region that covers an entrance to the steroid-binding pocket is shown as a *dashed green line*, and the dimer interface that holds two monomers together in a head to head manner to form the SHBG homodimer is also indicated. The position of the calcium atom (*yellow center*), the binding of which is essential for maintaining dimerization and the integrety of the steroid-binding is shown, as are the positions of the two zinc atoms (*blue center*); including the zinc atom that is held in position by a histidine in the flexible loop region of human SHBG and influences the specificity of steroid binding. The DHT is positioned in the steroid-binding site

The quaternary structure of the SHBG homodimer has never been resolved but there is evidence that it adopts a torpedo-like head to head conformation [43]. The N-terminal LG domain contains the steroid-binding site and the dimerization domain (Fig. 15.1b), and its crystal structure has been determined with several different androgens or estrogens in the steroid-binding site [44–46]. These structures show how different steroids are positioned within a hydrophobic binding pocket, and how critical amino acid residues participate in the steroid-binding interactions. In particular, they indicate that androgens and estrogens are accommodated very differently within the hydrophobic core of each SHBG monomer, and that the binding of androgens and estrogens exert differences in the positioning of specific residues on their surface [46], including those within a flexible loop region [47] that covers an entrance to the steroid-binding pocket (Fig. 15.1b). The positioning of this flexible loop has been proposed to influence the flux of androgens and estrogens into and out of the binding site differently, but an additional entrance or exit to the steroid-binding site for estrogens may also exist that is not accessible to androgens [36].

High-resolution crystal structures have also revealed the mechanism of how the SHBG monomers associate as a homodimer, and show that both monomers of the homodimer can bind a steroid ligand [48]. What remains to be determined is whether occupancy of the steroid-binding site of one monomer exerts allosteric effects that influence the steroid-binding affinity of the partner monomer, and whether androgens and estrogens exert different effects in this regard. If this is the case, it may have a different impact of the function SHBG in women in whom steroid occupancy of the binding sites is less than 20%, while in men ~50% of the sites are normally occupied by androgens [11, 28].

It has also been known for many years that calcium maintains the integrity of the SHBG homodimer and its ability to bind steroids with high affinity [49]. This explains why the steroid-binding activity of SHBG in EDTA plasma is disrupted by freeze thaw cycles. Structural resolution of the N-terminal LG domain of SHBG identified the calcium-binding site responsible for this (Fig. 15.1b), and provided evidence that calcium binding exerts long-range allosteric effects that integrate both dimerization and steroid-binding activities [44–46]. The crystal structures also revealed the presence of two zinc-binding sites in the N-terminal LG domain (Fig. 15.1b); one of which is highly conserved across species and is probably structurally important, although this has not been formally proven [50]. The other zinc-binding site is located within the loop region that covers the steroid-binding site, and a zinc atom is held in place by a histidine residue in this location (Fig. 15.1b). Importantly, occupancy of this zinc-binding site specifically reduces the binding affinity of human SHBG for estrogens but not androgens [50]. While this may not be relevant to the activities of SHBG in blood where free zinc concentrations are very low, it may be relevant in other extravascular tissue compartments where they are particularly high, such as in the prostate and male

reproductive tract.

Although the SHBG C-terminal LG domain structure has not been resolved, it can be modeled (Fig. 15.1b) on that of the N-terminal LG domain [12]. The C-terminal LG domain does not participate in steroid binding or dimerization, but it normally contains two sites for *N*-linked glycosylation. One of these *N*-glycosylation sites is invariably conserved across vertebrate species and is predicted to be functionally important [51]. There is also a relatively common human SHBG variant (SHBG D327 N) that contains an additional *N*-glycosylation site within the C-terminal LG domain [52].

Well before the structure of SHBG was resolved, reports appeared describing interactions between SHBG and other proteins, especially those within the plasma membranes of cells [53, 54], leading to proposals that the function of SHBG extends beyond those of a plasma transport protein [55]. These functions included cell-signaling activities that may explain in part the non-genomic actions of steroids [56] or the endocytotic delivery of steroids to their target cells [57]. The realization that SHBG was structurally related to the proposed receptor-binding domains of protein S and Gas6 [40] fueled interest in these ideas, as did the demonstration that the nonspecific endocytotic receptor megalin is capable of recognizing SHBG and internalizing it together with its ligand [58]. However, functional receptors for SHBG that exert activities in physiological contexts remain to be identified. The most likely reason for this is that early observations of interactions between SHBG and cell membrane proteins were made using purified preparations of SHBG and their potential targets, or tissue culture systems where purified SHBG was added in the absence of other plasma proteins. These experimental designs neglected the fact that SHBG is a trace protein in biological fluids, and there are many other proteins including those that have structurally related domains that could compete with its binding to membrane proteins in vivo.

However, an interaction between SHBG and the extracellular matrix-associated proteins, fibulin-1D and fibulin-2 has been identified under conditions that satisfy these experimental caveats [59]. These members of the fibulin family not only recognize and sequester SHBG from dilutions of plasma samples that mimic those in extravascular tissue compartment, but do so in a ligand-dependent and ligand-specific manner [59]. Moreover, the interaction was favored when the SHBG steroid-binding site was occupied by estradiol in vitro as well as in vivo in the endometrial stroma [59]. In mice expressing a human *SHBG* transgene, SHBG also leaves the blood vasculature in the endometrial stroma and accumulates in the stromal matrix when estrogens levels are elevated during the estrus cycle [59]. These observations at least provide direct evidence that SHBG is capable of acting in extravascular compartments of some tissues to modulate the accessibility of sex steroids to their target cells.

Tissue Specific Expression of Human SHBG

The *SHBG* locus is located within the short arm of human chromosome 17 [60], and comprises multiple transcription units defined by the utilization of different promoter regions that flank alternative exon 1 sequences [61–63]. These studies indicated that *SHBG* encodes a large number of transcripts, which all contain alternative exon 1 sequences spliced to a common exon 2 sequence followed by up to six other exon sequences, but only two of them have been shown to encode a functional SHBG protein (Fig. 15.2). The biologically most important of these is the mRNA in the liver that encodes the SHBG precursor polypeptide that includes the secretion signal polypeptide sequence encoded by exon 1 within an ~4.5 kb *SHBG* transcription unit [12]. By contrast, the major *SHBG* transcript in the testis [61] contains a noncoding alternative exon 1 sequence (Fig. 15.2), and there is evidence that an AUG translation initiation codon within exon 2 produces an N-terminally truncated SHBG isoform starting with Met30 in the mature SHBG sequence [64]. This SHBG isoform is produced in male germ cells and is not secreted, but it appears to bind steroids in same way as plasma SHBG and accumulates in the acrosome of sperm [64]. Although the function of this human sperm SHBG isoform is unknown, its concentration in the acrosome declines with age and correlates with sperm motility [65].

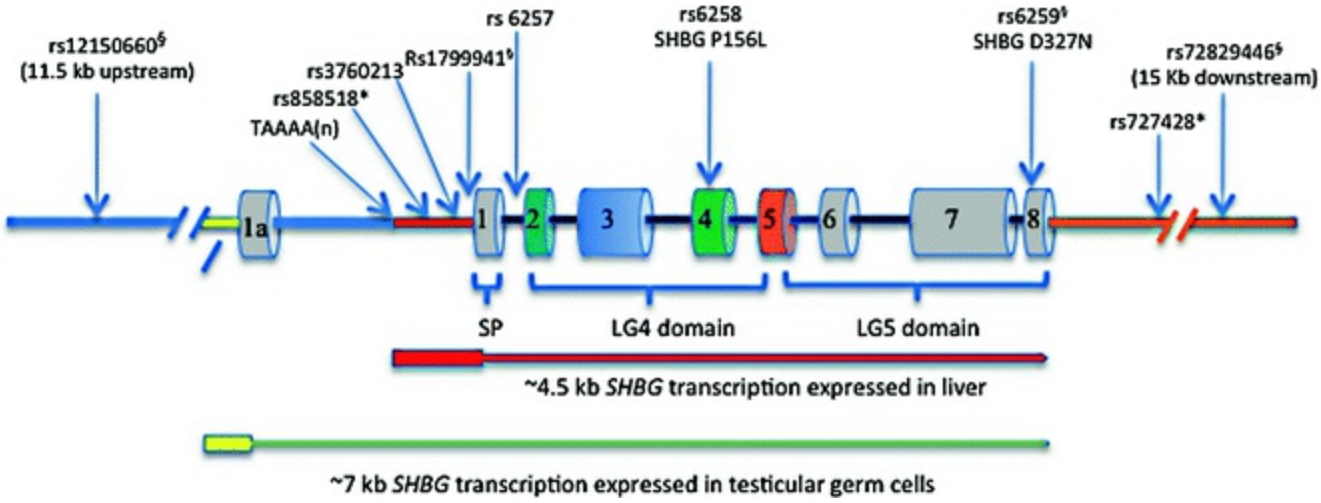

Fig. 15.2 Structural organization of the human *SHBG* locus, showing the two main transcription units expressed in the liver and male germ cells, together with the positions of common polymorphism associated with individual differences in the production or function of plasma SHBG. The ~4.5 kb transcription unit for the *SHBG* mRNA in the liver that encodes the SHBG precursor polypeptide (encoded by exons 1–8) includes ~800 bp of promoter sequence upstream of exon 1 that encode the secretion polypeptide (SP) that is remove co-translationally to yield the mature SHBG monomer of LG4 and LG5 domains. The ~7 kb transcription unit encodes the most abundant alternative *SHBG* transcript found in testicular germ cells, and it comprises an alternative exon 1a sequence spliced into exons 2–8. Exon 1a is noncoding but translation can initiate from the AUG codon (in exon 2) for Met30 in the mature SHBG polypeptide to yield an N-terminally truncated SHBG isoform that accumulates in the acrosome. Those SNPs in linkage disequilibrium with each other are indicated with symbols (* or §), respectively

Cell-specific expression of *SHBG* in the human testis differs from that observed in subprimate species in which the gene is expressed in Sertoli cells resulting in the

production of the SHBG homologue, known as the androgen-binding protein, which is secreted into the seminiferous tubules where it is thought to act to promote sperm maturation within the male reproductive tract [66]. Although SHBG is present in human seminal plasma [67], it does not originate from Sertoli cells because the human *SHBG* promoter contains a binding site for USF transcription factors that repress transcription in Sertoli cells, and these sequences are absent the *SHBG* genes of subprimate species [64]. This species difference in the cell type expression of SHBG in the testis is enigmatic but may be related to differences in the spatial organization of spermatogenic cells within the seminiferous tubules.

None of the other alternative exon 1 containing *SHBG* transcripts encodes a secretion signal polypeptide in frame with coding sequences for an SHBG-like polypeptide. However, "humanized mice" expressing an *SHBG* transgene have demonstrated that extrahepatic expression of *SHBG* occurs in some cell types, such as the androgen sensitive epithelial cells of the proximal convoluted tubules of the kidney, and much of the human SHBG produced by these cells remains within them [68]. Furthermore, the SHBG that accumulates within these cells is N-terminally truncated and incompletely glycosylated, much like the SHBG isoform in the acrosome [65], and it appears to act to sequester DHT from the extracellular environment and modify its access to the androgen receptor [69]. This raises the possibility that the alternative *SHBG* transcripts identified in human prostate cancer cells [62, 63] encode SHBG isoforms that function within the cells rather than having to be secreted to act, but this will only be resolved when these intracellular SHBG isoforms are actually isolated and characterized from human tissues.

Regulation of Plasma SHBG Production by the Liver

Early studies indicated that SHBG production by the liver is under hormonal, nutritional and metabolic control [29]. Reductions in plasma SHBG in boys during puberty were initially thought to reflect a negative effect of androgen on SHBG production, but this was difficult to reconcile with a lack of any increase in plasma SHBG levels in men after orchiectomy [70]. A normal pubertal decline in SHBG was also been reported in siblings with complete androgen insensitivity, implying that the substantial reductions in SHBG levels in boys during puberty are not androgen-dependent [71]. Importantly, these changes in SHBG levels during puberty in boys occur with advancing age irrespective of any changes in total testosterone [72], and cause increases in albumin-bound and free testosterone that likely contributes to male sexual development [73]. Clearly other factors account for reductions in plasma SHBG levels in girls during puberty, and there is evidence that increases in body weight and composition are the main operative agents of these changes [74]. Reductions in plasma SHBG are more pronounced in girls with premature puberty, many of whom are overweight, and SHBG

measurements have be useful as a prognostic indicator of risk for T2MD in these individuals [75], as highlighted in a recent review [76].

In healthy adults, SHBG levels remain relatively constant, and in elderly men and women changes in plasma SHBG are modest, with modest age-related increases in men during old age that has been attributed to GH deficiency [77]. Small reductions in SHBG levels in postmenopausal women are unlikely to be related to a loss of ovarian estrogen because transdermal hormone replace therapy does not influence plasma SHBG levels [78], and are more likely to associated with changes in body composition [79], hormones like leptin and adiponectin [80], thyroid hormone status [81], or the metabolic state of the liver [82].

Early reports that plasma SHBG levels are very low in obese men [83], but very high in patients with anorexia nervosa [84], provided the first indication that nutritional status or metabolic factors influence the production or plasma clearance of SHBG. It was also recognized early on that plasma SHBG levels are elevated in hyperthyroid individuals and in patients after treatment with thyroid hormone [29], which itself exerts profound effects on metabolic state.

The very low levels of SHBG in obese men and women remained unexplained until it was reported that treatment of human HepG2 hepatoblastoma cells with insulin decreased SHBG production in vitro [85]. This provided credence to the idea that elevated insulin levels were responsible for the low plasma SHBG levels in insulin-resistant obese individuals, but the cellular mechanisms responsible for this have never been identified. Although, SHBG levels are normal in children with type 1 diabetes [86], they are in the upper normal ranges in men and women with type 1 diabetes, and display an inverse correlation with fasting insulin levels [87]. While the latter observations were interpreted as confirmation of a negative effect of insulin on hepatic SHBG production, a strong association between SHBG and glucose levels was also observed in subjects with T2DM in the same study.

Hyperglycemia is the primary consequence of deficiencies in insulin production or action in patients with type 1 diabetes or T2DM, and the question of how nutrition and metabolic state might influence plasma SHBG production by the liver was therefore re-examined in HepG2 cells, as well as in humanized mice that express human *SHBG* transgenes in the liver under the control of the *SHBG* promoter [88]. These studies failed to confirm a direct effect of insulin on *SHBG* expression in the liver in vitro or in vivo. Instead they demonstrated that *SHBG* is regulated in the liver by the monosaccharides, glucose and fructose, rather than insulin. Moreover, they presented a detailed mechanism for how these effects are mediated at the level of *SHBG* transcription [88], thereby providing the first molecular explanation for why plasma SHBG levels are so low in patients with the MetS [4, 89]. The finding that monosaccharides, and fructose in particular, potently downregulated *SHBG* expression in hepatocytes also provided an explanation for why the wide-spread use of high

fructose corn starch as a food and beverage supplement has been associated with the remarkable increases in the prevalence of the MetS and T2DM worldwide.

The transcription unit responsible for SHBG production by the liver was originally identified through experiments using different human *SHBG* transgenes in mice [68], as well as with *SHBG* promoter-driven reporter constructs in HepG2 cells [90]. Together these studies demonstrated that the ~4.5 kb transcription unit, which includes an ~800 bp promoter flanking exon 1 (Fig. 15.2), is sufficient to drive *SHBG* transcription in the liver. The use of DNase I footprinting assays and reporter gene assays also allowed the mapping of *cis*-elements within the promoter that interact with hepatic nuclear protein extracts [90]. The latter studies revealed that the binding of HNF4α to a nuclear hormone receptor (NHR) response element close to the *SHBG* transcription start site compensates for lack of a TATA box, and substitutes for the TATA-binding protein in recruiting the transcriptional machinery to the proximal promoter of *SHBG*. At that time, the realization that HNF4α plays a key role in the regulation of *SHBG* expression was particularly enlightening because *HNF4α* had just been identified as the first gene to be implicated in Maturity Onset Diabetes of the Young [91].

As a member of the NHR family of transcription factors, HNF4α, binds to palindromic DNA response elements that also bind other members of the NHR family, including the COUP-TFs [92]. Importantly, HNF4α and COUP-TF1 compete for the same response element in the *SHBG* promoter and to exert opposing actions on *SHBG* transcription, with HNF4α increasing transcription while COUP-TF1 represses it [90]. This explained for the first time how HNF4α and COUP-TF1 might serve as the on-off switches of *SHBG* transcription in hepatocytes under a wide range of pathophysiological conditions related to the MetS.

The underlying mechanism of how oral glucose and fructose downregulate hepatic SHBG production involves their rapid hepatic metabolism into acetyl-CoA, the immediate precursor of palmitic acid: the first of the long chain fatty acids produced in the liver [88]. A buildup of palmitate in HepG2 cells after treatment with monosaccharides is indicative of increased lipogenesis, and occurs in concert with reductions in HNF4α and SHBG mRNA levels, and concurrent reductions in SHBG secretion [88]. A strong positive correlation between hepatic HNF4α and SHBG mRNA levels has been also observed in a study of male and female cancer patients [93]. Furthermore, in the later study, hepatic HNF4α and SHBG mRNA levels were also inversely related to hepatic triglyceride levels and decreased in relation to body mass index. Why HNF4α levels decrease in hepatocytes under conditions where lipogenesis is enhanced may be related to the fact that fatty acyl Coenzyme A thioesters and palmitoleic (C16:1) and oleic acid (C18:1) were originally identified as potential ligands of HNF4α [94–96], which may induce conformational changes in HNF4α [97] and somehow destabilize it. It is also possible that other dietary fatty acids, such as

linoleic acid, and long-chain fatty acid metabolites bind HNF4α and alter its levels or activity as a transcription factor [98]. This of interest because recent studies have indicated that diets rich in linoleic acid may increase plasma SHBG levels [99].

This interplay between HNF4α and COUP-TF as key regulators of *SHBG* expression in hepatocytes is emerging as a common mechanism that accounts for other links between abnormal plasma SHBG levels and clinical conditions related to an abnormal metabolic state. In patients with the MetS, an increase in free fatty acid flux due to a failure to suppress lipolysis in adipocytes will increase hepatic free fatty acids [100], and thereby contribute to reductions in hepatic HNF4α levels and *SHBG* expression. By contrast, high plasma levels of SHBG are usually found in individuals with hyperthyroidism, while low levels are associated with thyroid hormone deficiencies [29] and the reason for this is that thyroid hormone influences hepatic SHBG production indirectly by altering hepatic HNF4α levels [101]. Low plasma SHBG levels are also frequently observed in patients with hepatic steatosis that predisposes to T2DM [102], and have been independently associated with nonalcoholic fatty liver disease (NAFLD) in subjects with T2DM [103]. The low plasma SHBG levels in NAFLD patients likely reflects the substantial accumulation of fatty acids in their livers [104], including palmitic acid that is linked to reduced hepatic HNF4α levels [88].

Increases in the plasma levels of pro-inflammatory cytokines (TNFα and IL-1β) and reduced levels of adiponectin, which are typically seen in overweight individuals at risk for the MetS [105], have been recently shown to downregulate [106, 107] and upregulate [108] SHBG production by the liver, respectively, and they also do this by changing hepatic HNF4α levels. By contrast, the transcription factor PPARγ interacts with a different NHR response element in the *SHBG* proximal promoter to repress transcription [109], and this probably accounts for the lower plasma SHBG levels in subjects with the hyperactive 12 Ala PPARγ variant [110] that has been linked to low risk for T2DM and myocardial infarction [111]. However, the use of insulin sensitizers, like pioglitazone, that act by stimulating PPARγ, increase plasma SHBG levels in individuals with the MetS [112]. This may be explained by the fact that the NHR response element in the *SHBG* proximal promoter that binds PPARγ can also bind HNF4α [90], as well as several other orphan members of the NHR family, including CAR and LXR, which can all potentially compete for binding at this site. Improvements in hepatic metabolic state induced by pioglitazone and other insulin sensitizers, likely change the hepatic complement of these orphan NHRs, which may account for changes in *SHBG* expression after their use.

Lessons from Naturally Occurring *SHBG* Polymorphism

Individual differences in plasma sex steroid and SHBG levels were recognized as an

inherited trait in early studies [113], but the genetic basis for this was not appreciated until human *SHBG* was cloned and sequenced [61, 114]. The first SHBG variant to be characterized at the genetic level explained why SHBG monomers in the serum of some individuals migrate with an unusually large molecular size in denaturing polyacrylamide gels [38, 115], and was shown to be caused by an Asp327 to Asn substitution that introduces an additional consensus site for N-glycosylation and an extra N-liked oligosaccharide per monomer [52]. The nonsynonymous SNP (rs6259) responsible for the SHBG D327 N variant (Fig. 15.2) is common in most ethnic groups globally. While the steroid-binding activity of SHBG D327 N is normal [52], a reduction in its plasma clearance rate may account for why carriers of SHBG D327 N have slightly higher plasma SHBG levels [116], and in part explain reports that SHBG D327 N has a protective effect on T2DM risk [117, 118].

Several *SHBG* polymorphisms linked to differences in plasma SHBG levels are located in noncoding regions of the gene (Fig. 15.2). The first of these to be identified was the TAAAA(n) polymorphic repeat that is part of an *Alu* sequence positioned about 1 kb upstream from the transcription start site for the *SHBG* mRNA [119]. Variations in number of this pentanucleotide repeat per *SHBG* allele have been examined in relation to plasma SHBG levels in patients with a range of clinical conditions [120], and the emerging consensus is that men with low numbers of TAAAA repeats have lower SHBG levels are at greater risk for the MetS [121].

The molecular explanation for how the variable TAAAA repeat number influences hepatic SHBG production remains to be fully elucidated, but studies using reporter genes driven by *SHBG* promoter sequences in HepG2 cells have demonstrated that the pentanucleotide repeat number may influence *SHBG* transcription through interactions with other downstream elements within the promoter including an SP1 binding site [119]. However, the effect of variable TAAAA repeats on *SHBG* transcription observed in these in vitro studies does not match what is observed in terms of differences in plasma SHBG levels in patients [122], and this may be due to either the transformed nature of HepG2 cells or the fact that reporter constructs act as naked DNA without assuming the complex chromatin structure that the *SHBG* promoter normally adopts in vivo. This has precluded further in depth studies of the molecular basis of how variations in the TAAAA repeat number influence *SHBG* transcription, but the recent introduction of genome editing techniques should allow this question to be addressed more directly.

Other common SNPs within the *SHBG* promoter or its first intron have been linked to plasma SHBG levels in genome-wide association studies (GWAS) of sex hormone dependent diseases (Fig. 15.2), including those associated with the MetS and T2DM. One of these SNPs (rs1799941) that is linked to individual differences in plasma SHBG levels, in men [123], women [124, 125], and in children and adolescents [126], is located close (within 10 bp) to the *SHBG* transcription start site, with A and G alleles

being associated with higher and lower plasma SHBG levels, respectively [127]. However it has also been noted that rs1799941 is in linkage disequilibrium (LD) with other genetic variations within the *SHBG* locus [128], including rs6295 and rs72829446 that is located ~15 kb downstream from the coding sequences for *SHBG* transcripts (Fig. 15.2). The rs1799941 is also in strong LD with rs12150660 located about 11.5 kb further upstream within the *SHBG* locus (Fig. 15.2), and which is also strongly associated with plasma testosterone concentrations [10].

A common SNP (rs6257) within intron 1 of the *SHBG* gene (Fig. 15.2) was originally associated with lower plasma SHBG levels and breast cancer risk [129] but has also been reported as a risk allele for T2DM [117]. This is in contrast to rs6295 that produces the SHBG D327 N variant with a prolonged plasma half-life, which is considered to be protective of risk for developing the MetS [104, 117, 118]. Recently, another SNP (rs3760213) linked to higher levels of plasma SHBG has been associated with a decreased risk for the MetS [118], and it may be directly involved in increasing *SHBG* expression because it is positioned close to a footprinted region (DNase1 footprint 6) ~200 bp upstream (Fig. 15.2) of the *SHBG* transcription start site [90]. Another SNP (rs727428) that is associated with plasma androgen and SHBG levels is positioned ~1 kb downstream of the most 3' *SHBG* exon [129], and is in LD with an SNP (rs858518) within the promoter that appears to promote *SHBG* expression in the liver (Fig. 15.2).

Apart from the SHBG D327 N variant (rs6295), there is only one other relatively common non-synonymous SNP (rs6258) within the *SHBG* coding sequence (Fig. 15.2) that appears to have the capacity to influence plasma testosterone levels. This polymorphism encodes SHBG P156L that has a reduced affinity for testosterone, and men with this variant have lower testosterone levels in their blood, presumably because of an increase in the metabolic clearance of testosterone [10]. Several other naturally occurring SHBG variants have been identified recently with abnormalities in production or the ways they bind their sex steroids ligands, but these occur very rarely and their clinical significance is unknown [36]. However, one of them (SHBG G195E) with a secretion defect [36] has an amino acid substitution in the same position as another rare SHBG variant (SHBG G195R) recently identified in a man with no detectable SHBG in blood plasma [130].

Given the enormous numbers of plasma SHBG measurements performed worldwide for diagnostic reasons over several decades, it is remarkable that the young male adult patient homozygous for SHBG G195R represents the first verifiable case of a total absence of SHBG [130]. Like SHBG G195E, detailed molecular and biochemical analysis of SHBG G195R indicated that it is translated but is not secreted, most likely because of a folding defect [130]. As expected, the patient's plasma total testosterone concentration was well below the normal range, yet his free testosterone level was normal. Clinical assessments indicated fatigue, overt muscle weakness and low body

weight, and other symptoms of hypoandrogenism, but gonadal development and sperm production and function appeared to be normal. Although this report provides an indication that plasma SHBG is not essential for male reproductive development and sperm production, the proband's clinical phenotype suggests a more direct role for SHBG in supporting the anabolic activities of androgens [130].

SHBG: Biomarker or Agent in Disease Etiology

Measurements of SHBG concentrations in blood samples have been used for decades as a biomarker of androgenicity in conjunction with total testosterone measurements, especially in women suffering from symptoms of androgen excess. In these patients, many of whom are diagnosed with the polycystic ovarian syndrome (PCOS), the link between low plasma SHBG and elevated levels of free testosterone and clinical symptoms is easily drawn. There are several reports that the TAAAA repeat polymorphism in the SHBG promoter is associated with PCOS [120] but many other studies have failed to show consistent evidence for associations between any of the common variants in the *SHBG* gene and the development of PCOS, although they do confirm that many SHBG polymorphisms are associated with individual plasma SHBG levels [131]. The latter review also noted that discrepancies between studies examining the associations between *SHBG* polymorphism and risk of PCOS or T2DM may be explained by the genetic heterogeneity underlying both disorders, which may also be compounded by differences in ethnicity and variations in dietary preferences.

The most potent natural hormonal mediator of hepatic SHBG production is thyroid hormone, and very high plasma SHBG levels are observed in patients with hyperthyroidism or with poorly controlled exogenous thyroid hormone use [29]. In these cases, elevated plasma SHBG levels appears to be a biomarker of increased biologically active thyroid hormone, and it has been proposed that SHBG measurements may effectively discriminate between patients with thyrotoxicosis due to excessive thyroid hormone production/bioavailability and those with thyroid hormone resistance [132].

Increased SHBG levels in older men with osteoporosis have been associated with bone remodeling markers, hip bone mineral density and vertebral fracture risk, and serum SHBG measurements have been advocated as a useful biomarker for fracture risk in men [133], and especially in men with prostate cancer [134]. Given the established role that estrogen plays in maintaining bone mineral density, one interpretation is that increased plasma SHBG levels reduce the bioavailability of estradiol in both men and women [135]. However, under these circumstances, it should also be noted that reductions in free testosterone will decrease its availability as a substrate for estradiol synthesis in bone, and this may be much more relevant in males in whom high plasma SHBG levels associated with SNPs in the *SHBG* locus are linked to osteoporosis risk

[135, 136].

Many epidemiological studies have shown that low plasma testosterone and SHBG levels are strong risk factors for the MetS [13, 137, 138] and T2DM in men [139, 140]. While circulating testosterone levels in men are largely determined by the levels and activity of SHBG in the blood [10] it remains to be established if the health risks associated with low T are independent of SHBG. However, the association of free T levels and the MetS is much weaker, with substantial between-study heterogeneity [141]. Associations may differ according to age and BMI, as stronger associations have been reported in young [142] and non-obese men [4], and SHBG levels did not predict incipient diabetes among a group of subjects at high risk for developing diabetes [20].

On the other hand, while the inverse relationship between insulin resistance and reduced plasma SHBG levels is incontrovertible [143], the risk for T2DM in women with low SHBG levels is stronger than in men [19]. As discussed above, the molecular mechanisms responsible for this association clearly indicate that plasma SHBG production reflects hepatic metabolic state as well as the inflammatory conditions associated with insulin resistance. Moreover, it is apparent that SHBG measurements represent an independent predictive biomarker of these metabolic disturbances [13], as well as any responses to weight loss treatments [144].

New information from genetic studies linking SNPs with plasma SHBG levels and the relative risks for the MetS are beginning to raise questions of whether inherent differences in the hepatic production of SHBG, high versus low, protect against or predispose to the MetS in some individuals [117]. This is compatible with findings that higher testosterone and SHBG levels in aging males are associated with a reduced risk for the MetS, while the opposite hormonal profile increases risk, irrespective of body composition [8]. Furthermore, low plasma SHBG and testosterone levels, in association with symptoms of clinical androgen deficiency, are associated with increased risk of developing the MetS over time, especially in middle-aged men of normal body mass index [4]. It is unlikely that SHBG is directly implicated in these responses, and a recent study suggested that a reduced plasma estradiol:testosterone (E2:T) ratio may be protective against developing the MetS in men [7]. While a lower E2:T ratio may reflect lower aromatase activity, it may be influenced by the level of SHBG in that SHBG binds T more strongly than E2 and the ratio will rise as SHBG declines in insulin resistance. Nevertheless, these finding are of particular interest in light of evidence that testosterone replacement therapies improve insulin resistance, visceral obesity, dyslipidemia and sexual dysfunction along with improved quality of life [5, 145]. Importantly, since testosterone replacement is being increasingly used in middle-aged and elderly men, measurements of SHBG provide important baseline assessments of free testosterone levels that could help personalize therapies, or a biomarker of any improvements in metabolic state that are achieved over time during androgen replacement therapies.

SHBG and Free Testosterone Measurements

The concentrations of SHBG in serum and heparinized plasma are usually measured using immunoassays, and are used in algorithms together with total testosterone levels to calculate plasma free testosterone levels. For instance, calculated free testosterone levels may be particularly useful in assessing men with suspected hypogonadal symptoms [146]. However, the identification of SHBG variants that may not be recognized appropriately in immunoassays, or have abnormalities in their steroid-binding properties [10, 36], highlights the need for sensitive and specific direct measurements of free testosterone measurement using mass spectrometric approaches. Such measurements should ideally be conducted after separation of the free hormone fraction under physiological conditions, i.e. in undiluted serum or plasma samples at physiological temperatures, as has been conducted using radiolabeled steroids [147]. Although this presents technical challenges, relatively simple technologies exist that may allow this and they should be further developed.

Salivary steroid analysis have been used as a surrogate for measuring free steroid levels in the blood but have not been widely adopted because they are not easily adapted for routine clinical chemical laboratory use, and suffer from problems associated with plasma contamination of saliva from oral cavity wounds. However, as in the case of direct analyses of plasma free steroid levels, the interpretation of abnormal salivary steroid hormone levels is limited in the absence of knowledge of circulating SHBG or total testosterone levels. In addition, knowledge of changes in serum SHBG levels during the course of clinical conditions or in response to therapeutic interventions may be of value in of itself as a biomarker of improvements in metabolic state for instance. In the emerging era of personalized or precision medicine, knowledge of plasma SHBG levels, or SNP profiles that are associated with plasma SHBG levels and disease risk, could therefore be used to tailor individual hormone replacement treatments to optimize therapeutic dosages.

Conclusions

Plasma SHBG plays a central role in transporting and regulating the access of androgens and estrogens to their target tissues and cells. Production of SHBG by hepatocytes is regulated by hormones, cytokines, and dietary components that influence the metabolic state of the liver, and their effects are reflected in changes in plasma SHBG levels. Most of these effects are mediated by changes in levels of the transcription factor, HNF4α, which acts in concert with COUP-TF1 as the main “on/off” controls of *SHBG* transcription, respectively. The levels of HNF4α in the liver play a key role in regulating a network of genes involved in hepatic metabolism and homeostatic control mechanism, and it acts together other related members of the NHR

superfamily that also regulate metabolically important genes, including PPARγ, which may all influence *SHBG* expression directly or indirectly in the liver. As such, plasma SHBG measurements serve as a sensitive surrogate biomarker of abnormalities in liver metabolism that are associated with the MetS and its associated diseases. Several recent GWAS of patients with these latter diseases or at risk for them have also indicated that genetic polymorphisms linked to either higher or lower plasma SHBG levels protect or increase the disease risk, respectively. This raises the question of whether the substantial interindividual differences in *SHBG* expression in the liver participate more directly in the etiology of diseases associated with the MetS: for instance, by changing the relative balance of plasma androgen and estrogens that many studies have implicated as disease promoting agents. If so, SHBG may also emerge as a therapeutic target that could be manipulated to modify exposures to these steroids in individuals at risk for the MetS.

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16. An Ensemble Perspective of Aging-Related Hypoandrogenemia in Men

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Overview

Androgen deficiency in men is associated with reduced physical stamina, relative sarcopenia, osteopenia, visceral obesity, sexual dysfunction, depressed mood, reduced sense of well-being and detectable cognitive impairment [1–10]. Carefully titrated testosterone therapy to increase testosterone concentrations from low- to the mid-normal

young eugonadal reference range moderately improves sexual desire in otherwise healthy aging men with and without symptomatic low libido [11]. Impoverished testosterone production in the older male has been affirmed by (i) direct sampling of the human spermatic vein; (ii) cross-sectional analysis, including meta-analysis, of epidemiological data from many geographically diverse but predominantly Caucasian populations; and (iii) longitudinal investigations in some of these healthy populations [12–20]. For example, the European SENIEUR and Massachusetts Male Aging Cohort studies inferred that bioavailable (nonSHBG-bound) testosterone concentrations decline by 0.8–1.3% annually [14, 21]; the European Male Aging cohort showed a 1.3% annual decline in free testosterone [19]; and, a 15-year prospective analysis in New Mexico observed that total testosterone concentrations fall by 110 ng/dL per decade in men after age 60 [15, 22]. The rate of fall in testosterone can vary by as much as fourfold when the same cohort is examined longitudinally, instead of cross-sectionally. This discrepancy is only partly explained by concurrent longitudinal changes in weight [17, 19, 20]. Surgery, trauma, stress, systemic illness, medication use, and chronic institutionalization are also associated with heightened androgen depletion in elderly individuals [6, 8, 14, 23–26]. A possible unifying mechanism underpinning these diverse associations is that inflammation, in particular interleukin-2, induces androgen depletion through enhanced feedback inhibition of LH secretion in older men [27]. However, the fundamental mechanisms that mediate waning testosterone secretion in aging men are only partly understood. Indeed, a unified physiological concept has been difficult to develop in this arena, partly due to confounding by obesity or factors associated with obesity such as insulin resistance.

Available data point to an array of contributing mechanisms underlying relative androgen depletion in the older male [28–39]. Primary (nonexclusive) considerations include reduced hypothalamic GnRH outflow, limited gonadotrope secretory capacity, impaired Leydig-cell steroidogenesis, and anomalous androgen-directed feedback control [39–44].

As a unifying approach, the male gonadal axis is viewed as an adaptive neuroendocrine ensemble. In this broader concept, testosterone availability is adjusted on a minute-by-minute basis by repeated decremental and incremental signaling interactions among GnRH, LH, and testosterone (Fig. 16.1). Simplified biomathematical simulations based on this network-like perspective predict that hypoandrogenemia and altered LH secretion in aging could arise singly or jointly by way of: (i) attenuated hypothalamic GnRH feedforward drive (albeit not acting alone); (ii) impaired Leydig-cell steroidogenesis; and/or (iii) reduced negative feedback by testosterone [45–47]. Figure 16.2 highlights the foregoing primary mechanistic considerations.

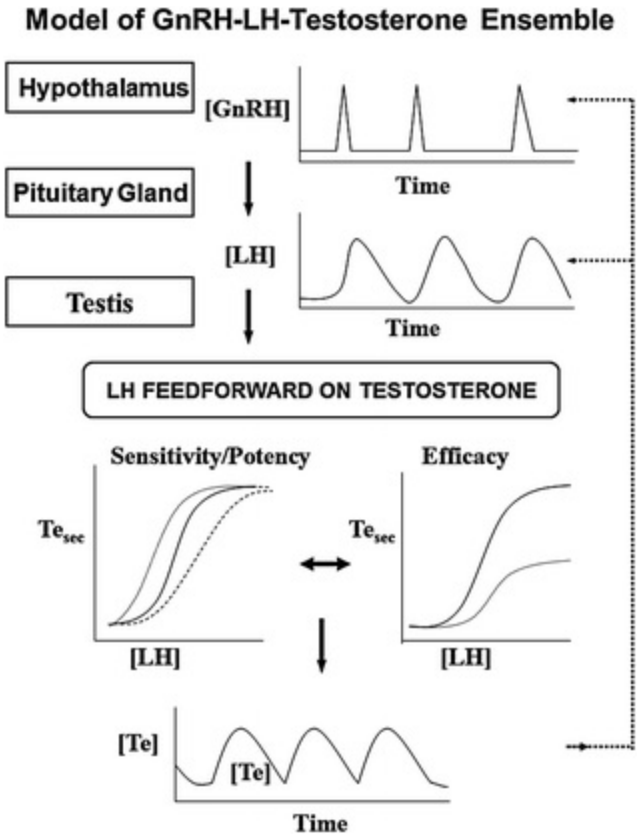

Fig. 16.1 Simplified schema of the GnRH-LH-testosterone (hypothalamo-pituitary-Leydig cell) axis with feedback (−, inhibitory) and feedforward (+, stimulatory) interactions mediated via specific interface (dose-response) functions

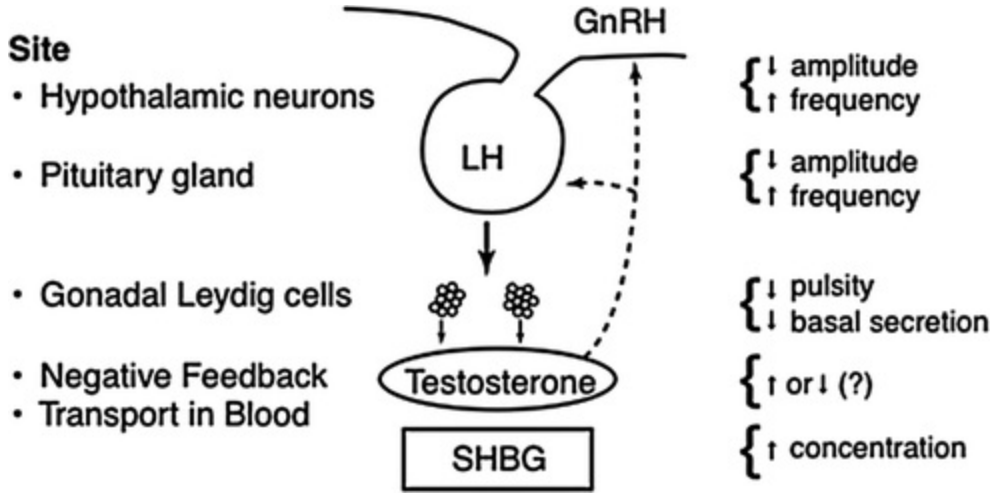

Fig. 16.2 Primary loci of postulated regulatory defects in the GnRH-LH-testosterone ensemble axis in aging men

Role of Intrinsic GnRH Deficiency in Mediating Low-amplitude LH Secretion

Experiments in the male mouse and rat support an important (but not exclusive) role of

GnRH-deficient hypogonadotropism in the hypoandrogenemia of aging [48–51]. Evidence to this end includes: (i) decreased castration-, naloxone-, and restraint stress-induced LH release in the older rodent [30, 49, 52–54]; (ii) diminished in vivo LH pulse amplitude in senescent animals [50, 55]; (iii) reduced in vitro hypothalamic GnRH secretion [56, 57]; (iv) altered GnRH neuronal synaptology [58]; (v) preservation of the stimulatory efficacy of GnRH [50, 59]; and (vi) restoration of sexual activity in the impotent animal by fetal hypothalamic neuronal transplantation [60]. Although such observations afford important clues for clinical investigation, the regulation of GnRH outflow in the laboratory animal is complex, multifactorial, incompletely defined, and not necessarily equivalent to that of the human male.

The intuition that hypothalamic GnRH deficiency subserves relative hypogonadism in elderly men has not been established or refuted [28, 32, 34, 61–64]. However, the notion is congruent with many clinical observations, as highlighted in Table 16.1. A necessary (but not sufficient) prediction is that GnRH action on gonadotropes is preserved. A recent prospectively randomized, interventional study used 14 days of pulsatile, i.v., infusion of GnRH versus saline to stimulate LH and testosterone secretion. Older and young men treated with this regimen achieved an equivalent elevation of 24-h LH concentrations (Fig. 16.3a) consistent with retention of gonadotrope secretory responsiveness in the older male [34]. In young men, the increment in mean LH concentrations elicited a proportionate rise in testosterone production. On the other hand, the same average increase in LH immunoreactivity in aged individuals failed to augment total (Fig. 16.3b) as well as bioavailable and free testosterone concentrations equivalently. The latter age-related contrast would point to an abnormal pulsatile LH signal (of equivalent mean concentration only), relatively defective Leydig-cell responsiveness and/or accelerated testosterone metabolism in older men. The last (kinetic) consideration is excluded by the age-related fall in the metabolic clearance rate of testosterone that is partly due to higher SHBG concentrations [40, 42, 43, 65].

Table 16.1 Indirect evidence for impaired GnRH drive in the aging male

	Human	Rodent
1. Low-amplitude spontaneous LH pulses	[28, 29, 32–34, 45–47, 62]	[31, 48, 50, 51, 55, 57, 152]
2. Normal or increased LH secretory responsiveness to, i.v., GnRH pulses	[34, 54, 61, 113, 153]	[40, 43, 59, 154]
3. Blunted unleashing of pulsatile LH secretion by sex-hormone depletion, opiate-receptor blockade, androgen receptor blockade or stress	[63, 86–88]	[29, 30, 34, 49, 50, 52, 54, 56, 58–61, 91, 152, 153, 155–158]
4. Age-comparable LH bioactivity	[32, 40, 63, 74, 87,	

	159–163]	
5. Quantifiably disorderly LH release	[34, 36, 46, 47, 88–90, 137]	
6. Asynchrony of neurohormone outflow	[37, 38, 136, 139]	
7. Mathematical ensemble predictions	[45–47, 97]	

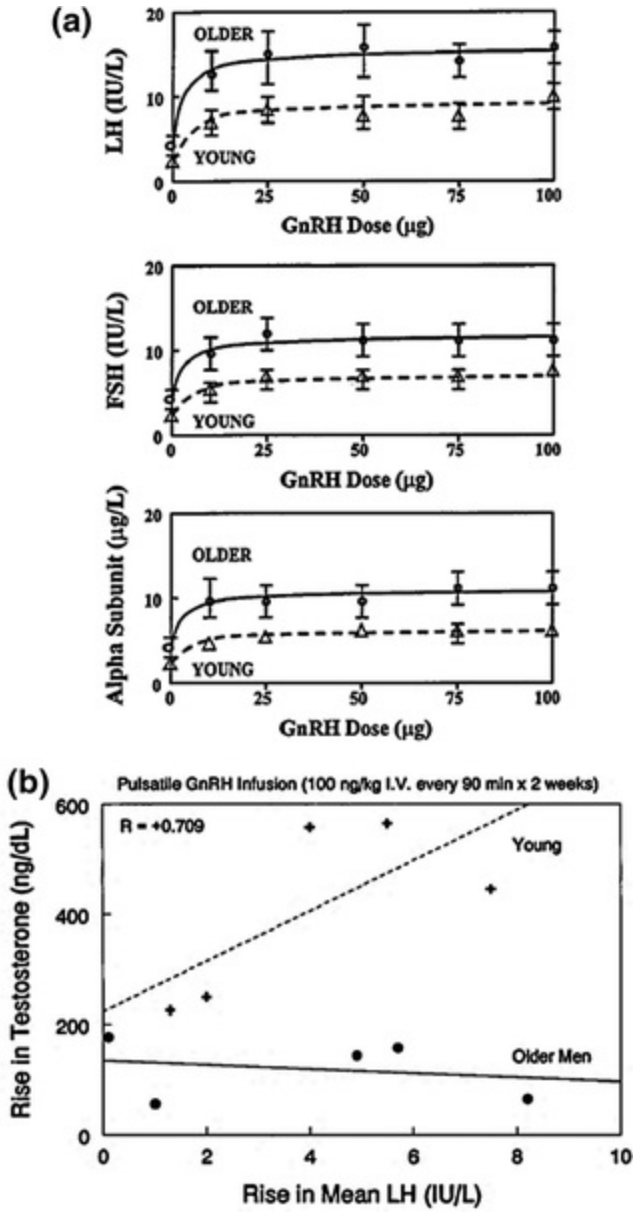

Fig. 16.3 **a** GnRH dose-response stimulation of (2-h mean) serum concentrations of LH (*top*), FSH (middle) and free (*uncombined*) alpha subunit (*bottom*) in young men (*interrupted lines*) and older volunteers (*solid lines*). Data are the mean ± SEM. Adapted with permission from [113]. **b** Relationship between the increment in 24-h mean concentrations of LH (*x* axis) and testosterone (*y* axis) induced by 14 days of uninterrupted pulsatile, i.v., GnRH infusion (100 ng/kg every 90 min) compared with saline in young men (*upper interrupted curve* and *plus signs*). There is no correlation in older men (*lower continuous line* and *solid circles*). Adapted from [34]

These earlier findings have been verified in a complementary experiment wherein 18 healthy men aged 23–72 years underwent graded suppression of LH secretion through four escalating doses of ganirelix (0, 0.1, 0.3, and 1 mg/m^2), a specific GnRH receptor antagonist [66]. Sixteen hours after subcutaneous administration of ganirelix, a submaximal dose of intravenous GnRH (100 ng/kg) was injected, and blood sampling was continued for a further 3 h. Increasing age attenuated the ability of ganirelix to decrease endogenous pulsatile LH secretion, but did not impact competitive interaction between ganirelix and injected GnRH. These joint outcomes suggest that age diminishes GnRH outflow without impairing GnRH action in healthy men. Further modeling confirmed that advanced age was associated with lower GnRH efficacy normalized to free testosterone and also revealed potentiated (maximal and graded) negative feedback by testosterone on hypothalamic GnRH secretion [67].

Contribution of Primary Leydig-Cell Steroidogenic Failure

Anatomical studies document an attrition of Leydig-cell number in the older male [68]. From a functional perspective, pharmacological stimulation with hCG does not induce maximal young-adult concentrations of testosterone in many elderly men [24, 28, 69–72]. Nonetheless, current hCG paradigms are difficult to interpret on experimental and physiological grounds; for example, (i) unequal concomitant (endogenous) LH concentrations among individuals may confound testicular responses to hCG [13, 24, 30, 71, 72]; (ii) the half-life of circulating hCG is approximately 20–30 h compared with 0.75–1.5 h for LH; indeed, the former kinetics abrogate normal intermittent stimulation of the testis [45, 73, 74]. This distinction is relevant, since each pulse of LH normally evokes a prompt burst of testosterone release, as monitored directly in the human spermatic vein [75, 76]; (iii) the nearly irreversible binding of hCG to the gonadal LH receptor downregulates steroidogenesis in vitro and in vivo [77, 78]; and (iv) the magnitude of hCG drive limits clinical evaluation to maximal Leydig-cell, responsivity. An equivalently sustained and potent lutropic stimulus is never achieved physiologically in vivo, except under maternal hCG exposure in the developing fetus [40, 42].

A second means of appraising Leydig-cell responsiveness is cross-correlation analysis of LH and (time-delayed) testosterone concentrations in paired hormone time series [79]. Comparisons by age disclose a young adult-like time lag of 30–40 min but significantly reduced feedforward coupling between LH and testosterone in older men. The latter distinction is reflected in a 50% reduction in the cross-correlation coefficient [33, 35].

A third experimental strategy to examine Leydig-cell steroidogenic capacity is to downregulate gonadotropin secretion with a GnRH analog and then add back pulses of rh LH, i.v., to emulate physiological patterns of gonadotropin stimulation. In this

paradigm, LH withdrawal enforced by pituitary down-regulation impairs testosterone secretory responsiveness profoundly in both age groups, albeit twofold more in elderly men [80].

A fourth-interventional model (Fig. 16.4) is to administer a maximal dose of a selective GnRH receptor antagonist (ganirelix) to suppress all endogenous LH secretion, and then infuse successive, i.v., pulses of rh LH to replicate normal LH on testosterone physiology [81–84]. Using this paradigm, pulsatile recombinant human (rh) LH infusions for 48 h designed to mimic both the pattern (every 2 h) and extent of young male LH secretion revealed lower mean and incremental and interpeak testosterone concentrations in 8 older (aged 60–73) compared with 13 young (aged 19–30) men [82]. These age relationships were confirmed in 15 healthy men aged 22–78 years across a tenfold range of amplitude varying, and not supraphysiological, pulses of rh LH administered across a 20-h period [83]. A further analysis of 92 healthy men aged 18–75 years who received various combinations of wide-ranging but not supraphysiological pulses of rh LH show that aging attenuates LH efficacy and testis sensitivity as well as augments Leydig cell downregulation [84].

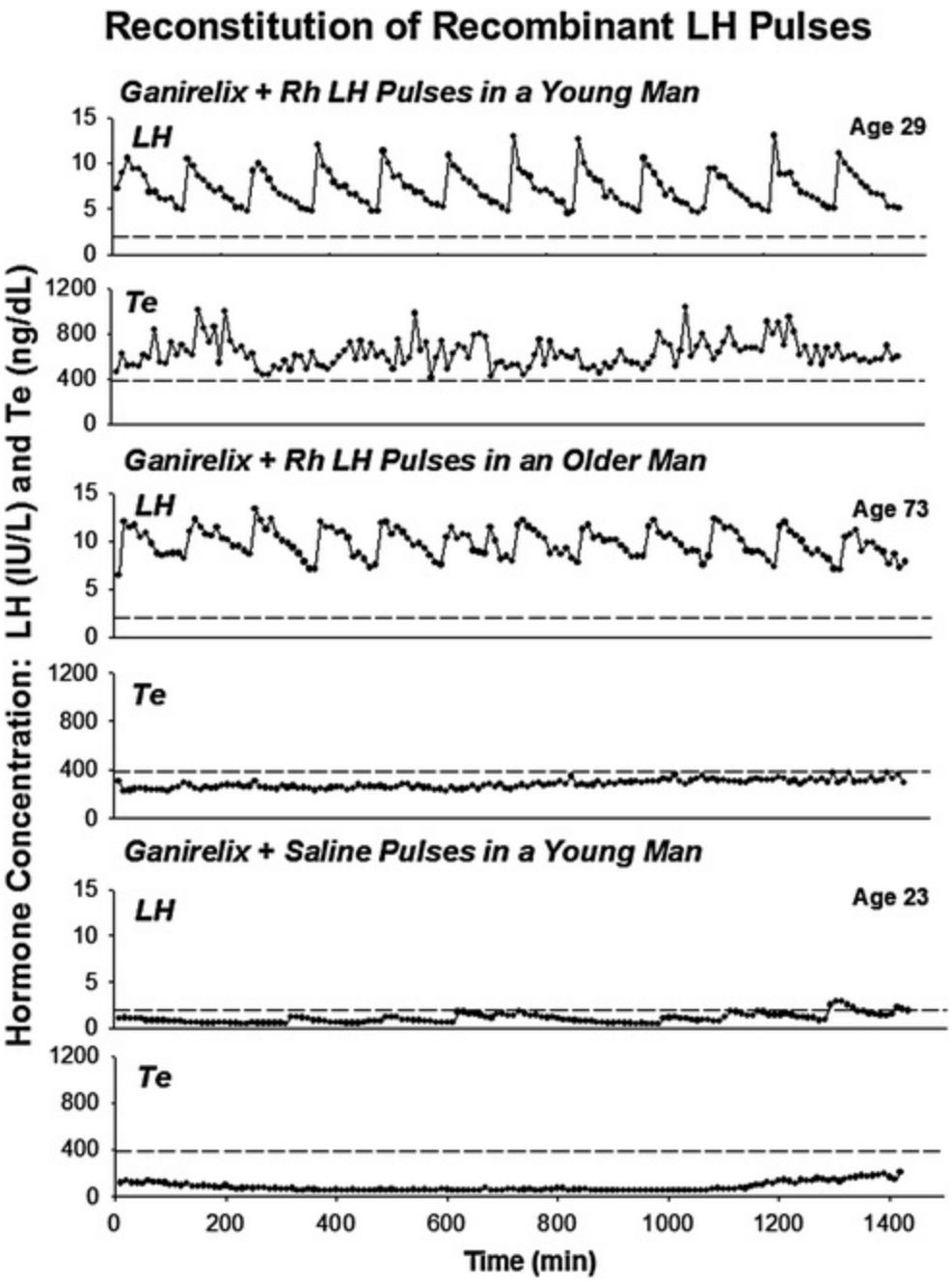

Fig. 16.4 Illustrative profiles of (paired) LH (*top*) and Te (*bottom*) concentrations sampled every 10 min for 24 h in a healthy young (*upper*) and older (*middle*) man on the second day of pulsatile iv infusion of rh LH and concomitant ganirelix administration [*see text*]. For comparison, LH and Te concentrations during ganirelix administration and iv addback of saline in a young subject are also shown (*lower*). Interrupted horizontal lines mark the lower 2.5% normal bounds of LH (2 IU/L) and Te (385 ng/dL) concentrations in young adults. Multiply Te values in ng/dL by 0.0347 to obtain units of nmol/L

Alterations in Testosterone Feedback Restraint

Whether or how sex-steroid negative feedback is altered in older men is controversial. For example, three clinical studies report heightened negative feedback by exogenous

androgens in the aging male. These analyzes were based on short-term, i.v., infusion of testosterone or dihydrotestosterone (over days), longer term transscrotal delivery of testosterone (for 11 months), and transdermal supplementation with 5 alpha-dihydrotestosterone (for 15 months) [39, 44]. Two other investigations describe reduced androgen feedback efficacy in the older male after several weeks of intramuscular injection of a high dose of testosterone [61, 85]. Using a novel matched testosterone addback paradigm, experiments showed that aging may impair *both* positive and the negative actions of systemic testosterone on pulsatile LH secretion [86]. Specifically, stepwise testosterone repletion across the hypogonadal to eugonadal range in 23 men aged 19–71 years revealed that age attenuated both the ability for increasing testosterone concentration to increase LH secretory burst mass and to decrease LH secretory burst frequency.

Two interventional studies using an estrogen- or androgen-receptor antagonist, and one biomathematical construct, infer reduced LH secretory adaptations to muted sex-steroid feedback in elderly men [63, 87, 88]. Recent studies show that aging mutes unleashing of LH secretion by androgen-receptor blockade using a fourth-generation biomathematical construct [89, 90]. From another vantage, cross-correlation analysis of 24-h (paired) LH and testosterone concentration profiles identifies impaired negative feedback by *endogenous* androgen in older volunteers [35]. And based on immuno-cytochemical detection, androgen (and estrogen) receptors may decline in the brain and pituitary gland of aged rats, and in genital fibroblasts of older men [91, 92].

Prominent adaptations to experimental androgen depletion in young men are accelerated frequency and reduced incremental amplitude of LH pulses (Fig. 16.5a) and quantitatively irregular patterns of LH release [88, 93–95]. The neuroendocrine phenotype in older men includes each of the foregoing feedback adjustments [33, 34, 36, 37, 62]. Thus, a facile hypothesis is that lower bioavailable and free testosterone concentrations in the elderly male are the proximate cause of unleashing anomalous LH secretion patterns. However, quite unlike the feedback-deprived state, basal, pulsatile, and total LH secretion remain normal in many healthy older men [45, 46] (Fig. 16.5b). Accordingly, an important but unresolved mechanistic issue is how neuroendocrine disturbances in aging individuals reflect expected feedback withdrawal due to hypoandrogenemia [43]. This question becomes important, inasmuch as testosterone repletion in androgen-deficient young men reverses each of rapid-frequency, diminutive-amplitude, and disorderly LH secretion [88, 93, 94].

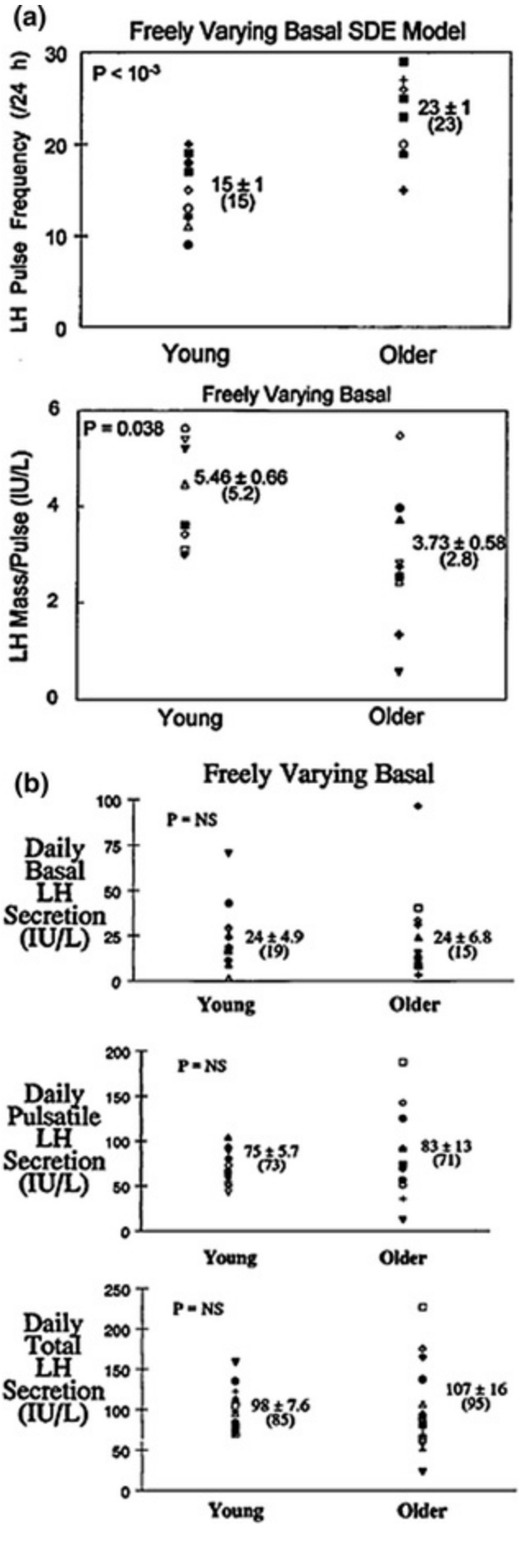
(a)
Freely Varying Basal SDE Model
LH Pulse Frequency (/24 h)
P < 10-3
15 ± 1
(15)
23 ± 1
(23)
Young
Older
Freely Varying Basal
LH Mass/Pulse (IU/L)
P = 0.038
5.46 ± 0.66
(5.2)
3.73 ± 0.58
(2.8)
Young
Older
(b)
Freely Varying Basal
Daily Basal LH Secretion (IU/L)
P = NS
24 ± 4.9
(19)
24 ± 6.8
(15)
Young
Older
Daily Pulsatile LH Secretion (IU/L)
P = NS
75 ± 5.7
(73)
83 ± 13
(71)
Young
Older
Daily Total LH Secretion (IU/L)
P = NS
98 ± 7.6
(85)
107 ± 16
(95)
Young
Older

Fig. 16.5 Comparison of LH secretory attributes in young and older men based on a variable-waveform model that allows a freely varying basal secretion rate. **a** Impact of age on LH pulse frequency (events/day) and LH secretory burst mass (IU secreted per pulse per L of distribution volume). **b** Deconvolution estimates of basal (*top*), pulsatile (*middle*), and total (*bottom*) daily LH secretion in young and older men. Numerical values are the mean ± SEM (median). *P* values are estimated by the rank-sum test. *P* = NS denotes <0.05. Adapted from [45]

One potential mechanism might relate to inflammation. Infusion of IL-2, which is an important inflammatory mediator, seems to potentiate the proportionate feedback inhibition of testosterone on LH in older men [27]. If verified, these data point to a possible interaction between age and concomitant comorbidities that cause inflammation. These data support the hypothesis that both age and comorbidities are associated with the age-related decline in testosterone.

Regulation of the Ensemble Hypothalamo-Pituitary-Leydig Cell Axis

An emergent thesis in neuroendocrine research is that early disruption of (negative) feedback and/or (positive) feedforward activation may occur without any demonstrable change in overall hormone production [34, 36, 41, 46, 47]. The foregoing insight is relevant to understanding early disarray of the hypothalamo-pituitary-gonadal axis in aging. Therein, we adopt the basic precept that repeated, dose-dependent, time-delayed adjustments among regulatory sites govern systemic androgen availability in a homeostatic fashion [45, 76, 96–103]. To aid intuition, we have formalized core feedback connections among GnRH, LH, and testosterone mathematically [45, 95–97]. A simplified model structure predicts that jointly impaired feedforward by LH, and feedback by testosterone (in part through aromatization to estradiol), would yield a high-frequency, low-amplitude, and disorderly pattern of LH release. A recent adaptation allows, but does not enforce, pulse-by-pulse hysteresis wherein the dose-response relationship during the ascending and descending phase of the response pulse peak is allowed to differ (Fig. 16.6) [104, 105]. On the other hand, isolated failure of GnRH outflow would explain only inappropriately low LH concentrations and attendant hypoandrogenemia, but not an elevated LH pulse frequency or irregular release patterns [46, 47]. Exploration of this ensemble concept requires more focused assessment of physiological regulation, as highlighted next.

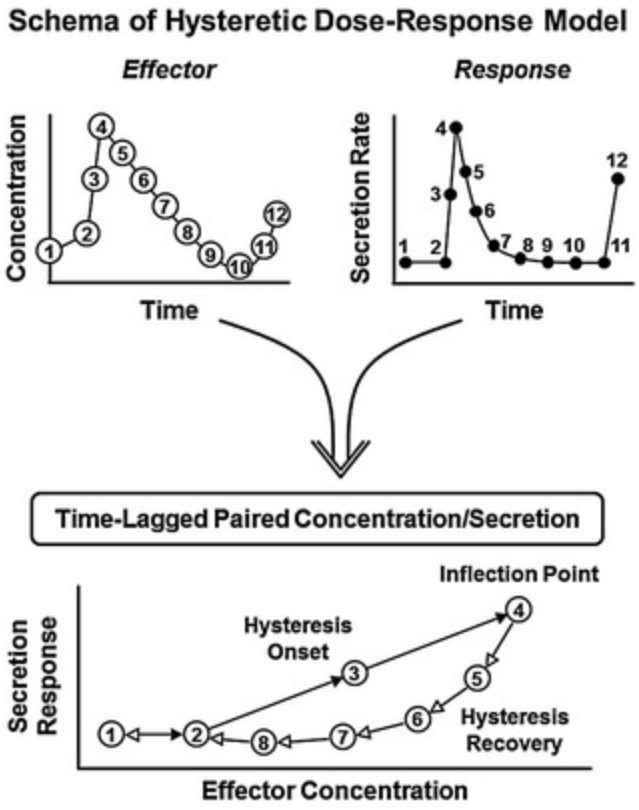

Fig. 16.6 Schema of dose-response hysteresis concept. Hormone (effector) concentrations within a pulse (*top left*) drive target-gland secretion rates (*top right*). The relationship between input (concentration) and output (secretion) is estimated as a dose-response logistic function (*below*). The onset (*solid arrows*) and recovery (*open arrows*) phases of the dose-response interface are estimated simultaneously with a hysteresis inflection point (delay time)

Sampling and Counting of Pulses

The frequency and amplitude of LH pulses provide a window to signals from the brain and pituitary gland. Withdrawal of blood at 5 or 10-min intervals for 12–24 h will capture the majority of discrete LH release episodes in healthy men [40, 106–110]. Suitable monitoring paradigms define an accelerated frequency and attenuated amplitude of pulsatile LH release in older men by independent methods of pulse detection, in separate gonadotropin assays and among different volunteer cohorts [34, 46, 47, 62, 111]. Figure 16.7 illustrates comparisons by age of immune-radiometric LH concentration times series collected every 10 min over 24 h [33]. Some, but not all, earlier analyzes using radioimmunoassay methods and/or less frequent (e.g., 20-min) blood-sampling protocols presaged the concept of lower amplitude and higher frequency LH peaks in older men [24, 40–42]. Recent biomathematical simulations establish that failure to detect either a loss of amplitude or a gain in frequency of LH pulses is a predictable technical artifact of insufficiently intensive blood sampling (Fig. 16.8).

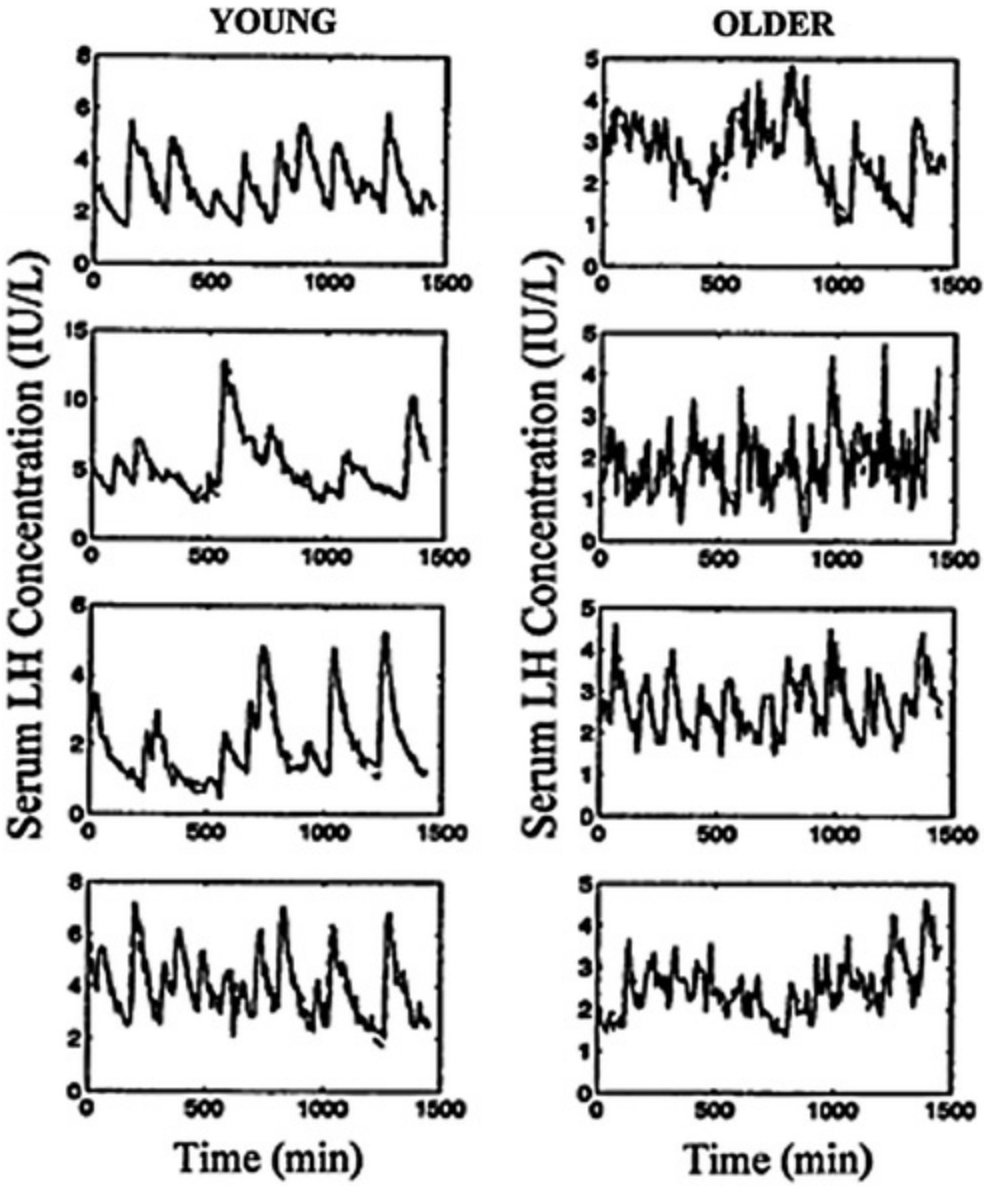

Fig. 16.7 Diminution in LH secretory burst mass and acceleration of LH pulse frequency in aging when compared with young men inferred by two deconvolution methods [47]. LH concentration profiles (± sample SD's) measured in serum collected every 10 min for 24 h and analyzed by immuno-radiometric assays and variable-waveform (stochastic differential equation (SDE) based) deconvolution analysis (predicted continuous curve) [45]

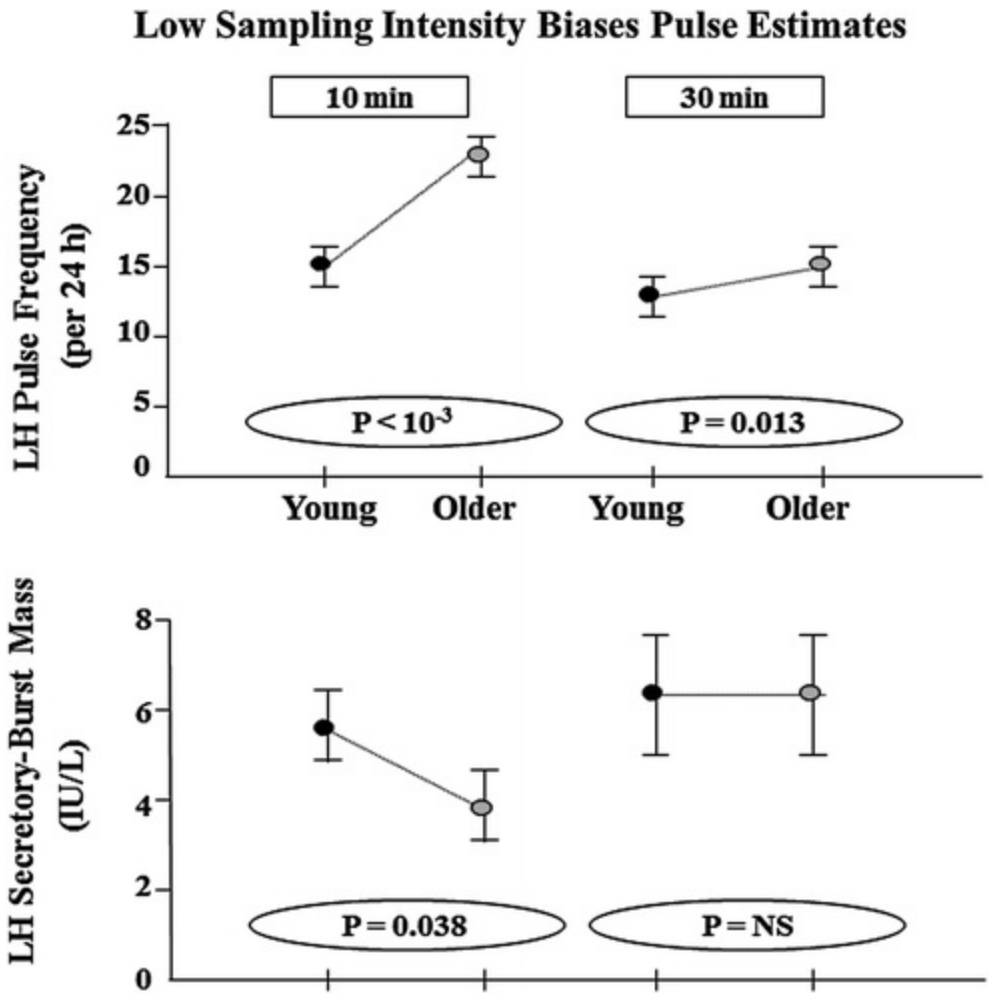

Fig. 16.8 Undersampling of LH release censors detection of LH pulses (*top*) and inflates estimates of LH secretory burst mass. The resultant technical bias limits the identification of age-related contrasts in pulse frequency and size. 24 LH concentration time series were edited from original 10-min sampling sets to yield 30-min daughter series (simple subsets) in 11 young and 13 older men. Unpublished reanalysis of data in [34, 35, 62] (Bradford, Kuipers and Veldhuis)

Gonadotrope Responsiveness to GnRH

Viewed mechanistically, a 50% reduction in incremental (peak minus nadir) LH pulse amplitude in aging men could denote a diminished amount of hypothalamic GnRH secreted per burst, an abnormal GnRH waveform and/or impaired gonadotrope-cell responsiveness to GnRH. Recent GnRH dose-response analyzes conducted in randomly assigned order on separate days establish normal maximal, and enhanced submaximal, acute and short-term (14-day) stimulatory effects of GnRH in the elderly male [34, 112, 113]. Heightened sensitivity to small amounts of GnRH occur predictably in the presence of augmented pituitary LH stores in older individuals, as inferred post mortem. Furthermore, pituitary sensitivity to a wide 300-fold range of intravenously administered pulses of GnRH is age-invariant when testosterone exposure is matched at a physiological concentration, or when a single submaximal GnRH dose is assessed across matching graded degrees of androgen repletion [86, 114].

Accelerated LH Pulse Frequency in the Aging Male

A basic, unresolved mechanistic issue is whether aging causes a primary hypothalamic disturbance that enforces a high frequency of GnRH pulses, and/or whether low bioavailable testosterone concentrations elevate LH pulse frequency via feedback withdrawal [33, 41–43, 47, 62]. This query is central to contemporary physiological assessments of aging, inasmuch as earlier investigations in healthy young men document that (i) infusion of testosterone or a nonaromatizable androgen suppress LH pulse frequency and elevate LH peak amplitude [30, 102, 115, 116]; and (ii) administration of ketoconazole (which depletes testosterone) or flutamide (which inhibits androgen-receptor binding) augments LH pulse frequency and reduces incremental LH peak (fractional) amplitude [93, 94, 117]. In the human, monkey, sheep and rat, a high frequency of GnRH stimuli reduces incremental (fractional) LH pulse amplitude [43, 55]. In view of this reciprocal control mechanism (albeit not fully understood), diminished androgen bioavailability in older men could contribute in principle to both more rapid and lower amplitude LH pulses. What remains unknown is whether hypoandrogenemia in aging fully accounts for the observed neuroendocrine changes.

The time pattern of LH secretion within the physiological range is now directly known to influence testosterone response [118, 119]. Nineteen healthy men aged 18–49 years received four infusions in random order: saline, and continuous, hourly and second hourly rh LH matched for total dose infused over the entire 12-h period. Hourly, rh LH exposure resulted in higher testicular secretory sensitivity, greater rh LH potency, greater pulsatile testosterone secretion and faster half-time of appearance of peak testosterone. These data show that the time pattern of LH secretion enforces quantifiable differences in testosterone secretory dynamics, and suggests but does not prove that the increased frequency of LH pulses with age is an adaptive response to maintain testosterone output.

Monitoring Unobserved Gonadotropin Secretion

Lower incremental LH peak amplitude in older men can be dissected further mechanistically under the premise that fluctuating hormone concentrations are driven by specific (unobserved) secretion and kinetic processes. Deconvolution analysis is a family of computer-assisted methods designed to quantitate underlying secretion and/or elimination. Some deconvolution procedures allow one to determine the following: (i) the frequency of secretory bursts; (ii) the amount of hormone released within each burst [mass of hormone discharged within the event normalized to unit distribution volume]; (iii) the subject- and condition-specific half-life of elimination of the hormone; and (iv) concomitant basal or (nonpulsatile) secretion.

There are several complementary deconvolution approaches to secretion analysis. Figure 16.9a illustrates one basic idea, wherein an unobserved Gaussian-shaped

secretory burst creates each peak in serum hormone concentrations [120]. In this *waveform-dependent* concept, hormone concentrations increase rapidly due to the sharp onset of an underlying secretory burst, and then fall gradually after the burst, as the hormone is eliminated slowly from the circulation. The Gaussian-burst model allows one to calculate the number, location, duration, and amplitude of secretory bursts and predict the hormone half-life at the same time [121, 122]. Application of this methodology in healthy men quantitates a lower mass and higher frequency of LH secretory events in older than young men, but a comparable LH half-life [33, 41, 62]. The lower mass (IU) of LH released per secretory burst in the elderly male is due to attenuation of the amplitude (maximal secretion rate attained) rather than abbreviation of the duration (min) of release episodes.

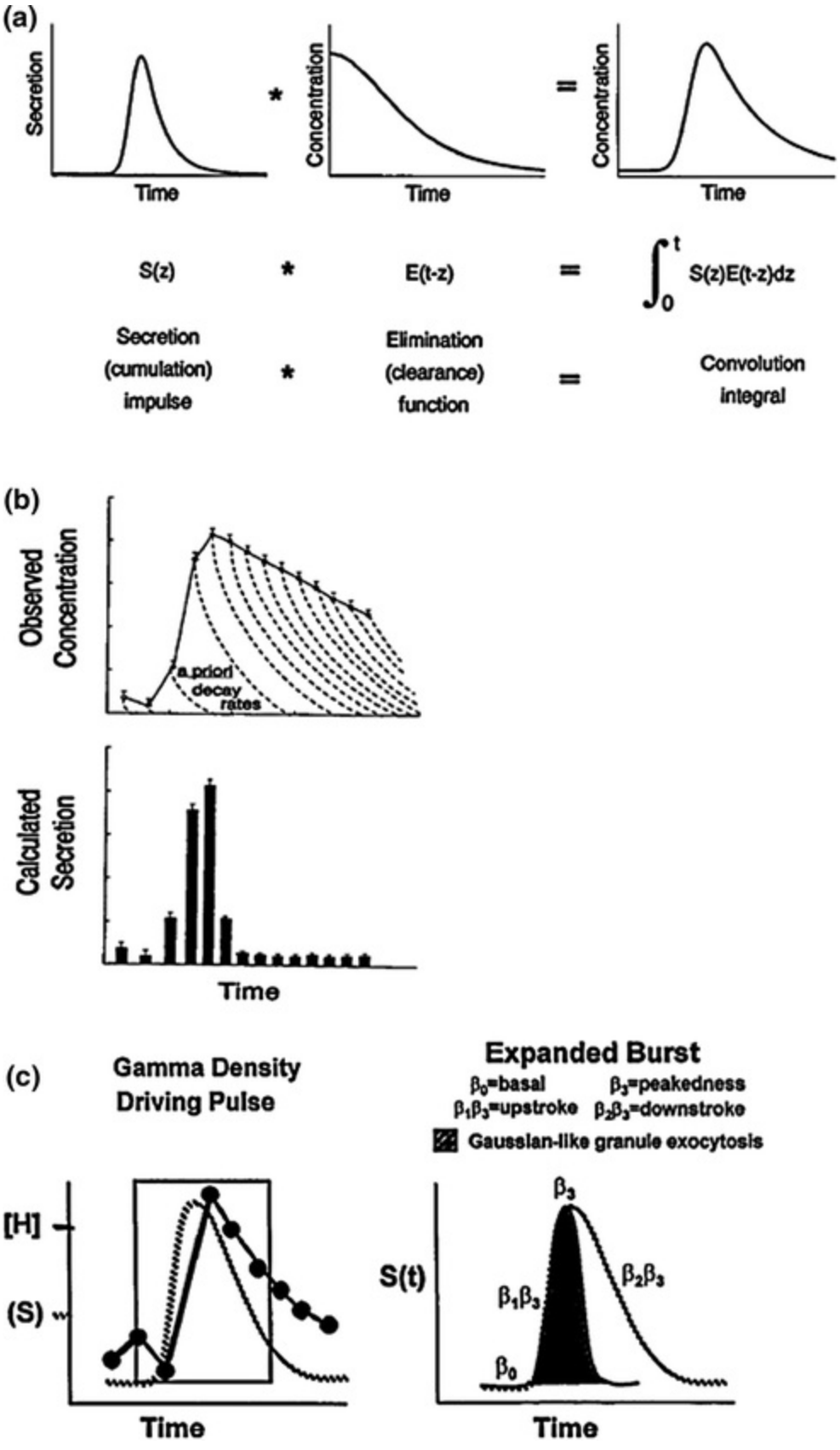

Fig. 16.9 Concept of deconvolution analysis as a family of methods designed to quantitate unobserved secretion and/or kinetics from fluctuating hormone concentrations. **a** A *Gaussian* or slightly skewed *secretory burst model* (*top left*) provides estimates of time-delimited, burst-like discharge of molecules with individual release velocities driving a

pulse. A secretory burst is defined by its position in time, maximum (amplitude), and half-duration (duration at half-maximal height) [120, 122]. The time integral of the convolution product of the secretory burst (*left*) and disappearance function (*middle*) yields the pulsatile hormone concentration (*right*). The reverse process of deducing underlying secretion and elimination from observed concentrations is termed deconvolution analysis. **b** A *waveform-independent approach* allows one to calculate sample secretion rates (*bottom*) from measured hormone concentrations (*top*) based on a priori biexponential kinetics (*interrupted decay curves*). Experimental errors inherent in hormone measurements and the populational half-life estimate are combined to determine statistical confidence limits for each sample secretion rate [121, 123]. **c** Flexible deconvolution construct of a *generalized Gamma-density (variable) secretory-pulse waveform* is utilized to encapsulate burst timing, number, shape and size, basal secretion, and rapid and slow-phase hormone elimination kinetics simultaneously with random effects [95, 97]. Random effects arise from sampling uncertainty, assay error, stochastic admixture of secreted hormone in the bloodstream, unpredictable pulse times and varying effector-response interface properties [46, 96]

A second-generation deconvolution technology defines secretion as an admixture of unknown basal output and superimposed events of any height, width, shape, and number [121] (Fig. 16.9b). This deconvolution construct is therefore defined as *waveform-independent*. For statistical reasons, optimal analysis in this methodology requires a priori knowledge of the two-component half-life of hormone elimination [123]. Because the true waveform of secretory bursts is (definitionally) unknown, definitive pulse counting is analytically difficult unless assumptions are made in fact about event shape, smoothness, location, or number [122]. However, this mathematical strategy likewise predicts lower peak rates of LH secretion in older men.

A third-generation deconvolution strategy uses coupled, stochastic differential equations to reconstruct simultaneously the number, shape, size, and timing of (unknown) secretory bursts, the basal release rate, biexponential disappearance kinetics and random effects driving hormone release [96, 97] (Fig. 16.9c). This comprehensive statistical platform predicts a lower mass and higher frequency of LH secretory bursts in older than young men with no age difference in LH kinetics [45, 47, 95]. Estradiol but not age per se reduces LH bioactivity in vitro and prolongs LH metabolic clearance rate in vivo [73, 124]. The diminution in mass and acceleration in frequency of LH pulses tend to counterbalance closely, thus leaving the total and pulsatile daily LH secretion rate unchanged in aging individuals.

The fourth-generation deconvolution strategy allows for computer automation by comparing all possible pulse sets and objectively choosing the best model fit using mathematically validated criteria (e.g., Akaike Information Criterion) [105]. This method has been mathematically and empirically validated in three mammalian species [125, 126]. Future advances being investigated include allowing for pulse-by-pulse flexibility in pulse shape [105].

Contemporary secretion-based insights explain some earlier observations. For example, one method of discrete hormone peak detection, Cluster analysis, identifies a pulse simply as a statistical jump and drop in the hormone concentration [40, 127]. This approach is termed model-free, since few assumptions are made in defining a pulse. Deconvolution analysis illustrates that the peak increment is proportionate to the mass

of hormone secreted in the burst *ceterus paribus* [122, 123, 128]. This point is important, since elderly men consistently exhibit a lower incremental LH peak amplitude compared with young counterparts [34, 95].

Regularity Analysis

Homeostasis within an endocrine axis requires repeated decremental and incremental adjustments in secretion [46, 96, 97] (Fig. 16.1). In the male gonadal axis, core homeostatic signals include (at least) GnRH, LH, and testosterone, which enforce adaptations over a rapid (minute-by-minute) time course [129]. Orderliness of the resultant subpatterns of hormone release can be quantitated objectively via a regularity statistic, approximate entropy (ApEn) [36, 130–132]. ApEn is calculated on a desktop computer as a single number, which exploits accurate probabilistic accounting to quantitate the reproducibility of successive measurements in a time series [133] (Fig. 16.10a). Higher ApEn denotes heightened irregularity of minute-by-minute hormone release, which in turn signifies deterioration or adaptation of feedback and feedforward adjustments [131, 134, 135].

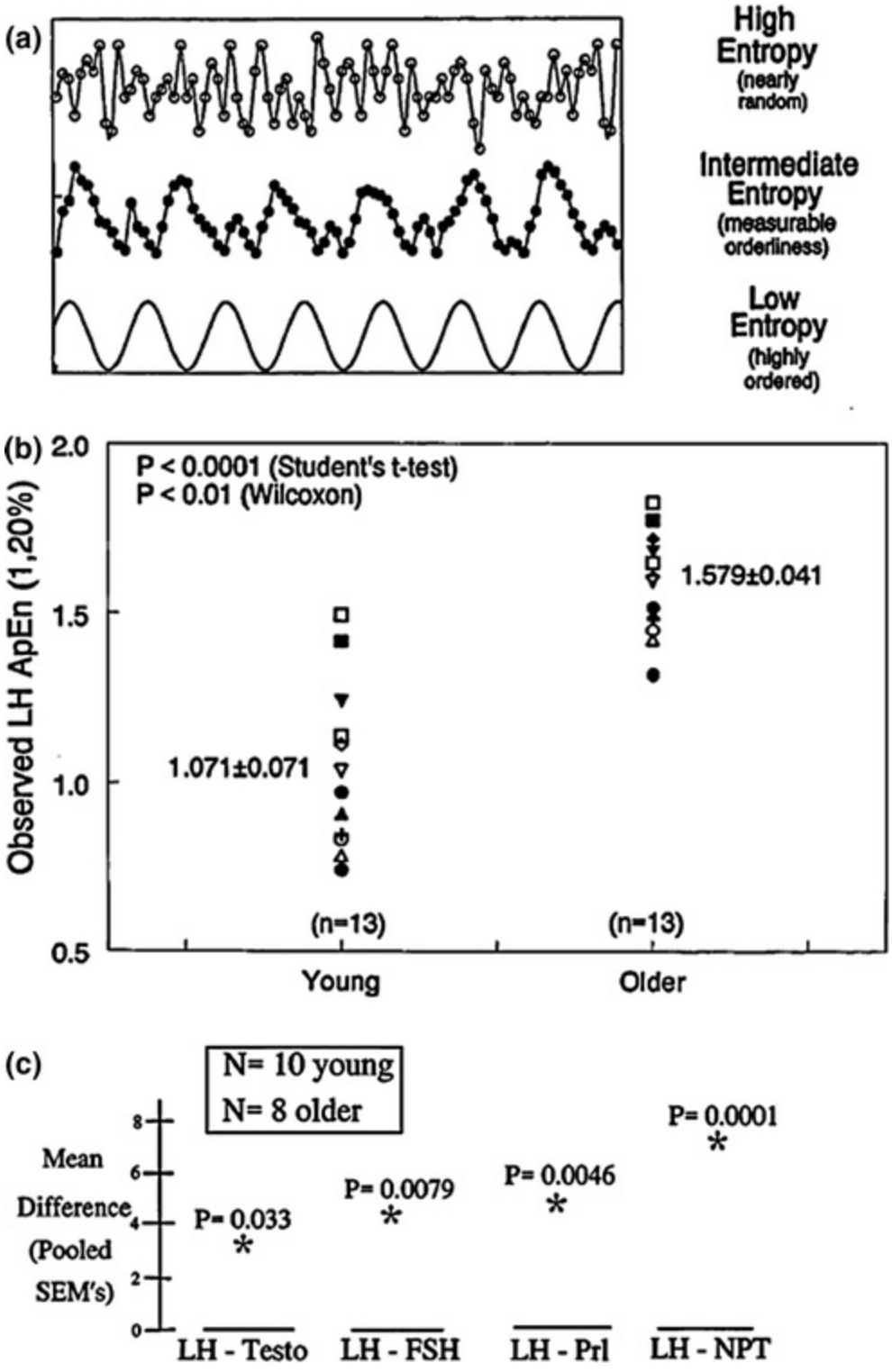

Fig. 16.10 **a** Notion of the approximate entropy (ApEn) statistic to quantitate relative regularity of serial (sample-by-sample) measurements. The bottom curve gives a cosine function (low ApEn) to illustrate a well reproduced pattern; the middle curve shows partial degradation of regularity (intermediate ApEn); and, the top frame depicts marked erosion of orderliness (high ApEn). **b** Increased ApEn of overnight (2.5-min) LH release profiles in healthy older compared with young men. Higher ApEn points to less orderly outflow of GnRH and/or LH, or disruption of the GnRH-LH feedforward interface. Numerical values are the mean ± SEM. **c** Cross-approximate entropy (cross-ApEn) differences in older and young men. Differences exceeding three SEM's (older minus young) denote significant deterioration of young-adult coupling between oscillations of LH and testosterone (*leftmost*), LH and FSH (*left*

middle), LH and prolactin (PRL, *right middle*) and LH and nocturnal penile tumescence (NPT) (*rightmost*). *P* values denote the probability of falsely rejecting the null hypothesis of equivalent two-hormone synchrony in the two age groups. Unpublished compilation of data reported in [36–38, 111]

In a clinical context, ApEn identifies disorderly patterns of hormone release, which denotes autonomy from normal regulatory adjustments [26, 131]. In addition, ApEn delineates irregular secretion of LH, testosterone, GH, ACTH, cortisol, and insulin in older compared with young individuals, thus defining age-related deterioration of adaptive control [34, 36–38, 41, 88, 136] (Fig. 16.10b). For example, elevated ApEn of LH and testosterone release patterns unmask disruption of one or more key signaling elements within the interlinked GnRH-LH-testosterone axis [36, 46, 47, 134]. Interventional studies are necessary to localize the individual and joint sites of statistically inferred regulatory defects. Under such a paradigm, aging attenuated the capability of graded testosterone addback to regularize LH secretion patterns under endogenous GnRH, but not under fixed exogenous GnRH [137]. Accordingly, these data point to impaired negative feedback at a hypothalamic locus.

The cross-ApEn statistic extends the concept of appraising single-hormone regularity to quantitating two-hormone (pairwise) synchrony [36]. Cross-ApEn unveils significant loss of bihormonal synchrony between LH and testosterone secretion in older men (Fig. 16.10c). This measure further documents deterioration of coordinate patterns of LH release and oscillations of each of prolactin, FSH, nocturnal penile tumescence (NPT), and sleep stage [37, 46, 47]. According to this idea, brainstem regulatory centers oversee synchronous secretion of GnRH, LH, FSH, and prolactin, sleep-stage transitions and autonomic control of NPT cycles. Aging symmetrically erodes both feedback (testosterone on LH) and feedforward (LH on testosterone) linkages in the unperturbed state, as well as when perturbed by graded GnRH receptor blockade [138, 139]. Thus, the foregoing findings establish multilevel disruption of CNS-dependent neurohormone outflow in older men.

Appraising Testosterone Signaling

To date, the majority of clinical studies have utilized the total testosterone concentration to assess androgen-dependent negative feedback on the hypothalamo-pituitary unit [42, 43]. However, in men total testosterone is distributed in plasma as free (approximately 2%), weakly albumin-bound (50–55%) and tightly globulin-bound (40–45%) steroid [140, 141]. Rapid dissociation of testosterone from low-affinity albumin (nominal unidirectional half-time 0.2 s at 37 C) would favor effectual tissue uptake within a brief (2–10 s) capillary transit time, so long as reassociation is minimized [142]. On the other hand, slow release of testosterone from high-affinity sex-hormone binding globulin (SHBG) [half-time 3.3 s at 37 C] would putatively restrict access to cells, at least when reassociation is limited. Protein-binding effects are important, since SHBG

concentrations increase as much as twofold, and bioavailable (nonSHBG-bound) testosterone concentrations fall by 30–50% in some older individuals [25, 143–146] (Fig. 16.11a). Advances in how SHBG should be measured and exactly how this binding should be modeled have occurred (see Chap. 15). Such modeling to determine calculated free testosterone will be increasingly important since recent epidemiological studies show that hypogonadism defined by dichotomization based on free, rather than total, testosterone is better associated with symptoms consistent with androgen deficiency [147]. However, current binding assumptions, analytical methods and models appear adequate when SHBG concentrations are within the normal male range [148–151]. Dynamic mechanisms of physiological control of free, SHBG- and albumin-bound testosterone are highlighted in the kinetic schema of Fig. 16.11b. Therefore, according to contemporary perspectives, carefully designed clinical studies will be needed to examine the age-dependence of free and bioavailable testosterone-mediated negative feedback on gonadotropin secretion [43].

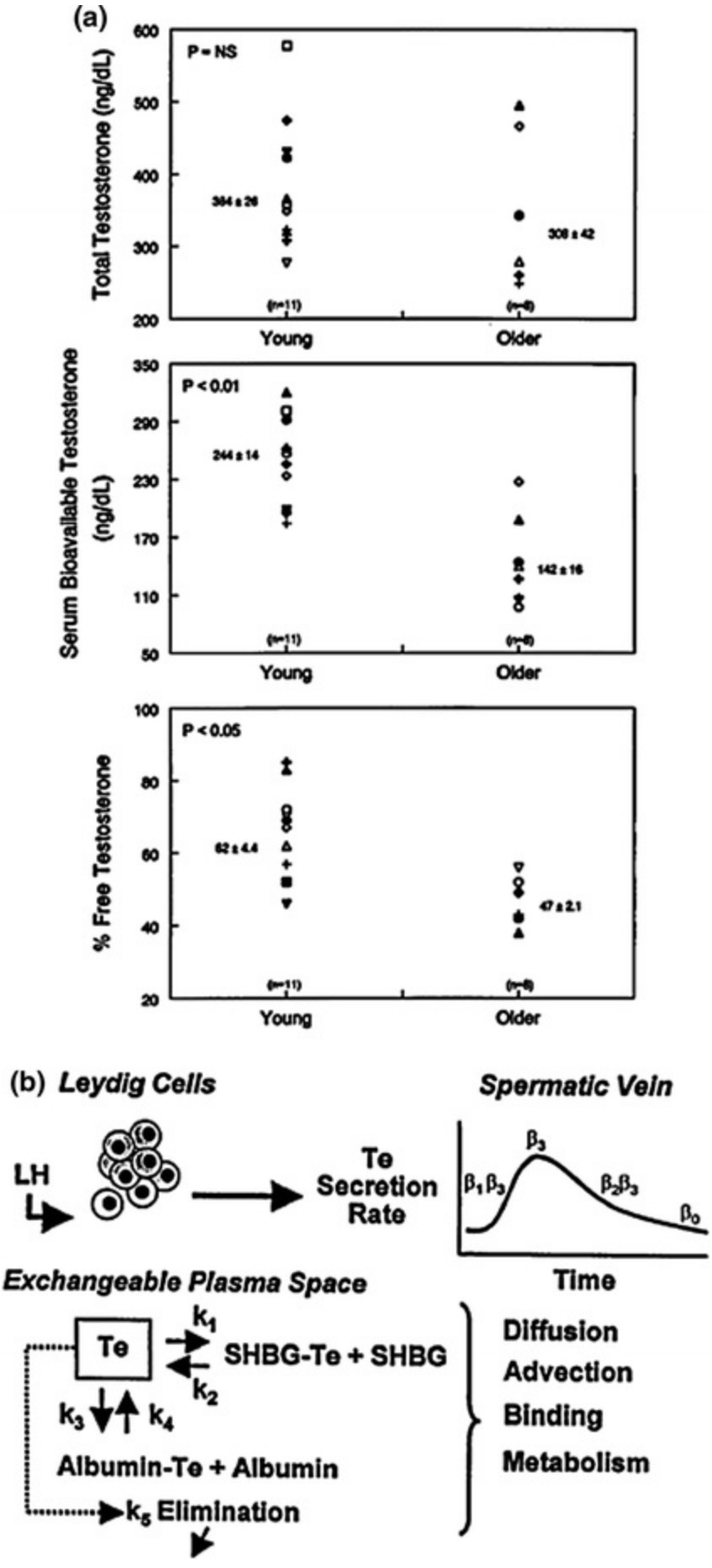
(a)
Total Testosterone (ng/dL)
P = NS
384 ± 26
308 ± 42
(n=11)
(n=8)
Young
Older
Serum Bioavailable Testosterone (ng/dL)
P < 0.01
244 ± 14
142 ± 16
% Free Testosterone
P < 0.05
62 ± 4.4
47 ± 2.1
(b) Leydig Cells
Spermatic Vein
LH
Te Secretion Rate
β3
β1β3
β2β3
β0
Time
Exchangeable Plasma Space
Te
k1
k2
SHBG-Te + SHBG
k3
k4
Albumin-Te + Albumin
k5 Elimination
Diffusion
Advection
Binding
Metabolism

Fig. 16.11 **a** Comparison of serum total, nonSHBG (bioavailable) and percentage free testosterone measurements in two cohorts of men; viz., young ($N = 11$) and older ($N = 8$). Similar mean total testosterone concentrations by age (*top*) belie a reduction in bioavailable and percentage nonSHBG-bound (% free) testosterone (*middle* and *lower*) in older individuals. Adapted from [38]. **b** Schema of fate of secreted testosterone in plasma

Summary

Developing concepts of time-adaptive control of testosterone secretion incorporate interactions among GnRH, LH, and testosterone. Homeostasis requires reversible, recurrent, and reciprocal (feedback and feedforward) adjustments to external stress (e.g., food deprivation, trauma) and internal needs (e.g., anabolism, procreation). In this ensemble perspective, no single gland acts alone. Clinical studies in the healthy aging male point to regulatory failure of GnRH-driven pulsatile LH secretion, LH-stimulated Leydig-cell androgen biosynthesis, and testosterone-enforced feedback on GnRH/LH outflow. In addition, a variety of morbidities are known to affect the testis, and the hypothalamic-pituitary unit. Accordingly, hypoandrogenemia in the older male is the final common consequence of a matrix of primary and secondary deficits in the hypothalamo-pituitary-gonadal ensemble.

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17. Obesity and Aging in Late-Onset Hypogonadism

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Introduction

It is well established that testosterone (T) deficiency is caused by pathologies affecting the hypothalamus or pituitary giving rise to secondary, AKA hypogonadotropic, hypogonadism (e.g., functioning or non-functioning pituitary adenomas, pituitary surgery, congenital GnRH deficiency, including Kallmann's syndrome), or the testis, resulting in primary, AKA hypergonadotropic, hypogonadism (e.g., Klinefelter syndrome, orchiectomy, chemotherapy, orchitis, testis injuries). During adult life, however, besides these pathological conditions of the hypothalamic–pituitary–testicular (HPT) axis, T

levels can gradually decline with increasing age [1], usually to the borderline or low end of the reference range for young men, but in a small minority of older men, to the very low levels usually found in pathological hypogonadism. The mechanisms for this decline are not completely understood; they seem to involve dysfunction of the testis, such as depletion of Leydig cells and impaired testicular response to luteinizing hormone (LH), as well as the hypothalamus-pituitary GnRH/LH outflow and feedback response to testosterone (see Chaps. 1 and 15). Conditions closely linked to aging, such as chronic illnesses and medications, can also affect HPT axis function as men age. Obesity, independent of age, is also associated with low T from low SHBG (Chap. 15) and from down-regulation of hypothalamic–pituitary gonadotropin secretion, the mechanisms for which may involve adipokines, pro-inflammatory cytokines, central insulin resistance, and a loss of GnRH- and inter-neurons. Insofar as the T decline with aging or obesity (so-called adult-onset, or late-onset hypogonadism, LOH) is generally relatively modest and its clinical consequences remain uncertain, the definition and criteria for the diagnosis of LOH are still under debate, with most guidelines recommending that low T be accompanied by cognate symptoms of androgen deficiency.

Testosterone and Aging

Morphological and Functional Alterations

It is recognized that the age-related progressive testicular dysfunction is underpinned by morphological derangements in the testis of aged men including a reduction in both number and function of Leydig cells [2, 3], germ cell degeneration, reduction in the number of Sertoli cells, thickening of basal membrane and appearance of areas of fibrosis [4]. Studies assessing Leydig cell steroidogenic capacity in healthy men pre-treated with GnRH-receptor agonists or antagonists showed that intravenous administration of exogenous LH in pulses can normalize T in younger men, whereas older men achieved T levels 30–50% lower than their younger counterparts [5, 6]. Furthermore, despite lower sperm quality with age [7, 8], most men can maintain a level of spermatogenesis sufficient for fertility during their entire life [9]. It is important to point out that the extent of testicular function impairment, in both the spermatogenic and steroidogenic compartments, associated with aging is often modest, partly due to the compensatory rise in gonadotrophins and somewhat variable due to the unpredictable consequences of comorbidities.

Although analysis of the pituitary found no specific morphological alteration attributable to aging, apart from interstitial fibrosis with high preservation of all cell types [10], several studies show that LH secretion in older men is blunted. Multiple concurrent mechanisms, including reduced hypothalamic GnRH outflow, impaired secretion from the gonadotrope cells or altered feedback by androgens, have been

hypothesized (for details, see Chap. 16).

Testosterone Declines with Age

Epidemiological studies in community dwelling men from different parts of the world showed a modest progressive decrease in T levels with increasing age [1, 11–14]. In the European Male Aging Study (EMAS), total T in the oldest (8th) decade was 8.6% lower than that in the youngest (4th) decade (Fig. 17.1) [1]. A more pronounced decline is observed for free T, with mean levels in the oldest decade being 33.1% lower than that in the youngest (Fig. 17.1) [1]. During a follow-up period of 4.4 years in EMAS, the subjects showed a small decline in T levels, with a mean decrease of 0.04 and 0.77% per year for total and free T, respectively [15]. Consistent with the cross-sectional data, the rates of T decline and LH rise were greatest for older men [15]. These data, in line with many other population-based studies from different parts of the world [11–13, 16–18], contrast with a few reports that did not find an age-related T decline [19–21]. The discrepancy may partly be explained by the health status of the subjects, since chronic diseases can abrogate HPT axis functions at multiple levels and accelerate the apparent aging-related fall in T [1, 22]. The loss of testicular function with aging in healthy men is moderated by the compensatory rise in LH such that the resultant pattern of a gradual but modest decline in T to the low physiological range over many decades stands in contradistinction with the relatively abrupt and inevitable loss of estradiol observed in women during the menopause. Hence, a real and universal "andropause" does not exist in men. However, in a small minority of elderly men (>70 year of age), T declines to the overtly hypogonadal range [12, 23–26], and if associated with symptoms, the term late-onset hypogonadism has been applied to describe this syndrome. In contrast, there is growing prevalence of symptomatic middle-aged men with obesity and insulin resistance [1, 15, 24, 27] who present with low total T and sex hormone-binding globulin (SHBG) levels, but normal or only modestly low free T. This group of men is largely responsible for the explosion in testosterone prescriptions over the past decade [28].

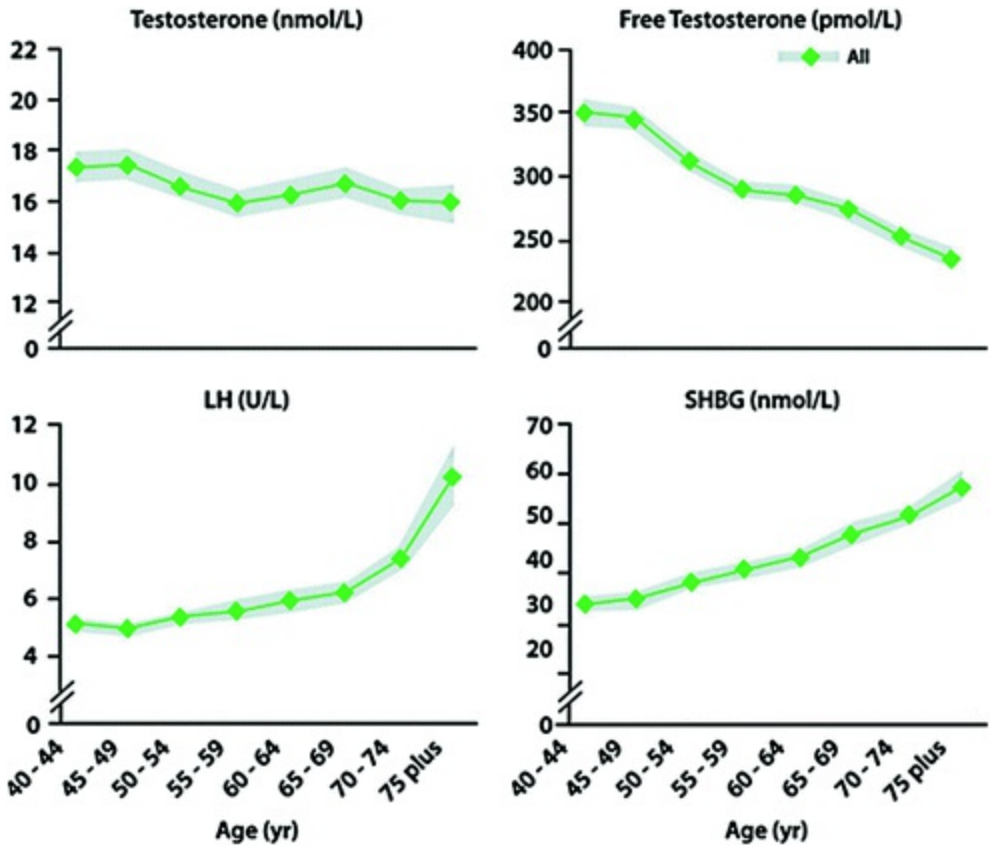

Fig. 17.1 Relationship between age and hormones. This figure shows mean hormone values at 5-year age bands with 95% confidence interval (shaded area) in 3220 European men. Mean hormone values with increasing age were interpolated to approximate the age trend. Total testosterone (T) and free T levels were significantly lower and luteinizing hormone (LH) and sex hormone-binding globulin (SHBG) was significantly higher in the older age groups. There was an apparent inflection point around 70 years for LH. This figure is reproduced from Wu et al. [1] with permission from Endocrine Society

Diagnosis of Late-Onset Hypogonadism (LOH) in Aging Men

Given the age-related decline in T levels, it is unfortunate that age-specific population reference ranges for T are not widely available. T thresholds for diagnosis are based on the reference intervals of healthy and young men [29], or their association with specific symptoms [30]. Hence, the cut-points for partitioning hypogonadal from eugonadal older men (and indeed eligibility for reimbursement) remain controversial, relying mostly on opined recommendations [31] and consensus statements of various professional societies [32–34] that, not surprisingly, do not completely agree with each other (Table 17.1). For example, the International Society of Andrology (ISA), the International Society for the Study of Aging Male (ISSAM), the European Association of Urology (EAU), the European Academy of Andrology (EAA) and the American Society of Andrology (ASA) recommended the diagnosis of LOH when total T is below 8 nmol/L or within 8 and 12 nmol/L, if free T is below 225 pmol/L [32]. The EAU, however, suggests the threshold of 243 pmol/L for free T [34]. The Endocrine Society

indicates a threshold between 9.8 and 10.4 nmol/L for total T, and for free T between 170 and 310 pmol/L while acknowledging that agreement on T thresholds is problematic and not truly evidence based [33]. There is agreement, however, on the requirement for two separate measurements of T for diagnosing LOH, based on the finding that subnormal T levels are not confirmed in almost 30% of repeat measurements [35]. Moreover, morning T levels are recommended (7.00–11.00 am) [32–34] because serum T exhibits a diurnal rhythm with peak levels in the morning [36].

Table 17.1 Clinical features of androgen deficiency and guidelines of the International Society of Andrology (ISA), International Society for the Study of Aging Male (ISSAM), European Association of Urology (EAU), European Academy of Andrology (EAA), American Society of Andrology (ASA) [32], the Endocrine Society [33], and the EAU [34]

	ISA, ISSAM, EAU, EAA, and ASA [32]	**Endocrine Society [33]**	**EAU [34]**
Testosterone thresholds			
Total testosterone	8 nmol/L 8–12 nmol/L: measurement of free T is recommended	10.4 nmol/L	8 nmol/L 8–12 nmol/L: measurement of free T is recommended
Free testosterone	225 pmol/L	170–310 pmol/L: suggested in men with total T near to the lower limit of normal range and in those with suspected alterations of SHBG	243 pmol/L
Symptoms and signs			
Primary and secondary sexual characteristics		Gynecomastia and breast discomfort Testis hypotrophy Infertility Oligo-azoospermia	Gynecomastia Small testes Male-factor infertility
		Reduced body hair and frequency of shaving	
		Hot flushes, sweats	Decreased body hair
		Mild anemia	Hot flushes
Sexual	Low libido	Reduced sexual desire Reduced sexual activity	Reduced sexual desire Reduced sexual activity
	Erectile dysfunction		Erectile dysfunction
		Decreased spontaneous morning erections	Fewer and diminished nocturnal erections
Psychological	Decreased vitality	Decreased energy Decreased motivation Decreased initiative Decreased self-confidence	Fatigue

	Depressed mood	Depressed mood Feeling sad or blue Dysthymia Poor concentration and memory	Changes in mood
		Sleep disturbance Increased sleepiness	Sleep disturbances
			Anger Diminished cognitive function
Bone		Height loss	
		Low trauma fracture	Low trauma fractures
	Decreased bone mineral density Osteoporosis	Low bone mineral density	Decrease in bone mineral density (osteoporosis)
Body composition and metabolic disorders	Decreased muscle strength Decreased muscle mass	Reduced muscle bulk and strength Diminished physical or work performance	Decrease in lean body mass and muscle strength
	Increased body fat	Increased body fat	Visceral obesity Metabolic syndrome Insulin resistance and type 2 diabetes mellitus

SHBG Sex hormone-binding globulin

A further important issue is the method of T measurement. The most commonly used for clinical practice are platform-based immunoassays, but their accuracy and precision for T immunoassays have been questioned, especially at the low levels in children, women and severely hypogonadal men [37–40]. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is now considered the gold standard for steroid measurement, but its costs and technical demands do not make it universally available for routine clinical practice. With improved quality control and methodological stringency, immunoassays are capable of providing T results in good agreement with LC-MS/MS in men [41]. Assessment of free T (non-protein-bound fraction of T) remains a controversial aspect of the biochemical evaluation of gonadal status, and its direct measurement is burdened with even more challenging analytical issues than for total T. The usefulness of evaluating free T arises from the “free hormone hypothesis” which suggests that only free unbound hormones are biologically active in target tissues. This hypothesis, though widely accepted in clinical practice, has not to date been corroborated by robust experimental evidence. In addition, there is also some evidence

that T bound to SHBG can be internalized into cells by endocytosis through megalin protein [42], thus suggesting the possibility of a direct biological action also for protein-bound T. How to assess free T is also a matter of debate. The gold standard is measurement of free T by equilibrium dialysis, a technique that is expensive, time-consuming and not widely available. Alternative methods of measurement based on a labeled T analogue are unreliable and should not be used [43]. To circumvent these methodological issues, algorithms for calculating free T, based on the law of mass action and binding stoichiometry, have been introduced by Vermeulen [44], Södergård [45], Mazer [46] and Zakharov [47]. Other equations, published by Nanjee [48], Ly [49] and Sartorius [50], are empirical estimates obtained through statistical extrapolation to laboratory FT measurements by equilibrium dialysis. The equations based on the law of mass action are strongly dependent on the association constant (K_a) for binding of T to SHBG and albumin. However, various K_a are used in different methods [44, 45, 51, 52], so that these equations carry intrinsic systematic errors based on their assumptions. Furthermore, their reliability is dependent on the accurate determination of total T and SHBG [53]. A study comparing the results of free T obtained by different equations based on the law of mass action (Vermeulen and Södergård) [44, 45] found that these equations systematically overestimate the value obtained by equilibrium dialysis [54]. The small overestimation of percentage free T may be attributed to errors in the measurement of T and SHBG with immunoassays, incorrect T-binding stoichiometry for SHBG, or the use of different K_a values [50]. Equations based on empirical assessment of free T may demonstrate better accuracy, but their applicability and transportability to different laboratories using different methods of hormone measurement is problematic. Despite the small systematic error associated with the mass action equations, a very high agreement between the calculated and the measured free hormone has been consistently found [44, 49, 55, 56]. Thus, despite the systematic, but nonetheless predictable, overestimation, the widespread application, accumulated credible clinical experience for more than 25 years, and convenience of the mass action calculations make it an acceptable method currently for evaluating free T in clinical practice until better alternatives are available. Accordingly, the U.S. Endocrine Society's Expert Panel states that the use of equations based on the law of mass action provides the best approach for the estimation of free T concentration [57].

Based on these considerations, pending further studies demonstrating the clinical applicability of free T in the assessment of the androgenic status in men, total T measured by reliable methods, is still favored for the initial assessment and diagnosis of hypogonadism in men [57, 58]. However, recent data from EMAS showed that men with low total T levels do not have symptoms and signs of androgen deficiency if free T is normal, but when free T is low, even when total T is in the normal range, symptoms and signs of androgen deficiency are prevalent [59]. These results support the pivotal

importance of measuring free T in conditions associated with alterations in SHBG levels, such as obesity or T2DM (low SHBG), or HepC or alcoholic liver cirrhosis, HIV infection or use of certain anticonvulsants (high SHBG) [32–34]. In these situations, the measurement of free T is essential for the accurate identification of LOH and to minimize its over-diagnosis [32–34].

Besides the uncertainty of T thresholds for hypogonadism in the elderly, the lack of precise knowledge on specific clinical features or consequences arising from pathologically low T levels in older men makes the diagnosis of LOH ever more challenging. Recommendations from professional societies require that the diagnosis of LOH requires low T levels together with androgen deficiency symptoms [32–34] (Table 17.1). However, symptoms of androgen deficiency are generally non-specific and overlap substantially with those of aging, depression and ill health. An attempt to create a working definition of LOH was based on the strength of the association between low T levels and the presence of symptoms [30]. After considering a large set of possible symptoms of hypogonadism, the presence of three sexual symptoms (i.e., erectile dysfunction, decreased sexual desire and impaired morning erections) was found to form a strong syndromic clustering with low T. Accordingly, LOH was defined as the simultaneous presence of erectile dysfunction, decreased sexual desire, and impaired morning erections in men with total and free T below 11 nmol/L and 220 pmol/L, respectively [30]. The specificity of sexual symptoms, but not physical and psychological ones, to low T, has been confirmed in the longitudinal extension of the EMAS, which showed that the fall of total T into the hypogonadal range during 4.3 years of follow-up is associated with the development of new sexual symptoms or the worsening of those already present at baseline [60]. Interestingly, testosterone replacement therapy (TRT), compared to placebo in 800 older hypogonadal men in a randomized clinical trial (RCT), has recently been shown to improve sexual symptoms, with variable to negative results on physical and psychological outcomes [61]. Another RCT, involving more than 700 adult men (>18 years of age) receiving T gel or placebo for 3 months, showed that TRT improved sexual symptoms, but not energy level or mood [62].

LOH, as defined by low T and three sexual symptoms, has an overall population prevalence of 2.1% [30]. The prevalence increases with age, BMI, and comorbidity (Fig. 17.2) [30]. This prevalence is much lower than that reported (5.6 and 12%) in other surveys of similar populations [25, 63], presumably due to the inclusion of less stringent or specific clinical features.

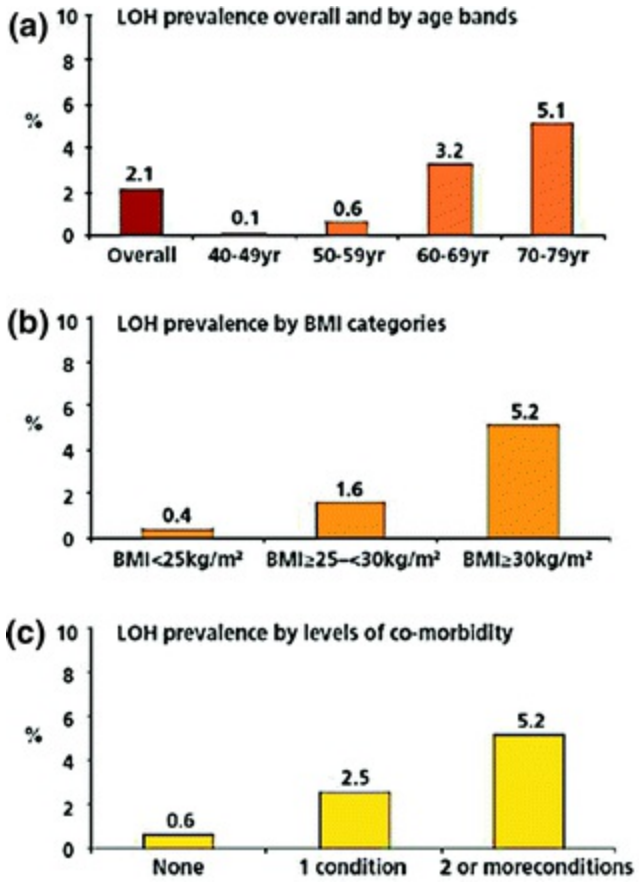

Fig. 17.2 Prevalence of the late-onset hypogonadism (LOH) in EMAS, as defined by at least three sexual symptoms and total testosterone levels of <11 nmol/L and free testosterone of <220 pmol/L, overall and stratified by age (**a**), body mass index [BMI; Panel (**b**)] and comorbidity (**c**). The figure is reproduced from Wu et al. [30] with permission from Massachusetts Medical Society

Obesity-Related Testosterone Decline and Potential Underlying Mechanisms

HPT Axis Dysfunction

Obesity, independently of age, is probably the most important condition associated with low T in men. This is an increasingly common clinical problem due to the rapidly rising prevalence of obesity. In a number of population-based studies [1, 15, 24, 27], obesity, independently of age, was associated with a substantial decrease in both total and free T levels. In the EMAS population, the mean total and free T in obese (BMI ≥30) men was about 5 nmol/L and 55 pmol/L lower, respectively [1], compared to the non-obese, approximating to a 15-year difference in age-stratified T distribution (Fig. 17.3). In contrast to the age-related decline, the fall in T in obese men is not accompanied by a compensatory rise in LH (Fig. 17.3) [1]. This pattern is consistent with secondary hypogonadism suggesting a derangement in HPT axis at the central level (hypothalamus-

pituitary).

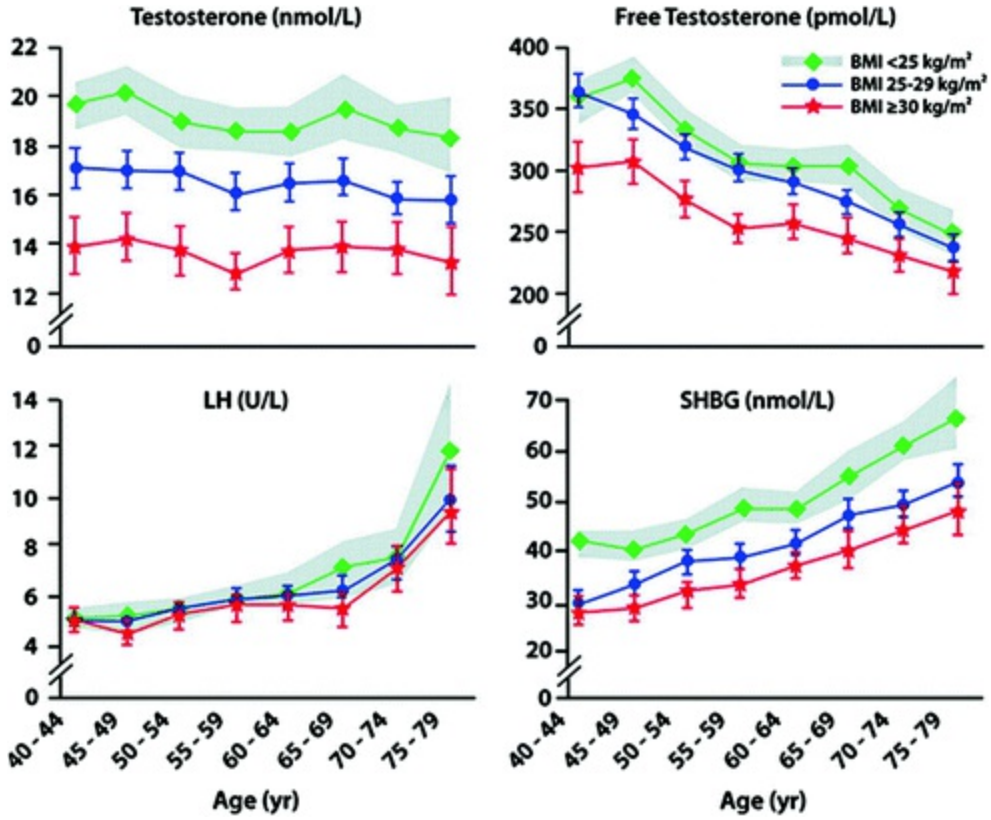

Fig. 17.3 Relationship between age, body mass index (BMI), and hormones. The EMAS cohort was stratified according to BMI into three groups: non-obese (BMI <25 kg/m^2), overweight (BMI ≥25 to <30 kg/m^2), and obese (BMI ≥30 kg/m^2). Mean (95% confidence interval in shaded area and *vertical lines*) total and free testosterone (T) and sex hormone-binding globulin (SHBG) were significantly lower in the overweight and obese at all ages, compared with non-obese. The total T and SHBG age trends in the three BMI categories were similar (indicating no interaction between BMI and age); the free T age trend in the obese group was less steep than in the other two groups (indicating an interaction between BMI and age). Mean LH was not significantly different among the three groups at the median age of 60 years. LH was higher in the older than 70 years non-obese, compared with the overweight and obese groups, due to a negative BMI age interaction. This figure is reproduced from Wu et al. [1] with permission from Endocrine Society

The finding of T rising after weight loss offers evidence supporting the causative role of obesity for hypogonadism rather than the reverse [15]. A meta-analysis of the available RCTs has shown that, in obese men, weight loss, obtained with either low calorie diet or bariatric surgery, was associated with a significant increase in total and free T levels as well as gonadotropins [64] (Table 17.2). The improvement in hormone profile was proportionate to the extent of weight loss, thus demonstrating an apparent graded effect of weight loss on gonadal function [64]. Interestingly, the weight loss-related T increment was attenuated in older men [60, 64], exemplifying the multiple mechanisms at play in the aging HPT axis [vide supra]. Increased body fat and deterioration in health as well as age contribute to the suppression of HPT axis function

at multiple levels, resulting in lower T in older men.

Table 17.2 Mean weighted differences and 95% confidence intervals of hormone levels before and after intentional weight loss obtained with low calorie diet or bariatric surgery

	Mean difference between post and pre-treatment	***p***
Total testosterone (nmol/L)		
Low calorie diet	2.87 (1.68–4.07)	<0.0001
Bariatric surgery	8.73 (6.51–10.95)	<0.0001
Free testosterone (pmol/L)	72.73 (41.86–103.60)	<0.0001
SHBG (nmol/L)	14.49 (10.04–18.94)	<0.0001
LH (U/L)	1.33 (0.75–1.91)	<0.0001

Data are derived from the meta-analysis of the available studies published in Corona et al. [64]

Obesity is not a mechanism, and the precise mechanism(s) by which obesity can suppress HPT axis function and induce hypogonadism is largely unknown. A preponderant role is attributed to the expansion of visceral adipose tissue, a source of many signaling molecules that can disrupt function and metabolism at multiple sites. Some of the most important are discussed below.

Leptin

Leptin is a peptide produced by adipose tissue (adipokine) whose circulating levels change according to nutritional status, being directly correlated with the amount of fat mass [65]. Leptin is involved in several functions, such as insulin sensitization, regulation of the balance between hunger and satiety, and hypothalamic–pituitary–gonadal function [66, 67]. Leptin signaling is one candidate to explain the link between nutritional status and gonadal function [68]. Animal models of leptin deficiency are characterized by hypogonadotropic hypogonadism with lack of pubertal development [69]. In women with hypothalamic amenorrhea induced by excessive physical exercise, or the weight loss of anorexia nervosa, low leptin levels are associated with gonadotropin deficiency [70], and treatment with exogenous leptin is able to increase LH pulse frequency and amplitude as well as improve ovarian function [71]. However, in obese subjects, leptin is increased, in the presence of hypogonadotropic hypogonadism, suggesting possible leptin resistance [72–74]. GnRH neurons lack leptin receptors [75, 76], and the effect of leptin on GnRH secretion is thought to be mediated via kisspeptin neurons, which express leptin receptors [77]. In support of the role of Kiss1 neurons as mediators of leptin action, ob/ob mice (carrying inactivating mutations of the leptin gene) have a lower hypothalamic expression of kisspeptin mRNA that is

increased by the administration of leptin [77]. Furthermore, the intra-cerebroventricular administration of kisspeptin in pubertal rats pre-treated with an anti-leptin antibody restored the secretion of LH [78]. Short-term kisspeptin administration increased LH pulse frequency and amplitude as well as testosterone levels, indicating functional reversibility at or proximal to hypothalamic GnRH neurons, in obese men with low T levels [78].

TNFα

Obesity is associated with chronic low-grade systemic inflammation with increased expression of pro-inflammatory cytokines. Circulating TNFα is increased in obese men, and is a possible candidate linking visceral obesity with hypogonadotropic hypogonadism [79]. In an experimental model of high fat diet-induced metabolic syndrome, rabbits develop secondary hypogonadism and hypothalamic inflammation, with increased hypothalamic expression of IL-6, IL-8 and COX2 and a macrophage infiltrate that correlated with a reduction in circulating gonadotropins [80]. In this model, circulating TNFα is increased, and treatment with infliximab, an anti-TNFα mono-clonal antibody unable to cross brain-blood barrier, is associated with a decrease in hypothalamic inflammation [80]. Besides TNFα, other pro-inflammatory cytokines might be involved in the pathogenesis of obesity-related T decline. Recently, the acute administration of low to moderate doses of IL-2 reduced LH and T levels over a period of 24 h in 32 healthy volunteers, suggesting an inhibitory effect of this cytokine, involved in several systemic inflammatory conditions, on HPT at the hypothalamic/pituitary level [81].

Insulin Resistance

Another possible mechanism involved in the down-regulation of the HPT axis function in obese subjects is insulin resistance. Obesity is associated not only with peripheral but also with central insulin resistance [82]. Besides playing an essential role in glucose metabolism, insulin is involved in signaling satiety to the central nervous system [83]. Both GnRH neurons and kisspeptin neurons express insulin receptors [84, 85], and mice with a selective neuronal knockout for insulin receptors show an increased body mass and hypogonadotropic hypogonadism [86], suggesting a role for insulin in regulating the HPT axis. Accordingly, in vivo treatment of mice with increasing doses of insulin in hyperinsulinemic clamp studies was associated with an increase in circulating LH levels [87]. In addition, treatment of GnRH neurons with insulin in vitro induced a dose-dependent secretion of GnRH [88]. Obesity-related central insulin resistance could therefore negate the action of insulin on HPT axis, leading to secondary hypogonadism.

Estrogens

Estrogens have been, for a long time, the main candidate to explain the relationship between increased fat mass and down-regulation of the HPT. The hypothesis is that expanded adipose tissue in obese subjects leads to greater aromatase activity and thereby increased circulating estrogens, and an exaggerated feedback inhibition of GnRH-gonadotropin secretion. Accordingly, estrogen-antagonist [89] and aromatase inhibitor [90] treatment of overweight hypogonadal men increased LH and testosterone levels. However, the estrogen excess hypothesis is not entirely supported by data in obese diabetic [91, 92] and non-diabetic men [26], whose estradiol levels were low, rather than high, and showed a correlation with T but not with BMI. Only in cohorts of extremely obese men (candidates for bariatric surgery) estrogens are inappropriately high for the levels of T [93, 94] and show a positive correlation with BMI [95].

The Role of Low Testosterone in Development of Obesity

The relationship between T and obesity is complex and bidirectional. If a role of obesity in inducing hypogonadism is well recognized, some data also suggest that low androgen levels can lead to the development of obesity. Important information on the role of androgens in metabolism has been provided by the studies on androgen receptor (AR) knockout mice (ARKO). ARKO mice develop obesity with an increase in both subcutaneous and visceral fat, and they show a derangement in brown adipose tissue characteristics, which suggest a shift to the white adipose tissue profile [96]. Furthermore, ARKO mice display an increase in circulating triglycerides as well as in those stored within skeletal muscle and liver, and an impairment of glucose tolerance with hyperinsulinemia, hyperleptinemia, and hypoadiponectinemia [97]. In vitro studies demonstrated that androgens could inhibit the differentiation of pre-adipocytes in mice and in humans [98, 99]. Similar results have been provided by in vivo studies [100, 101]. In addition, androgen treatment of pluripotent mesenchymal cells increased myogenic but inhibited adipogenic differentiation [102]. Taken together, these data suggest a role for androgens in regulating both central and peripheral insulin sensitivity and body composition, which is best shown when androgen action is abolished rather than diminished.

A human model for the metabolic derangements arising from chemical castration is represented by men with metastatic prostatic cancer who are treated with androgen deprivation therapy (ADT). Several prospective studies demonstrated that ADT for prostate cancer is associated with significant weight gain and changes in body composition from the first months of therapy [103]. A meta-analysis of longitudinal studies shows that ADT is associated with a mean increase of 7.7% in fat mass and a decrease of 2.8% in lean mass from baseline, befitting the clinical feature of

"sarcopenic obesity" [104]. These changes in body composition were accompanied by impairment in lipid profile and insulin sensitivity [103], and patients are at higher risk for developing metabolic syndrome and diabetes [105]. The role of T and androgen deficiency in modifying body composition was assessed in a study conducted in more than 400 healthy young men aged 20–50 years in whom severe hypogonadism was experimentally induced by a GnRH analogue [106]. Volunteers were then randomly assigned to receive for 16 weeks placebo or different doses of T gel with or without an aromatase inhibitor. Results showed that experimental androgen deprivation is associated with a reduction in lean mass and leg-press strength as well as an increase in total and subcutaneous fat mass, *but no change in visceral fat* [106]. Testosterone replacement with aromatase inhibition (pure T effect) maintained lean mass and muscle strength, but not fat mass [106]. These data suggest that the adverse effects of severe hypogonadism on lean mass are mediated by testosterone, and in adipose tissue, by estrogen deficiency.

It should be highlighted that hypogonadism induced by GnRH analogues, as in ADT in men with prostate cancer or experimental hypogonadism in eugonadal men [106], is associated with castrate T levels, which are much lower than those commonly found in LOH. A few longitudinal observational studies in the general population have evaluated the relationship between T and obesity with conflicting results. In the Rancho Bernardo study [107], a negative linear relationship between T or SHBG at baseline and waist-to-hip ratio at follow-up was found even for T levels within the eugonadal range (mean baseline total T of 18 nmol/L for those in the highest quartile of waist-to-hip ratio at follow-up). This observation is supported by a longitudinal study of 110 second-generation Japanese–American healthy male volunteers followed for 7.5 years [108]. In this study, the increase in intra-abdominal fat, as assessed by computed tomography, showed a linear negative relationship with total T at baseline, with a mean baseline total T of 14.8 nmol/L in the group with the highest increase in intra-abdominal fat [108]. More recently, longitudinal data from the EMAS showed that although low T at baseline is associated with incident metabolic syndrome, it was not associated with development of visceral obesity (determined by waist circumference) after 4.3 years [109]. Taken together, unlike the short-term changes associated with castration, chronic exposure over many years to moderately low T may predispose to abnormalities in central fat metabolism and body composition in men, but a causal relationship cannot be assumed from these observational data.

Testosterone Treatment (TRT) and Obesity

A number of uncontrolled registry studies appear to support the effect of TRT in improving body composition [110–115]. Several small RCTs of the metabolic outcomes of T therapy have been conducted. Meta-analysis of these results showed that T

treatment was not associated with any change in BMI, body weight, or waist circumference [116]. However, a significant decrease in total fat mass and an increase in lean mass were consistently found in men treated with T, irrespectively of their gonadal status [116] (Table 17.3). The improvement in body composition by treating hypogonadal men with TRT is not necessarily accompanied by a substantial improvement in insulin sensitivity. In another meta-analysis of double-blind RCTs conducted in hypogonadal men with metabolic syndrome and/or diabetes mellitus [117], TRT showed only a modest, although significant, improvement in HOMA-IR as compared to placebo, whereas the effect of TRT on glycated hemoglobin was not different from placebo (Table 17.3). A recent RCT of 44 men with type 2 diabetes mellitus and low free testosterone with normal or low LH levels showed that i.m. T treatment for 24 weeks improved insulin sensitivity, as assessed by hyperinsulinemic-euglycemic clamp and fasting glycemia, without any change in glycated hemoglobin [118].

Table 17.3 Summary of the results from meta-analyses evaluating the effect of testosterone replacement therapy on body composition and metabolic outcomes as compared to placebo

Meta-analysis	Outcome	# Studies	# Subjects	Follow-up (weeks)	Age	Effect size used	Effect size and 95% confidence interval
Corona et al. [116]	Fat mass	11	715	12–168	52–77	Standardized difference in means	−0.39 (−0.61; −0.17)
	Lean mass	10	1000	12–168	52–71	Standardized difference in means	0.45 (0.26; 0.63)
Grossmann et al. [117]	HOMA index	7	833	12–48	44–64	Standardized difference in means	−0.34 (−0.51; −0.16)
	HbA1c	6	649	12–48	44–64	Difference in means (%)	−0.15 (−0.39; 0.10)

In order to allow a better comparison, for Corona et al. [116], the results from the meta-analysis of randomized clinical trials (RCTs) involving only hypogonadal men are reported, as shown in the original article. These results do not differ from those obtained from the meta-analysis of the RCTs enrolling men with both normal and low T levels [116]

In summary, currently available data suggest that TRT can improve body composition (total lean mass and fat mass) in hypogonadal men, but its benefits on insulin sensitivity and glycemic control remain uncertain. Based on available evidence, TRT is not recommended as a treatment for obesity, metabolic syndrome, or diabetes but is indicated only for the treatment hypogonadal men for the relief of symptoms of androgen deficiency.

Hypogonadism and Mortality

Several longitudinal studies showed that low T is associated with increased all-cause mortality [119]. Although low T did not predict incident cardiovascular (CV) events, it was associated with an increased CV mortality in several cohorts [119]. In the EMAS population, LOH, as defined by the presence of low free T levels and three sexual symptoms (see above), independently predicted overall and CV mortality [120].

The pathogenic mechanism(s) and cause-and-effect relationships underlying these associations are not clear. T is involved in vasomotion as well as in hemostasis and anti-inflammatory mechanisms, and its deficiency can alter these functions thus leading to atherosclerosis [121, 122]. In animal models, TRT was demonstrated to restrain fat storage in the arterial wall and to limit restenosis post-angioplasty [123, 124]. However, as discussed above, low T is associated with obesity and several related metabolic disorders, which may confound the apparent relationship between low T and CV mortality. The lack of association with incident CV events and the higher overall mortality suggest that low T is likely to be an epiphenomenon of poor health leading to mortality for different causes, rather than a factor with a direct or indirect detrimental effect on CV health. These considerations may be relevant to the recent controversy regarding CV safety of TRT in elderly men with pre-existing comorbidities [http://www.fda.gov/Drugs/DrugSafety/ucm436259.htm; 28].

Conclusions

Testosterone declines with increasing age due to depletion of Leydig cells and an impaired testicular response to LH, superimposed upon dysregulated GnRH/LH secretion. These changes are further aggravated by changes due to aging-related chronic illnesses. Obesity, independently of age, is associated with low T due to low SHBG, and from apparent down-regulation of hypothalamic–pituitary gonadotropin secretion. In contrast to classical pathological male hypogonadism, the T decline associated with aging/obesity (so-called LOH) is relatively modest, often in the borderline rather than the pathological range, and its clinical consequences are uncertain. According to current practice guidelines, LOH can be recognized when low T is accompanied by cognate symptoms of androgen deficiency. However, the threshold(s) for subnormal T (total or free) and the nature or severity of symptoms are not well defined. Among the common symptoms in aging men that potentially may arise from low T, sexual dysfunction is considered the most specific for hypogonadism, consistently occurring when T falls and while mounting evidence from recent RCTs confirmed that T replacement in older men with LOH is effective in improving sexual function. Although LOH is often associated with higher central fat mass and insulin resistance, particularly in type 2 diabetics, there is insufficient evidence that T treatment results in clinically significant improvements in

glucose homeostasis or glycemic control. Low T, independent of obesity, is a predictor of increased overall and cardiovascular mortality, the most likely interpretation of which is that low T represents a marker of poor health, rather than an actual path variable leading to adverse clinical outcomes. This is an important caveat when rationalizing T replacement as symptomatic treatment in older men among whom the underlying causes of low T should always be sought and managed.

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18. Androgen Replacement Therapy in Hypogonadal Men

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Introduction

The preceding chapters have discussed the various etiologies of male hypogonadism. In this chapter, we will discuss the criteria for embarking on androgen replacement, the benefits and potential adverse effects of androgen replacement, and current and future

treatment options. There has been significant progress in the field of androgen replacement for hypogonadal men in the past decade including a better understanding of who to treat, new and confirmed treatment targets, and new delivery systems, but there are also new controversies concerning long-term cardiovascular and prostate safety.

Who Should Receive Androgen Replacement Therapy? How Is Male Hypogonadism Diagnosed?

The T prescription rate for men over 40 years of age in the US tripled from 2001 to 2011. Of those receiving T therapy, only 74.7% had T levels measured, and the common diagnoses for T treatment included hypogonadism, erectile dysfunction and psychosexual dysfunction, and fatigue [1]. This increase in testosterone prescription may be due to increased awareness of hypogonadism and the benefits of T treatment by physicians and/or direct-to-consumer marketing of new T products. The United States Food and Drug Administration stated that T replacement therapy should be administered for men with classic hypogonadism that is caused by specific well-recognized medical conditions, and that available evidence does not support testosterone treatment for late onset, age-related, or idiopathic hypogonadism [2].

Men with androgen deficiency, defined as persistently low serum T concentrations, may present with decreased libido, impaired erectile function, decreased body and facial hair, easy fatigability, decreased muscle mass and strength, increased body fat, bone pain or fractures due to low bone mineral density (BMD), and increased negative mood parameters such as irritability, nervousness, inability to concentrate, and poor quality of life. Data from the European Male Aging Study (EMAS) and The Testosterone Trials (The T Trials), reveal that sexual symptoms including sexual activity, erectile dysfunction, loss of morning erections, and decreased sexual desire are best predictive of low T levels and are the commonest symptoms reported in middle-aged and older men [3, 4]. Because most of these symptoms are nonspecific, initiation of treatment should not be based on symptoms alone, but must include a persistently low serum T level [5].

For confirmation of the diagnosis of male hypogonadism, the total serum T should be measured in the morning because of the known diurnal variation of serum T concentrations with highest levels in the morning hours. It has been recognized that there are large variations between methods and laboratories in measured serum T levels by direct immunoassays (radio-immunoassays, enzyme-linked immunosorbent assays, and chemiluminescent immunoassays) which are performed without sample extraction and chromatography. These assays are precise but not accurate, and some lack specificity and sensitivity. The preferred method for T assessment is by liquid chromatography–tandem mass spectrometry (LC-MS/MS) especially for samples in which low T

concentrations are anticipated [6–8]. The Center for Disease Control in the United States established a program for harmonization of hormone assays. The program qualifies the laboratory and then provides continuous external testing to ensure that T results reported by the laboratories are accurate, irrespective of the methods used [9–11]. Similar hormone standardization programs exist in the European Union and Australia. The reference ranges for adults may differ for the various methods. Therefore, clinicians should carefully review the reference range quoted by each laboratory to make a diagnosis of hypogonadism based on serum T levels [7, 12]. The reference range should be based on serum values from healthy young adult men. Serum total T assays in which the reference ranges are significantly different from 300 to 1000 ng/dl (10.4–35 nmol/l) may need further validation by accuracy-based proficiency testing.

It is generally accepted that, based on a reference range of serum T levels in healthy adult young men of 300–1000 ng/dl (10.4–34.8 nmol/l), if the serum T level is <250 ng/dl (8.7 nmol/l) and there are symptoms suggestive of hypogonadism, the patient is most likely hypogonadal, and the cause for the hypogonadism should be identified (Fig. 18.1). If the serum T level is over 350 ng/dl (12.2 nmol/l), the patient is likely to be eugonadal and other causes of his symptoms should be investigated. If a morning total serum T level is in the lower normal or slightly below the normal range, a repeat test is suggested together with the measurement of free or bioavailable (non-SHBG bound) T [13–18].

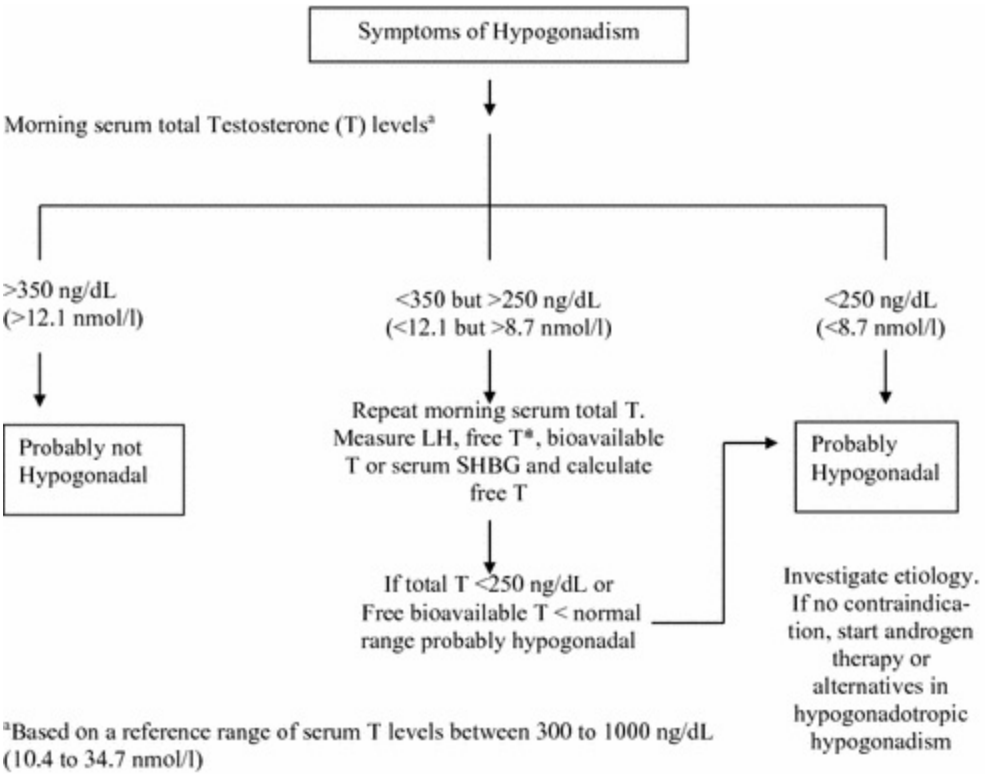

Fig. 18.1 Who should be treated with androgen replacement therapy?

Variations in sex hormone binding globulin (SHBG) gene polymorphisms and SHBG levels directly affect T levels (see Chap. 15). In genome-wide association studies, two single nucleotide polymorphisms were independently associated with serum T levels. One of the polymorphisms affects the ability of SHBG to bind T [19]. Most studies indicate that SHBG levels in men increase with age, which may mask hypogonadism because total T levels may be normalized by higher SHBG with aging [20]. In contrast, in overweight and/or insulin resistant men, total T may be low because the level of SHBG is low [21, 22]. Measuring free and/or bioavailable T in these situations is helpful.

Serum free T should be measured by equilibrium dialysis [16] which is available from most reference laboratories. If the free T measured by equilibrium dialysis is not available, the physician may request a measurement of sex hormone binding globulin (SHBG) and calculate the free T according to an established formula [23] (http://www.issam.ch/freetesto.htm). However, the applicability of mathematical estimation models to all patients has been questioned as it is based on a predefined affinity of SHBG to bind T, and this affinity may vary [24–26]. Many clinical laboratories continue to measure serum free T using analog displacement assays using automated platforms with chemiluminescent labeled reagents. Such assays of free T do not accurately assess the free T fraction [27, 28], the levels are very low compared to measurements by equilibrium dialysis, and should not be used clinically [29]. Bioavailable T is the fraction of T in the serum that is the sum of free and albumin bound. This can be calculated or measured after ammonium sulfate treatment of the serum. The SHBG-bound hormone is precipitated and the supernatant containing the free and albumin bound fraction is quantitated by a serum T assay [14, 15]. Assays for bioavailable T are not available in many laboratories, and reference ranges have to be established by each laboratory.

If the repeat morning serum T level and/or the serum free T or bioavailable T levels are below the reference range of the laboratory, and the patient presents with symptoms consistent with hypogonadism, he may be considered for T replacement therapy if there are no contraindications; individuals with comorbid conditions that could produce transient stress may have treatment delayed, as treatment for the underlying condition should first be addressed. Next, the levels of LH and FSH should be determined. The finding of an elevated serum LH level is confirmatory for primary hypogonadism in men with low or borderline low serum T levels. Patients with functional or structural hypogonadotropic hypogonadism will have low or inappropriately normal serum LH concentration coupled with low serum T concentrations. While the only therapeutic option for primary hypogonadism is testosterone replacement, patients with hypogonadotropic hypogonadism may have intact testicular function with appropriate

gonadotropic stimulation, and they may benefit from alternative treatment modalities that stimulate testosterone secretion and sperm production such as gonadotropins and antiestrogens or aromatase inhibitors. Given the important function of estrogens in bone mass, fat accumulation, and perhaps sexual function in healthy men [30, 31], agents that decrease serum estrogens to low levels are probably not appropriate for the long-term management of hypogonadism [32].

What Are the Contraindications to Androgen Replacement Therapy in Hypogonadal Men?

The absolute contraindications to androgen replacement therapy are untreated or active prostate and breast cancer, as these are androgen-dependent tumors. The other contraindication is an elevated hematocrit or hemoglobin level (e.g., hematocrit >54%). Androgen treatment may cause fluid retention, and in unusual circumstances precipitate or aggravate heart failure. In older patients with symptoms or signs of moderate or of severe congestive heart failure, androgens should not be used until the heart failure has been treated.

While there is minimal evidence to implicate T in the development or aggravation of benign prostate hyperplasia (BPH), if a patient has significant lower urinary tract obstructive symptoms due to BPH then the symptoms should be controlled before instituting T replacement. Obese and older subjects who may be at risk of sleep apnea should be carefully questioned about their ventilatory disturbances during sleep. Patients with moderate or severe obstructive sleep apnea symptoms should be appropriately evaluated or treated before the start of T replacement therapy. On the other hand, recent studies also reveal that testosterone deficiency is associated with obstructive sleep apnea especially in obese subjects [33].

What Safety Tests Should Be Done Before Start of Androgen Replacement?

A digital rectal examination and a serum prostate specific antigen test (PSA) should be performed to assess prostate abnormalities before a middle-aged or older patient is started on androgen treatment, or if the patient presents with a history of lower urinary tract symptoms. If the serum PSA level is elevated, or nodules or irregularities are found, the patient should be referred for Urological assessment. In general, a PSA concentration of >4 ng/ml indicates that the patient should be referred to an Urologist before considering androgen replacement. A complete blood count, liver function tests, HbA1C, and a lipid panel should be performed to ensure that the subject does not have an elevated hemoglobin or hematocrit and to assess the baseline liver enzymes and

serum total, LDL and HDL-cholesterol since NAFLD, diabetes and dyslipidemia are common in men seeking treatment for adult onset hypogonadism.

What Are the Benefits and Risks of Androgen Replacement Therapy?

Androgen administration induces pubertal changes in patients who have not undergone puberty. In adult subjects, androgen maintains and restores secondary sex characters including facial, body, and pubic hair, the lower tone voice, external genitalia appearance, and the growth of the prostate. The risks and benefits of testosterone replacement therapy are summarized in Table 18.1 and described in the following sections.

Table 18.1 Benefits and risks of androgen therapy

Benefits	Risks
• Improves sexual function	• Acne, oily skin
• Maintains secondary sex characters	• Decreased testis volume and suppressed spermatogenesis
• ↑ Bone mass, muscle mass, and strength	• ↑ Hematocrit/hemoglobin
• Decreased fat mass (visceral adiposity)	• Gynecomastia (with some preparations)
• Improves mood in hypogonadal men?	• Weight gain, fluid retention
• Cognitive function?	• ↓ HDL, ↑ LDL: HDL ratio
• Metabolic benefits?	• Sleep apnea?
• Coronary vasodilation?	• Increased cardiovascular disease risk?
• Decrease cardiovascular disease risk?	• Prostate dysfunction (benign prostatic hyperplasia, prostate cancer)?

Sexual Function

Androgen replacement has been shown to improve sexual function more in younger than in older hypogonadal men. These include sexual desire (libido), sexual fantasies, sexual enjoyment and frequency of sexual thoughts, sexual activities, and erectile function in hypogonadal men. In younger hypogonadal men, sexual performance including erectile dysfunction is improved by androgen replacement therapy [34–38]. In older men, erectile dysfunction is frequently multifactorial, other causes such as vascular, neurogenic, psychogenic, medication-induced, and cavernosal problems may coexist. Therefore, improvement in erectile function following T treatment of androgen deficient older men may be less than in younger subjects [39, 40]; however, several controlled trials have shown some beneficial effects [41–43]. While objective data are limited and controversial, it is possible that androgen deficient older men whose erectile dysfunction has been improved by phosphodiesterase V inhibitors (e.g., Sildenafil, Vardenafil, Tadalafil), sexual performance may benefit from cotreatment with T through

improvement in libido [42, 44]. When T was administered to older men with low or low normal T levels (<400 ng/dl, 13.9 nmol/l) for three years, there was no improvement in sexual desire, erectile dysfunction, or overall sexual function compared to placebo [45]. In a recent randomized, double-blind, placebo-controlled trial in hypogonadal older men with serum T levels <275 ng/dl (9.5 nmol/l), testosterone replacement resulting in physiological T concentrations moderately improved sexual activity, sexual desire, and erectile function scores as assessed by standardized questionnaires in both men with and without symptoms of sexual dysfunction. This is confirmed by the global impression of increase in libido [43]. It should be noted that androgen replacement to restore serum T to the low normal range appears to induce maximum sexual function improvement in hypogonadal men [35, 36, 38, 46]. Once a threshold level of serum T is achieved with androgen replacement therapy, it has been suggested that further increase in serum T levels may not increase sexual motivation or performance [17, 47].

Mood and Well-Being

While there is no definitive answer whether quality of life improves after androgen replacement in young or older hypogonadal men, improvement in sexual function and mood, working in synchrony may improve quality of life in hypogonadal men. Anecdotal reports have indicated that androgen treatment may cause increased anger and “rage” attacks in men. Moreover, higher salivary T levels were found in athletes and other subjects who were engaged in competitive activities [48]. In a placebo-controlled study in normal men who were administered testosterone at a supra-physiological dose for up to 20 weeks, there was no significant change in aggression and mood parameters [49, 50]. Uncontrolled studies of hypogonadal men who were administered different T preparations have demonstrated enhanced positive mood parameters such as well-being, energy, friendliness, and a reduction in negative mood parameters including anger, fatigue, irritability, and nervousness [49, 51–53]. Thus, in contrast to anecdotal reports, T treatment in placebo-controlled and uncontrolled studies in hypogonadal and eugonadal men either has no effect or appears to improve mood [54]; however, the data remain conflicting. There are no data on beneficial effects in men with established clinical depression [41, 55]. Recently published results of randomized placebo-controlled trials of testosterone replacement (The Testosterone Trials) suggested slight improvement in mood and depressive symptoms in those receiving T replacement compared to placebo- treated older men [43].

Cognitive Function

Studies of cognitive function in hypogonadal younger men are lacking except for uncontrolled studies in men with Klinefelter syndrome in whom T replacement

improved verbal skills [56–58]. In younger eugonadal men rendered hypogonadal by exogenous administration of a progestin, verbal memory was reduced, which corrected when T was coadministered with the progestin [59]. In older hypogonadal men, several well-controlled studies with limited numbers of subjects reported better performance on spatial ability, and in some studies, verbal memory also improved [60–63] after androgen replacement. Thus, whether T improves cognitive function remains unanswered.

Bone Mineral Density (BMD)

Androgens are required to achieve peak bone mass in adolescence, and are responsible for the higher BMD in men compared to women. Hypogonadism results in a progressive decrease in bone mass, and is one of the causes of osteoporosis in men. With aging, a progressive loss of BMD is associated with an increased fracture rate. Interestingly, BMD in older men is more strongly positively correlated with serum free estradiol than serum free testosterone levels [64, 65]. The few case reports of estrogen receptor mutations and aromatase deficiency in males all described the presence of severe osteoporosis [66–68]. Thus, the current hypothesis is that estrogens are required for maintaining peak BMD in men [69, 70]. A recent study in induced hypogonadism in which graded doses of T replacement were administered in the presence or absence of an aromatase inhibitor showed a consistent decline in BMD when an aromatase inhibitor was coadministered with T, and this decline was independent of T replacement dose. While it is apparent that estrogen is the primary sex steroid regulator of bone mass in men [71], T also has positive effects on BMD possibly through both estrogen and androgen receptors [72]. The level of estrogen activity in the target tissues required to maintain BMD is not known, but in one study, serum estradiol >10 pg/ml (36.7 pmol/l) and testosterone levels >200 ng/dl (6.9 nmol/l) prevented increases in bone resorption and loss in BMD [71]. In the MrOs study of community dwelling older men, higher SHBG levels were associated with a higher likelihood of prevalent vertebral fracture, and men with serum estradiol levels equal to or less than 17 pg/ml (62.4 pmol/l) had a slightly higher likelihood of prevalent fracture than those with higher levels. Moreover, new or worsening vertebral fracture was associated with higher serum SHBG but not with serum T and estradiol [73].

Androgen replacement by injections, oral, or transdermal preparations have been shown to increase BMD in younger hypogonadal men [38, 74, 75]. The increase in BMD is accompanied by early elevations of bone formation markers and decreases in bone resorption markers [37, 38, 74, 76]. In older men, androgen administration resulted in increased BMD in some studies [77] but not others [78–80]. A randomized controlled trial in hypogonadal men older than age 50, with frailty and osteoporosis, showed significant improvement in lumbar and femoral neck BMD after 12 months of T

replacement [81]. The peak increase in bone mineral density has been observed after 12–24 months of androgen replacement [75, 78]. In general, the lower the BMD and serum T level before treatment, the greater the improvement in BMD with T replacement [38, 78]. Though significant changes in BMD have occurred in hypogonadal men after long-term androgen replacement, randomized controlled studies to examine the effect of androgens on bone fractures have not been performed.

Body Composition

A meta-analysis of changes in body composition after T administration to middle-aged and older men including 29 randomized, controlled trials showed that T decreased fat mass (−6.2%), increased fat-free mass (+2.7%), but caused no change in overall body weight [82]. The conclusion is supported by a more recent meta-analysis of T treatment on body composition in 69 randomized placebo-controlled studies (Fig. 18.2) [83]. It is well established that androgens cause nitrogen retention in the body. Androgen treatment increases lean body mass and muscle mass by increasing muscle protein synthesis [84] to induce muscle fiber hypertrophy [85]. Androgens administered as sublingual tablets, injections or transdermal gels have increased muscle (lean) mass assessed by dual energy X-ray absorptiometry (DEXA) scans in both younger and older hypogonadal men [37, 38, 74, 78, 86, 87]. The increase in muscle mass is associated with increased muscle strength in younger hypogonadal men in both the upper and lower limbs assessed by a number of different techniques [37, 38, 74, 78, 86]. Muscle strength is difficult to quantify in a standardized manner, especially in older men [88], but improvement in strength has recently been demonstrated [40, 77, 87]. Several randomized controlled trials have shown improvements in specific strength measurements after T therapy, such as isometric knee extension, gastrocnemius muscle thickness [89], leg press strength, chest press strength, and climbing power [90] but not in other measures such as grip strength [91, 92], carried load, or gait speed [90, 93]. The increase in muscle mass and strength is directly related to the amount of testosterone administered and to serum T concentrations [46]. However, these increases in muscle mass and strength do not increase walk speed [43] or other daily activities of older men. The only study of T and coordination was in adolescent soccer players in whom serum T levels were positively correlated with soccer-specific performance laboratory tests [94].

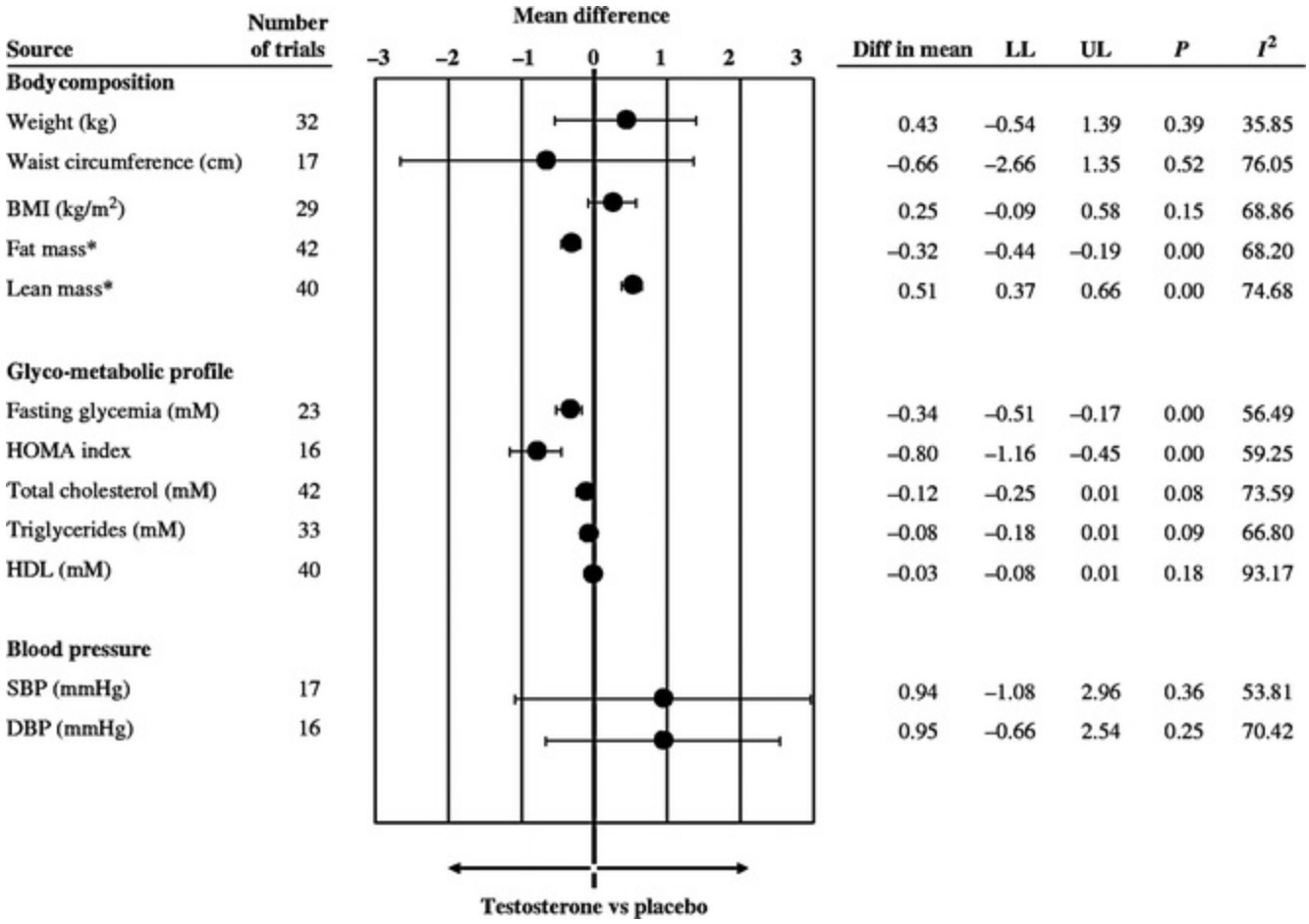

Fig. 18.2 Effect of testosterone therapy on body composition and other metabolic parameters. Weighted mean differences (with 95% CI) of body weight, waist circumference, BMI, fat and lean mass, fasting glycemia, HOMA index, total cholesterol, triglycerides, systolic (SBP), and diastolic (DBP) blood pressure at end point studies used in the meta-analysis. From Corona et al. [83]. Reprinted with permission from Bioscientifica Ltd.

Concomitant with increases in lean mass after androgen substitution in hypogonadal men, are consistent decreases in fat mass and percent body fat measured by DEXA, or visceral fat measured by abdominal CT or MRI scan. Decreases in subcutaneous fat, as well as adiponectin levels were observed in elderly men after six months of testosterone gel treatment [95]. Decrease in fat mass has been demonstrated with injectables and transdermal testosterone but not with sublingual T [37, 38, 74, 78, 86], and the effect of oral TU was less than that of intramuscular T injections [96] which may be related to the amount of T delivered and the serum T levels achieved. A dose-response study in normal men showed that the decrease in fat mass is directly related to the serum T level achieved and the dose of T administered [46]. Decreases in fat mass are also observed in middle-aged or older men receiving T [59, 77, 97]. The decrease in visceral fat observed in some studies has been suggested to result in decreased insulin resistance. A recent meta-analysis showed increases in lean mass and decreased fat mass while using either injectable or oral T formulations [98]. Another study showed a decrease in fat mass and increase in lean mass resulting in no net change in

total body mass [99]. Similar positive effects were observed in patients with metabolic syndrome and type 2 diabetes [42, 100, 101].

Metabolic Parameters

A recent meta-analyses of 59 randomized controlled studies showed that T supplementation resulted in a reduction of fasting glucose and decreased insulin resistance (Fig. 18.2) [83]. T replacement has been reported to decrease insulin resistance and improve glycemic control in some studies of hypogonadal men with diabetes [100, 102–104]; however, improvement has not been consistent across all studies [105]. A recent study in hypogonadal men with diabetes and/or metabolic syndrome reported a decrease in insulin resistance after 6 months with no further benefit after 12 months of T replacement using a testosterone gel [42]. While another study of testosterone treatment in patients with type 2 diabetes failed to show significant effects on insulin resistance, a very small but significant reduction in HbA1c was noted, with most improvement in poorly controlled patients [41].

Testosterone replacement also improved serum inflammation markers in patients with type 2 diabetes and metabolic syndrome [100, 106, 107]. The metabolic benefits of T treatment in obesity, type 2 diabetes and metabolic syndrome, and the impact on cardiovascular disease risks are complex [108]. This field remains controversial and the results are inconclusive, and large randomized controlled studies are needed in men with low T levels with close attention to covariates.

Lipid Profile

Supra-physiological doses of T esters administered to healthy men in male contraceptive trials resulted in significant suppression of serum HDL-cholesterol levels and a decrease in HDL to total cholesterol ratios [109]. On the other hand, serum HDL-cholesterol levels were not significantly changed in hypogonadal men administered transdermal T in patches or gels [38, 110–112]. A meta-analysis of 51 testosterone replacement studies has shown a slight decrease in HDL-cholesterol [113] while another meta-analysis of studies in men with total T <346 ng/dl (12 nmol/l) showed reduction of total cholesterol and triglycerides without any effect on HDL-cholesterol [83]. The decrease in serum HDL-cholesterol was dependent on the dose of T administered and the serum T level achieved [114]. Thus, during androgen replacement of hypogonadal men with near physiological doses of testosterone, significant changes in serum lipid levels are uncommon. Moreover, the possible negative effect of a slight lowering of serum HDL-cholesterol levels needs to be considered together with effects of T on other lipoproteins, coagulation, and fibrinolytic factors. The role of HDL in cardiovascular disease has been recently revisited, and the levels of HDL-cholesterol may not be as important as the type and metabolism of HDL [115, 116].

Cardiovascular Disease Risk

Low T levels are associated with established cardiovascular risk factors, such as obesity, metabolic syndrome, and type 2 diabetes. In men undergoing coronary angiogram, men with demonstrable coronary artery disease had a significantly lower serum free androgen index and bioavailable T levels than those who did not have evident coronary artery disease [117, 118]. A number of studies have shown that testosterone replacement may improve risk factors for cardiovascular disease, such as fat mass, insulin resistance, inflammatory markers, and other features of metabolic syndrome (see Section "Metabolic Parameters," Fig. 18.2). Other large epidemiological studies have shown that men with lower serum T levels have a higher risk of cardiovascular events and all-cause mortality [119–124] (Table 18.2).

Table 18.2 Cardiovascular outcomes in clinical studies

Study	Design	Treatment	No. of subjects	Age	T cutoff	Duration	CVD Outcomes	A r
Shores et al. [124]	Retrospective analysis of VA database	None	858	>40	N/A	Avg. 4.3 years	No CVD data	
Khaw et al. [120]	Nested case control	None	825 died to 1489 alive of 11,606	40–79	N/A	Avg. 7 years	Lowest quartile versus highest ↑CV mortality, OR 1.89 (1.16–3.13)	
Laughlin et al. [119]	Prospective observational	None	794	50–91	N/A	Avg. 11.8 years	Low T↑ CVD mortality, HR 1.38 (1.02–1.85)	

Shores et al. [224]	Longitudinal cohort study	None	1032	66–97	N/A	Median 9 years	Low DHT but not T ↑incident CVD when adjusted for other CV risk factors	
Shores et al. [225]	Retrospective VA database study	Various T formulations (88.6% IM)	1031 (TRT in 398)	>40	≤250 ng/dl (8.7 nmol/l)	Avg. TRT duration 20 months	N/A	
Muraleedharan et al. [226]	Retrospective cohort (TIMES2 follow up)	Various T formulations (93.8% gel)	64 on TRT versus 174 no TRT with Type 2 diabetes	Avg. 60.3 ± 11.5	T < 300 ng/dl (≤10.4 nmol/l)	Avg. TRT duration 41.6 months	N/A	
Vigen et al. [132]	Retrospective analysis of VA database	Various T formulations (63.3% patches)	1223 started TRT, 7486 did not; undergoing coronary angiogram	Avg. 63.8 ± 9	T < 300 ng/dl	~10 months of TRT	TRT ↑ CVD, HR 1.29 (1.04, 1.58). Nonsignificant difference in absolute risk	
Finkle et al. [136]	Health-care prescription database study	Various T formulations (most common—gel)	55 593 men prescribed TRT	Avg. 54.4		1 year pre versus 90 days post prescription	TRT ↑Post/pre-prescription rate ratio for MI—1.36 (1.03–1.81), in >65 years—2.19 (1.27–3.77), in <65 with CV history—2.9 (1.49–5.62)	
Baillargeon et al. [135]	Retrospective cohort Medicare database	IM T	6355 at least 1 T injection versus	>66	N/A	Avg. follow up 49.8 months	TRT not associated with MI, HR 0.84 (0.71–	

			19065 matched controls				1.05)	
Basaria et al. [131]	RCT multicenter, 3 centers	Trans-dermal T titrated to 500–1000 ng/dl	209	>65 limited mobility	TT 100-350 ng/dl (3.5-12.1 nmol/l) or FT <50 pg/ml (173 pmol/l)	6 months	TRT ↑ CV related AE, HR 5.8 (2.0–16.8); highest T quartile versus others, HR 2.4	
Calof et al. [227]	Meta-analysis		1084 (651 in TRT and 433 placebo) in 19 trials			Avg. 10 months	No TRT effect on CV events, OR 1.14 (0.59–2.20)	
Haddad et al. [228]	Meta-analysis		CV events reported in 6 trials (161 TRT and 147 placebo)				No TRT effect on CV events, OR 1.82 (0.78–4.23)	
Fernández-Balsells et al. [229]	Meta-analysis		2679 in 51 trials			3mo-3 years	No TRT effect on MI, RR 0.91 (0.29–2.82)	
Corona et al. [121]	Meta-analysis	Cross-sectional studies	5153 patients with and 7513 without CVD in 54 studies				T ↓ in CVD patients. Low T associated with CVD, HR 1.31 (1.28–1.34) for 1 nmol/l ↓ in T	
		Longitudinal:	12,375 subjects in 10 studies				Baseline T ↓ in those with incident CVD; no T difference between case and controls	
		RCTs in CHD patients	128 pts with CHD received T and 129 -			Avg. 23 weeks	TRT ↑ treadmill test duration and time to ST	

			placebo in 6 RCTs				depression
Toma et al. [230]	Meta-analysis	RCTs in HF patients	198 men with HF in 4 studies			12–52 weeks	TRT ↑ 6-minute walk test, incremental shuttle walk test, and peak oxygen consumption
Xu et al. [137]	Meta-analysis		2994 in 27 trials			At least 12 weeks	TRT ↑ CV event risk 1.54 (1.09–2.18) overall, in pharma non-funded studies 2.06 (1.34–3.17) but not in pharma funded 0.89 (0.5–1.6)
Corona et al. [138]	Meta-analysis		5464 in 74 trials (MACE in 26)			Avg. 34 weeks	TRT not associated with MACE incidence, OR = 1.01 (0.57–1.77); In men with metabolic disease ↓ CVD
Albert et al. [140]	Meta-analysis		5328 in 45 trials	63.3 ± 7.9		10.6 ± 8.6 months	TRT not associated with CV risk overall, RR 1.10 (0.86–1.41). ↑ rate during first 12 mos, 1.79 (1.13–2.83); >65 years, 2.9 (1.35–6.21). IM T—0.96 (0.462–1.98), oral 2.28(0.60–8.59), transdermal

							2.80 (1.38–5.68)

HR Hazard ratio, *OR* Odds ratio, *TRT* Testosterone replacement therapy, *IM* Intramuscular, *CV* Cardiovascular, *CVD* CV disease, *CHD* Coronary heart disease, *MACE* Major CV event, *MACE* Major adverse cardiovascular event

In patients with cardiovascular disease, short term administration of T showed variable results. A study showed decreased ST segment depression and anginal symptoms in men with cardiovascular disease treated with T [125, 126]. Acute administration of T to men with exercise-induced myocardial ischemia reduced ST segment depression and increased exercise testing time compared to placebo [127, 128]. Because of this acute action of T, the effect may be ascribed to a direct coronary vasodilatory effect of the steroid. A subsequent study confirmed this hypothesis by showing acute T infusion increased coronary artery diameter and coronary blood flow in men with established coronary artery disease compared to vehicle administration [129]. However, others failed to demonstrate a beneficial effect on acute stress-induced myocardial ischemia [130].

Concerns that T replacement in hypogonadal men may increase adverse cardiovascular events were heightened when a randomized controlled trial in elderly hypogonadal men with mobility limitations and high prevalence of chronic disease was discontinued early due to higher incidence of cardiovascular adverse events in T-treated men compared to the placebo group [131]. These elderly men received 1% transdermal T gel up to 15 g/day. This study, however, was not designed nor powered to evaluate cardiovascular outcomes. The recorded cardiovascular adverse events were not adjudicated by independent committee and were highly variable. Included in the reported major cardiovascular adverse effects were: syncope, elevated blood pressure, tachycardia, and peripheral edema. Despite another randomized controlled study in older intermediate-frail men that did not demonstrate an increase in cardiovascular adverse events [89], the former study engendered a number of reports raising concerns about the cardiovascular safety of T replacement. These more recent reports included case-cohort studies, analyses of prescription data from large databases, and meta-analyses.

A retrospective cohort study in veterans with low T levels (<300 ng/ml) and a high burden of comorbidities who underwent a coronary angiography showed that T use was associated with increased mortality and myocardial infarction and stroke. The absolute difference in cardiovascular event rates was 5.8% higher at 3 year after the coronary angiogram in men who had filled at least one T prescription compared to those who were not treated. In this cohort, 82.4% of the men filled more than one prescription [132]. In another study also of veterans with low T, replacement with T was associated with lower mortality compared with no T treatment [133]. These authors showed in a

longitudinal study that serum T and calculated free T were not significantly associated with cardiovascular disease or mortality, but serum dihydrotestosterone (DHT) and free DHT had significant curvilinear association with incident cardiovascular events [134]. In a large case-cohort study of older men, T therapy did not increase the risk of myocardial infarction, and in men at high risk for myocardial infarction, T use was moderately protective [135]. Another cohort study, using a large health-care database, showed that men who received a T prescription have a higher rate of myocardial infarction in the 90 days following the prescription compared to the rate 12 months prior to the prescription. The increase in myocardial infarction rates was observed in men over the age of 65 years. The post/pre-prescription rate difference in myocardial infarction was not observed in men instead prescribed phosphodiesterase 5 inhibitors [136]. It should be noted that neither the patient diagnosis nor the reason for the prescription of T was known.

An older meta-analysis of randomized controlled trials found that T treatment of hypogonadal men was not associated with increased cardiovascular adverse events [113]. A more recent meta-analysis of 27 randomized, controlled trials found increased cardiovascular events risk in T-treated hypogonadal men. Interestingly, increased cardiovascular disease risk was only found in trials that were not funded by the pharmaceutical industry [137]. This was followed by another meta-analysis of T supplementation in men from 75 placebo randomized controlled trials which showed that T treatment was not related to cardiovascular adverse events [138] especially when hypogonadism is properly diagnosed and T replacement correctly performed [139]. Another meta-analysis of 45 trials showed that overall T supplementation was not associated with increased cardiovascular events risk, however, there was a slight increase in risk within the first 12 months of treatment, especially in men >65 years. Intramuscular T appeared to have no effect while oral or transdermal T treatments were associated with increased cardiovascular disease risk [140].

Despite the controversial results, major limitations, and questionable validity of these studies, such reports led the Food and Drug Administration to alert physicians to discuss cardiovascular disease risks with their patients before prescribing androgens (FDA January 2014) and required manufacturers to amend the product label to indicate a possible increased risk of heart attacks and strokes in patients treated with testosterone (FDA March, 2015) [2]. None of the randomized, placebo-controlled testosterone studies to date including The Testosterone Trials were of sufficient power to evaluate cardiovascular adverse outcomes [93]. Thus, the FDA Drug Safety Communication March, 2015 stated “We are also requiring manufacturers of approved testosterone products to conduct a well-designed clinical trial to more clearly address the question of whether an increased risk of heart attack or stroke exists among users of these products.” (http://www.fda.gov/Drugs/DrugSafety/ucm436259.htm)

In summary, there are no reliable data showing that T administration has either an

adverse or beneficial effect on coronary artery disease risk, and further research in this area is necessary. There are no data to suggest that T replacement is contraindicated in certain groups of patients; however, when treating older men with multiple comorbidities who are at high cardiovascular risk, extreme care should be exercised. A recent AACE/ACE position statement recommends avoiding T treatment of the frail elderly [141], and risk versus benefits should be discussed with the older patient with testosterone deficiency. A large prospective, randomized, controlled intervention trial in older men with higher risk of major cardiovascular adverse events is being planned by the pharmaceutical companies in response to the request from the FDA to address the question whether T replacement in hypogonadal men changed their risk of cardiovascular disease.

Prostate Disease

Prostate growth and development are known to require the presence of androgens from childhood to adulthood. In younger hypogonadal men in whom prostate diseases are uncommon, androgen replacement results in an increase in the size of the prostate from the smaller volumes at baseline to the range observed in eugonadal men. Progressive increases in prostate volume do not occur with continued T replacement [142, 143]. Serum PSA levels may increase significantly with T replacement, but mostly remain within the normal range [34, 77, 110, 143]. In middle-aged and older men, androgen replacement has resulted in few instances of lower urinary tract obstructive symptoms (LUTS) and increased serum PSA levels resulting in urological referral and ultrasound-guided prostate biopsy [38, 40]. In most of the reports, these prostate-related adverse events occurred early in the course of androgen replacement suggesting that androgen replacement may have unmasked an existing latent or histological cancer by increasing serum PSA levels to above the reference range triggering an early prostate biopsy and diagnosis. More recent studies, however, have not reported worsening of LUTS symptoms in patients with underlying symptoms at baseline [81, 91, 100, 144–146]. A recent meta-analysis of 14 testosterone replacement trials in which LUTS were evaluated by IPSS showed no significant difference between those receiving T and placebo [147]. Most Urologists agree that androgens do not induce BPH. Currently, we recommend that androgens should not be used in subjects with lower urinary tract obstructive symptoms until these symptoms have been assessed and treated.

There is no evidence in hypogonadal young or older men that androgen replacement induces formation of prostate cancer or converts a latent, histological prostate cancer to a clinically significant or metastatic cancer [148]. However, if a hypogonadal patient had a history of, or is at high risk of developing prostate cancer, then androgens should be avoided. Use of calculators to estimate risk of developing prostate cancer (such as http://deb.uthscsa.edu/URORiskCalc/Pages/calcs.jsp) has been recommended [5]. On

the other hand, there are instances in which a patient with a distant completely resected intra-prostatic prostate cancer and long standing nearly undetectable PSA levels with severe symptoms and signs of T deficiency seeks T replacement. Treatment of such patients may be justified only with careful surveillance and well documented informed consent [149]. Coadministration of a 5α-reductase inhibitor with testosterone appeared to spare the prostate from androgenic stimulation during T replacement in older, hypogonadal men with symptomatic benign prostatic hyperplasia [150].

Hematocrit, Hemoglobin, and Liver Function Tests

Androgens increase erythropoiesis by multiple mechanisms including: acting directly on bone marrow cells by increasing telomerase activity and extending the length of telomeres [151–153]; an action on the kidney to produce erythropoietin [154]; and by suppressing hepcidin leading to increased iron absorption and incorporation into red blood cells [155, 156]. Androgen therapy in hypogonadal and eugonadal men results in an elevation of hematocrit and hemoglobin. The effect of T to increase red cell indexes has been demonstrated in many androgen replacement studies [38, 86, 110]. The increase in hemoglobin and hematocrit occurs within three months which increase progressively if the dose of T is adjusted upwards. Direct dose-response relationships have been shown between T and hematocrit and hemoglobin concentrations [46]. A greater increase in hemoglobin was seen in older compared to young men with induced hypogonadism [157, 158]. A substantial increase in hemoglobin was found in men with obstructive sleep apnea [92] who are often polycythemic. Thus, androgen therapy must be used with caution in men with baseline hematocrit of 50% or over. When the hematocrit rises above 50%, the subject must be carefully monitored and the T replacement dose adjusted downwards to prevent hyperviscosity and increased risk of thrombosis. The Endocrine Society suggests stopping the therapy if hematocrit is >54% until it normalizes, and reinitiating with a reduced dose [5, 131]. Alternatively, phlebotomy may help decrease hematocrit to safe levels without compromising the treatment plan, and genetic testing for polycythemia vera should be considered.

Abnormal liver function tests have been reported with the use of 17 alkylated androgens [159, 160]. Modifications of the steroid ring were necessary to allow these 17 alkylated androgens to be active after oral administration. Because of the possibility of liver toxicity and the increase in LDL and decrease in HDL-cholesterol with oral 17 alkylated androgens, they are not recommended for use as androgen replacement. It should be noted that native T and T esters administered as replacement therapy do not result in abnormalities of liver function [37, 38, 86, 110].

Other Possible Adverse Effects

Increased oiliness of the skin, and acne, are common complaints after T replacement,

especially when high doses are administered. These conditions are treated by topical measures or by either changing the T preparation or reducing the T replacement dose. Because T esters, like native T, are aromatized to estrogens, changes in the androgen to estrogen balance may occur after T replacement which may cause gynecomastia. Testosterone replacement also causes fluid retention during the early weeks of treatment and should be used with caution in older patients with congestive heart failure or poor myocardial function. It has been recommended not to initiate T replacement in patients with poorly controlled heart failure [5]; however, no negative effect has been observed in patients with stable heart failure [161].

Earlier studies reported that sleep-related breathing disorders may be aggravated after T replacement [162]; however, a more recent randomized control study in aged obese men with untreated obstructive sleep apnea showed that while T replacement therapy did increase oxygen desaturation index and hypoxemic sleep time at seven weeks, no differences were observed at the study end point of 18 weeks, and there were no differences in apnea episodes [92]. In addition, no significant worsening of sleep apnea symptoms was observed in relatively healthy hypogonadal patients randomized to T treatment in other studies [144], thus the significance of sleep apnea as an adverse effect of T treatment should be reevaluated. A detailed history for symptoms of sleep apnea before and during T treatment should be obtained. High-risk patients (obese, elderly) should be investigated and treated for sleep apnea before commencement of androgen replacement therapy (see Chap. 19).

Testosterone administration suppresses gonadotropin secretion, and leads to reduced intratesticular T levels and hypospermatogenesis. These changes are reversible after withdrawal of treatment. The rate of recovery of spermatogenesis depends on the type of androgen used (longer or shorter acting androgen), duration of treatment, sperm concentration at baseline, ethnicity, and age, as shown in healthy men administered androgens in male contraceptive trials (Fig. 18.3) [163]. Because suppression of spermatogenesis may be prolonged with long acting T undecanoate injections, men with hypogonadotropic hypogonadism seeking future fertility should instead be treated with gonadotropins.

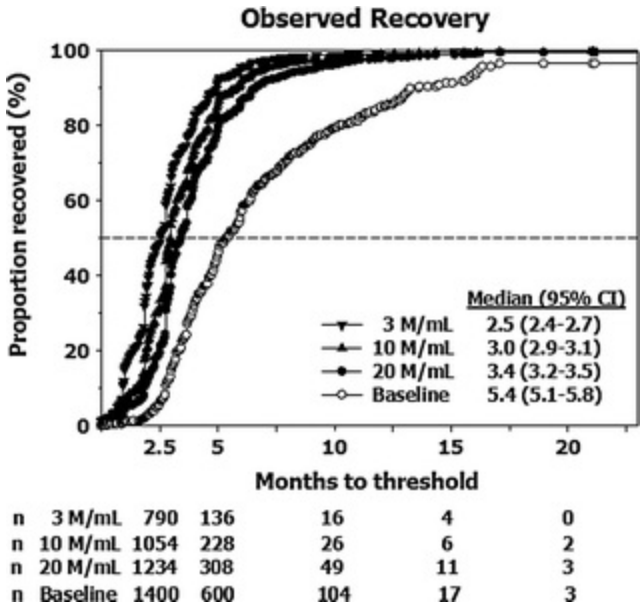

Fig. 18.3 Kaplan-Meier observed plots showing recovery to sperm density thresholds of 3 (*inverted triangle*), 10 (*triangle*), 20 (*circle*) Mil/mL, and back to individual baseline (*open circle*). Median (95% CI) recovery times for each of the thresholds are shown. The number of subjects (n) remaining at 2.5, 5, 10, 15, and 20 months for each of the above thresholds are shown on the bottom of the graph. From Liu et al. [163] and the Hormonal Male Contraception Summit group. Reprinted with permission from Elsevier

What Are the Available Androgen Preparations?

Table 18.3 shows the currently available androgen preparations and those under development. Numerous new options have been developed and introduced into clinical practice in the past 15 years. The most popular methods are long acting intramuscular injections and transdermal gel preparations. Traditional intramuscular formulations are low cost; however, gels present a more convenient option for many patients. The long acting T undecanoate injection with one injection every 10–12 weeks is preferred by many patients and physicians. Gel tablets for buccal and nasal administration of testosterone have also been developed, but these have to be administered two to three times a day. Future goals of the pharmaceutical industry are to develop modified androgen and synthetic androgen receptor modulators to avoid potential adverse effects (for example, stimulatory effects of androgens on prostate growth) while maintaining the beneficial effects on bone, muscle mass, sexual function, and mood.

Table 18.3 Androgen preparations and delivery systems

	Currently available	**Under development**
Injectable	T enanthate T cypionate T undecanoate	T microspheres
Oral	T undecanoate (not available in the US)	T undecanoate (other formulations)

		Dimethandrolone Selective androgen receptor modulators (SARMS)
Transdermal	T nonscrotal patch T gels/lotions	Other T gels, creams DHT gel
Transmucosal	Trans-buccal T Intranasal T	
Implants	T pellets (available as 75 mg in US; 100 or 200 mg in Europe or Australia)	7α methyl 19 nor-T (MENT)

Injectables

Injectable intramuscular solutions have been the mainstay of T replacement therapy since the 1940s. Injectable T is usually esterified, which makes the molecule more oil soluble to extend the rate of resorption of T from the injection site so that a longer duration of action is achieved. Because testosterone is released from the injection site, the metabolism of T is not affected. Therefore, different T esters result in different durations of action. Testosterone enanthate and cypionate are T esters usually administered as a deep intramuscular injection. The usual recommended dose is 200 mg in one mL oil administered every two weeks. The pharmacokinetics (PK) of TE is graphically shown in Fig. 18.4. Serum T levels peaked within 1–3 days after administration, and reached a trough after two weeks. In many subjects, the level of serum T achieved may reach a concentration which is higher than the normal adult male reference range for the first few days after an injection. The PK of T injections has been well studied [164, 165]. Most patients can be taught to self-administer injections. In some patients, the high peaks and low troughs of serum T levels may result in mood swings and acne. In such patients, the dose may be decreased and the frequency of the injections increased, for example, T enanthate or cypionate may be administered at 100 mg every 7–10 days.

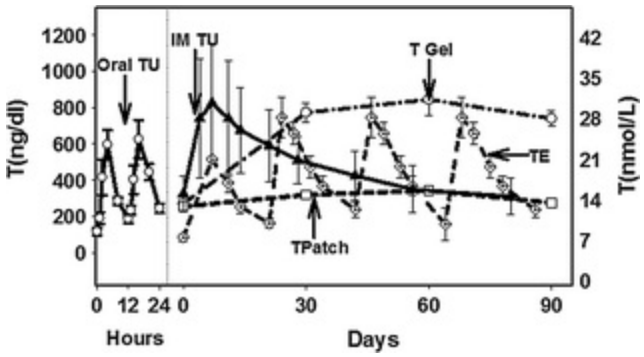

Fig. 18.4 Graphical presentation of pharmacokinetic profiles of different testosterone preparations. *TE* Testosterone enanthate 200 mg IM once every three weeks; *TU IM* Testosterone undecanoate 750 mg IM once in ten weeks after 3 or 4 injections; *T patch* Trough levels after application of 5 mg T patch; *T gel* Trough levels after application of 10 g

T gel; and *Oral TU* Levels after oral administration of TU 200 mg twice a day [170, 183, 222, 223]

Testosterone undecanoate (TU) was developed and marketed initially in China for the treatment of hypogonadism [166]. The depot preparation (TU 250 mg/mL, 1000 mg per injection) is widely used in Europe, Middle East, South America, and Australia. The recommended regular dosing interval is 10–14 weeks; a booster dose at four to six weeks after the first injection helps to achieve steady state levels. After administration of 1000 mg TU, the serum T level peaked in the first or second week, and remained within the normal range for as long as 12 weeks [167, 168]. A lower dose formulation is available in the United States (250 mg/mL, 750 mg per 3 ml injection), is dosed approximately every 10 weeks, with the second booster injection administered 4 weeks after the first injection [169, 170].

The injectable preparations may pose a greater risk of polycythemia than with transdermal preparations, as the T concentration peak is higher, especially with shorter acting formulations [171]. There have been reports of bouts of coughing after IM injections, which has been ascribed to pulmonary oil microembolism, especially with depot TU, as well as rare possible anaphylactic reactions. These observations have led to a requirement in the United States that TU must be administered in a clinic or physician's office, and patients must be observed for 30 min after the injection. With the use of long acting T, it is recommended that serum T levels should be measured prior to an upcoming injection (trough level)—with a goal of serum T being within the lower normal range. In older men, it may be prudent to start with a lower T dose and use short acting preparations before transitioning to longer acting injectables in case adverse events occur [172].

T microspheres are T incorporated in biodegradable polylactide glycolide. When administered as a single injection, T microspheres can maintain serum T levels within the normal range for 10 weeks in hypogonadal men [173]. Recent studies reaffirmed the long acting properties of T microsphere injections with a brief pronounced early peak of serum T followed by low normal T levels for 10–11 weeks [174]. Further development of such a formulation has not occurred.

Oral

Oral 17 alkylated T preparations are not recommended for long-term androgen replacement because of the potential adverse effects on liver function and on lipids. TU is a T ester with a long fatty acid side chain. TU is manufactured dissolved in castor oil. When TU is administered by mouth, it is absorbed mainly through the lymphatics, avoiding a first pass effect on the liver. Thus, absorption of TU is substantially influenced by the ingestion of food [175]. When TU is administered in the fasting state, serum T levels remained low. Serum T levels rose to peak levels 4–5 h after administration with food, and remained in the low normal range 8–12 h after oral

administration [176–178]. The usual dose is TU 80 mg twice per day or 40 mg three times per day. There is large intra-and inter-subject variation in serum T levels mainly because absorption is dependent on ingestion of food and the fat content of the meal. Moreover, after oral TU (in castor oil) administration, serum T levels are often below the normal reference range prior to the next dose [179, 180]. TU has been used in many countries throughout the world except in the United States for many years, and has long-term safety data [181]. Several new formulations of TU are being developed, including a self-emulsifying drug delivery system which produces higher serum TU levels that result in higher and more consistent serum T levels [182]. However, as the 5α-reductase enzyme is expressed in the intestine and liver, and converts TU to DHT undecanoate, the serum DHT to T ratio may be higher than with other formulations [183].

Transdermal Gels, Lotion, and Patches

Steady serum T levels in the normal range mimicking circadian variation are attained after T transdermal patch application (Fig. 18.3). A scrotal T patch was first to become available in the early 1990s. It was 60 mm in diameter, required shaving or clipping of scrotal skin hair, and produced high levels of DHT because of the higher activity of 5α reductase in scrotal skin [184, 185]. This patch has been superseded by other transdermal preparations, and is not currently marketed in the US. The only trans-dermal patch currently available in the US is a permeation enhanced patch (Androderm®), which delivers 5 mg T per day, and produces serum T levels in the low normal range [184–188]. This patch is associated with skin irritation in up to 60% of subjects, leading to discontinuation in up to 10–15% of subjects [184–188]. Pre application of a corticosteroid cream may reduce the skin irritation. Another larger non-scrotal matrix patch (Testoderm TTS®) was developed, which produced much less skin irritation; however; poor adhesion to the skin and frequent patch dislodgement led to marketing discontinuation of this product.

Numerous transdermal T gels have been developed, and they are popular due to their convenience of use. They are currently the most common form of T replacement in the United States, United Kingdom, and many other countries [189]. The gel is applied to a wide area of skin (usually on the arms and shoulders), and is absorbed into *stratum corneum*, which, along with subcutaneous tissues, serve as a reservoir from which T is constantly absorbed for about 24 h. Only about 10% of the applied gel is absorbed into the systemic circulation during this period. Skin in different locations exhibits different absorption properties, which may result in different serum T levels [190]. Additionally, a study in older hypogonadal men showed large serum T fluctuations between applications and among patients which may be attributed to skin structural differences. This leads to difficulties in predicting treatment effect, as well as determining the appropriate time for testing serum T levels and assessing the adequate treatment dose

[191, 192].

Application of T gel results in dose-dependent increases in serum T levels, and as with other T preparations, positive effects on libido, mood, muscle size and strength, and body fat have been demonstrated in hypogonadal men treated with T gel. Additionally, there are positive effects on bone mineral density [193] that persist for over 3 years of treatment duration [194]. Because of the ease of application and flexibility of dosing, this method of T replacement is acceptable to many hypogonadal men. Most currently available formulations are T hydroalcoholic gels, which dry in a few minutes after application; however, they are flammable, therefore precautionary measures are recommended. A potential problem is the possibility of transfer of T to women and children during close contact of skin surfaces. This can be avoided by wearing clothing or removal of any residual T on the skin by showering before anticipated skin to skin contact. Washing clothes that patients wore after T gel application with other laundry resulted in only minimal transfer of T [195]. Skin irritation is the most common adverse event with transdermal gels; however, the irritation is much milder than that with T patches, and usually does not warrant discontinuation of treatment [196].

In addition to T gel, a gel containing the potent androgen, dihydrotestosterone (DHT) has been studied for treatment of younger [197] and older hypogonadal men [198–201]. DHT appeared to improve sexual dysfunction in older men with late onset hypogonadism but had minimal effect on muscle mass and strength [198, 200]. It is controversial whether DHT has any significant benefits over T as an androgen replacement therapy. Moreover, DHT is a nonaromatizable androgen, and unlike T is thus not converted to estradiol. Since some of the beneficial (as well as adverse) effects of T are attributed at least in part to its metabolite estradiol, it is not clear whether DHT will have any positive effects on bone mass, cognitive function, and fat mass [202] or be free from problems of T induced gynecomastia. Two years of DHT gel treatment did not cause undesirable prostate effects; however, it resulted in a decreased spine but not hip bone mineral density [203].

Transmucosal Preparations

Transmucosal delivery systems for T have also been developed. As mucous membranes are more permeable than is skin, absorption rate is higher, and a lower dose is required to achieve the same serum T levels. Two formulations have been approved, and are currently marketed in the United States—an intranasal gel and a trans-buccal system. Due to its short duration of action, the intranasal gel has to be dosed 2–3 times per day, and results in somewhat fluctuating serum T levels. However, its noninvasive approach and avoidance of first pass metabolism in the liver may prove this to be a convenient alternative to currently available options [204]. A trans-buccal system is a tablet, which

after application to the gum, forms a gel tablet, and delivers steady serum T levels for 8–12 h. Thus, it is administered twice per day [205]. A report of two years of continuous use of the system revealed that 62 percent of subjects had at least 80% of their T measurements within normal limits. Treatment was generally well tolerated, with local adverse events (gum edema, blistering, and gingivitis) leading to discontinuation in 4.3% of the patients [206].

Implants

Crystalline T pellets have been inserted into the abdominal subcutaneous fat for androgen replacement in hypogonadal men for some time. In Europe and Australia, where T pellets are available in 100 and 200 mg doses, they were frequently used to treat hypogonadal men before very long acting TU injections became available [207, 208]. Four 200 mg implants often maintain normal serum T levels for 16–20 weeks. Insertion of the pellets requires a minor surgical procedure under local anesthesia. Extrusion of the T pellets has been observed in 8–11% of subjects [209], although with insertion by a very skilled operator, the extrusion rate is much lower. Implants are available commercially in the United States. This method for androgen replacement has not been widely used but is gaining popularity among some U.S. urologists.

Selective Androgen Receptor Modulators (SARMs)

SARMs are being developed as drugs for sarcopenia, cachexia, osteoporosis, and hypogonadism, among other indications. SARMs bind to androgen receptors, and may be agonistic or antagonistic in action depending on the target tissues and the modulating effects of coactivators or co-inhibitors of androgen receptor signaling [210]. An example of a steroid SARM is 7α methyl 19 nor-testosterone (MENT). MENT can be aromatized to an active estrogen but is not 5α reducible. In rodents and monkeys, MENT has a greater stimulatory effect on muscle relative to prostate [211, 212]. A clinical study showed that MENT could maintain sexual function and muscle mass in hypogonadal men. MENT is being developed as a long acting implant [52]. Production of non 5α reductase reducible androgens through modification of the steroid ring may be clinically preferable as they might pose less prostate risks than with T. However, they may not be aromatized to active compounds, which raise long-term safety issues with regards to bone, fat mass, and perhaps sexual function. Other agents include 7α-methyl-19-nortestosterone [213, 214] and dimethandrolone (7α-11 β-dimethyl-19 nortestosterone) [215]. These modified androgens appear to be more potent than T, and exhibit no liver toxicity. Early phase 1 studies of dimethandrolone were reported [216]. These androgens may not be aromatizable to estrogenic compounds, and thus bone and fat mass, as well as sexual function must be monitored if they are used for long periods as androgen replacement therapy in hypogonadal men.

Nonsteroidal orally active SARMs, which have potent actions on muscle and the brain, but little or markedly lower stimulatory effect on the prostate, are the goals of product development of a number of pharmaceutical companies [217, 218]. SARMs may suppress gonadotropin secretion and testosterone production, and it may be difficult to accurately assess the adequacy of androgen replacement. Some SARMs have less gonadotropin suppressive effect, and thus do not alter endogenous hormone levels. Current clinical studies have focused on the treatment of sarcopenia in patients with chronic comorbidities, cancer, and the elderly [90, 219, 220]. These drugs may prove to be advantageous in clinical practice [90, 213, 216, 221].

How Do We Monitor Androgen Replacement Therapy?

The goal of androgen replacement therapy is the alleviation of symptoms. In order to monitor and adjust the dose of T, serum T levels should be measured at an appropriate time after drug administration based on the pharmacokinetic characteristics of the specific preparation. For example, with T patches serum T levels peak at 12–16 h and return close to baseline by 24 h. Monitoring is recommended about 12 h after application, and levels should be in the mid normal range. Since serum T levels are maintained in a relatively steady state by transdermal gels, serum T can be measured at any time after the levels have plateaued, usually 3–4 days after initial application. In older men, considerable within-individual variation of serum T occurred when serum T was measured on different days. Serum levels of T 8–12 h after application of transdermal T gel appeared to best reflect average serum T over 24 h [191]. The injectable T preparations, T enanthate and cypionate result in peaks at 2–3 days and troughs at 10–14 days; serum T is measured before the next injection to ensure trough levels are near the lower end of the reference range for adult men, and that there is no accumulation of T. Serum T should be periodically measured at day 7 to ensure that serum T levels are within normal limits. Once a stable dose is determined, frequent measurement of serum T levels is generally not necessary unless dose adjustments are made. In subjects using long acting TU injections, blood should be drawn prior to the next injection in order to establish the timing of further injections. If free or bioavailable T (either calculated or directly measured after equilibrium dialysis for free T and ammonium precipitation for bioavailable T) are used for the diagnosis of hypogonadism in men with low SHBG, monitoring should also use these parameters rather than total T. However, age-adjusted reference ranges for free and bioavailable T are not generally available, and the clinicians may use the range provided usually by reference laboratories.

Since administration of T may unmask histological prostate cancers by increasing serum PSA levels, measuring PSA early after treatment initiation, e.g., one to three months, is recommended. Thereafter, PSA should be checked on a yearly basis

according to the urological practice applicable to each man. Hemoglobin and hematocrit should be checked at three months and then after each dose adjustment followed by yearly intervals. Subjects whose hemoglobin level is high before treatment should be monitored more carefully. As with other patients on replacement therapy, a yearly liver function test and lipid profile should be performed.

Conclusions

Androgen replacement therapy should be considered for all hypogonadal men with persistent low serum T levels and symptoms of T deficiency. Before commencement of replacement, contraindications to androgen treatment should be identified. In hypogonadal men, androgens improve sexual function, energy, and mood, increase muscle mass and strength, as well as bone mineral density. Androgen therapy can be tailored to each patient's needs and preferences. There have been many new androgen delivery systems introduced in the past few years, and many more are in development. Selective androgen receptor modulators may have the potential to optimize beneficial effects while minimizing potential adverse effects. In older men, monitoring of prostate dysfunction and red cell indexes is necessary and important. With care, androgens can be used efficaciously and with minimal side effects.

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19. Androgen Therapy for Hypogonadism in Men with Chronic Illnesses

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Introduction

Mild androgen deficiency and inflammation are common features of many systemic illnesses, particularly metabolic disorders such as obesity, diabetes mellitus, and nonalcoholic fatty liver disease [1–5]. Recent studies in humans now directly show that interleukin-2, a hallmark of inflammation, induces androgen depletion though enhanced feedback inhibition of LH secretion [6, 7]. These findings suggest a potential unifying mechanism whereby many diverse chronic illnesses could lead to mild androgen deficiency through low-grade inflammation.

In addition to mild androgen deficiency, many chronic illnesses also feature reduced energy, impaired sleep, sarcopenia, and osteopenia. This clinical picture is suggestive of male hypogonadism. Sufficiently prolonged or severe androgen deficiency could plausibly contribute to the clinical presentation of many chronic diseases. Accordingly, androgen therapy could ameliorate those features that are due to inadequate androgen exposure, most likely those features arising from dysfunction of organs that are unequivocally androgen-responsive such as bone, striated muscle, cardiac muscle, vascular smooth muscle, brain, and fat [8].

Recent studies in normal men have shown effects of testosterone therapy on erythropoiesis, skeletal muscle mass and strength, bone and fat mass, and sexual desire that are undeniably dose-related [9–11]. Applying these dose–response relationships from normal men to men with chronic illnesses is not prudent because coexisting inflammation or other factors may potentiate, retard, or invalidate these relationships, making decisions to treat that are benchmarked to eugonadal reference ranges in healthy individuals unreliable. For these reasons, simply assuming that testosterone therapy is indicated and can be titrated to maintain certain blood testosterone targets derived from reference ranges for eugonadal healthy individuals is presumptive. Instead, the efficacy of adjunctive androgen use in specific chronic illnesses should be firmly established by randomized placebo-controlled trials, first considering efficacy, and then establishing safety, within the limits imposed by the natural history of the underlying illness. Such an approach grounded in randomized controlled trials is the standard that would be required for any xenobiotic drug.

This review focuses on such high quality clinical studies, specifically those that assess effects on recognized androgen-sensitive tissues. Effects of androgens in acute illnesses (burns, surgery, trauma), psychiatric illnesses (schizophrenia, major depression), and in physiological processes (aging, growth, mood) are intentionally excluded due to space constraints. Certain chronic diseases such as cancer (including hematological malignancies and related conditions) and its treatment, and metabolic disorders, such as diabetes mellitus and nonalcoholic liver disease, are addressed elsewhere in this monograph.

Respiratory Diseases

Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is associated with weight loss and muscle wasting as well as decreased blood testosterone levels [12]. Reduced systemic testosterone exposure has been postulated to occur due to chronic systemic inflammation, glucocorticoid use, abdominal obesity, hypoxemia, hypercapnia, age, and smoking [13]. Cardiopulmonary rehabilitation is a core component of the management

of COPD and associated congestive heart failure (CHF) [14], and androgen therapy could improve overall performance in either condition directly through increased skeletal muscle strength, or indirectly through increased respiratory muscle strength and oxygenation. Seven placebo-controlled, parallel group studies examining the impact of androgen therapy on body composition, overall performance (walking) and respiratory specific parameters, including respiratory muscle strength and oxygenation in those with COPD, are summarized in Table 19.1 [15–21]. These studies of aromatizable (testosterone) and nonaromatizable (stanozolol and nandrolone) androgens show an increase in muscle mass with either androgen type, and a reduction in fat mass in two of the three studies of testosterone therapy, but generally no effect on total weight, physical performance (walking), respiratory function or oxygenation. These findings seem independent of underlying wasting and nutrition status and co-interventions such as cardiopulmonary rehabilitation, but the paucity of studies and small sample sizes limit generalizations. Moderate and inconsistent improvements in health related quality of life [21] and sexual quality of life [18] have been reported. These findings are consistent with a study of 122 adults, including 31 women, with chronic respiratory failure on long-term oxygen therapy or home ventilation [22]. The underlying cause for respiratory failure was COPD in 70–75% of those recruited; participants were randomized to receive home health education or a multimodal program consisting of health education, nutritional supplements, exercise, and oral testosterone decanoate (80 mg bid for men and 40 mg bid for women). Multimodal therapy increased weight and fat-free mass, but did not result in functional improvements in walking (6 min walking distance).

Table 19.1 COPD

Study	N M/F	Inclusion criteria	Intervention	Co-intervention	Tx Dur Wks	Resp Muscle Strength	Oxy Pa02	Exercise Capacity (6MWT)	Body composition		
									Total	Muscle	Fa
Ferreira (1998)*	23	BMI < 20 PImax < 60%	T 250 mg IM once + St 12 mg qd	None* then IMT then ET	27	PImax ↔ FEV1 NR	NR	↔	↔	↔	←
Schols (1995)[a] (depleted)	110?/?	iBW < 90%	ND IM (50 mg M/25 mg F) q2wks	ET	8	PImax ↑ FEV1 ↔	NR	↔[b]	↑**	↑**	↓
Schols (1995)[a] (nondepleted)	107?/?	iBW ≥ 90%	ND IM (50 mg M/25 mg F) q2wks	ET	8	PImax ↔ FEV1 ↔	NR	↔	↑**	↑**	←
Creutzberg (2003)	63	BMI < 30	ND IM 50 mg q2wks	PR	8	PImax ↑ FEV1 NR	NR	NR	↔	↑	←
Svartberg	29	BMI nml	T IM	None	26	PImax	↔	↔	↔	↑	↓

(2004)			250 mg q4wks			NR FEV1 ↔					
Casaburi (2004)***	53	75–130% iBW	TE IM 100 mg qwk	PRT	10	PImax ↔ FEV1 ↔	↑	NR	NR	↑	↓
Sharma (2008)	16 9/7	BMI normal	ND IM (50 mg M/25 mg F) q2wks	PR nutrition ET	16	PImax ↔ FEV1 ↔	↔	↔	↔	↔	←
Daga (2014)	32	BMI normal	ND IM 25 mg qwk	nutrition	6	PImax NR FEV1 ↔	↔	↑	↔	NR ↑ arm circum	N

St Stanozolol, *T* Testosterone, *TE* Testosterone Enanthate, *ND* Nandrolone Decanoate
IMT inspiratory muscle training, *ET* exercise training, *PR* pulmonary rehabilitation, *PRT* progressive resistance training *BW* body weight, *iBW* % of ideal body weight, *BMI* Body Mass Index (kg/m^2)
PImax maximal inspiratory pressure, *FEV1* forced expiratory volume in 1 s, *Oxy* oxygenation, *PaO* $_2$ partial pressure oxygen, *6MWT* 6 min walking test
NR No Result
*Co-intervention consisted of none weeks 1–9, IMT weeks 9–18 then ET weeks 18–27
**nutrition +ND vs placebo
***Randomized to co-intervention of PRT in two-by-two factorial design
[a]randomization was stratified to depleted and nondepleted groups a priori, and were randomized to 3 groups: placebo injections, placebo injections with nutrition and nandrolone injections with nutrition
[b]12 min walk

Two studies are notable [15, 19]. The first, by Schols et al. examined 217 adults with stable, moderate to severe, and bronchodilator-unresponsive pulmonary disease, and randomized them to receive placebo injections, nutritional supplementation plus placebo injections, or nutritional supplementation plus nandrolone injections [15]. Randomization was stratified according to baseline muscle depletion (ideal body weight <90% with or without lean mass <67%) and all subjects underwent a rehabilitation program (9 week blocks of no co-intervention, inspiratory muscle training, and then exercise training successively). Both nutritional supplementation with or without nandrolone increased body weight compared to placebo, with combined therapy also increasing lean body mass in all individuals and respiratory muscle

strength in depleted individuals. However, walking distance did not increase. In contrast, none of these parameters differed between those randomized to nutritional supplementation alone versus those who received nutritional supplementation plus nandrolone therapy. The lack of blinding with respect to the nutritional supplementation is a study limitation. After 4 years of follow-up, survival did not differ between those receiving nandrolone and nutritional supplementation, and either of the other two treatment groups [23]. One limitation of this study was that women were recruited, since the number, and whether there were gender differences in outcomes was not reported. The other study, from Casaburi et al. administered testosterone 100 mg or placebo every week to 47 male COPD patients, with or without progressive resistance training, 3 times each week for 10 weeks in a two-by-two factorial design, thereby allowing additive effects to be assessed [19]. Testosterone treatment was associated with a significant increase in body weight, increase in lean mass, and decrease in fat mass. Testosterone therapy with progressive resistance training (PRT) improved PaO_2 relative to placebo therapy with resistance training, but this was not observed between those randomized to testosterone or placebo in those who were not randomized to resistance training. Subjects receiving testosterone and resistance training tended to have a greater increase in lean mass, but this was not statistically significant. A small and significant increase in peak oxygen uptake, peak work rate, and lactic acidosis threshold was observed only in the testosterone and resistance training group (compared with the no exercise and no testosterone group). Generally, however, the combination of testosterone with PRT was no better than either alone. This study provides some evidence that supplemental testosterone might be utilized together with rehabilitation programs to improve muscle strength and improve respiratory function in COPD patients.

In conclusion, the management of COPD should be multidisciplinary. Nutrition should be optimized and combined with cardiopulmonary rehabilitation. Testosterone therapy increases lean muscle mass, but this has not been demonstrated to translate into meaningful functional improvements thereby limiting widespread adoption. Further research in specific subgroups that are more likely to benefit from androgen therapy may be warranted.

Obstructive Sleep Apnea

Obstructive sleep apnea is associated with reduced blood testosterone, with lower blood testosterone concentrations being particularly related to greater degrees of hypoxemia [24]. The putative mechanisms underpinning reduced systemic testosterone exposure include hypoxemia-induced alterations in the hypothalamo-pituitary pulsatile secretion of luteinizing hormone [25–27], but detailed appraisal of the entire gonadal axis has not been systematically performed and direct testicular effects, from increased

interleukin 2 or other factors, have not been excluded. Expert guidelines caution the use of androgen therapy in men with obstructive sleep apnea [28]. Two sets of two randomized controlled trials have examined the influence of testosterone therapy on sleep and/or breathing during sleep in young hypogonadal [29, 30] or older eugonadal men [31, 32]. In the first study [29], 11 hypogonadal men already receiving testosterone (testosterone enanthate 200–400 mg every two weeks) were studied, once approximately 3–7 (mean 3.5) days after a testosterone injection, and again at least 30 (mean 53) days after a testosterone injection in random order. Testosterone therapy increased the number of apneas and hypopneas by, on average, 9 events/h. In the other study [30], 10 men were rendered acutely hypogonadal with leuprolide, and were randomized to receive testosterone enanthate 200 mg or oil placebo every 2 weeks for 4 weeks. Testosterone only lengthened time in slow wave sleep, and effects on breathing were not reported.

In the other set of two studies performed in older eugonadal men, in the first study, 108 men were randomized to receive a dose-titrated testosterone patch (about 6 mg/day) or matching placebo for three years [31]. Sleep breathing did not deteriorate, but the portable device was relatively insensitive, could only detect hypoxemia but not breathing, and sleep architecture was not examined. In the other study [32], 17 older men were randomized to receive either three injections of intramuscular testosterone esters at weekly intervals (500, 250 and 250 mg) or matching oil-based placebo, and then crossed-over to the other treatment after eight weeks washout. Polysomnography occurred 2–4 days after the last injection. Testosterone shortened sleep by an hour, induced sleep disordered breathing by 7 events/h, and increased the duration of hypoxemia by 2%. These studies in men without sleep disordered breathing suggest that high dose testosterone esters, but not lower dose more steady-state testosterone therapy, may have short-term effects on sleep and disordered breathing.

Only one study has purposely treated men with obstructive sleep apnea with testosterone, utilizing the hypothesis that lower dose more steady-state therapy may have time-dependent effects on sleep disordered breathing [33–36]. Sixty-seven middle-aged obese men with obstructive sleep apnea were placed on a hypocaloric diet and randomized to additionally receive three intramuscular injections of either testosterone undecanoate 1000 mg or matching placebo every 6 weeks. Testosterone therapy worsened the oxygen desaturation index by 10 events/h and the duration of hypoxemia by 6% after the second injection, but not after the third [33]. These data are consistent with putative time-dependent effects where adaptations in the hyperoxic ventilatory recruitment threshold to carbon dioxide reduced the propensity to sleep disordered breathing with time [35]. Despite these adverse effects on breathing during sleep, 18 weeks of testosterone therapy increased muscle mass, reduced liver fat, and improved insulin sensitivity and sexual desire compared with placebo therapy [34, 36]. Further studies examining the risks and benefits of testosterone therapy in men with

obstructive sleep apnea over the longer term are required.

Heart Diseases

Systolic Heart Failure

Blood testosterone concentrations are reduced in men with severe systolic heart failure or coronary artery disease (CAD) irrespective of etiology [37]. The mechanisms by which this occurs are multifactorial. Cardiopulmonary rehabilitation is a key component of the care of men with systolic heart failure [14], and androgen therapy could improve overall performance either directly, through increased skeletal muscle strength and cardiac contractility, or indirectly, through improved systemic oxygenation and blood flow. Five randomized placebo-controlled trials of 12–52 weeks of testosterone therapy are summarized in Table 19.2 [38–43]. All these studies, except Stout et al.'s [42], show that testosterone improves physical performance (walking). On the other hand, cardiac function (Left Ventricle Ejection Fraction) and biochemical markers of congestive cardiac failure (brain natriuretic peptide) are consistently unaffected by testosterone therapy. Accordingly, the improved physical performance is presumably secondary to direct skeletal muscle effects that are independent of changes in cardiac contractility. One methodological issue is that these walking tests are volitional, and hence can be altered by a placebo effect. Although all of these studies (Table 19.2) are placebo-controlled, only Malkin's study utilized a matched placebo [40], with the remaining being possibly unblinded due to the use of a saline-based placebo [38, 39, 41–44]. Nevertheless, the unequivocally blinded study showed concordant findings [40], which were replicated in another unequivocally blinded trial of 36 women with CHF who were randomized to receive testosterone (300 μg) or placebo transdermal patch [45].

Table 19.2 CHF

Study	N M/F	Trial type	Intervention	Co-intervention	Duration (weeks)	Performance measure	Cardiac Function (LVEF)	Biochemical (BNP)
Malkin (2003)/Pugh (2004)	20	PG	T* 100 mg IM q2wks	None	12	ISWT ↑	↔	↔
Malkin (2006)	76	PG	T patch 5 mg qd	None	52	ISWT ↑	↔	↔
Caminiti (2009)	70	PG	TU 1000 mg IM q6wks	None	12	6MWT ↑ Muscle Peak Torque ↑	↔	NR
Schwartz (2011)	58	PG	TU	None	12	NR	NR	NR

			1000 mg IM q6wks					
Stout (2012)	41	PG	T 100 mg IM q2wks	Exercise	12	ISWT ↔	↔	↔
Mirdamadi (2014)	50	PG	TE 250 mg IM q4wks	None	12	6MWT ↑	↔	NR

PG parallel group
T testosterone, *TU* Testosterone Undecanoate, *TE* Testosterone Enanthate
ISWT-incremental shuttle walk test, SWT-shuttle walk test, 6MWT-6 min walk test
NR no result, *LVEF* Left Ventricular Ejection Fraction, *BNP* Brain natriuretic peptide
*T ester mixture commercially available as Sustanon

The one study (by Stout et al.) which failed to show improved physical performance, was also the only study to select men with a low testosterone level (<15 nmol/L) and to concurrently enforce an exercise program consisting of aerobic and resistance training two times each week [42]. In this small study, the baseline shuttle walk distance of men randomized to receive testosterone was substantially lower than that of the placebo group, and 32% of subjects enrolled did not complete the study. Whether this really means that androgen therapy has no additional impact in men already undergoing an exercise program will require more and larger studies utilizing a two-by-two factorial design.

In addition to improvements in physical performance, two studies by Malkin et al. and Schwartz et al. have also shown beneficial electrocardiographic effects of testosterone therapy, namely on QT dispersion [38] and reduced QTc interval [41]. Both findings imply improved electrical conductance. Another study also examined immediate hemodynamic effects of testosterone therapy [46]. In this study, 12 men with CHF received 60 mg of buccal testosterone or matching placebo pills in random order on consecutive days. Testosterone therapy increased cardiac output measured by thermodilution, and reduced systemic vascular resistance 180 min after buccal application. Reduced systemic vascular resistance with testosterone therapy within this time frame is suggestive of peripheral arterial vasodilation. Whether the changes in cardiac output are real, or secondary to reduced vascular resistance, is not clear. Even if real, these immediate changes do not appear to be sustained. The most parsimonious interpretation of this study in conjunction with the studies shown in Table 19.2 would be that improvements in physical performance due to testosterone do not seem to be mediated by improved cardiac function.

Coronary Artery Disease

Three studies in men with coronary artery disease show that testosterone can rapidly improve cardiac ischemia (see Table 19.3) [47–49]. Improved cardiac ischemia within this timeframe is most consistent with coronary vasodilation, and this is consistent with animal studies which show that testosterone causes endothelium-independent coronary vasodilation through decreased smooth muscle tone. All studies were placebo-controlled, crossover in design, and objectively measured cardiac ischemia by stress ECG or imaging. Testosterone therapy improved cardiac ischemia in the first two studies by Rosano 1999 et al. and by Webb et a.l; these studies also discontinued anti-anginal therapy prior to enrolment and were performed 10 or 30 min after IV doses which raised testosterone concentrations up to 22-fold higher than normal [47, 48]. The third study by Thompson et al. was the largest, but did not show improved cardiac ischemia. In this study, anti-anginal therapy was not discontinued and dose titration occurred to carefully raise testosterone 2 and sixfold higher than baseline [49]. Whether the discordant findings relate to dose or concomitant medications requires further investigation. Nevertheless, only intravenous testosterone infusions that result in very high concentrations can acutely enhance brachial artery flow-mediated dilatation [50]. Not surprisingly, angina was not improved, likely owing to the short treatment duration.

Table 19.3 CAD

Study	N	Intervention	Design	Tx Dur	Washout Interval	Effect	Ischemia	Angina
Rosano (1999)	14	T 2.5 mg IV once	XO	5 min	2 days	I	↓	↔
Webb (1999)	14	T 2.3 mg IV once	XO	10 min	7 days	I	↓	↔
Thompson (2002)	32	T IV Titrated	XO	20 min	7 days	I	↔	↔
Jaffe (1977)	50	TC 200 mg IM qwk	PG	8 weeks		S	↓	↔
Wu and Weng (1993)	62	TU 120 mg qd for 2wk then 40 mg qd for 2 wk	XO	4 weeks	14 days	S	↓	↓
English (2000)	46	T patch 5 mg qd	PG	12 weeks		S	↓	↔*
Webb (2008)	22	TU 80 mg BID	XO	8 weeks	None	S	↓[a]	↔
Cornoldi (2010)	87	TU 40 mg TID	PG	12 weeks		S	↓	↓

T Testosterone, *TC* Testosterone Cypionate, *TE* Testosterone Enanthate, *TU* Testosterone

Undecanoate
PG parallel group, *XO* crossover
I immediate, *S* sustained
*Pain perception was improved by testosterone therapy
[a]Reduced ischemia only in myocardium supplied by non-obstructed arteries

Five randomized placebo-controlled trials show that testosterone therapy for 1–3 months results in sustained improvements in measured cardiac ischemia (see Table 19.3) [51–55]. Wu's and Cornoldi's studies both report reduced angina [52, 55], and English's study reports that perceived pain intensity, and physical role limitation measured by SF-36 questionnaire were improved by testosterone therapy [53]. These data, in combination with studies of immediate androgen action, suggest that improved cardiac ischemia can reduce symptoms when sustained. Blinding appeared to be adequate in all studies except possibly in one [52], in which another report inconsistently described the placebo treatment [56].

An unresolved issue is whether and how testosterone therapy would affect the process underlying coronary artery disease, and whether, over the longer term, such therapy would reduce, increase, or have no effect on cardiovascular events or mortality. Studies of androgen therapy of adequate duration in men with coronary artery disease are not available to address this issue, but data in men without coronary artery disease are available. In a study of 308 relatively healthy men over the age of 60, titrated testosterone gel therapy for 3 years did not increase common carotid artery intima media thickness or coronary calcification compared with placebo [57]. Although atherosclerosis progression was not demonstrated in this study that was adequately powered to do so, the possibility that effects may differ in those with preexisting or advanced atherosclerosis limits generalization to that population. Clinical trial data support this hypothesis. In one study that randomized 22 men to receive 8 weeks of testosterone undeconate 80 mg bid or placebo pills in random order, imaging studies showed increased myocardial perfusion only in areas supplied by unobstructed coronary arteries [54]. Nonrandomized observational studies of men without known coronary artery disease also report that testosterone therapy decreases [58], increases [59, 60] or does not change [61] cardiovascular events. Findings from randomized controlled trials are also contradictory [62, 63], with one study showing an excess of cardiovascular events [62], but not the other slightly larger study [63], despite both studies being nearly identical in study design. Meta-analyses of randomized placebo-controlled trials also show contradictory results [64, 65]. The first reported that testosterone therapy increases cardiovascular events [64], but a later meta-analysis that included more studies [65] did not.

In conclusion, testosterone therapy decreases cardiac ischemia and improves symptoms when sustained. Effects on the underlying atherosclerotic process are ill-

defined, and concern remains regarding long-term effects on cardiovascular events and morbidity. Until the cardiovascular safety in otherwise healthy individuals is established, there does not appear to be a role for androgen therapy in those with coronary artery disease, given the many other treatment options that are disease modifying and/or anti-anginal.

HIV/AIDS

Hypogonadism is common in men with HIV especially in those with AIDS wasting. Testosterone production is adversely affected by HIV due to poor nutrition, opportunistic infections and the medications used to treat HIV/AIDS [66]. The availability of effective antiretroviral combinations has revolutionized the treatment and outcomes for patients with HIV/AIDS [67]. Both beginning therapy earlier, and effective preventative strategies, have reduced the progression of this disease so that wasting is much less common [68]. Randomized placebo-controlled trials of testosterone and the nonaromatizable androgens nandrolone, oxandrolone and oxymetholone are summarized in Tables 4 and 5, respectively [69–87]. These studies all showed that androgen therapy does not increase CD4 count or reduce viral load, except two studies showing either increased CD4 count [77] or reduced viral load [76], but not both. Nevertheless, androgen therapy for HIV/AIDS may still be useful to improve weight loss, weakness, mood, and quality of life. This is highly relevant because loss of fat-free mass [88], as well as cachexia in general [89], and specifically in those with HIV [90], predicts mortality. Accordingly, androgens or other agents such as megestrol or growth hormone that increase appetite and/or body weight may delay death, but no study has been designed to assess this outcome. Nevertheless, the degree of wasting may also be an important factor that modifies response.

Randomized placebo-controlled trials that examined the effect of testosterone, which is aromatizable (Table 19.4), or nonaromatizable androgens (Table 19.5) on weight, muscle mass, strength, and fat mass are summarized, showing wasting status. Most studies recruited men with wasting, and almost all studies recruited exclusively men, except two non-testosterone studies that included women [78, 85]. Three studies are not included due to design issues: one because it randomized between nandrolone alone with or without progressive resistance training [75], meaning that nandrolone alone effects could only be assessed within group, another because all subjects received megestrol, which is known to induce hypogonadism and may have confounded results [91], and the third because it only compared megestrol with oxandrolone [92]. Some studies of testosterone, and all studies of other androgens, recruited men with any baseline testosterone level, and even those that specified an upper testosterone value [70–72, 79, 86, 87] included men with total testosterone concentrations of up to 400 ng/dL. Irrespective of baseline testosterone and wasting status, androgen therapy

increases muscle mass, and more consistently so with nonaromatizable androgens. In this regard, the two studies by Grinspoon are particularly notable because both assessed muscle comprehensively by three modalities (fat-free mass by DEXA, lean mass by total body potassium and muscle mass by urinary creatinine excretion), and all three measures were increased by 6 months of testosterone therapy compared with placebo [71, 76]. Furthermore, these changes were sustained during the 6-month open-label extension of the study [93]. Two studies by Grinspoon 2000 and Bhasin 2000 of testosterone therapy have additionally examined specific peripheral muscle area [76, 79]. Both show an increase in CT or MRI measured leg and/or arm muscle area.

Table 19.4 HIV aromatizable androgens

Study	N	T status (ng/dL)	Wasting (Y/N)	Androgen intervention	Co-intervention	Weeks	Strength	Body Composition			B
								Total	muscle	fat	
Coodley (1997)**	39	Any	Y WL > 5%	TC 200 mg IM q2wk	None	12	↔	↔	NR	NR	C V
Bhasin (1998)	41	<400	N	T patch 5 mg qd	None	12	UE ↔ LE ↔	↔	↔	↓	C V
Grinspoon (1998)	51	Any[a]	Y iBW < 90% ± WL > 10%	TE 300 mg IM q3wk	None	24	6MWT ↔	↔	↑	↔	C V
Dobs (1999)	133	<400[b]	Y WL 5-20%	T scrotal patch 6 mg qd	None	12	NR	↔	NR	NR	C V
Bhasin (2000)	61	<350	Y WL ≥ 5%	TE 200 mg IM qwk	PRT thrice weekly*	16	UE ↔ LE ↔	↑	↔ Thigh (MRI) ↑	↔	C V
Grinspoon (2000)	54	Any	Y iBW < 90% ± WL > 10%	TE 200 mg IM qwk	PRT thrice weekly*	12	UE ↑ LE ↔	↑	↑ arm (CT) ↑ leg (CT) ↑	↓	C V
Bhasin (2007)	88	<400[c]	N Abdominal obesity	T 1% gel 10 g qd	None	24	NR	↔	↑	↓	C V
Knapp (2008)	61	<400	Y BMI < 20 ± WL ≥ 5%	TE 300 mg IM qwk	None	16	LE ↔ Physical function ↔	↔	↑	↔	C V
Sardar (2010)	104	Any	Y BMI < 20 ±WL 5–10%	ND 150 mg IM or T 250 mg IM q2wk	None	12	NR	↑	NR	↔	C V

				(2:2:1)							

TC Testosterone Cypionate, *T* Testosterone, *TE* Testosterone Enanthate. *ND* Nandrolone Decanoate

NR no result

iBW ideal Body weight, *WL* weight loss, *BMI* body mass index (kg/m^2), *PRT* progressive resistance training

6MWT 6 min walk test, *LE* lower extremity, *UE* upper extremity; *(CT)* measured by CAT scan; *(MRI)* measured by MRT scan; *Viral* serum viral load

*also randomized to co-intervention in two-by-two factorial design

**Crossover design with no washout period

[a]and free T < 12 pg/ml

[b]or free T < 16 pg/mL

[c]or free T < 40 pg/mL or bioavailable T < 115 nd/dL

Table 19.5 HIV nonaromatizable androgens

Study	N M/F	T status	Wasting (Y/N)	Androgen Intervention	Co-intervention	Duration (weeks)	Strength	Body Composition			Bioc
								Total	Muscle	Fat	
Berger (1996)	63	Any	Y WL ≥ 10%	Ox 5 mg or 15 mg qd	None	16	↔	↑	NR	NR	CD4 Vira
Strawford (1999)	24	>225	Y WL ≥ 5%	Ox 20 mg qd	PRT + T 100 mg qwk	8	UE ↑ LE ↔	↑	↑	↔	CD4 Vira
Batterham (2001)[a]	15	Any	Y WL ≥ 5%	ND 100 mg IM q2wk	None	12	N/A	↑	↑	↓	CD4 viral
Hengge (2003)	89 79/10	Any	Y WL 5–10%	Oxymetholone 50 mg BID or TID	None	16	NR	↑	NR	↔	CD4 Vira
Shevitz (2005)*	50 35/15	Any	Y WL 5–10% ±BMI < 20	Ox 10 mg BID	Nutrition	12	6MWT ↔ UE ↔ LE ↑	NR	↔	NR	CD4 Vira
Storer (2005)**	82	Any	Y WL 5–15% ±BMI 17–19	ND 150 mg IM q2wk	None	12	↔	NR	↑	↔	CD4 viral
Grunfeld (2006)	262	Any	Y WL 10–20% ±BMI ≤ 20	Ox 20, 40 or 80 mg qd (1:1:1:1)	None	12	↔	↑	NR	↔	CD4 Vira
Gold	303	Any	Y	ND 150 mg or	None	12	N/A	↑	↑	↔	CD4

(2006)			WL 5–15% ±BMI 17–19	T 250 mg IM q2wk (2:1:1)							Vira
Saha (2009)[b]	73	Any	Y WL 5–10%	ND 150 mg IM q2wk	None	12	NR	↔	↔	↔	CD4 Vira
Sardar (2010)	104	Any	Y WL 5-10% ± BMI < 20	ND 150 mg or T 250 mg IM q2wk	None	12	N/A	↑	NR arm circumference ↑	↔	CD4 viral

WL weight loss
T Testosterone; *Ox* Oxandrolone, *ND* Nandrolone Decanoate
NR no result
viral load; *PRT* progressive resistance training
UE upper extremity, *LE* lower extremity
*randomized to oxandrolone, placebo or progressive resistance training
**randomized to nandrolone, placebo or growth hormone, *BMI* body mass index (kg/m^2)
[a]randomized to nandrolone, medroxyprogesterone or dietary counseling
[b]randomized to nandrolone or no treatment

Effects on fat mass have been more difficult to demonstrate. First, no study of nandrolone, oxandrolone, or oxymetholone has shown a reduction in body fat (Table 19.5). However, this may be explained by the fact that these androgens are minimally aromatizable, and aromatization is important for body fat [10]. In contrast, the larger studies of testosterone therapy that have utilized accurate methods to measure fat have generally shown a reduction in fat mass. Bhasin's 2007 study is notable for three reasons: it treated the largest number of men with testosterone and placebo, men were specifically recruited for abdominal obesity, and body fat was examined comprehensively by both DEXA and abdominal CT [86]. Testosterone gel therapy decreased total body fat, including trunk and extremity fat, as well as total abdominal subcutaneous fat, rather than visceral fat, compared to placebo. Since most studies examine men with HIV wasting, and no other study has recruited only abdominally obese men, it is not possible to determine if the lack of effect on visceral fat was specific to this population.

These improvements in body composition, namely increased muscle mass with (testosterone) or without (nonaromatizable androgens) reduced fat, have resulted in corresponding increases or no change in total weight (Tables 19.4 and 19.5). Despite demonstrating increased muscle mass in participants, most studies have failed to demonstrate an increase in muscle strength or other performance measurements. When

measured, improvements in quality of life have either not been demonstrated or inconsistently improved [70, 72, 73, 79, 80].

Two studies by Gold et al. and Sardar et al. directly compared nandrolone with testosterone [83, 84], and a third study by Storer et al. compared testosterone with recombinant human growth hormone (rhGH) [81]. The first two studies randomized men to receive intramuscular injections of nandrolone decanoate 150 mg, testosterone 250 mg mixed esters as Sustanon®, or oil-based placebo every two weeks for 12 weeks, and assessed body fat only by bioimpedance, which is a relatively inaccurate method. In the smaller study by Sardar et al. 104 men were randomized 42:42:20 (nandrolone: testosterone: placebo). Nandrolone was superior to testosterone for increasing BMI, but was not significantly different for total body weight, waist circumference, triceps skinfold thickness, or any other anthropometric measurement. In the larger study by Gold et al. 303 men were randomized 157:66:80, and nandrolone was superior to testosterone for both BMI and total body weight. The lack of reliable body composition information, particularly for muscle, is a major limitation. The third study by Storer et al. randomized 82 men with HIV-associated weight loss to receive intramuscular injections of nandrolone decanoate 150 mg, matching placebo every 2 weeks, or daily subcutaneous rhGH for 12 weeks. Both nandrolone and rhGH increased muscle and decreased fat (both determined by DEXA) compared with placebo. However, nandrolone was inferior to rhGH for fat loss, but equivalent for muscle gains. Despite these body compositional changes, muscular strength and insulin sensitivity measured by minimal model were no different from placebo.

Five studies incorporated a standard progressive resistance training (PRT) program three times per week into the study design [74–76, 78, 79]. A placebo for PRT was not used in any of these studies, and subsequently this intervention should be considered unblinded. In the first study by Shervitz et al. 50 adults with AIDS wasting all received a personalized and intensive nutrition program, and were additionally randomized to receive placebo tablets, oxandrolone 20 mg daily, or PRT for 12 weeks [78]. Oxandrolone was not directly compared against PRT in this study so that the relative benefits of androgen therapy versus PRT were not assessed. The additional effect of combination therapy has been examined in the remaining four studies [76, 79]. The first two studies by Grinspoon et al. [76] and Bhasin et al. [79] utilized a two-by-two factorial design, and reported that the combination either did not promote further gains [79], or neglected to report the effect of combination therapy [76] over testosterone therapy or PRT alone (see Table 19.4). In the third study, 30 men with stable weight were treated with supraphysiological weekly doses of intramuscular nandrolone decanoate (200 mg for the first dose, 400 mg for the second dose and 600 mg for all subsequent doses), and then randomized to additionally receive PRT or not for 12 weeks [75]. Combination therapy augmented gains in muscular strength and lean body mass measured by DEXA and bioimpedance, but not total body weight, perhaps

because supraphysiological doses of nandrolone were administered. In the final study by Strawford et al. (see Table 19.5), 24 men with HIV-associated weight loss were all treated with PRT and testosterone enanthate 100 mg weekly "to suppress endogenous testosterone production" and then randomized to also receive oxandrolone 20 mg each day or placebo tablets for 8 weeks [74]. The addition of oxandrolone increased muscle and upper limb strength, but the lack of a no-treatment control and the concurrent use of two androgens limit interpretation.

Together, these studies indicate that androgen therapy increases muscle and that aromatizable androgens probably reduce fat, with corresponding effects on total weight. Only inconsistent effects on muscle strength and quality of life have resulted from these body compositional improvements. The separate and adjunctive roles of exercise and rhGH have been inadequately studied.

Chronic Kidney Disease

Hypogonadism is common in men with chronic kidney disease, and is likely to contribute to their low libido, anemia, sarcopenia, and low bone mass [94]. The underlying mechanisms are multifactorial in origin, and include suppression of gonadotropins due to elevated prolactin arising from decreased renal clearance, comorbidities, and concurrent medications. Hypogonadism rarely improves with dialysis but typically does so after renal transplantation. Direct renotrophic effects of androgens have been suspected for decades [8]; however, data in humans showing that androgens can directly improve renal function in those with impaired renal function either did not report benefit [95, 96], or were uncontrolled and therefore unreliable [97]. On the other hand, sarcopenia and anemia are recognized features of end stage renal failure, and muscle and erythropoiesis are recognized androgen targets.

Five randomized placebo-controlled trials consistently showed that weekly intramuscular injections of 100–200 mg of nandrolone for 3 months [98] or 6 months [99], or 6 months of oral oxymethenolone 50 mg bid [100–102] increases DEXA-measured lean body mass, compared with placebo (Table 19.6). Of these, the three largest studies, (Johansen's study [98], Aramwit's study [100], and Supasyndh's study [102]), also reported a reduction in fat mass [98, 100, 102]. These body compositional changes translated into functional improvements. Oxymethenolone therapy increased handgrip strength in 41 dialysis patients, including 16 women [102], increased insulin sensitivity by HOMA in 44 patients (including 19 females) [100], and improved global nutritional status in 24 adults, including 4 women [101]. Significant elevations in liver enzymes with oxymethenolone compared to placebo were highlighted in all three reports [100–102], although Aramwit's study in 2010 [101] may actually be from a subset of a larger study [100].

Table 19.6 Renal

Study	N M/F	Study group Dialysis	Wasting (Y/N)	Intervention	Co-intervention	Tx Dur (Weeks)	Strength	Body composition		
								total	muscle	fat
Johansen (1999)	29 23/6	HD or PD	Y	ND 100 mg IM qwk	None	24	Walk/stair climb ↓ Treadmill ↔	↔	↑	↔
Johansen (2006)	79 49/30	HD	N	ND 200 mg IM qwk (male) 100 mg IM qwk (female)	Resistance training	12	Walk/stair climb ↔ LE ↔	↑	↑	↓
Aramwit (2009)	44 25/19	HD	N	Oxymetholone 50 mg BID	None	24	NR	↑	↑	↓
Aramwit (2010)	24 20/4	PD	N	Oxymetholone 50 mg BID	EPO	24	NR	↔	↑	NR
Supasyndh (2013)	41 25/16	HD	NR	Oxymethalone 50 mg BID	None	24	Handgrip ↑ muscle area ↑	↔	↑	↓

HD hemodialysis; *PD* peritoneal dialysis
ND Nandrolone Decanoate, *EPO* recombinant human erythropoietin, *NR* no results

The increase in lean body mass with nandrolone therapy also translated into modest functional improvements. In Johansen's study [99], weekly intramuscular injections of nandrolone 100 mg for 6 months in 29 patients, including six women, improved some measures of physical performance when compared with saline placebo injections, including timed walking and stair climbing and self-reported fatigue, but not treadmill testing time and oxygen consumption or handgrip strength [99]. Given these improvements, the authors conducted a study to assess the synergistic effect of exercise in a two-by-two factorial design [98]. Seventy-nine adults, including 30 women, were randomized to 3 months of weekly intramuscular injections of nandrolone, lower limb exercise three times each week, both interventions simultaneously, or matched placebo injections supplied by the nandrolone manufacturer. Each injection of nandrolone was 200 mg for men and 100 mg for women. The main effects analyses showed increases in lower limb strength with exercise in a training-specific manner, but not with nandrolone therapy. Quadriceps muscle cross-sectional area increased with exercise and with nandrolone, without interaction between the two main effects. In these two studies, excess elevation of liver enzymes was not observed in those who received nandrolone. However, women did become virilized.

All five studies of nandrolone and oxymethenolone showed increases in lean muscle

mass. Two other studies of nandrolone therapy confirm this finding: one in those not yet requiring dialysis [95], and the other which utilized a no-treatment control group consisting of those who declined to participate in the randomized dose-finding trial [103]. In these studies, however, increased lean muscle mass was not always associated with increased muscle strength, and treatment improved some, but not all, measures of physical function. Non-pharmacological interventions, such as exercise training or nutritional supplementation, may be as, or more, effective. There is a paucity of studies looking at combination therapy.

The anemia of end stage renal failure has multiple contributing factors such as micronutrient deficiency (iron, folate, pyridoxine), blood loss and hemolysis, reduced red cell survival, EPO or androgen deficiency, and impaired EPO or androgen action [104]. A link between androgens and erythropoiesis has been suspected for over 75 years [105]. Recent physiological studies establish a linear dose–response relationship across the hypogonadal and eugonadal range that is augmented in older men [9]. Potential mechanisms include increased erythropoietin and reduced hepcidin [106], although effects on erythropoietin may be transitory [9, 107, 108]. Whether effects on hepcidin are mediated through recently identified master regulators such as erythroferrone requires further investigation [109].

It is well established that androgen therapy increases hemoglobin concentrations in men with end stage renal failure. The three largest randomized placebo-controlled studies have shown that nandrolone treatment (100–200 mg weekly) for 3–5 months increases hemoglobin concentration by 10–15 g/L [110–112] and decreases transfusion requirements [110]. These findings contrast with three smaller studies that failed to show an increase in hemoglobin in patients with end stage renal failure which may be explained by inadequate dosing and dropouts from the study [96, 113, 114]. Two other randomized controlled crossover trials concluded that injectable androgens were superior to oral androgens in supporting erythrocytosis [115], and that erythrocytosis occurred only in those with remnant renal function [116]; however, methodological problems make these finding less convincing. There was a large proportion of dropouts in both studies, and neither study was blinded due to a lack of an appropriate placebo drug. The finding that remnant renal function was required was based on a subgroup analysis in anephric and non-anephric patients that appeared to be decided post hoc [116], and the differences between injectable and oral androgens may have been due to inadequate oral dosing: testosterone enanthate (4 mg/kg/wk) and nandrolone decanoate (3 mg/kg/wk) versus oxymetholone (1 mg/kg/day) and fluoxymesterone (0.4 mg/kg/day).

Current guidelines recommend recombinant human erythropoietin and analogues (EPO) but not androgen replacement [117], despite two randomized placebo-controlled trials showing equivalence between nandrolone and EPO [118, 119]. However, those two trials were small, each recruiting less than 30 men over 50 years of age, so that only large differences between therapies could have been recognized. Combination

therapy with both androgen and EPO is also not recommended [117], but the hemoglobin targets on which these guidelines were based have been criticized and hence, these recommendations can be challenged [120, 121]. Three randomized controlled trials examined the combination of nandrolone with EPO versus EPO alone [122–124]. The first study was underpowered with only 12 adults in total, and up to only 4 months in treatment duration [122]. The other two studies were comparable, but only one showed that the combination was superior [123]. In that study, 19 adults, including eight women, were randomized to receive nandrolone (100 mg weekly) plus EPO (4500 U/wk) or EPO alone for 6 months. The addition of nandrolone to low dose EPO resulted in a significantly greater rise in hematocrit. However, a larger study in which 32 adults, including 12 women, were randomized to receive the same total dose of nandrolone (50 mg twice weekly) for the same duration of time (6 months) with or without a lower dose of EPO (3000 U/wk) showed no significant differences in hematocrit or hemoglobin (124). How the different doses of EPO affected these contradictory findings depends on the effect of androgen therapy on EPO deficiency and/or action. However, circulating EPO is neither consistently increased by androgens nor related to the resultant increases in hemoglobin [125]. On the other hand, the mean ages in the combined treatment groups were 54 [12] and 44 [124] years; this could conceivably account for these differences, since the induction of erythropoiesis by androgens is enhanced in older men [9]. In both studies, baseline hematocrit (23–25%) and the proportion of women (37–42%) were comparable. Virilization resulted in half of all women in the combination arm discontinuing from the study [124].

Two other randomized studies examined the combined effects of 6 months of androgen therapy with EPO, and also reported divergent results [101, 126]. Unlike the studies utilizing nandrolone, these two studies utilized placebo controls. The first study utilized an inadequate dose of transdermal testosterone, which only modestly and inconsistently increased serum testosterone or DHT in 40 uremic men with low testosterone levels, of whom 22 discontinued by the end of the 6-month treatment period [126]. The second study took 24 adults, including 4 women, on a stable dose of EPO and randomized them to oxymetholone 50 mg bid or placebo tablets [101]. Combined therapy further increased hematocrit from 0.32 to 0.38 and hemoglobin from 10.8 to 12.9 g/dL, with highly significant between group differences at 6 months, and no changes in EPO dose. In contrast with the earlier studies using nandrolone [123, 124], these individuals had much less anemia, were treated with a much higher EPO dose (on average 4000 U/wk), and included fewer women. The use of this 17α-alkylated androgen was also associated with a significant increase in liver enzymes, although no subject discontinued for this reason or developed transaminitis that exceeded 5 times the upper limit of normal.

Together, these five randomized controlled trials provide contrasting and complementary information regarding the utility of combining EPO with androgen

therapy for the anemia of end stage renal disease [101, 122–124, 126]. Although androgen therapy is considerably cheaper than EPO, it should not be used in women and children, and 17α-alkylated androgens may result in hepatotoxicity. One area of potential usage could be in older men [118, 127] who respond poorly to EPO, or for whom EPO is contraindicated or unavailable. Restricting use to non-17α alkylated androgens, particularly in older men [9, 118, 127], may avoid most of the hazards. Other than anemia, testosterone deficiency in men with CKD may also contribute to low bone mass, increased cardiac disease, and increased mortality. Studies are needed to establish these potential benefits in this population, even in those who would appear to be unequivocally hypogonadal.

Alcoholic Liver Disease

Men with alcoholic liver disease show features of hypogonadism including small testes, gynecomastia, soft smoother skin, and reduced bone and muscle mass. Direct hepatotrophic effects of androgens have been described [8, 128]; however, the systematic use of androgens in alcoholic liver disease is now of historical interest, and information on the use of androgens in non-alcoholic fatty liver disease is very limited (see Chap. 11). The effects of androgen therapy could differ when applied acutely for alcoholic liver disease [129–132], compared with more prolonged therapy for those with alcoholic cirrhosis [133–135].

Three studies have examined whether one month of oxandrolone 80 mg daily during acute alcoholic hepatitis can improve short or long-term survival or other clinical endpoints. The first study separately randomized 132 men with moderate disease and 131 men with severe disease to 30 days of oxandrolone, prednisolone (60 mg daily initially) or placebo [129]. There were no statistically significant differences in short-term (30 day) or long-term survival among the three treatment groups, irrespective of disease severity. Recent data indirectly strengthen this finding by showing no statistically significant difference in short-term (28 day) or long-term (90 days or 12 month) mortality between men and women treated with prednisolone (40 mg daily) or placebo for 28 days [136]. A second study showed no overall survival benefit in the intention-to-treat analysis amongst 271 undernourished men with severe alcoholic hepatitis randomized to combined oxandrolone with high calorie enteral supplementation, although a post hoc subgroup analysis showed a survival benefit with combined therapy in those with moderate malnutrition [130]. In any case, the study design did not allow for separation of effects due to improved nutrition from those due to oxandrolone. This is relevant because improved nutrition alone may reduce mortality [137]. The third study did not include placebo controls and only examined biochemical endpoints [131, 132].

Systematic studies in men with alcoholic cirrhosis are equally sparse and also now

of mainly historical interest. Findings from small short-term studies treating <50 men with androgens for <1 month are inconclusive [133, 134], and there is only one relevant study, which was conducted by the Copenhagen Study Group for Liver Disease and powered to detect a 35% decrease in mortality. In this randomized parallel group study, micronized testosterone (200 mg tid, $n = 134$) compared with placebo ($n = 87$) administered for 3 years did not improve mortality [135], liver histology [138], liver function [139], or sexual dysfunction [140]. These studies of oral testosterone and oxandrolone resulted in proportionally higher hepatic than systemic exposure. Overdosage presumably resulting in detrimental effects, including portal vein thrombosis occurred [141].

Together, these studies show little benefit of adjunctive androgen use in men with alcoholic hepatitis or cirrhosis, although it is possible that overdosage to the liver may have adversely affected overall outcome. These data should not be extrapolated to non-alcoholic fatty liver disease, where other biological processes may be more androgen-responsive.

Muscle and Bone Disorders

Although skeletal muscle and bone are known androgen targets, the use of androgen therapy in disorders of muscle and bone is limited. Conceptually, simply increasing the mass of dysfunctional muscle or bone may not be beneficial. Randomized placebo-controlled trials of androgen therapy in bone disorders such as osteopetrosis, osteomalacia, or other metabolic bone diseases are not available. On the other hand, androgen therapy may be useful for idiopathic osteoporosis where there is no underlying abnormality in bone architecture. Two randomized controlled trials are available [142, 143], but neither was adequately powered to examine fracture rates, and neither showed consistent or sustained improvements in bone mineral density. The largest study involved 327 patients treated with methandienone 2.5 mg daily or matching placebo for 9 months with 1 year of follow-up [142], and the other study randomized only 21 men to receive either weekly injections of nandrolone decanoate 50 mg or no treatment for 12 months [143]. Neither androgen utilized is aromatizable, which is problematic since aromatization is critical for action on bone [11].

Randomized controlled trials have examined the role of androgen therapy in men with muscular dystrophies such as myotonic dystrophy [144] and Duchenne muscular dystrophy [145]. In the first study, 40 men were randomized to receive intramuscular injections of testosterone enanthate (3 mg/kg) or placebo each week for 12 months [144]. In the other study, 51 boys aged 5–10 years were randomly assigned to receive oxandrolone (0.1 mg/kg/day) or placebo for 6 months [145]. Neither study showed consistent improvements in functional measures of performance such as skeletal or respiratory muscle strength, or walking.

Conclusions

The wide-ranging effects of androgens on multiple key organs important for survival and quality of life have long been recognized. Striated muscle, cardiac muscle, respiratory muscle, bone, fat, hemopoietic tissue, vascular smooth muscle, and neurological tissues are all androgen-responsive. By improving function, androgen therapy could plausibly ameliorate the morbidity associated with many chronic illnesses that arise from sarcopenia, low energy, impaired sleep, and osteopenia. These improvements could plausibly impact both the natural history and outcome of many non-gonadal disorders.

Despite over 50 randomized controlled studies in multiple organ systems, androgen therapy has not increased survival or altered the natural history of any chronic illness. Increased lean muscle mass (with any androgen) and decreased fat mass (particularly with aromatizable androgens) has been universally observed in various lung diseases, heart diseases, renal diseases, and in HIV/AIDS with and without wasting. However, specific improvements in respiration, cardiac contractility or muscular strength have been much more difficult to show. Despite this, subjective functional improvements have often been demonstrated in the absence of objective changes in performance. Relaxation of vascular smooth muscle leading to improved circulation has been documented, but the impact on atherosclerosis and on cardiovascular morbidity and mortality are safety concerns that need to be addressed once efficacy is established.

Cardiopulmonary rehabilitation is a core component of the management of chronic obstructive pulmonary disease and congestive heart failure, and would seem to be important for at least some wasted individuals with kidney disease or HIV/AIDS. Careful clinical trials are required to properly establish the role of androgen therapy, but can be, and have been, performed in other settings such as aging [146]. Renotrophic and hepatotrophic effects are plausible, but have not been demonstrated with androgen therapy. Further research for these indications has been essentially abandoned because of the development of renal and liver transplantation. An adjunctive role for appropriately dosed androgen therapy to prolong the survival or function of renal and liver transplants is plausible, but would require careful investigation since the immunomodulatory effects of androgens could be harmful. Androgen therapy for renal anemia, although once a standard therapy, has long been supplanted by modern erythropoietin analogues. However, an adjunctive role could be considered as new therapies based on new molecular targets (such as erythroferrone) are developed. Combining new with long established agents such as androgens that have a well-characterized mechanism of action and wide-ranging targets may be advantageous in certain clinical settings. As this review highlights, androgen therapy remains promising, even as new drugs come to market.

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20. Stimulation of Spermatogenesis in Hypogonadotropic Men

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Introduction

The hypothalamic–pituitary–gonadal (HPG) axis coordinates two functions essential for male reproductive capacity: synthesis and secretion of sex steroid hormones, primarily testosterone, and production of spermatozoa. Gonadotropin-releasing hormone (GnRH), a decapeptide produced in hypothalamic GnRH neurons, is released into the portal blood in discrete pulses, binds to specific receptors on gonadotrophs in the anterior pituitary, where it elicits gonadotropin secretion. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are glycoprotein hormones that control the development, maturation and function of the gonads [1]. To induce and maintain quantitatively and qualitatively normal spermatogenesis, both gonadotropins are required [2]. While LH binds to the LHCG receptor of Leydig cells, thereby stimulating testosterone synthesis and secretion, FSH binds to its receptor on Sertoli cells and

induces spermatid maturation (spermiogenesis) during spermatogenesis [1].

Knowledge of the impact of the patient's genetic background and our understanding of the physiologic principles of male reproductive health have substantially increased in recent years, enabling physicians not only to identify causes of hypospermatogenesis among a wide spectrum of possible disorders affecting maleness and reproductive capacity, but also to establish therapeutic strategies. Men with hypothalamic or pituitary disorders have an excellent prognosis for fertility restoration and for paternity by natural intercourse or using assisted reproductive techniques.

Pretreatment Diagnostic Workup in Male Hypogonadotropic Hypogonadism

Hypogonadotropic hypogonadism (HH) is suspected in teenage boys with delayed, absent or partial (and then arrested) puberty which needs to be distinguished from the far more common constitutional pubertal delay (see Chap. 4). In adulthood, suspicion is raised by symptoms of androgen deficiency and/or infertility. The diagnosis of permanent HH has to be based upon a meticulous pretreatment diagnostic workup.

Previous medical history should include the presence of an undescended testis at birth (with age at possible orchidopexies) and previous diseases, including eating disorders, chronic diseases leading to weight loss, malignancies with chemo- and/or radiotherapy, inflammatory diseases (meningitis, orchitis) and testicular trauma or surgery. Inquiries should be made into the ability to smell and on somatic features that may be associated with various causes of congenital HH/Kallmann syndrome, including visual disturbances, renal agenesis, dental and digital anomalies, cleft lip and palate, coloboma, synkinesia/mirror movements and ataxia. In addition, information on exercise, recent medication (including opioids) and drug abuse (including anabolic steroids) should be sought.

Family history ought to assess the parent's age at entering puberty, age at maternal menarche and signs of hypogonadism or infertility in relatives.

Physical examination includes auxological measurements and pubertal Tanner staging with measurement of testicular volumes using a Prader orchidometer or ultrasound.

While *hormone investigations* of the hypothalamo–pituitary–gonadal axis focus on LH, FSH and testosterone levels, measurement of TSH, free T4, GH, IGF1, IGFBP3, prolactin, and cortisol enables investigation of associated pituitary and adrenal hormone deficiencies.

Other laboratory analyses and function tests with measurement of inhibin-B levels and GnRH agonist testing (buserelin) may help to differentiate HH from CDGP (see Chap. 4).

Further diagnostic workup includes imaging procedures, such as magnetic resonance imaging (MRI) of the hypothalamo–pituitary region to rule out intracranial malformations, neoplasms or infiltrating diseases. Scrotal ultrasound provides information on parenchymal structure and testicular size [3]. Determination of bone age according to Greulich and Peyle [4] by a left-hand carporadiogram is used to estimate residual longitudinal growth potential in adolescents with absent or arrested puberty. Semi-quantitative olfactometry [University of Pennsylvania Smell Identification Test (UPSIT) or Sniffin'sticks] may be performed to distinguish between normal, partially or totally defective olfaction. Semen analysis is relevant to evaluate spermatogenesis and function of accessory glands, especially if paternity is desired. Karyotyping (+FISH) may be undertaken to rule out associated chromosomal anomalies. Sequencing of candidate genes in subjects with CHH (see Chap. 6) with description of mutations or polymorphisms is indicated for investigation of the genetic origin of HH. Results may also be helpful for genetic counselling prior to hormone replacement for fertility induction.

Hormone Substitution Modalities for Stimulation of Spermatogenesis in HH

GnRH Replacement

GnRH is effective therapy in men with hypothalamic GnRH deficiency provided that no additional pituitary pathologies are present [5–8], and that there is no GnRH-R mutation. The latter condition cannot be distinguished from GnRH deficiency unless genetic testing is done. GnRH has to be delivered in a pulsatile fashion by a portable mini-pump to successfully stimulate gonadotropin secretion, and consequently testosterone secretion and spermatogenesis. Continuous application of GnRH results in downregulation of pituitary GnRH receptors and blockade of signal transduction [9]. GnRH replacement is costly and inconvenient for patients as they have to carry a mini-pump and subcutaneous catheter for an extended period. Therefore, this modality—although physiologically appealing—is reserved for very motivated patients [10]. The needle of a catheter linked to the portable GnRH mini-pump is placed into the patient's abdominal subcutaneous tissue. It has to be changed every 2 days. The pump is programmed to deliver GnRH boluses every 120 min, as this frequency was shown to be most effective [11]. After a starting dose of 4 µg per pulse, increases of 2 µg may be performed every 4 weeks, aiming to produce testosterone levels in the normal adult range after 3–12 months (depending on the maturity of the testes achieved previously). Dosages necessary to induce spermatogenesis vary considerably between individuals with HH, ranging from 5 to 20 µg GnRH per bolus (or 25–600 ng/kg GnRH per bolus). To induce spermatogenesis sufficient to induce a pregnancy, 1–3 years of treatment may

be required. Patients with previously undescended testes may require considerably longer treatment for induction of spermatogenesis than patients with scrotal testes [5].

Gonadotropin (hCG/FSH) Replacement

In patients with hypogonadism of either hypothalamic or pituitary origin, subcutaneous or intramuscular hCG/FSH replacement is most often successful in stimulating endocrine and spermatogenic testicular functions and growth of previously immature testes.

Effective regimens of subcutaneous injections of hCG and FSH have been developed (Table 20.1).

Table 20.1 Therapeutic options licensed for puberty induction and fertility treatment in male HH

Drug	Trade name	Application	Dose
Human chorionic gonadotropin (hCG)	Brevactid®[a] Pregnyl[b] Novarel[b]	s.c. or i.m.	1000–2500 IU twice weekly (on Mondays and Fridays)
Recombinant FSH (rFSH)	Gonal F®[a], Puregon®[a] Follistim[b]	s.c. or i.m.	75–150 IU three times weekly (on Mondays, Wednesdays, Fridays)
Pulsatile GnRH	LutrePulse®	Mini-pump s.c.	4–20 μg per pulse every 120 min

[a]Trade names of drugs marketed in Europe
[b]Trade names of drugs marketed in the US

Human chorionic gonadotropin (*hCG*) purified from the urine of pregnant women has been used to replace LH since 1952 [12]. It contains almost exclusively LH-like bioactivity [13], and effectively stimulates testosterone production by Leydig cells.

Follicle-stimulating hormone (*FSH*) is required for spermatid maturation (spermiogenesis) during initiation and for maintenance of quantitatively normal spermatogenesis at puberty and thereafter [2, 14, 15]. *Urinary human menopausal gonadotropin* (hMG) has been used as a source of FSH since 1966 [16], *highly purified urinary FSH* has been available since 1997/98 [17, 18] and *recombinant FSH* (*rFSH*) since 1995 [19–22]. The putative risks of transmission of prion disease from urinary-derived gonadotropin preparations have not been documented in over 50 years of use. rFSH, not being of human or animal origin, harbours no such risk; however, a minor risk of infection transmission remains, as foetal calf serum is used in the cell culture media in rFSH production [23, 24]. Currently, there are no data concerning the

efficiency of *recombinant LH* (*rLH*) or *recombinant chorionic gonadotropin* (*rCG*) for stimulation of spermatogenesis in male HH patients, as these substances are licensed only for assisted reproductive techniques in women. Likewise, *corifollitropin alfa*, a long-acting recombinant FSH-like substance, necessitating only one injection every two weeks to achieve therapeutic levels (instead of thrice weekly FSH injections), is licensed only for use in women [25]. However, this long-acting follicle 'FSH-CTP' (Org 36286) [consisting of the α-subunit of human FSH and a β-subunit composed of the beta-subunit of human FSH and the C-terminus (CTP) of the β-subunit of hCG to prolong its half-life] was found to increase inhibin-B levels in hypogonadotropic males [26], but has not been studied further.

Gonadotropin treatment is generally initiated by administering hCG for 3–6 months [27] to stimulate the synthesis of testosterone (and potentially other Leydig cell products) which is the pre-requisite for spermatogenic induction. In some patients with partially preserved gonadotropin secretion, hCG alone may initiate spermatogenesis [28]. Intramuscular (i.m.), as well as subcutaneous (s.c.), hCG injections are possible, the latter allow for self-injection. If men remain azoospermic, FSH is added. To induce physiologic adult FSH levels, doses of 75–150 IU per injection are required [29]. Large studies using combined hCG/FSH treatment in adult male patients with HH are listed in Table 20.2.

Table 20.2 Studies using hCG/FSH treatment for spermatogenic induction in adults with hypogonadotropic hypogonadism

Studies using hCG/FSH treatment in more than 20 adult HH patients	Number of adult HH patients	Age (years)	Gonadotropin preparations and sequence of applications	Duration of substitution (months) mean or median (range)	Previous gonadotropin exposure (endogenous or exogenous) or previous TRT	Mean ± median (range) final single TV (ml) reached	Spermatoge achieved
Burgués and Calderón [17]	60	18–45	hCG → hCG + purified FSH	hCG: 1 hCG + FSH: 6 (+3)	47 (78%) previous TRT 8 (13%) post-pubertal-onset HH 36 (60%) previous gonadotropins	11 ± 1	48/60 (80%)
European Metrodin HP study group [18]	28	17–42	hCG + purified FSH	hCG: 3–6 hCG + FSH: 18	11 (39%) previous TRT 4 hCG 1 hCG/MHG	10 ± 4	89% after 18 month FSI
Liu et al. [68]	26	26–52	hCG → hCG + rFSH or uFSH (+further cycles)	hCG: 3–6 hCG + FSH: 4 (0–10)	26 no previous gonadotropins	n.a.	n.a.

					9 post-pubertal HH 19 no TRT 6 with sperm at start of hCG 16 with more than one cycle		
Bouloux et al. [20]	49	31 ± 7 (18–60)	hCG, hCG → hCG + rFSH	hCG: 4 hCG + FSH: ≥11	7 GnRH 19 hCH/FSH 8 no previous tx	12 ± 7	23/49 (47%): (18%) on hC alone 14/30 (47%) additional 6 months FSI
Matsumoto et al. [22]	36	18–55	hCG → hCG + rFSH	hCG: 3–6 hCG + FSH: 18	5 hCG 2 hCG/FSH 5 GnRH 32 TRT	15 ± 4 13 (7–28)	22/29 (76%)
Warne et al. [21]	100	16–48	hCG → hCG + rFSH	hCG: 3–6 hCG + FSH: 18	n.a.	12 (15) ± 5	68/81 (84%)
Rohayem et al. [60]	51	22–47	hCG → hCG + FSH (urinary derived or purified or rFSH)	hCG: 11 ± 8 hCG + FSH: 9 ± 7	10 with pubertal arrest 10 post-pubertal HH 0 with previous hCG/rFSH 42 with previous TRT	27 ± 15	50/51 (98%)

In recent years, the question has been raised whether the sequence of gonadotropin stimulation should be reversed, and replacement should begin with FSH. This notion was triggered by the hypothesis that Sertoli cells may proliferate better upon FSH stimulation if not previously exposed to testosterone [30, 31]. In a recent trial, rFSH administration for 4 months prior to the initiation of pulsatile GnRH treatment resulted in spermatogenesis in 7/7 HH patients after 24 months at which time only 4/6 subjects treated with pulsatile GnRH alone had sperm in their ejaculates [32]. The groups were too small for a definite conclusion, and larger trials are required before this regimen may become routine. It should also be remembered that in HH adolescents presenting primarily for induction of puberty, use of FSH without hCG will not stimulate testosterone secretion or induce pubertal virilisation in contrast to protocols beginning with hCG. As rFSH is costly, the sequential approach starting with hCG has also been preferred for financial reasons.

Hormone Replacement in Prepubertal-Onset HH

A treatment regimen beginning with testosterone for induction of puberty stimulates normal linear growth, pubertal virilization and psycho-sexual maturation in congenital HH. However, this traditional approach neglects testicular growth and the acquisition of fertility as components of normal puberty. The testes remain immature and small, i.e. in a prepubertal state, and spermatogenesis is not initiated. Meanwhile, there is sufficient evidence that complete male puberty, comprising pubertal virilisation in concert with testicular growth and initiation of spermatogenesis, can be successfully achieved during adolescence by replacement with gonadotropins (hCG/FSH) [7, 8, 30, 33–36] (Table 20.3).

Table 20.3 Studies using hCG/FSH in adolescent patients with HH, with assessment of puberty (incl. increase in testicular volume (TV)) and spermatogenesis

Studies using hCG/FSH in adolescent patients with HH, with assessment of puberty (incl. increase in TV) and spermatogenesis	Number of adolescent HH patients	Age (years)	Gonadotropin preparations and sequence of applications	Duration of substitution (months)	Virilisation/adult T levels achieved (%)	Mean ± medi (range) final single TV (m reached
Liu et al. [7]	3 (subset of cohort)	16–17	hCG/HMG		100	9 ± 1
Schopohl [8]	9 (subset of cohort)	18–24	hCG/MHM	20 ± 2	100	n.a. (8–30)
Barrio et al. [33]	14 (IHH: 7 panhypopit: 7)	13–21	hCG + rFSH	31	100	IHH: 10 ± 4 panhypo: 15 ±
Raivio et al. [30]	14	10–18	rFSH → rFSH + hCG	rFSH: 2–34 hCG + FSH: n.a.	100	6 (2–37)
Sinsi et al. [34]	10 (subset of cohort)	11–25	hCG → hCG + rFSH	hCG/rFSH: 12 (−24)	100	10 (7–15)
Zacharin et al. [35]	7 (subset of cohort)	16–22	hCG → hCG + rFSH	hCG/rFSH: 9	100	12 ± 7 10 (5–2
Rohayem et al. [36]	60 A: 34 B: 26	14–22	A: hCG → hCG + rFSH B: Testo → hCG → hCG + rFSH	A: hCG: 31 ± 6 hCG/FSH: 25 ± 9 B: hCG:	A: 100 B: 100	A: 17 ± 3; 15 (8–40) B: 16 ± 3 17.5 (2–30)

				30 ± 7 hCG/FSH: 25 ± 9		

Gonadotropin treatment may be suggested for pubertal induction, even if fertility is a matter for the future. Even though rFSH is costly, normalization of testicular size and initiation of spermatogenesis at a peer-related time may positively affect body image and fertility concerns, and thus provide self-assurance and confidence for the future in teenage boys with HH [36, 37]. In addition, spermatogenesis, once driven to full maturation, can be re-stimulated much faster when fertility is desired later in life [5, 38]. Subsequent treatment with testosterone does not seem to jeopardize the outcome of later stimulation therapy [36]. This is important for prepubertal boys who are unwilling to commit to five s.c. injections per week for 2–3 years. However, some of these young patients may be more agreeable to gonadotropin stimulation once pubertal induction with testosterone is accomplished [39].

In adolescent patients with open epiphyses (i.e. with a bone age < 16 years), the dose of hCG should be escalated in small increments over the first year (until serum testosterone levels are in the normal adult range). The goal is a pubertal growth spurt and attainment of final height in the range of mid-parental expectations. Adverse effects, including severe acne or gynecomastia, can generally be avoided by this regimen. Subsequent combined hCG/rFSH replacement over two to three years is required for testicular growth and to fully activate the individual's spermatogenic potential. Over 70% of adolescents will thereby reach normal adult testicular size, and sperm will appear in semen in over 90% of patient [35, 36].

In view of the challenging differential diagnosis of constitutional delay of puberty (CDGP), this regimen bears an important further advantage: low hCG doses have only weak suppressive effects at the hypothalamic or pituitary level; therefore, the pubertal GnRH pulse generator may activate in cases of unrecognized CDGP. Patients misdiagnosed as HH may thus be recognized if special attention to a potential rise in serum LH levels is given during the first phase of gonadotropin replacement.

In patients with lack of pubertal development but a late diagnosis of CHH in adulthood, or those with previous testosterone treatment, final height may already have been attained. In these adult men, higher initial hCG replacement doses and faster dose escalations can be applied than in HH boys with open epiphyses/immature growth plates. Success rates for spermatogenic induction in adults are slightly below those of adolescents, ranging from 65 to 90% [5, 17, 18, 40].

Treatment Protocol for Testosterone-Naïve Prepubertal Patients

A starting dose of (250−) 500 IU hCG, injected subcutaneously on Mondays and

Fridays, with increments of 250–500 IU hCG per injection every 6 months, to a maximum of 3 × 2500 IU hCG s.c./week, is recommended. The aim is to achieve pubertal levels [serum testosterone ≥ 1.5 ng/ml, (5.2 nmol/l)] after approximately 6 months, and levels in the mid-normal adult range [testosterone > 3.5 ng/ml, (12 nmol/l)] by one year. rFSH (follitropin alpha) 3 × (75−)150 IU s.c./week (injected Mondays, Wednesdays and Fridays) is added when pubertal serum testosterone levels (>5.2 nmol/l) are reached. Subsequent rFSH dosage modifications above 150 IU per injection are not recommended as they do not further stimulate spermatogenesis.

Treatment Protocol for Testosterone-Virilized Adolescents with Prepubertal-Onset HH

A full hCG starting dose of 1500 IU s.c. may initially be applied twice weekly. hCG dose reduction is recommended if polycythemia, gynecomastia or excessive acne occur. If testosterone levels remain below the normal adult range (<12 nmol/l) after 6–9 months, the hCG dose can be increased by increments of 500–1000 IU per injection (to a maximum of 3 × 2500 IU s.c./week). rFSH (follitropin alpha) 3 × (75−) 150 IU is additionally injected thrice weekly after 3–6 months of hCG without subsequent dose modifications.

Hormone Replacement in Post-pubertal-Onset HH

In patients with HH acquired post-pubertally, testicular maturation has already been completed, as indicated by adult testicular size. If the patient has been gonadotropin deficient for a prolonged period, however, paused spermatogenesis may have led to a reduction in testicular volume. Hormone replacement can be initiated either with testosterone or gonadotropins. Since gonadotropins are costly, this therapeutic investment is justified only if paternity is desired. In all other cases, testosterone is the preferred modality for replacement. When stimulation therapy for fertility induction is initiated in patients with previously accomplished testicular maturation, Leydig cell response (as indicated by a rise in serum testosterone levels to the adult range) to hCG occurs within 1–3 months. In some cases this is sufficient for initiation of spermatogenesis [27]. However, prolonged treatment with hCG alone will suppress endogenous residual FSH secretion. Therefore, addition of FSH is often required. Spermatozoa appear in semen after 3–9 months of combined hCG/FSH replacement. Stimulation of spermatogenesis will not proceed further when the sperm concentration in the ejaculate reaches a plateau that is indicative of the individual's spermatogenic capacity. Once spermatogenesis is fully activated by combined hCG/FSH replacement, sperm production may be maintained with hCG alone for several months (in patients with residual endogenous gonadotropin secretion) [41, 42].

It has been proposed that extended periods of hCG replacement could predispose to the development of hCG antibodies with loss of its effectiveness [43–45], as well as a risk for hypothyroidism [46] due to the structural homology between hCG and TSH. However, this concern has not been substantiated in clinical practice. Nevertheless, recombinant LH (rLH), so far not licensed for use in males, might be used as a therapeutic "rescue strategy" for restimulation of spermatogenesis in such cases.

Treatment Protocol for Adult-Onset HH

A full hCG starting dose of 1500 IU s.c. is applied twice weekly. HCG dose reduction is recommended in cases of polycythemia, gynecomastia or excessive acne. If testosterone levels remain below the normal adult range (<12 nmol/l) after 3–6 months, the hCG dose can be increased by increments of 500–1000 IU per injection every (3)–6 months to a maximum of 3 × 2500 IU s.c./week. In the great majority of patients who remain azoospermic on hCG alone after 6 months, rFSH (follitropin alpha) 3 × 150 IU is additionally injected thrice weekly after 6 months of hCG, without subsequent dose modifications.

Monitoring

Measurement of serum LH, FSH, testosterone levels and testicular volumes (via Prader orchidometer and/or ultrasound) ought to be performed at 3–6 monthly intervals during gonadotropin substitution, along with annual bone age estimations in adolescents until a bone age of 16 years (and thus near final height) is reached. Special attention should be given to LH levels, as a spontaneous increase above >1 U/L would indicate spontaneous HPG axis activation during gonadotropin substitution, which would question the diagnosis of persistent HH. In such cases, cessation of hormone replacement is indicated. Regular measurement of serum testosterone levels (e.g. every three months) is useful not only to monitor the Leydig cell response, but also, along with FSH levels, adherence to treatment. Once maturity for semen analysis is attained in previously prepubertal subjects, ejaculates can be collected and analysed according to WHO guidelines [47] after at least 48 h of sexual abstinence. Thereafter, semen analyses may be repeated every three months until a plateau of sperm concentrations is documented in at least two follow-up visits or until pregnancy is achieved. Sperm cryopreservation may be offered before gonadotropin replacement is stopped and patients are switched to testosterone replacement. However, restimulation of the testes with gonadotropins by a second treatment cycle is known to be successful in most subjects, and spermatogenic induction is achieved faster than during the initial phase of gonadotropin stimulation, even after a long period of testosterone substitution [5]. Therefore, sperm cryopreservation is recommended only in patients with a very poor primary spermatogenic response or in subjects necessitating assisted reproductive techniques for

impregnation of their partners.

Caveats During Hormone Replacement

Misdiagnosis of CDGP and CHH Reversal

Lifelong hormone replacement is indicated in nearly all cases of congenital hypogonadotropic hypogonadism. Once gonadotropin substitution (in adult doses) is begun, the GnRH pulse generator is suppressed, making spontaneous activity of the GnRH pulse generator improbable. Because of clinical overlap between CHH and constitutionally delayed puberty, however, CDGP might be misdiagnosed as HH. Moreover, GnRH deficiency seems to resolve in approximately 5% of patients with Kallmann syndrome or CHH (with *CHD7*, *FGFR1*, *GNRHR*, *TACR* mutations) [48–52]. Therefore, continuous attention should be given to LH levels (the parameter reflecting the patient's endogenous HPG axis activity), in order to recognize spontaneous GnRH pulse generator activation. However, during hCG/FSH replacement, an LH surge is probable only in patients regularly omitting injections. Of note, if reversal of HH occurs, it may not be sustained [51, 52]. The vulnerability and plasticity of the reproductive axis emphasizes the need for lifelong attention to symptoms of androgen deficiency and surveillance of HPG axis hormones.

HPG Axis Suppression/Inhibition: Drug-Induced Hypogonadotropic Hypogonadism

Prolonged abuse of anabolic androgenic steroids (AAS) (including testosterone preparations) suppresses the HPG axis [53, 54]. As shown in clinical trials of testosterone-based hormonal male contraception, suppression is reversible if androgen administrates ceases [55]; however, reactivation of the GnRH pulse generator may be delayed up to 3–24 months [56]. In these cases, cessation of exogenous hormone administration or abuse is the primary recommendation. In clinical practice, a variety of approaches have been described to overcome AAS-induced hypogonadism, such as hCG or antiestrogens [53]. However, these may produce unwanted side effects such as gynecomastia and liver dysfunction so that the best advice is to expect spontaneous recovery of HGP axis function after cessation of abuse.

GnRH secretion is also suppressed with the use of morphine, heroin and methadone, each of which activates hypothalamic inhibitory neurons [57–59]. Iatrogenic HPG axis downregulation also occurs as a result of GnRH agonist treatment (for precocious puberty in boys and during anti-androgenic treatment of metastatic prostate cancer in adults). Therefore, inquiries on recent medication use and drug abuse are essential to recognize this potentially reversible cause of HH.

Therapeutic Outcome of Central Hormone Replacement Strategies

Efficacy of Spermatogenic Induction: Comparison GnRH Versus Gonadotropins

Pulsatile GnRH replacement mimics physiology by stimulating the pituitary to release endogenous pulses of LH and FSH. In contrast, subcutaneously administered hCG induces two, and FSH injections produce three peaks per week in the patient's bloodstream. Nonetheless, the efficacy of spermatogenic induction is comparable with both therapeutic strategies [5, 7]. Success rates in adults range from 65 to 90% [5, 16, 18, 40].

Factors Influencing Response to Gonadotropins or GnRH

GnRH and gonadotropin substitution regimens are more successful in cases without additional testicular damage. Testicular growth and full spermatogenic activation are achieved less quickly, and less effectively in patients with Kallmann syndrome or CHH with absent puberty, compared to those with arrested puberty or HH that is acquired after puberty [60] (Fig. 20.1). Likewise, the time needed to achieve adult testosterone levels following Leydig cell stimulation with hCG is longer in these groups. The pretreatment serum level of the Sertoli cell marker inhibin B, and baseline testicular volumes both mirror the degree of seminiferous tissue development and endogenous maturation, with low values in subjects with severely impaired maturation and higher values in less severe cases [61, 62]. Cryptorchidism, known to induce changes in the germinal epithelium [63], is a negative predictor of outcomes in HH patients seeking fertility who are treated with GnRH or gonadotropins [5, 21, 38, 63–65]. Kallmann syndrome patients have a high prevalence of undescended testes at birth [60, 66]. Nevertheless, a history of previously cryptorchid testes does not preclude fertility, especially in unilateral cases, but testicular responses require longer stimulation [5]. Patients with congenital multiple pituitary hormone defects also tend to have poorer semen quality in response to treatment [60, 67].

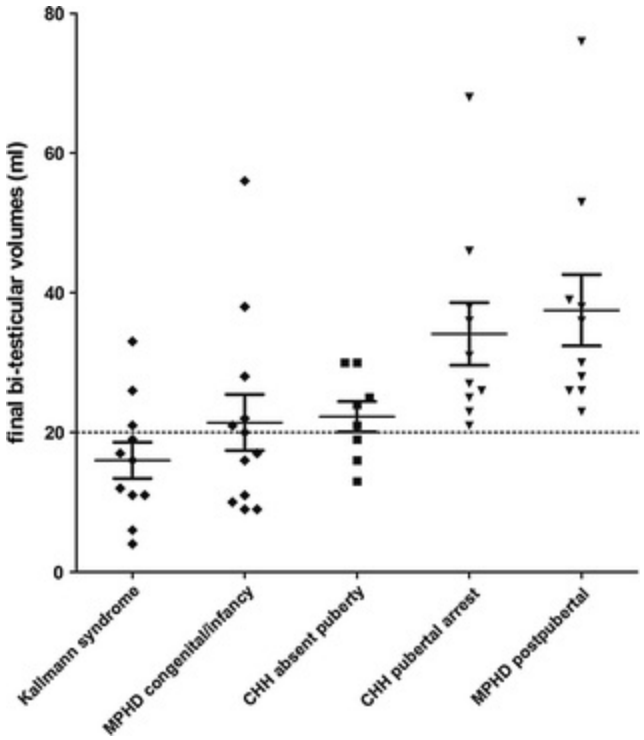

Fig. 20.1 Maximal final bi-testicular volumes (BTVs) achieved by gonadotropin replacement: Testicular growth reflects the putative onset and severity of HPG axis functional impairment in different HH subgroups, with better results in patients with post-pubertally acquired hypogonadotropic disturbances compared to those with congenital or early-onset HH. MPHD congenital/infancy = multiple pituitary hormone deficiencies, either congenital or acquired during infancy or childhood; CHH absent puberty = congenital hypogonadotropic hypogonadism with absent pubertal development; CHH pubertal arrest = congenital hypogonadotropic hypogonadism with spontaneous initiation of pubertal development, but subsequent pubertal arrest; MPHD post-pubertal = multiple pituitary hormone deficiencies, acquired after puberty. From Rohayem et al. [60]. Reprinted with permission from John Wiley and Sons

Pregnancy Outcomes

Pregnancies are achieved in 40–55% of partners of HH men wishing paternity who have undergone central hormone replacement [5, 38, 60, 68]. Spontaneous pregnancies can occur with subnormal sperm counts, as spermatozoa have an excellent fertilizing potential [5, 33, 60, 64, 65, 68] (Fig. 20.2). However, in 1/5 to 1/8 patients, assisted reproductive techniques (ART), i.e. in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), are required to achieve conception [60, 68, 69]. Even if no sperm appear in the ejaculate after prolonged treatment, testicular sperm extraction (TESE) may provide viable spermatozoa. However, pregnancies via TESE-ICSI in partners of HH patients have not been reported in the current literature, except for a HH patient also suffering from adrenal hypoplasia due to *DAX-1* (*NROB1*) mutation [70].

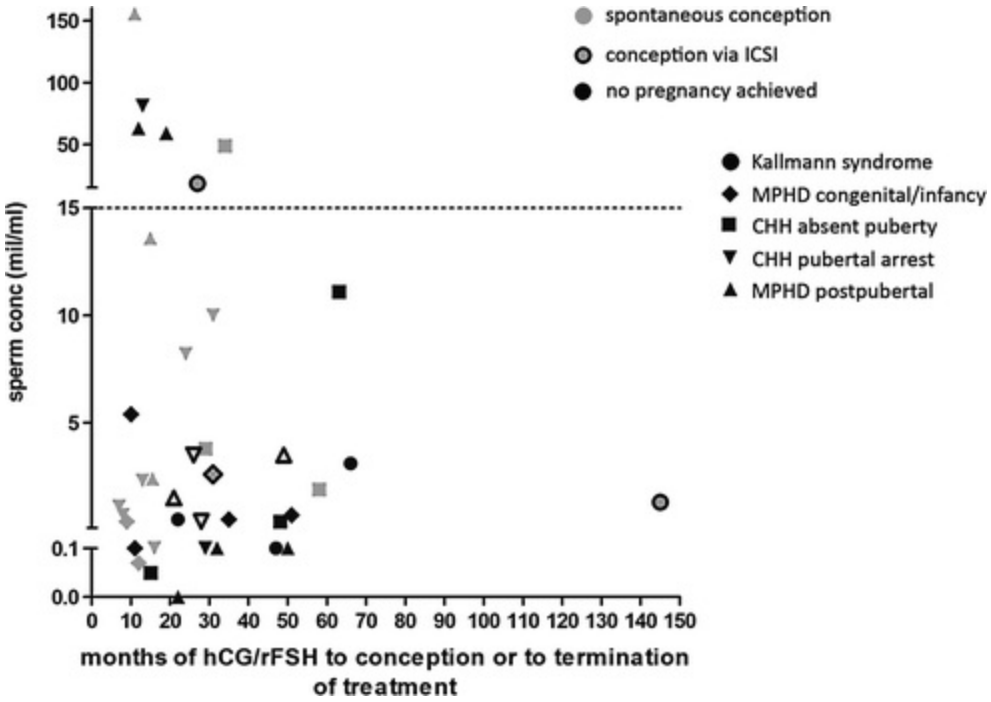

Fig. 20.2 Sperm concentrations at the time of achievement of pregnancy in different HH subgroups are plotted over months of hCG/FSH treatment to conception in successful patients. In those unsuccessful, maximal sperm concentrations are plotted over months of hCG/FSH until treatment was terminated. The majority of conceptions occurred despite subnormal sperm concentrations (<15mill/ml) in the ejaculate. MPHD congenital/infancy = multiple pituitary hormone deficiencies, either congenital or acquired during infancy or childhood; CHH absent puberty = congenital hypogonadotropic hypogonadism with absent pubertal development; CHH pubertal arrest = congenital hypogonadotropic hypogonadism with spontaneous initiation of pubertal development, but subsequent pubertal arrest; MPHD post-pubertal = multiple pituitary hormone deficiencies, acquired after puberty. From Rohayem et al. [60]. Reprinted with permission from John Wiley and Sons

Inheritance of Congenital Hypogonadotropic Hypogonadism

Patients with CHH are usually infertile; therefore most cases occur sporadically, as a result of de novo mutations or—in case of recessive inheritance—from heterozygous (often consanguineous) parents. With successful fertility treatments, vertical disease transmission may increase, especially in case of dominant heredity. Given that the genetic basis of the majority (around 70%) of HH patients remains unknown, even if molecular genetic testing is performed, it is difficult to provide accurate counselling. Nevertheless, information on the risk of disease transmission to the patient's offspring should be given prior to fertility treatment and genetic counselling provided to all subjects with identified mutations. Pre-implantation diagnostics (PID)/pre-implantation genetic diagnosis (PGD) and foetal ultrasound [71] may be options for the future to select embryos without disease-causing mutation.

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