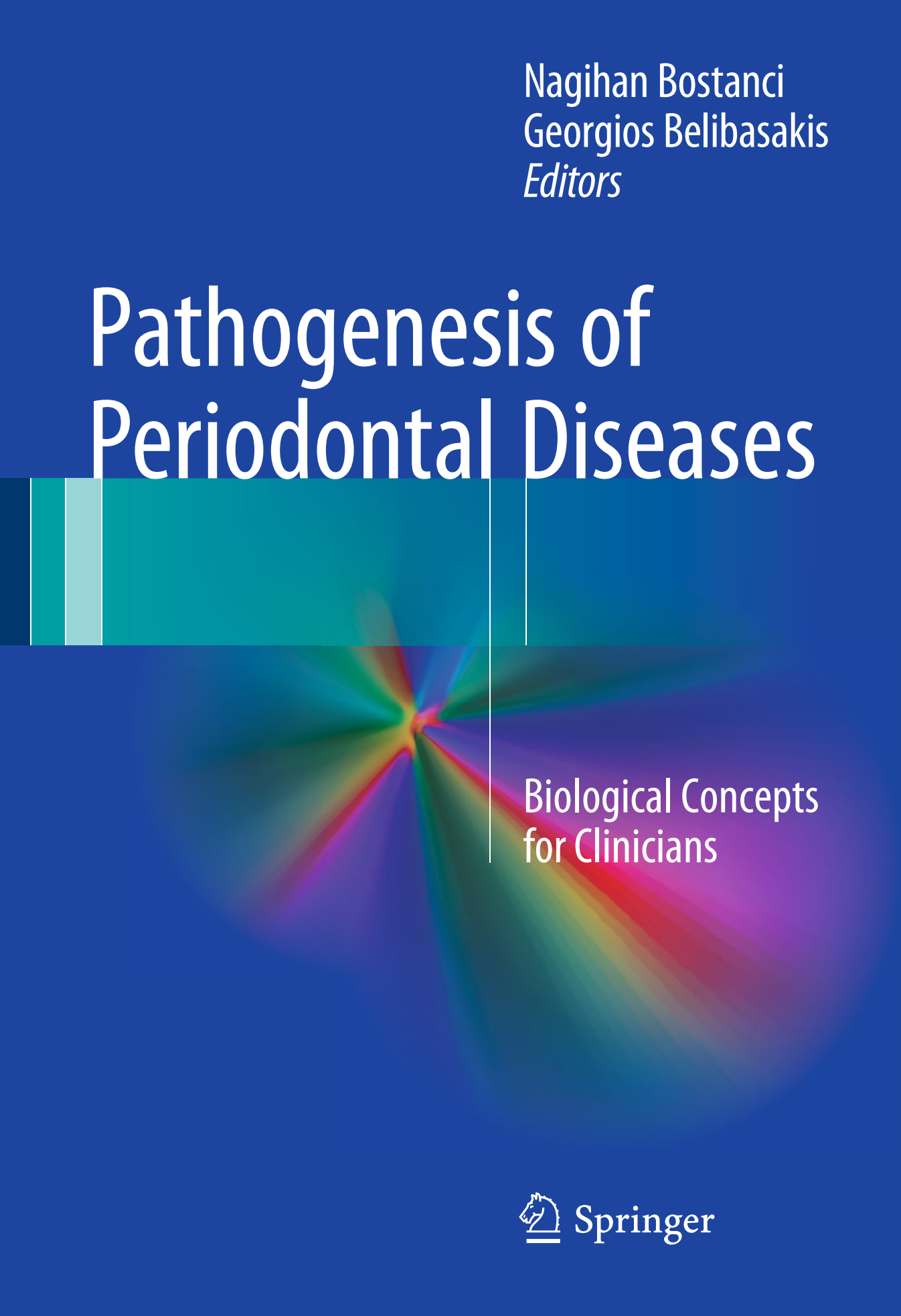
Nagihan Bostanci
Georgios Belibasakis
Editors

Pathogenesis of Periodontal Diseases

Biological Concepts for Clinicians



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1 Periodontal Pathogenesis: Definitions and Historical Perspectives

Nagihan Bostanci and Georgios N. Belibasakis

1.1 Introduction: Realizations from an Epidemiological Perspective

Periodontal disease, or periodontitis, is a globally widespread pathology of the human oral cavity. Indeed, approximately 10% of the global adult population is highly vulnerable to severe periodontitis. Another 10–15% appears to be completely resistant to it, while the remainder vary between these two extremes [1, 2]. Moreover, the prevalence of periodontitis is peaking at the fourth decade of life and increasing to 70–85% in the age group of 60–65 [3]. Strikingly, despite major improvements in oral hygiene practices today, these proportions are not far from what was reported in possibly the first epidemiological report of periodontitis in humans back in 1918 (then defined as *periodontoclasia* or *pyorrhea alveolaris*). According to that, the prevalence of the disease in the Chicago area was 13% in the age range of 20–24, 68% in 30–39, and 88% over 50. Much recent data from USA showed that there is not a drop but rather an increase in these numbers among the older individuals [4]. That means that periodontitis is an inevitable oral pathology of the human population, and its prevalence increases with age. Taking also under consideration the increasing life expectancy, periodontitis is a growing health problem.

Despite the persistence of severe periodontitis even in the twenty-first century, the last 100 years have witnessed a significant progress in our understanding of its pathogenesis, that is, the conglomerate of biological processes that lead to the disease. Nevertheless, the actual "coordinator(s)" of the disease is still an issue of intense debate. Microbiology researchers place emphasis in seeking species, or combinations of them, associated with different clinical forms of the disease. On the other side, immunologists are in pursuit of cells and molecules that orchestrate the tissue-destructive inflammation, as a result of the bacterial challenge. These are the two sides of the same coin, and only when studied together can we appreciate the complexity of the periodontal pathogenesis. Understanding the variants, uniqueness, and redundancies of these biological mechanisms is a preamble for thinking in new ways for preventing and managing these diseases.

1.2 Definition of Periodontal Disease from a Historical Perspective

The human interest in defining and understanding periodontal disease spans over the centuries, and many paradigm shifts occurred concerning its

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epidemiology and pathogenesis. Pioneers in the field have left us with a heritage of historical knowledge that has been transforming over the years. Their views and contributions were direct reflection of the knowledge that was available at the time and an important testament for the generations to come. Although it won't be possible to single out all great contributors, we will try to summarize some hallmarks in chronological order.

1.3 The Eras of "Pitius" and "Calculus"

While ancient Greeks knew nothing about the nature of periodontal disease, let alone their pathogenesis, they used their senses to "diagnose" it by its malodor and proposed the etiological factors. Hippocrates wrote in his scripts that the "evil malodor" is as result of "pitius." He even a proposed a therapy, which involved rinsing the mouth with a solution containing oil from anise seeds and white wine, possibly one of the pioneer mouthwashes [5]. Moving into the Roman Era, the disease was still not named. "Wobbly" (mobile) teeth were observed as a result of "calculus," a Latin word meaning "a pebble or stone." It was possibly then when calculus was placed on the map as an etiological factor of periodontal disease, something that emerged again in the early eighteenth century.

1.4 The Era of "Periodontosis" and "Pyorrhea Alveolaris"

While there were no known scientific attempts to prove the causative relationship between calculus and the disease for over a millennium, this was the dominating dogma due to the casual observation that routine calculus removal improves this condition. French pathologist Pierre Fauchard, the "father of modern dentistry," was possibly the first to discuss this periodontal pathology in detail and to describe it as "a distinct type of scurvy" in 1746. He supported that the disease does not have in systemic causes, since he observed that "internal remedies" were not successful as a cure. Hence, it had to be a local or accidental origin that caused the disease [6]. More than half a century later, physiologist and surgeon John Hunter proposed that alveolar bone around the teeth is dissolved due to inflammation occurring in the gingiva, coining the term "periodontosis" to the disease [7]. In 1882, American dentist John Riggs historically named the disease "pyorrhea alveolaris," later known as "Riggs' disease," which he described as a suppurative inflammation of the gums and alveolar process [8]. Although inflammation of gingival tissue was acknowledged, Riggs still advocated for calculus being the local etiological factor of the disease, based on the observation that it was cured following the removal of calculus by a scaler.

1.5 The Eras of Histopathology and Microbial Causation

The period between 1880 and 1920 was marked by an expansion of the understanding of the scientific discipline of microbiology and the recognition of resided oral bacteria as a causative factor for pyorrhea alveolaris. With that, the notion of dental plaque being a causative factor for the disease was on the rise. In 1890, W.D. Miller hypothesized that several remote causal factors weakened periodontal tissues, rendering them susceptible to challenge by bacteria inhabiting the mouth [9], a concept of great resemblance to the ecological plaque hypothesis that developed more than a century later [10]. During the same period, G.V. Black, a renowned restorative dentist, detailed the structure of the periodontal tissues and named their pathological destruction "periodontoclasia-calcic inflammation of the periodontal membrane." His descriptions were based on clinical characteristics, and he was the first to use a primordial-type periodontal probe and X-rays to assess the disease [11].

From the mid-1920s to early 1950s, the understanding of periodontal pathology shifted toward the degeneration of the periodontium with oral bacteria considered merely as secondary invaders of degenerated periodontium [12]. Hence, this period was dominated by histopathology observations, with the two most profound breakthroughs being (a) the description of the epithelial attachment on the tooth as a "sealer tissue" to protect the underlying connective tissue and (b) the definition of gingival pocket [13]. These findings paved the way for modern periodontal pathogenesis research, with implications in routine clinical practice.

From the mid-1940s onward, dental researchers once again rediscovered bacteria in the etiology of periodontal disease, with the predominant notion of a nonspecific infection. This provided the first seeds for the conception of the nonspecific plaque hypothesis [14]. It was suggested that microorganisms within the mass of dental plaque, rather than the calculus, were responsible for causing the disease. They were not thought to be exogenous pathogens but rather overgrown indigenous oral species. Because it was unclear which organisms were pathogenic, treatment approaches during that era were directed at suppressing all of them, a concept largely applicable in today's preventive techniques.

1.6 The Era of "Nonspecific Plaque" Hypothesis and Experimental Gingivitis

In the era of "nonspecific plaque hypothesis," several epidemiological studies showed a close relationship between poor oral hygiene and periodontal disease [14]. In the mid-1960s, the landmark studies of Harald Löe convincingly demonstrated that plaque accumulation directly preceded gingivitis, in a volunteer human experimental model known as "experimental gingivitis" [15]. According to this, gingival inflammation occurs as short as 3 days following abstinence of oral hygiene, and an inflammatory exudate of the periodontal tissues (gingival crevicular fluid) accompanies the clinical signs of inflammation. Within days of reinstating oral hygiene practices, the inflammation subsides, and gingival tissue health is restored. Since then, experimental gingivitis studies in humans have been the hallmark of clinical, microbiological, and histopathological investigations, more recently also in conjunction to cutting-edge proteomic techniques [16]. Although many believed that gingivitis was harmful and would indifferently lead to the destruction of the periodontal tissues, today we know that not all gingivitis cases will progresses to periodontitis. Additionally, the clinical signs of gingivitis alone are not adequate to identify the risk of transition to periodontitis [17, 18], and it is still debatable whether gingival inflammation in response to plaque accumulation is an interim stage between health and periodontitis.

1.7 The Era of "Specific Plaque" Hypothesis and Periodontal Pathogenesis

In the mid-1960s and 1970s, the next major breakthrough came by attempts to demonstrate microbial specificity of subgingival plaque at sites with "periodontosis" [19]. A number of pioneers, including Max Listgarten [20, 21], Sigmund Socransky [22], and Jorgen Slots [23], have identified and implicated specific microorganisms as etiologic agents for periodontal disease. These findings led to the revision of older favorites, such as *Capnocytophaga* spp., and the emergence of new species as more significant contributors to the disease, such as *Bacteroides gingivalis*, *Bacteroides forsythus*, and *Actinobacillus actinomycetemcomitans* (today known as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Aggregatibacter actinomycetem-*

comitans, respectively). The specific plaque hypothesis was further corroborated by studies on the quality and quantity of serum antibodies, showing, for instance, that patients with severe forms of periodontitis have high serum antibody titers to selected species [24].

The unravelling of the microbial constituents of supra- and subgingival plaque has naturally led to the investigation of their role in the initiation and establishment of the disease. It was now clear that microbial plaque initiated a series of as-yet-undefined events that led to the destruction of the periodontium. The efforts to understand the pathogenic mechanisms of periodontitis were intensified in the 1970s, as favored by the boost of immunology and immunopathology fields and the establishment of the concept of "host response." It was now becoming evident from several in vitro and in vivo studies that bacteria or their products may affect the gingival epithelium, the underlying connective tissue, as well as the enclosed immune cells. The instigated pathological alterations are reflected by physical breach of the epithelium, impairment of phagocytosis by polymorphonuclear neutrophils (PMNs), breakdown of the connective tissue extracellular matrix, activation of resident macrophages and osteoclasts, and destruction of the alveolar bone [25, 26].

1.8 Periodontal Pathogenesis Based on Description of Histopathological Features

In vivo experimental studies in dogs have enabled the study of the conversion of established gingivitis to destructive periodontitis, leading to pioneering concepts in the pathogenesis of periodontal disease, which hold up to date [26]. These studies have shown that gingival tissues respond within 2–4 days from the beginning of the accumulation of microbial plaque, as an acute exudative vasculitis in the plexus of venules lateral to the junctional epithelium. This response coined the term "initial lesion" and includes features, such as migration of PMNs via the junctional epithelium into the gingival sulcus, co-exudation of fluid from the sulcus, and loss of perivascular collagen. This subsequent stage is the "early lesion," which develops within 4–10 days. It is characterized by a dense infiltrate of mainly T lymphocytes (T cells) and other mononuclear cells, as well as pathologic alteration of fibroblasts. The early lesion is followed by the "established lesion," which develops within 2–3 weeks. It is dominated by activated B cells (plasma cells) and accompanied by further loss of the marginal gingival connective tissue matrix, but no bone loss is yet detectable. A number of PMNs continue to migrate through the junctional epithelium, and the gingival pocket is gradually established. The established lesion, clinically manifesting as moderate to severe gingivitis, may remain stable for years or even decades, or it may progress to a destructive "advanced lesion." In the "advanced lesion," plasma cells continue to predominate as the architecture of the gingival tissue disturbed, together with destruction of the alveolar bone and periodontal ligament. In summary, the conversion from the established to the advanced lesion is characterized by (a) conversion of junctional (eventually ulcerated) epithelium to pocket epithelium, (b) formation of denser inflammatory infiltrate composed of plasma cells and macrophages, (c) loss of collagen attachment to the root surface, and (d) resorption of the alveolar bone [26]. The initial, early, and established lesions represent sequential stages in gingivitis, whereas the advanced lesion clinically manifests as periodontitis. These events are summarized in Fig. 1.1.

Fig. 1.1 Histopathological stages of periodontal disease according to Page and Schroeder (1976)

1.9 Current Paradigm of Periodontal Pathogenesis

According to the most well-established pathogenesis paradigm, periodontal disease is the result of a complex interplay between microbial challenge, host response, and other modifying factors [26]. The microbial challenge triggers an inflammatory response by the host, which is meant to be protective in first place. Yet, if it becomes excessive, it will lead to gingival connective tissue and alveolar bone damage, culminating to periodontitis. Environmental/habitual and genetic host factors (risk factors) render some patients more susceptible than others to the inflammatory response and consequently to the disease. This may explain the observed large variations in the destruction patterns and susceptibilities to the disease. However, many individuals with disease have only one, or none, of the classic risk factors. Although the proposed model is reasonable, our understanding of how inflammatory host response is regulated is far from complete. New discoveries are needed to augment the information obtained from traditional indicators and to better illuminate the disease mechanisms. As a result, there is still an intense interest in applying emerging technologies to achieve a better understanding of the disease processes.

1.10 Pending Clinical Questions in the Era Systems Biology

Based on the progress in our understanding of etiology and pathogenesis of the disease, several classification systems have been proposed and came to clinical use [27–34]. Yet, based on clinical parameters alone, we still cannot predict when a periodontal pocket is about to be formed,

whether it will progress over time, or whether indeed it will resume clinically healthy levels once treated. Additionally, the current classification system in practice refers to "different" forms of periodontitis that, paradoxically, can have similar clinical presentation. We therefore need more robust diagnostic and prognostic tools, which can only come with the incorporation of molecular parameters into the daily practice. It is very much anticipated that the enormous advances in genomic and proteomic technologies will have an impact in bridging these gaps between our scientific knowledge and clinical practice.

One of the most crucial open questions in periodontal pathogenesis is to understand and capture the conversion from gingivitis to periodontitis. A major challenge is that periodontitis is "silent" in its nature, meaning that the disease does not cause pain and can progress unnoticed. Understanding the molecular events that occur during this transition could give us a breakthrough diagnostic and risk assessment tool for evaluating susceptibility to periodontitis. It is also likely that several immune regulation mechanisms exist, all leading to similar or comparable clinical manifestations of periodontitis. Understanding the uniqueness and redundancies among these mechanisms via the application of genomics, proteomics, and structural biology holds strong promise in providing targets for periodontal monitoring, prevention, and personalized treatment.

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Diversity of Oral Biofilms in Periodontal Health and Disease

2

Purnima S. Kumar

2.1 The Oral Real Estate

Our knowledge of the human microbiome is changing on an almost daily basis. Although the number of bacteria that colonize the human biome is still being debated [1], we now have evidence that areas in the human body that were normally considered sterile under conditions of health, for example, the placenta, joint cavities, and brain, do in fact contain bacteria [2, 3].

The oral cavity is one of the first portals of entry for bacteria into the human body; therefore, at any given time, there are over two million organisms in this environment, representing nearly 700 distinct species [4]. Each individual carries about 70–120 different species in different oral niches. Several of these species are transient (allochthonous) members, while most are stable colonizers (autochthonous species). Within the mouth, bacteria form organized, cooperating communities linked through energy flow, nutrition, and metabolic networks. These communities are called biofilms, and the bacterial species and all their genes in a biofilm community constitute a microbiome. Biofilms can be found on abiotic surfaces such as the tooth, dental implants, and dental restorations, as well as biotic environments such as the subgingival crevice, tongue, buccal and alveolar mucosa, and tonsils. The subgingival crevice provides 12 cm^2 of surface area for bacterial colonization [5], while the oral mucosa hosts a real estate of more than 200 cm^2 [6]. Together with the tooth surfaces, there is half a square foot of space available for bacterial colonization.

2.2 Bacterial Diversity in Health

Colonization determinants: Colonization of the tooth surface is a complex interplay between both inter-bacterial and host-bacterial interactions. Inter-bacterial interactions promote colonization by providing structural and metabolic support. For example, *Streptococcus*, *Actinomyces*, *Haemophilus*, *Neisseria*, and *Veillonella* are considered pioneer organisms, because they adhere to the acquired salivary pellicle on enamel by specific and non-specific molecular interactions between adhesins on the cell and receptors on the surface [7]. Streptococci contain antigen I/II receptors for salivary agglutinin glycoprotein, which allow them to bind to salivary pellicle, dentin, and collagen as well as to *Actinomyces naeslundii* [8]. *Veillonella* and *Streptococcus*, two of the earliest and most abundant genera to colonize oral biofilms, share a nutritional syntrophy, in that the *Veillonella* utilize the lactate that is produced by the *Streptococcus* as a food source

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[9]. Also, *Streptococcus sanguinis* and *S. oralis* exhibit synergy in degrading mucins [10], as do *Streptococcus mitis*, *S. gordonii*, *Streptococcus cristatus*, and *A. naeslundii* [11], thereby allowing efficient utilization of host glycopolysaccharides for nutrition.

The innate immune responses of the host also play an important role in determining the bacteria that colonize each individual. The sulcular epithelium provides physical, chemical, and immunological barriers against bacterial invasion and, in doing so, determines the community structure of the subgingival biofilm. Chemical mediators of innate immunity include antimicrobial peptides (AMPs). Defensins (alpha and beta) are probably the most well-studied AMPs. Early colonizers have been shown to upregulate AMPs but not cytokines and demonstrate a tolerance to these protein molecules [12]. In contrast to these pioneer organisms, the "orange complex" (described later in this chapter) intermediate colonizers induce high levels of both AMPs and IL-8 and are highly susceptible to both peptide-mediated and phagocyte-mediated killing. The immunological barriers in the sulcus are provided by neutrophils, T cells, dendritic cells, macrophages, and mast cells, which reside in the sulcular epithelium and underlying lamina propria. Complement, which bridges innate and adaptive immune responses, also plays a major role in shaping this indigenous microbiome.

Colonization sequence: Bacterial acquisition into the subgingival crevice occurs in a series of well-orchestrated, hierarchical events, beginning with conditioning of the tooth surface with salivary proteins. Coaggregation and coadhesion are two important mechanisms that play a role in this colonization. Coaggregation is defined as the attachment of genetically distinct bacteria through specific molecular interactions. Coadhesion is the attachment of planktonic cells or coaggregates in suspension to already adherent cells or onto a virgin surface. Weak, long range, van der Waals-type forces create reversible attachment between bacteria and the tooth surface. These forces allow the development of adhesion-mediated attachment to occur. In gram-positive bacteria, adhesin I/II, pilus [13], and surface proteins known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) target fibronectin, collagen, and host extracellular matrix (ECM) [14]. Gram-negative bacteria use pili, autotransporters, and ECM-binding proteins for adhesion [15]. Coadhesion allows for co-localization of metabolic and structural partners within the plaque biofilm. Together, this phase of coaggregation and coadhesion contributes to microbial succession within the biofilm [8].

During the next phase, the biofilm matures through development of an ECM. This interbacterial matrix is highly variable; when gram-positive bacteria predominate, it is very fibrillar due to the presence of dextrans and levans. On the other hand, the gram-negative matrix is very regular and contains trilaminar vesicles, which are filled with endotoxins and proteolytic enzymes and are probably involved in adherence. ECM not only reinforces the physical structure of the biofilm but also creates fluid and communication channels and chemo-osmotic and oxygen gradients. Together with the proximity of specific organisms, this structural configuration triggers specific gene expression patterns and inter-bacterial interactions. During this maturation phase, elaborate food chains are established, and cell-to-cell communication, quorum sensing, and DNA transfer abilities (competency) are enhanced. The final phase of biofilm development is when planktonic cells or coaggregates detach from the parent biofilm and metastasize to other sites.

Benefits of biofilm lifestyle: The biofilm lifestyle changes the phenotype of certain organisms and gene expression patterns. Thus, the functional potential of a biofilm is not merely the sum of the potentials encoded in the species in a planktonic state.

- The biofilm provides a secure means of attaching to the tooth or mucosal surface and protection from friction and shearing forces. This allows for colonization by species that lack attachment abilities as well as those that have fastidious growth requirements. For example, the presence of *Streptococcus cristatus* (a facultative aerobe) promotes colonization and

survival of *Fusobacterium nucleatum*, an obligate anaerobe, since *S. cristatus* uses up the oxygen in the environment. Thus, the biofilm environment creates more "generalists" (organisms that require few host-associated benefits and so can occupy a wide range of habitats, e.g., *Streptococcus*, *Veillonella*, *Fusobacterium*, *Neisseria*, and *Prevotella*) out of species that would normally behave as "specialist" (species that are confined to a single or narrow range of habitats).

- The biofilm provides a barrier against environmental changes. The presence of a robust intercellular matrix creates a diffusion gradient, with minimal diffusion occurring in the interior regions. This creates a buffered region within the biofilm that is not influenced by pH changes induced by food and lifestyle (brushing, mouthwash, carbonated and sugar-rich diet, etc.). This also is a mechanism for the antibiotic and antimicrobial resistance that has been observed in biofilms.
- Transfer of genetic material is crucial to maintaining genetic diversity. Apart from acceptance of DNA (competence), genetic material can be transferred through conjugation, transformation, plasmid transfer, and transposon transfer. Gene transfer has been demonstrated as a mechanism of antibiotic resistance among commensal species like *Streptococcus* and *Neisseria* [16]. Quorum sensing is another benefit conferred on a species through biofilm living [17, 18]. Quorum sensing is regulation of expression of specific genes through accumulation of signaling compounds that mediate intercellular communication and is typically mediated through competence-stimulating peptide (CSP) in streptococci and autoinducers (AI) 1 and 2 in many other species [19, 20]. The *LuxS* gene encodes for AI, which is secreted by both gram-negative and gram-positive organisms. AI-1 and AI-2 turn on in response to cell density. Commensal bacteria produce and respond to low levels of AI-2, while pathogens produce AI-2 in high levels. Thus, the levels of AI-2 may encourage growth of beneficial species and may determine switch from commensal to pathogenic community.
- Antibiotic resistance in dense biofilms. Biofilm bacteria are 1000–1500 times more resistant than planktonic cells [21]. Several reasons have been attributed to this incredible resistance. The primary reason is that antibiotics that target DNA require actively dividing cells for efficacy; and biofilm existence drastically reduces cell turnover rate [22]. These slow growers express non-specific defense mechanisms and make more exo-polymers which retard diffusion by size selection or by binding to the charged antimicrobial agent (diffusion-reaction theory). This ion-exchange mechanism prevents highly charged molecules from reaching deeper zones. The biofilm is also rich in extracellular enzymes (beta-lactamases, formaldehyde dehydrogenase, formaldehyde lyase), which inactivate antibiotics [23]. Importantly, the biofilm lifestyle changes the bacterial phenotype, in that these bacteria express different genes and may demonstrate modification of drug targets. Also, there may be a subpopulation of "persister" organisms within the biofilm, which are specialized survivor cells that neither grow nor die in the presence of microbicidal agents [24].

Benefits of hosting an indigenous microbiome: Biofilm existence also benefits the human host, since bacteria in a biofilm play an important role in preventing exogenous colonization. This colonization resistance may occur by means of effective competition for nutrients and attachment sites, the production of inhibitory factors, and creation of unfavorable growth conditions by resident microflora [7]. Thus, by using very specific mechanisms of aggregation and signaling, as well as by partitioning the available resources within the biofilm, a health-compatible community can prevent colonization by pathogens. This phenomenon, called niche saturation, has been observed in several biofilm systems in the human body, e.g., the gastrointestinal tract, vagina, etc. Recent evidence indicates that bacteria in dental plaque also play an important role in maintaining a healthy biofilm by preventing adhesion of pathogenic species [25]. There is also evidence to indicate that periodontitis is

associated with loss of beneficial bacteria, for example, species belonging to *Veillonella* and *Streptococcus* within the biofilm [26–28].

Evidence has shown some commensal oral bacteria have antagonistic activity against periodontopathogens [29]. Specific examples of bacterial antagonism by means of producing metabolites in the oral cavity include hydrogen peroxide production by streptococcal species to inhibit growth of periodontopathogens [30] and lactic acid production to prevent *Pseudomonas aeruginosa* incorporation into the biofilm [31]. Evidence has shown streptococci exhibit antagonistic properties toward certain staphylococci in the oral cavity as well [32]. Some indigenous microbiota take colonization resistance a step farther by producing specific antibiotics, such as bacteriocin production in strains of *Streptococcus salivarius*, which act on specific pathogens to prevent their colonization of the community [33]. Hillman and Socransky also demonstrated that plaque from periodontally healthy individuals was capable of inhibiting growth of certain periodontal pathogens [30]. It is also being recognized that these bacteria provide immense health benefits to the host, ranging from immune education and homeostasis to prevention of pathogen expansion. The microflora tends to remain stable over time ("microbial homeostasis"), which results from a dynamic balance of microbial interactions, including commensalism, symbiosis, synergism, and antagonism. This stability develops a sense of "familiarity" in the host immune system and establishes a benchmark for "normalcy." The immune system recognizes deviations in community membership (loss or gain of species) or community structure (change in relative abundances of species) and responds to it with an upregulation of immune-inflammatory responses, in an effort to neutralize these deviations from the norm (Fig. 2.1).

Bacterial diversity in health: In examining bacterial diversity in health, it is important to remember that the state of health persists through three dentitions states: primary, mixed, and permanent. Relatively few studies have examined the alteration of microbial profile in a changing dentition through primary, mixed, and permanent stages of dentition. The studies, methodologies, and findings are summarized in Table 2.1. The subgingival plaques of children aged 4–5 years with mixed dentition tend to have a multiform

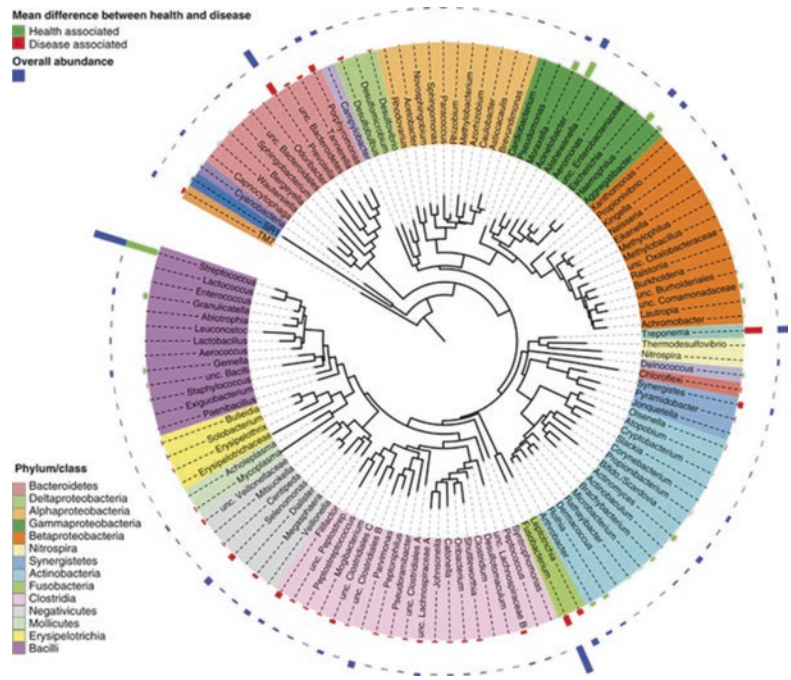

Fig. 2.1 Circular maximum likelihood phylogenetic tree at level of genus. The inner band shows genera colored by phylum or class, the next band shows significant mean differences between healthy controls and deep pockets (*colored green* for genera higher in healthy pockets and *red* for genera higher in disease pockets), and the outer band shows overall relative abundance. Figure is published in Griffen et al. ISME J 2012; 6(6):1176–85. doi: 10.1038/ismej.2011.191

Table 2.1 Evolving concepts in the bacterial etiology of periodontal diseases

Premise	Limitations
Based on Koch discovering a single organism as the etiological factor of tuberculosis	Organisms seen in both health and disease
Concurrent with advances in microscopy	Older individuals had more extensive and severe periodontitis
Fusiforms, spirochaetes, streptococci, amoeba seen in mouth	No single organism could be identified (poor resolution of identification methods)
All bacteria have the potential to cause disease	Plaque levels did not correlate with extent and severity of attachment loss in chronic periodontitis
Disease occurs when the host cannot neutralize bacterial products or toxins	Minimal plaque formation and severe attachment loss characteristically seen in aggressive (juvenile) periodontitis
Successful prevention and treatment dependent on wholesale plaque removal	Plaque control did not prevent continued attachment loss in recurrent and refractory periodontitis
Microscopic and culture-based studies demonstrating that microbial profiles of heathy subjects different from subjects with disease	No single organism or consortia could be associated with disease initiation or progression
Specific bacteria associated with disease progression and recurrence	Several putative periodontal pathogens (e.g., *P. gingivalis*, *T. forsythia*) identified in healthy sites and individuals
A. actinomycetemcomitans recognized as the principal pathogen in localized aggressive periodontitis	Subjects with localized aggressive periodontitis did not always demonstrate *A. actinomycetemcomitans*
Local factors determine the composition of the microbiome	Periodontitis is a multifactorial disease, and the effects of environmental shift cannot be established as a single factor in altering microbial ecology
Change in environment plays a critical role in altering ratio of beneficial bacteria and pathogens	Unlike dental caries, the effect of environmental changes on a single species is not easy to establish
Specific bacteria modulate the host response to improve their survival	Keystone species are not definitively identified
These bacteria, known as keystone species, promote growth of other species (accessory pathogens)	Emerging evidence indicates that disease sites are functionally similar and different species contribute to similar functions
Keystone species do not have to be in large numbers to effect their changes	Functional genes, rather than species, may be hallmarks of disease and health

gram-negative, anaerobic bacterial composition, some of which are suspected periodontal pathogens [34, 35]. It has been reported from culture studies that *Gemella morbillorum* and *Peptostreptococcus magnus* are statistically significantly more frequently detected in incisors, while *P. micros*, *S. intermedius*, *B. forsythus*, *Fusobacterium nucleatum*, *Prevotella loescheii*, *P. melaninogenica*, and *Selenomonas sputigena* are more frequently detected in molars. Periodontal pathogens can be detected in children under 3 years old [36] as well as in young, periodontitis-free children at a carrier state with no signs of destructive periodontitis [37]. Several investigators have found, using culturing and DNA-DNA checkerboard, that *Actinomyces*, streptococci, and *Veillonella* may be indigenous, host-compatible organisms [38]. These findings are in agreement with the results of other culture studies that examine the prevalence of subgingival species in sites of gingival health and other clinical conditions. Ximenez-Fyvie et al. reported a significantly larger proportion of *Actinomyces* species occurred in subjects that were periodontally healthy [39]. Molecular studies using culture-independent approaches (e.g., 16S rRNA amplification, FISH) have demonstrated considerable diversity of subgingival microflora in health [21]. *Veillonella* sp. oral clone X042, a gram-negative bacterium, was found to be the most common bacteria detected in a study using 16S cloning and sequencing in a study of adults who were periodontally healthy [27]. In another study, Kumar et al. [26] found higher proportions

of *Streptococcus*, *Abiotrophia*, *Gemella*, and *Veillonella* in plaque of periodontally healthy adults. Aas et al. [4] reported that in periodontally healthy adults (age range 23–55), the most common bacteria found by 16sRNA gene and PCR detection were *Gemella*, *Granulicatella*, *Streptococcus*, and *Veillonella* in a variety of sites sampled, including the tongue, buccal fold, hard and soft palate, labial gingiva and tonsils of soft tissue surfaces, and supra- and subgingival plaque. Ledder et al. [40] reported incidence rates of 52 bacteria in healthy and diseased adults (mean age 40.1 years, range 20–55 years) using multiplex PCR analysis. Bacteria that were found with the highest incidence were *Pseudomonas* sp. (56%), *Streptococcus mitis* (50%), *Streptococcus sanguinis* (44%), and *Neisseria* sp. (44%). Additionally, four bacteria were associated exclusively in healthy patients at an incidence rate of 17% and were non-detectable in diseased subjects: *Staphylococcus* sp. (AB167056), *Sphingobium yanoikuyae* (AJ627009), *Corynebacterium matruchotii* (X82065), and *Streptococcus mutans* (AE014854). The association of *C. matruchotii* with healthy gingiva has been previously reported [41]. Ledder et al. was unable to correlate biodiversity with either health or disease, and no significant differences were found between the groups ($p > 0.05$). All of these findings support the hypothesis that bacteria play an important role in immune education; the host immune system recognizes a biofilm composed of a specific ratio of organisms as "friend." Periodontal health is the result of this immune tolerance. When the proportions of organisms within a community change, this balance is upset and leads to an immunoinflammatory response, which results in disease.

2.3 Bacterial Diversity in Periodontal Disease: Historical Perspective

The presence of bacteria in dental plaque has been known since 1683, when Antonie van Leeuwenhoek first described his observations of "animalcules" in dental plaque in letters to the Royal Society of London. However, for several centuries plaque was thought of as an amorphous collection of bacteria. In 1882, Koch published a treatise describing a specific bacterium as the etiological agent of tuberculosis along with criteria for establishing an organism as the etiological agent of a disease [42]. This began the search for species involved in the etiology of several other communicable diseases. The time period from 1880 to 1930 became known as the golden age of microbiology. During this period, many microbial pathogens were linked to specific infections in the body. This led oral health researchers to seek out specific pathogens for the etiologic agent of dental caries and periodontal disease, and the age of the specific plaque hypothesis was born. Several possible agents were isolated; however, no specific bacterium could be identified as the causative agent. Further, no organism could be isolated only from diseased individuals; on the contrary, organisms found in disease were also found in health.

Another important evidence came from epidemiological data indicating that the prevalence of periodontal disease was higher in older individuals [43]. While it was later realized that this was because of the cumulative destruction from the disease, at the time it suggested that the presence of plaque, and not necessarily specific organisms, resulted in disease. As a result, the 1930s brought a new view—the non-specific plaque hypothesis [44]. This new hypothesis evolved around the idea that all plaque was considered pathogenic and diseases associated with plaque arose from "elaboration of noxious products by the entire plaque flora" [44]. Large amounts of plaque would lead to production of toxins that could overwhelm the host defenses and result in disease. This hypothesis led to wholesale removal of plaque by surgical or nonsurgical methods and rigorous homecare as therapeutic options for periodontal disease.

The 1960s brought another change with many advances in bacterial detection and characterization. An important discovery was that mutans streptococci (*S. mutans* and *S. sobrinus*) were the primary pathogens in the etiology of dental caries

[45]. Evidence also suggested that subgingival plaque behaved differently when certain species were present [46]. Also, longitudinal studies revealed that many individuals with significant amounts of plaque accumulation never developed destructive disease [47]. Further, within any individual, the disease was seen to be site specific, that is, only certain teeth demonstrated large amounts of destruction and these sites were seen in close juxtaposition to normal sites. The development of better techniques for microbial characterization demonstrated significant differences in the microbial profiles of periodontal health and disease. Thus, the specific plaque hypothesis was again adopted, as it was believed specific microbes were responsible for disease and once identified would allow for targeted treatment of the disease. This concept gained acceptance when *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* was recognized as the predominant pathogen in localized aggressive periodontitis.

Investigations on the influence of the oral environment on the structure and composition of microbial communities led to the development of the ecological plaque hypothesis [7]. This hypothesis suggests that the resident microflora undergoes a transformation from a commensal to a pathogenic population due to environmental perturbations, for example, pH, oxygen tension, flow of gingival crevicular fluid, and presence of blood and blood products. While the ecological hypothesis is similar to the specific plaque hypothesis in recognizing the varying pathogenic potentials of individual bacterial species, it maintains that perturbation in the microbial homeostasis is the result of environmental shifts. Thus, treatment of disease does not only require targeting specific species, it is also important to alter the environment from one that promotes pathogen enrichment to one that is compatible with commensal growth.

A more recent line of thinking has resulted in the polymicrobial synergy and microbial dysbiosis theory [48]. According to this, certain species (called keystone pathogens) modulate the host immune response and, therefore, the local ecosystem [48, 49]. These species do not have to be in high abundances to effect this change. This modulation not only allows these organisms to manipulate resources to survive in the environment, it also encourages the growth of other selected members of the indigenous microbiome, which now act as accessory pathogens. This polymicrobial synergy that is orchestrated by species such as *P. gingivalis* leads to the development of a dysbiotic microbiome, which results in disease.

The identification of specific causative species, or periodontopathogens, has been hampered by some of the unique features of periodontal diseases. The foremost of these is that disease occurs in a site already colonized by an indigenous microbial community. Thus, it is difficult to differentiate between species that cause the disease and species that are present as a consequence of the disease. For example, it has been shown that *Capnocytophaga* spp. are seen in high levels prior to the onset of gingivitis, while *Prevotella* spp. are detected in areas with established gingivitis. Thus, *Capnocytophaga* is more likely an etiological agent, and *Prevotella* species are present as a consequence of the disease process [50]. Colonization by exogenous pathogens is thought to contribute to the episodic nature of disease progression [51], i.e., the fact that not all sites with baseline attachment loss demonstrate the same rate of disease progression or disease activity at the same time points.

2.4 Specific Microorganisms Associated with Periodontal Health and Disease

Different periodontal diseases have fairly unique profiles of associated bacteria. This, along with the fact that disease occurs in sporadic bursts in the mouth, strengthens the evidence for the role of specific microorganisms in disease causation and progression.

Periodontal health: Bacteria that are associated with periodontal health include primary or early colonizers such as *Streptococcus sanguinis*,

Streptococcus mitis, *Gemella* spp., *Atopobium* spp., *Fusobacterium nucleatum*, and *Capnocytophaga* spp. [41, 52, 53]. Species belonging to the genera *Veillonella*, *Streptococcus*, and *Capnocytophaga* are thought to be beneficial to the host [53]. Molecular analysis has shown the presence of certain uncultivated species such as *Bacteroides* oral clone BU063 strongly associated with periodontal health [54] (Fig. 2.2).

Gingivitis: Gram-positive species, for example, *Streptococcus* spp., *Actinomyces viscosus*, and *Parvimonas micra* (formerly *Peptostreptococcus micros*), as well as gram-negative species such as *Campylobacter gracilis*, *F. nucleatum*, *Prevotella intermedia*, and *Veillonella*, have been associated with gingivitis [55–57]. Pregnancy-associated gingivitis, however, has a microflora predominated by *P. intermedia* [58].

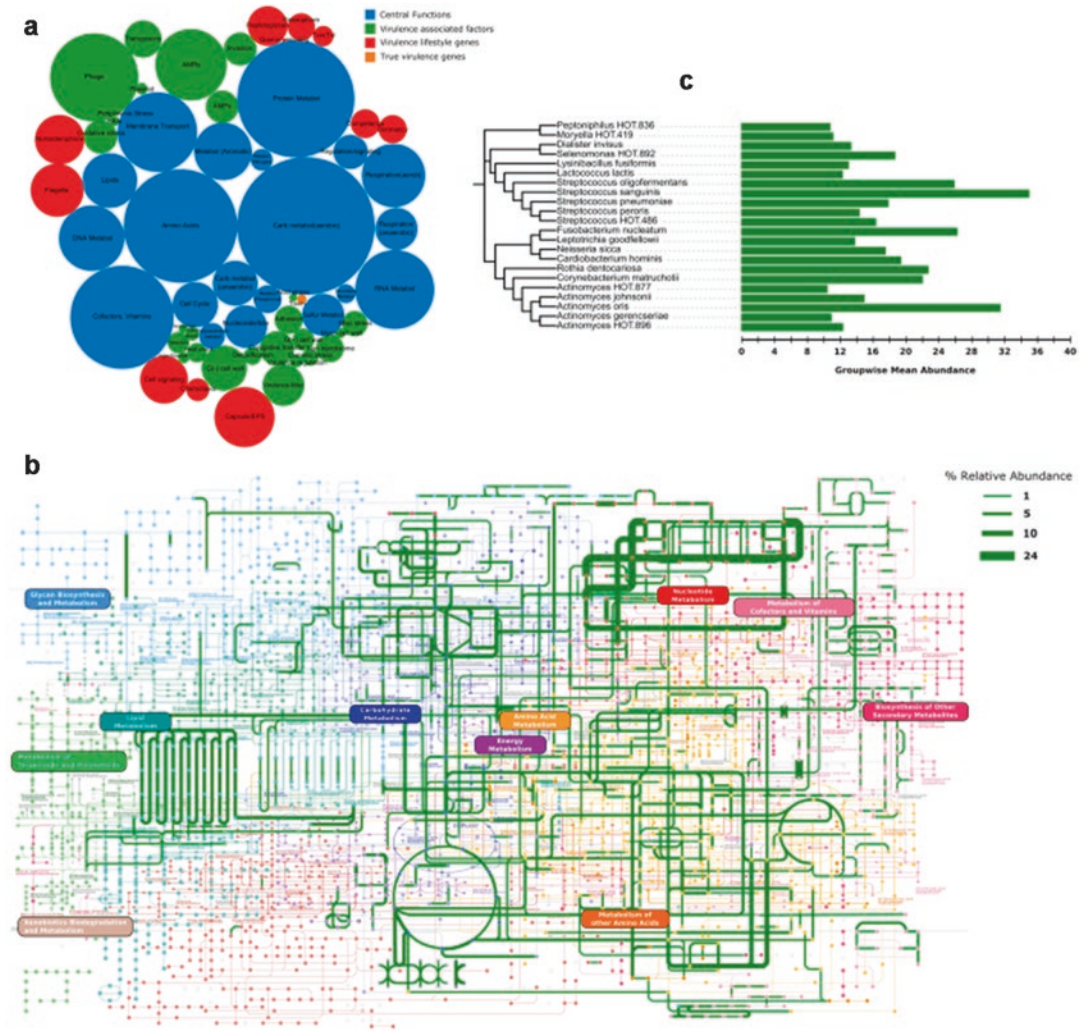

Fig. 2.2 Graph of core genes grouped into higher order functions. *Circles* are sized by relative abundances of genes contributing to each function (**a**). Core metabolic pathways in the health-associated microbiome. The *lines* are sized by log fold abundances (**b**). A selected group of species that contributed to these functions. The species shown here belonged to the core microbiome (80% or more of healthy individuals). The *green bars* represent the relative abundances of the species in all samples (**c**). Figure is published in Dabdoub et al. Sci Rep 2016; 6: 38993. doi: 10.1038/srep38993

Chronic periodontitis: The bacterial profile of chronic periodontitis has been explored in cross-sectional and longitudinal studies. The effect of various treatment methods on changing the microbial ecology has also been investigated. *P. gingivalis*, *T. forsythia*, *P. intermedia*, *Campylobacter rectus*, *Eikenella corrodens*, *F. nucleatum*, *A. actinomycetemcomitans*, *P. micros*, and *Treponema* spp. have been most commonly found. *P. gingivalis*, *T. forsythia*, *P. intermedia*, *C. rectus*, and *F. nucleatum* are found in higher levels in sites with active disease or with progressing disease [59–62]. Clinical resolution of disease is also associated with a decrease in the levels of these species. More recent molecular approaches have found uncultivated bacterial species, such as *Desulfobulbus* sp. oral clone *R004*, *Deferribacteres* sp. oral clones *BH017* and *D084*, and *Bacteroides* sp. oral clone *AU126*, to be significantly associated with periodontitis [41, 63].

Localized aggressive periodontitis (LAP): Studies have implicated *A. actinomycetemcomitans* as an important organism in the etiology of LAP [64–66]. This species has been found as the predominant cultivable species in as many as 90% of sites with LAP. However, it should be noted that not all studies support the association of *A. actinomycetemcomitans* in aggressive periodontitis. This organism is not always found in disease sites; further, it has been found in healthy children, suggesting that it is a member of the healthy microbial flora [67]. Other species such as *P. gingivalis*, *E. corrodens*, and *C. rectus* have also been found in high levels in certain cases of LAP [68]. Viruses such as Epstein-Barr virus (EBV-1) and human cytomegalovirus (HCMV) have also been associated with this disease [69].

Generalized aggressive periodontitis (GAP): The microbial etiology of generalized aggressive periodontitis is not as well defined as other forms of periodontal diseases due to multiple transformations in disease nomenclature. The disease now encompasses entities such has periodontosis, prepubertal periodontitis, and rapidly progressing periodontitis. Nevertheless, available evidence suggests that the bacterial profile of generalized aggressive periodontitis is not significantly different from that of chronic periodontitis [70–72].

Necrotizing ulcerative gingivitis: The bacterial flora of necrotizing ulcerative gingivitis has been demonstrated to be composed, for the most part, of fusobacteria and spirochetes. Recent studies have isolated previously unsuspected spirochetes, e.g., *Treponema putidum*, a proteolytic treponeme, from lesions of necrotizing ulcerative gingivitis [73]. Other bacteria reported in these lesions include *Rothia dentocariosa*, *Treponema* spp., *Achromobacter* spp., *Propionibacterium acnes*, *Capnocytophaga* spp., and *P. intermedia* [52].

Periodontal abscess: A periodontal abscess is a localized purulent infection within the tissues adjacent to the periodontal pocket. *F. nucleatum*, *P. intermedia*, *P. micra*, *T. forsythia*, *C. rectus*, and *P. gingivalis* have been recovered from these lesions [74, 75].

Conclusion

Bacteria are acquired in subgingival biofilms in a sequential manner to form organized, cooperating communities called biofilms. These biofilms are composed largely of organisms with low inflammatory potential, and they play an important role in preventing expansion of inflammophilic organisms (or pathogens). They also educate the immune system to recognize "friend and foe." This immune tolerance is the basis for health and is dependent on continuous crosstalk between a stable microbiome and the host immune system. Changes in the local environment (short term, such as food intake, or long term, such as smoking) can alter these ecosystems, resulting in dysbiosis, which forms the etiologic basis for all oral diseases.

Clinical Implications

- Oral biofilms are formed by sequential acquisition of pioneer species, followed by secondary and tertiary colonizers.
- Commensal organisms play important role in reducing pathogen colonization and immune education.
- Stable bacterial communities are important for health.
- Biofilms are difficult therapeutic targets, since they are resistant to chemo-mechanical disruption.
- Disease occurs due to dysbiosis within these communities, with decrease in levels of commensals and pioneers and increase in levels of secondary and tertiary colonizers.
- Change in the oral environment induced by smoking, uncontrolled hyperglycemia, pregnancy, etc. leads to the creation of dysbiotic communities.

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Subgingival Biofilms as Etiological Factors of Periodontal Disease

3

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3.1 Definition of Subgingival Plaque as a Biofilm

A biofilm is a structured community of microbial cells embedded in a self-produced (hydrated) matrix extracellular polymeric substance (EPS) and adherent to an inert or living surface, as defined by Costerton [1] and modified in 2012 by IUPAC [2]. Microbial cells growing in a biofilm differ physiologically from planktonic cells of the same organism, which are swimming or floating single cells in a liquid medium. Although the fact that microorganisms are able to grow attached to solid surfaces was reported already in 1936 by Zobell [3], it took more than 40 years until it was recognized that in nature most bacteria grow in biofilms attached to a surface rather than growing planktonically [1, 4]. A cell switching to the biofilm mode of growth undergoes a phenotypic shift in behavior with many genes being differentially regulated [5]. Biofilms may be formed in response to factors such as recognition of attachment sites on a surface, nutritional signals, or protection from harmful conditions [6–8]. Living in a biofilm represents a universal survival strategy of microorganisms on our planet. It allows microorganisms to colonize new ecological niches and survive in hostile environments thereby adopting biofilm structure in response to environmental conditions [9, 10]. The dense and perplexed structure of a biofilm not only hampers diffusion of molecules, but it also forms a barrier against the host's defense mechanisms such as antibodies, lysozyme, or against other antimicrobial agents.

The formation of a biofilm takes place in five sequential stages, namely, initial attachment, irreversible attachment, maturation I, maturation II, and dispersion [11, 12]. During initial attachment, free-floating microorganisms attach to a surface. While still not fully understood, it is thought that the first colonists of a biofilm adhere to the surface initially through weak, reversible adhesion via van der Waals forces and hydrophobic effects [13]. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili (irreversible attachment). Some species are not able to attach to a surface on their own but are instead able to anchor themselves to the matrix or directly to earlier colonists. It is during this colonization (maturation I) that the cells are able to communicate via quorum sensing (QS) using small diffusible signal molecules [14–16]. Once colonization has

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begun, the biofilm grows through a combination of cell division and recruitment (maturation II). Extracellular polymeric matrices consisting mainly of polysaccharides, proteins, nucleic acids, and lipids typically enclose bacterial biofilms [17]. The EPS matrix is an important key to the evolutionary success of biofilms. One reason is that it traps extracellular enzymes and keeps them in close proximity to the cells. Thus, the matrix represents an external digestion system and allows for stable synergistic microconsortia of different species [17]. The final stage of biofilm formation is known as dispersion and is the stage in which the biofilm is established and may only change in shape and size. Dispersal of cells from the biofilm colony is an essential stage of the biofilm life cycle. Dispersal enables biofilms to spread and colonize new surfaces [18]. Enzymes that degrade the extracellular matrix, such as dispersin B and deoxyribonuclease, may play a role in biofilm dispersal [19, 20]. Biofilm matrix-degrading enzymes may therefore be useful as anti-biofilm agents [21]. Biofilms are formed on various surfaces, e.g., on boat hulls, water pipelines, artificial heart valves, or on teeth, the latter commonly known as dental plaque.

3.2 Oral Biofilms: Microenvironment and Etiological Role in Oral Disease

Biofilms on the tooth surface can grow under both aerobic and anaerobic conditions, depending on the concentration and partial pressure of the available oxygen. Hence, both aerobic and anaerobic microorganisms can be encountered in these biofilms. The rich diversity of the oral microbiota allows for ample combinations of different microorganisms to be present within a biofilm and define its composition. This microbial affluence can result in very complex microbial combinations that vary between individuals or even between sites of the same individual. However, this should not be perceived as a random colonization event, as the local microenvironmental conditions actually define which of the microorganisms are best fit to grow as a biofilm, within a given site. Throughout the mass of the biofilm, "microgradients" for physicochemical parameters are established, including temperature, redox potential, oxygen partial pressure, pH, and diffusion of nutrients. Hence, the bacteria most well adapted to these conditions are eventually going to survive and grow.

The "ecological plaque hypothesis" summarizes the current concept on the relationship between oral bacteria, or biofilms, and the development of common oral infectious diseases, such as dental caries and periodontal disease [22]. This hypothesis proposes that under normal conditions, the oral bacteria and the host tissues are in a dynamic health-compatible balance. The microenvironment may undergo local changes, causing a breakage in this homeostatic balance and subsequently shifts in the biofilm's microbial composition. Under the newly established conditions, quiescent opportunistic pathogens can now become prolific and more virulent, leading to oral disease. Microbes typically associated with the disease may still be present at a healthy site, albeit at too low numbers and proportions to be deleterious.

3.2.1 Subgingival Biofilms and Their Association with Periodontitis

The dental plaque that grows on the tooth surface in the abstinence of oral hygiene is indeed a polymicrobial oral biofilm, comprised of hundreds of different microbial species. A biofilm growing on the surface of the dental enamel and above the free gingival margin is termed supragingival, whereas the one that grows on the dental cementum surface underneath this margin is termed subgingival. The development of periodontitis, which is the primary cause for tooth loss in adults, is associated with the formation of subgingival biofilms within the periodontal pocket, a clinical sign of the progressing disease. The mechanisms of pathogenesis of periodontitis are

centered around the inflammation caused to the periodontal tissues by subgingival biofilms. Before describing in more detail the composition of subgingival biofilms, it should be noted that species characterized as "common periodontal pathogens" have been isolated and detected more frequently and in higher numbers in periodontal disease than health [23].

3.2.2 Composition and Structure of Subgingival Biofilms: General Concepts

The first microorganisms that colonize the oral cavity are called pioneer species. The predominant pioneer organisms in the mouth are streptococci, in particular, *S. salivarius*, *S. mitis*, and *S. oralis* [24]. A key element for initial colonization and subsequent biofilm formation is communication among microorganisms [25]. By and by the environment changes due to metabolic activity of the pioneer organisms affecting, e.g., pH, redox potential, or nutrient supply, enabling colonization of other microorganisms. This succession eventually leads to a stable situation with increased species diversity [26].

The microenvironment of the periodontal pocket is an anaerobic one, with high protein amounts, due to the constant presence of the inflammatory exudate of the tissue in periodontal disease (gingival crevicular fluid). Therefore, it is rational that the types of bacteria favored to colonize, grow, and form subgingival biofilms in this milieu are anaerobic and proteolytic. It is now well established that the switch from periodontal health to periodontal disease is associated with the conversion of a Gram-positive aerobic and nonmotile microbes to Gram-negative, anaerobic, and motile ones [23, 27]. The changes in the composition of biofilms during the conversion from health to disease have also been described according to the shapes of the observed bacteria (i.e., morphotypes), using dark field microscopy techniques [28, 29]. These have documented that biofilms sampled at periodontally healthy sites are primarily colonized by cocci and a few rods, but almost no fusiform, coiled (i.e., screwlike), or motile bacterial types. However, the gradual conversion to gingivitis and subsequently to periodontitis is marked by an increase in fusiforms, long filaments, and coiled and motile bacterial types (such as spirochetes).

The three red complex species according to the classification by Socransky (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*) [27, 30] are the most extensively studied and discussed putative periodontal pathogens to date (Fig. 3.1). All three are highly proteolytic anaerobes and highly associated statistically with deeper periodontal pockets and higher prevalence in periodontal disease. Additional bacterial species present in subgingival biofilms and closely associated with periodontal disease are *Prevotella intermedia*, *Eikenella corrodens*, *Campylobacter rectus*, *Fusobacterium nucleatum*, and *Aggregatibacter actinomycetemcomitans* [27]. Otherwise, the presence of *Streptococcus sanguinis*, *Streptococcus mitis*, and various *Actinomyces* spp. in the biofilms is associated with periodontal health rather than disease [31–33]. Recent studies using cutting-edge molecular detection methodologies are pointing to specific "microbial signatures" in subgingival biofilms that can distinguish between periodontal health and disease [34]. These will be elaborated in more detail in the following section of this chapter.

The microbial vitality varies throughout the biofilm as confocal laser scanning microscopy (CLSM) analyses of live/dead stained biofilms prove, with the most viable bacteria present in the central part of biofilms [35]. Regarding individual bacterial localization within subgingival biofilms, in situ studies demonstrated that putative periodontal pathogens are located at the outer extremity of the biofilms, in close proximity to the gingival tissue and cells of the immune system (e.g., neutrophils). Regarding the structure of the biofilms, lactobacilli are centrally located within the bacterial aggregates, and streptococci together with *Candida albicans* yeast form corncob structures within the biofilms. Moreover, putative periodontal pathogens may already colonize mature biofilms and form microcolonies therein [36].

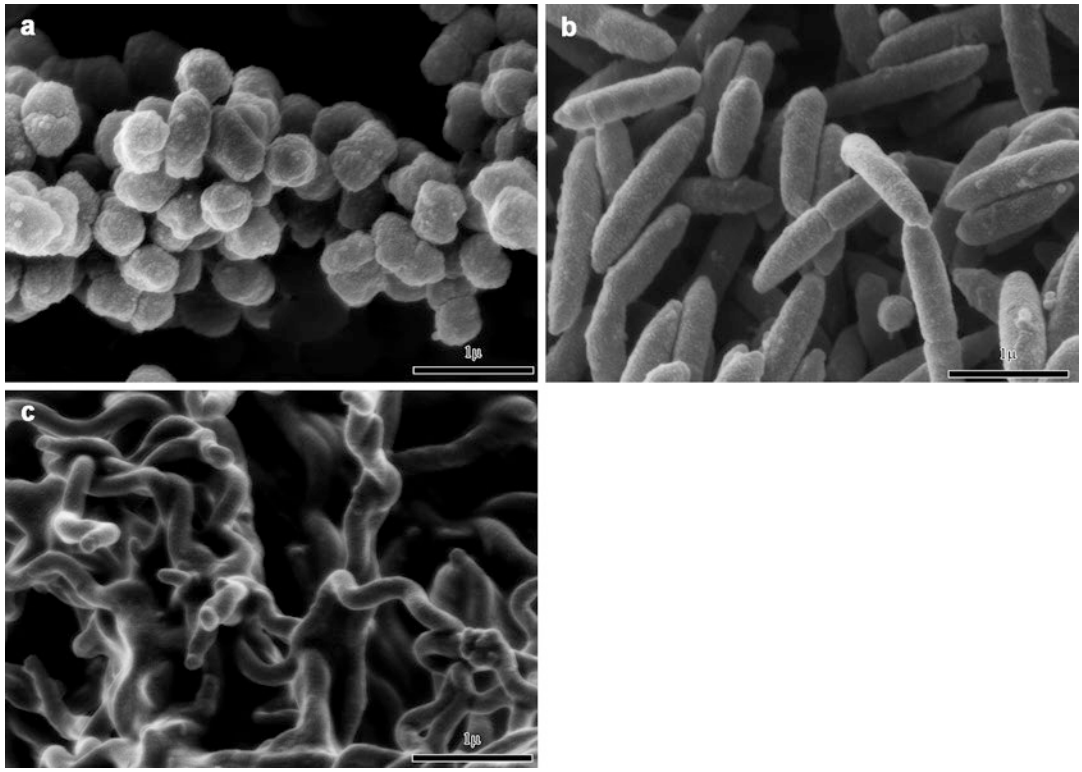

Fig. 3.1 Scanning electron microscope images of the three members of the "red complex" bacteria, *Porphyromonas gingivalis* (**a**), from the phylum *Bacteroidetes*, is a nonmotile, asaccharolytic, Gram-negative, obligately anaerobic rod, forming black-pigmented colonies on blood agar plates; (**b**) *Tannerella forsythia*, formerly *Bacteroides forsythus*, belonging also to the phylum *Bacteroidetes*, is a Gram-negative, obligately anaerobic, nonmotile fusiform rod; (**c**) *Treponema denticola*, a member of the phylum *Spirochaetes*, is a Gram-negative, obligate anaerobic, motile, spiral-shaped, and highly proteolytic spirochete bacterium

3.2.3 Subgingival Biofilm Composition as Revealed by Metagenomics

Our established knowledge on the microbial composition of the subgingival biofilms and the association of certain bacteria with periodontitis comes mostly from studies using conventional bacterial cultures. However, most microorganisms of the oral cavity cannot be cultured in the laboratory [37]. Therefore, culture-independent detection methods applied in oral samples allow for the discovery even of species that we are not yet able to cultivate, which are crucial in understanding the composition of oral biofilms.

Many of these commonly used molecular methods were advent of different 16S ribosomal RNA-based phylogenetic methods, which originated from the 1980s [38], such as the "checkerboard" DNA-DNA hybridization [39], polymerase chain reaction (PCR) [40], and microarray chip [41]. These ever-evolving molecular methods are rapidly increasing our understanding of the biofilm composition. Recently, researchers started to shear entire DNA samples by the "shotgun" approach, followed by the "next-generation sequencing" (NGS) technologies [42]. This "shotgun" approach could clearly distinguish closely related subgingival species using complete reference genomes provided from National Center for Biotechnology Information (NCBI), Human Microbiome Project (HMP), or Human Oral Microbiome Database (HOMD). Using combined "shotgun" and 16S rRNA sequencing for analyz-

ing subgingival biofilms, one study predicted periodontal disease cases with an accuracy of 81.1% and revealed the potential of different microbial taxa for monitoring disease progression [43]. However, all these techniques are not without limitations. DNA-DNA checkerboard hybridization or microarray chips normally can only provide analytical input for a limited set of species, methods requiring PCR amplification can be biased due to different chemistry settings [44], and NGS-based technologies highly rely on the available databases and computational abilities.

We now know that the microbiota constituting the subgingival biofilm is far more complex than initially thought based only on bacterial culture-dependent methods. A recent study has estimated that as many as 19,000 different bacterial taxa can be identified in the human oral cavity [45], a number which is far higher than previously reported results (around 700 species) using culture-based or cloning methods [46]. Contemporary research in periodontal microbiology is now stepping into the fast-evolving field of metagenomics. By definition, metagenomics is the study of genetic material recovered directly from environmental samples using large-data analysis methods. Complex subgingival biofilms obtained from periodontal pockets may well be considered as such environmental samples.

Using metagenomics for analyzing subgingival biofilm samples, researchers were able to identify less-known uncultivable microbes associated with periodontal disease, such as *Peptostreptococcus stomatis*, *Filifactor alocis*, *Desulfobulbus*, *Dialister*, *Megasphaera*, *Synergistetes*, *Deferribacteres*, and TM7 [38, 39, 47–49]. Furthermore, a systematic review of the literature highlighted at least 17 species or groups of microbes, not previously considered to be associated with periodontal disease [44]. In recent years, the contribution of phages in the formation of subgingival biofilm was also brought into light with the help of metagenomic technologies [42, 50, 51].

Distribution of subgingival species present in different conditions or niches is another major advance brought in by metagenomics. From more than 774 genera identified in dental biofilms, Tsai et al. distinguished eight and six of those to be significantly enriched in healthy individuals and severe chronic periodontitis patients, respectively [52]. Ge and his colleagues also found that bacterial abundances were significantly altered between shallow and deep sites of periodontal patients [53]. Species diversities between healthy and periodontal individual were also reported in other different studies [54]. *Porphyromonas*, *Tannerella*, *Treponema*, and *Filifactor* numbers were found to decrease after treated by antimicrobials and root planning [55]. However, another metagenomic study showed that antibiotics have great influence on the subgingival biofilm composition 3 months after therapy, but these changes do not remain effective as long as 6 months [56]. Interrelationships between periodontal infections with other diseases or conditions were also revealed by metagenomics. A recent study has showed higher level of *Fusobacteriaceae* and lower level of *Prevotellaceae* in healthy elder individuals, compared to matched individuals with dementia [57]. Further studies have reported that both smoking and pregnancy are also affecting the overall microbial compositions of subgingival biofilms [58–60].

In summary, metagenomics and the associated technologies for microbial detection have proved to be a powerful tool for the understanding of the composition of subgingival biofilms, providing higher accuracy and efficiency compared to previous approaches.

3.3 In Vitro Modeling of Subgingival Biofilms

Artificial subgingival biofilms can be generated in the microbiology laboratory in order to study their behavior in vitro and deduce conclusions for their behavior in vivo. That can be clinically very useful, for instance, in understanding the role of the different biofilm bacteria in the pathogenesis of the disease but also for testing the antimicrobial efficiency of different agents, prior to being applied for patient treatment. According to our

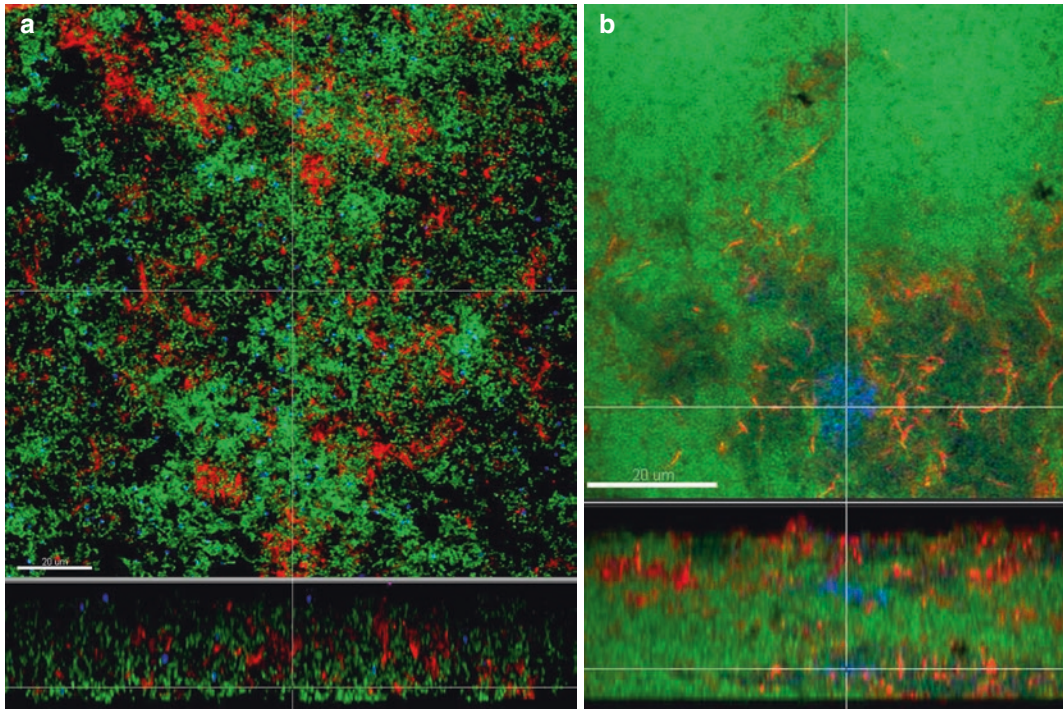

Fig. 3.2 Confocal laser scanning microscopy images (CLSM) of a fluorescence in situ hybridization (FISH)-stained supragingival (**a**) and subgingival (**b**) biofilm, respectively. Bacteria appear green and *Fusobacterium nucleatum* in particular appears *red* in (**a**) and (**b**), whereas streptococci appear *blue* in (**a**) and *Porphyromonas gingivalis* appears *blue* in (**b**). The biofilm base in the cross section is directed toward the top view. Scale bars: 20 µm

earlier studies [61], there are several requirements that should be met by a good model system. Most importantly, results generated by the model system should be reproducible. Due to the enormous microbial complexity of periodontal disease, there are several obstacles to overcome in order to successfully build a model system. The overwhelming amount of different species as well as the diversity of their appearance is a limiting factor that restricts the prospect of matching the in vivo situation accurately with a model system.

The Zürich biofilm model has been established more than a decade ago and was designed as a fully defined, in vitro batch model system first as supragingival model consisting of six oral microorganism characteristics for supragingival plaque [62–65], which later was extended to a subgingival model [66–71]. The ten bacteria used in that model were selected according to published observations concerning biofilm formation and periodontal disease. An aim was to incorporate the main disease-associated, "red complex" species [27]. To facilitate their incorporation, other species were selected with the goal to provide a suitable matrix in terms of attachment receptors [32] and redox potential, while further nutritional conditions still remained to be optimized. In Fig. 3.2, CLSM images of the supragingival and the subgingival model are shown. The model system proved to produce stable and reproducible biofilms, which in proximity to cultured human epithelial cells induced cellular apoptosis [66], and a number of histopathological [70, 72] and protein changes known to be associated with periodontal diseases [73].

Conclusive Remarks

Subgingival biofilms are complex microbial communities that colonize the periodontal pocket during the progression of periodontal disease. The bacteria that manage to thrive within the biofilm are the ones best adapted to do so in microenvironment conditions that govern the periodontal pocket. Although some of the included species are understandably labeled as "pathogens" due to their high association with periodontal disease, they can better be described as "putative" or "opportunistic" pathogens, since they can be occasionally found in health, albeit at low numbers. No one of these bacteria should be single-handedly considered as the etiological factor for the disease, as they all act in concert within the biofilm community to instigate periodontal inflammation. The disease itself is a result of a disadvantageous interaction between the subgingival biofilm and the host inflammatory immune response, which becomes insufficient to tackle the establishing chronic infection and destructive.

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Bacterial Virulence Factors that Contribute to Periodontal Pathogenesis

4

Anders Johansson and Gunnar Dahlén

4.1 Overview

In this chapter, the role of different microbial virulence factors in relation to the pathogenesis of periodontal diseases is addressed. These factors are molecules produced by pathogens and contribute to their pathogenicity by promoting colonization and affecting host response. The importance of different virulence factors in the life of the oral biofilm and the interplay with the host's response is exemplified here by two of the major, and most well studied, periodontal pathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. Both of these microbes have great genetic intraspecies diversity and express a number of different virulence factors, which have the capacity to cause imbalance in the host's response. *A. actinomycetemcomitans* is the major pathogen in aggressive forms of periodontitis (Fig. 4.1) that affect young individuals, while *P. gingivalis* is frequently detected in periodontal pockets of individuals with the chronic forms of the disease (Fig. 4.2). However, the role of these two bacteria in periodontal breakdown is still not entirely clear.

4.2 Pathogenesis of Periodontal Disease

Periodontitis is a microbe-induced inflammatory disease that affects the tooth-supporting tissues, bone and connective tissues. The commensal oral microbiota directly colonizes the surfaces of the oral mucosa and the teeth or the microbiota colonizes an already established biofilm. These surfaces of the oral cavity provide specific receptors for ligand binding and glycolipid structures, which serve as targets for more unspecific bacterial adhesion. The role of specific pathogens for this degenerative disease is gradually being elucidated, but it is generally accepted that it accounts for the combination of the total bacterial load, the release of specific virulence factors, and the effects on the host's response. Therefore, the periodontal bacterial species that are associated with periodontal disease could be described as "opportunist pathogens." In a clinically healthy situation, there is a balance between the periodontal microbiota in a biofilm and the host response of the gingiva. That balance results in a protective immune response from the host that

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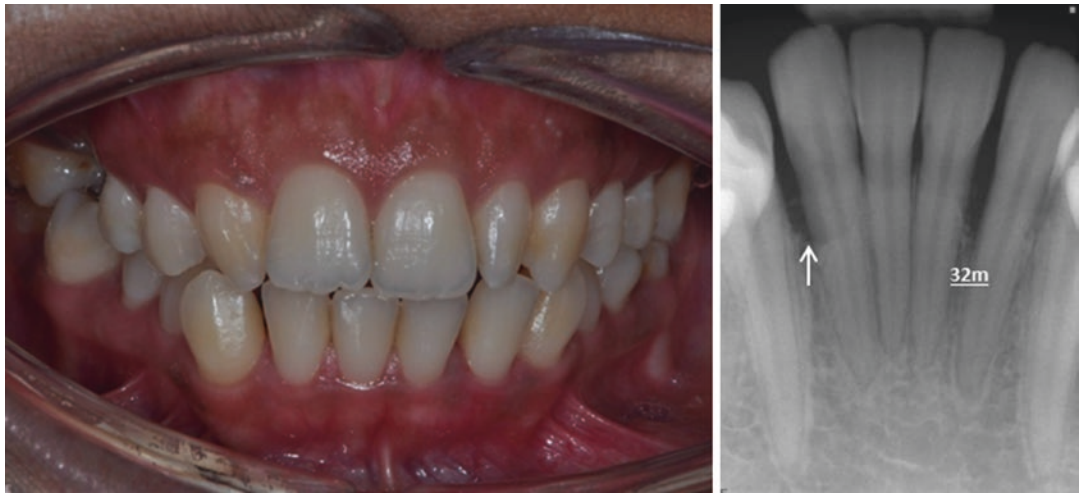

Fig. 4.1 Clinical presentation of localized aggressive periodontitis. A 16-year-old female presenting with radiographic alveolar bone loss associated with bony defects (marked with *arrows*) and probing attachment loss at the lower incisors. The clinical presentation shows sparse plaque accumulation and localized gingival inflammation with 4–8 mm periodontal crevices with bleeding on probing in the affected region. Microbiological analysis, by cultivation technique, confirmed the presence of high levels (7.7×10^6) and proportions (92%) of *A. actinomycetemcomitans* in the sampled lesion (32 m). By courtesy of Dr. Carola Höglund Åberg

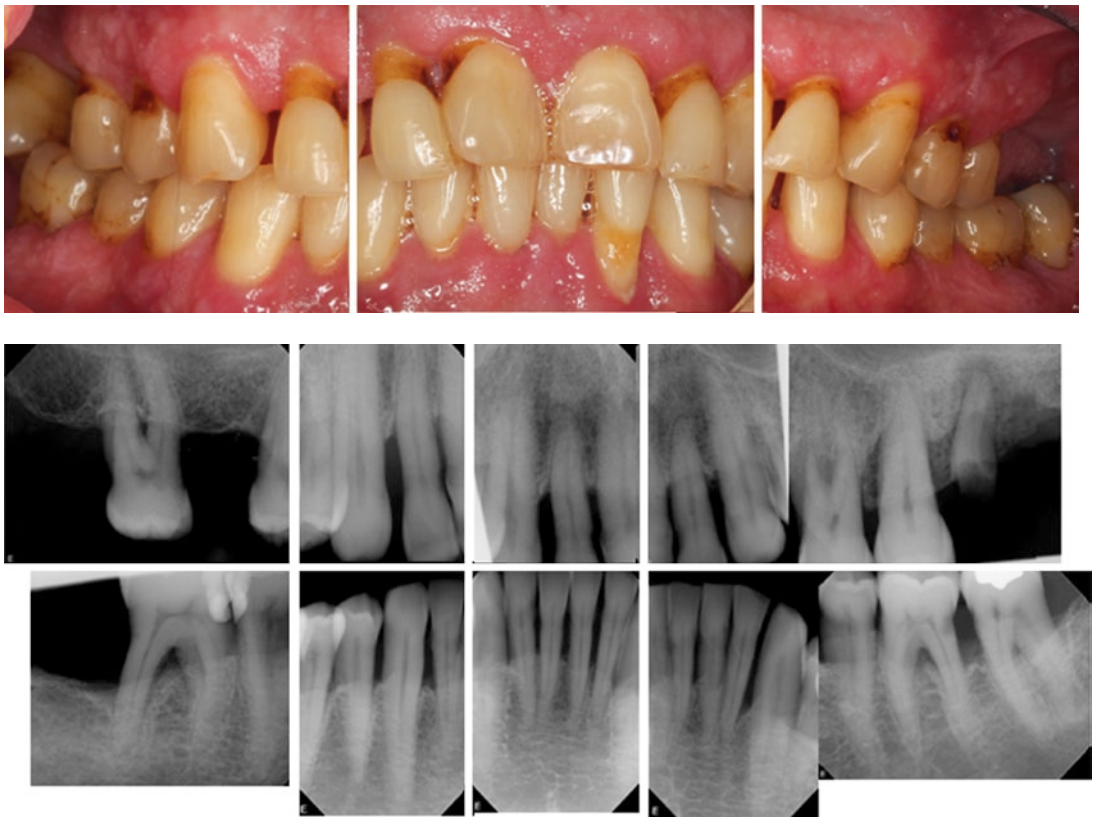

Fig. 4.2 Clinical presentation of chronic periodontitis. A 56-year-old male patient with severe periodontitis with pathological pocketing around most teeth. Note on radiographs that almost all teeth show bone loss of >7 mm. By courtesy of Dr. Giovanni Serino

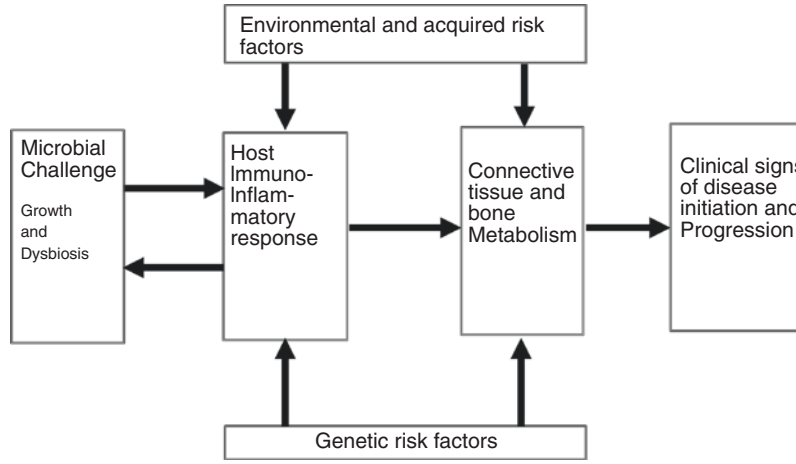

Fig. 4.3 Pathogenesis of periodontitis according to the classical model by Page and Kornman [1]. *LPS* lipopolysaccharides, *MMP's* matrix metalloproteinases, *PMN's* polymorphonuclear neutrophils

does not affect the equilibrium in the tissue regeneration. The amount and composition of the molecules that are released from the oral biofilm determine the effect on the host's response. Invasiveness of bacteria or bacterial products further contributes to a rapid disease progression.

The tooth supporting, or else *periodontal*, tissues—connective tissue and bone—are under constant renewal through processes involving death, proliferation, and differentiation of cells, as well degradation and production of matrix molecules. The tissues constantly fluctuate between destructive phases and healing or tissue repair (granulation tissue formation). Loss of periodontal ligament and bone resorption are irreversible processes that lead to a net loss of attachment, epithelial down growth, and pocket formation. The pathogenic process follows the outline that was proposed by Page and Kornman [1], and it is still the model or hypothesis that is believed to be correct today [2, 3], see Fig. 4.3.

It is well established that the microbial challenge induces and maintains the immune-inflammatory response of the host. However, the specific role of each bacterial species in the disease progress and the change in connective tissue and bone metabolism is less clear. There is reason to believe that the magnitude of the inflammatory response is dictated by genetic risk factors in the individual host, and this causes some individuals to be much more susceptible than others to progression of the disease. Environmental factors (e.g., smoking) or acquired risk factors (e.g., systemic diseases such as diabetes) may further modulate the severity of that response. There is also reason to believe that certain bacteria (periodontopathogens) associated with the periodontal disease progression play an active role in the modulation of the immuno-inflammatory response through their virulence factors, making the periodontal process more chronically destructive, according to the recent "keystone" pathogens hypothesis [4, 5].

Hence, this chapter goes on to describe the role of bacterial virulence factors in the development of periodontitis, and elaborates how they participate in the initiation and modulation of the host's responses in the periodontal tissues. This is described with special reference to the two most recognized periodontopathogens, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis,* which have distinctly different strategies for their establishment, survival, growth, and pathogenic role in periodontitis.

4.3 Periodontal Bacteria and Their Virulence Factors

Infection occurs when the virulence, the number, and the exposed time supersede the local and general host's defense, and that leads to a pathological reaction in the host's tissues [6]. The virulence can be divided into three parts: the ability to establish (colonization/infectivity), the ability to invade (invasivity), and the ability to cause tissue

damage (pathogenicity). Virulence means the degree of pathogenicity of a microorganism as indicated by the severity of the disease that is produced and the ability to invade the tissues of the host [7]. Thus, virulence is a microbial property that can only be expressed in a susceptible host. Hence, virulence is not an independent microbial property, because it cannot be defined independently of a host. Logically, the dependence of virulence factors on virulence implies that the definition of a virulence factor requires a functional definition for microbial virulence [8].

Factors involved in adhesion and persistence. The bacterial colonization of the oral cavity takes place at birth and continues throughout the whole life of the host. The colonization is mainly orchestrated by the host and its surface receptors. Thus, the microbial adhesins that specifically fit to the host's receptors determine the selection of microorganisms and thereby constitute and build the commensal microbiota living in harmony and balance (microbial homeostasis) with the host [9–12]. Periodontopathogens need to compete with the commensal primary colonizers and pioneer species in order to establish, grow, and cause pathology. Most periodontal bacteria are less efficient in adhesion, some are even motile (*Treponema* spp., *Campylobacter* spp., *Selenomonas* spp.), and the motile ones normally do not colonize smooth surfaces. They are rather dependent on the co-adhesion with other species already established in a biofilm. Since most of the recognized periodontopathogens are microaerophilic or strict anaerobic, they need an environment with low redox potential (Eh) to survive and establish. In healthy individuals, most periodontopathogens are outcompeted, unless these bacteria find niches that fulfill the requirement of mechanical forces (retention) and anaerobic conditions. Such conditions are found on the dorsum of the tongue and around and between the teeth where these bacteria may persist without being eliminated by the salivary flow in periodontal healthy individuals. Adhesins may also be important factors in the initial step of intracellular invasion (see below) in cells such as epithelial cells, fibroblasts, and leukocytes.

Microbial metabolic activity and growth (Fig. 4.4). Another mechanism by which the periodontal bacteria can evade the natural elimination and cleaning forces is by increased growth. The gingival pocket is a perfect niche for anaerobic, fastidious, low adherent, motile bacteria such as the red complex bacteria (*P. gingivalis,*

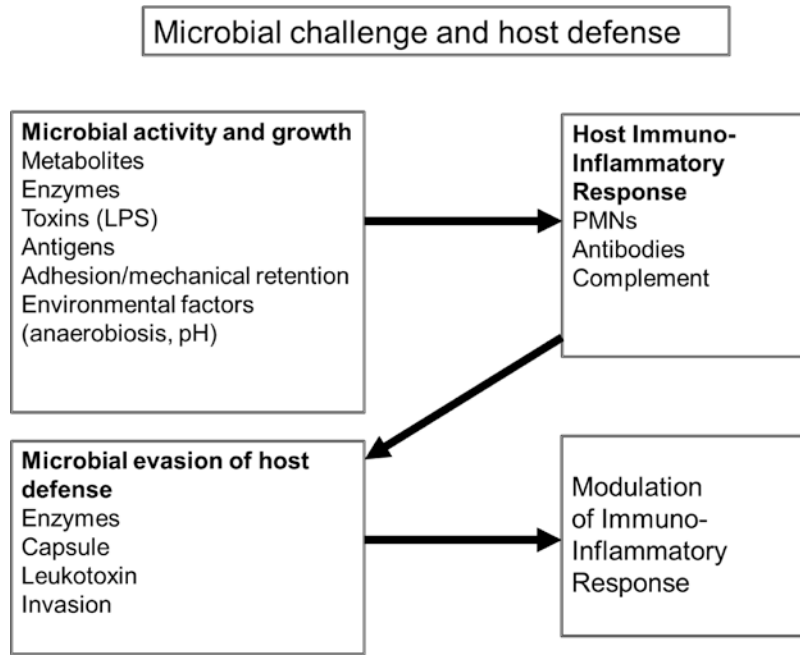

Fig. 4.4 Microbial challenge and host defense. Factors involved in host–parasite interaction in periodontitis

Tannerella forsythia, Treponema denticola, according to [13]) and the orange complex bacteria (*Fusobacterium* spp., *Prevotella* spp., *Campylobacter* spp., anaerobic streptococci, and more). The simultaneous inflammatory response that is elicited in the gingival connective tissue and subsequent deepening of the gingival pocket gives these bacteria important advantages over the primary tooth colonizers such as the saccharolytic, facultative streptococci and *Actinomyces* spp. [9, 14]. The gingival pocket offers the periodontal bacteria mechanical persistence (including motile bacteria), anaerobiosis, and nutrients including specific growth factors such as hemin, vitamins, and hormones and other serum factors in the gingival exudate [15]. The main nutrient factors in the exudate are proteins, peptides, and amino acids, which are the targets for the strongly proteolytic red and orange complex bacteria. Simultaneously, many of these periodontal bacteria produce various virulence factors that may help them to evade or escape the host's defense system (see below). The increasing subgingival bacterial growth and changed ecology (dysbiosis) are the major factors that enable a simultaneous development and increase of the inflammatory reaction. The higher metabolic activity produces toxic metabolites such as short carboxylic acids (butyric acid, propionic acid, valeric acid, capronic acid, and phenylacetic acid), ammonia, and hydrogen sulfide [14]. These small molecules are produced in high amounts during growth, penetrate through the pocket epithelium, enter the connective tissues, initiate, and maintain the inflammatory reaction. Subsequently, the periodontal bacteria begin to multiply. The vast majority of the periodontal bacteria are proteolytic and Gram-negative, and during growth they release proteolytic enzymes and endotoxins (lipopolysaccharides, LPS), which interact with the tissues and the host's response. This increase in metabolic activity, increase in bacterial number, and adaptation of new bacterial species lead to a continuously changing ecology, which lasts until a balance (homeostasis) is created between the bacterial community and the host. Such homeostasis may continue for long periods, or even lifelong for low-periodontitis susceptible individuals with chronic gingivitis or periodontitis. Note that the etiology of periodontitis always starts with an intact periodontium, where the proteolytic anaerobes have few advantages as compared with the unique milieu in the inflamed deepening periodontal pocket. The role of this group of Gram-negative anaerobes is discussed more in detail below, exemplified by *P. gingivalis*.

Evading the host's defense. The host reacts extensively to the microbial challenge by employing the inflammatory reaction and assembling defense factors such as neutrophils, complement activation and antibodies in the exudate and gingival pocket. Early studies [16] showed that 95% of the leukocytes moving by chemotaxis into the gingival pockets are neutrophils and the rest are macrophages. In the pockets, neutrophils that are loaded with phagocytized bacteria are constantly removed out from the pockets. However, some bacteria have specific means to escape phagocytosis (leukotoxin) or survive or overcome (capsule) intracellular killing once they have been phagocytized [17, 18].

Leukotoxin is a well-known virulence factor, which is found in several pathogenic bacteria such as the PV-leucocidin in *Staphylococcus aureus* [19] and *Fusobacterium necrophorum* [20]. This explains the invasive character and abscess-promoting character of these pathogens. Therefore, the leukotoxin-producing microorganism, *A. actinomycetemcomitans*, among the periodontal bacteria, has been extensively studied due to this virulence factor [21, 22]. The leukotoxin explains the specific ability of this bacterium to survive or escape the host's defense (neutrophils), but it does not explain the periodontal breakdown that is associated with this bacterium in aggressive forms of periodontitis in children and young individuals. The specific role of *A. actinomycetemcomitans* and its virulence factors are extensively discussed below in a separate section.

Capsule formation is a well-known virulence factor of many pathogenic microorganisms. The capsule is produced to escape phagocytosis and intracellular killing by neutrophils and macrophages. The inability of the host's defense to kill

the pathogens may result in spread of infections and to complications such as sepsis [18]. Proteolytic enzymes may split or degrade the host's defense molecules such as immunoglobulins and complement [23].

Cell and tissue invasion. Invasion is often regarded as a key event when colonization is transformed into infection. Invasion has also been claimed to be a key event in periodontitis [24–26]. Invasion has clearly taken place if the epithelial barrier (junctional epithelium) is disrupted in case of ulceration. In acute necrotizing ulcerative gingivitis (ANUG) spirochetes are shown to invade the underlying connective tissue by motility (Listgarten [27, 28]). A similar picture is also found in peri-implantitis where the epithelium does not cover the connective tissues of the apical part of the peri-implant pocket [29]. The periodontal abscess is another example of invasion into the tissues by bacterial growth, and the abscess can sometimes even lead to fistula formation. Invasion in these examples is due to bacterial multiplication and expansion, and it is associated with heavy neutrophil attraction and suppuration and sometimes symptoms, which all are characteristic of an acute infection. In the chronic forms (chronic gingivitis, chronic periodontitis) it is more controversial whether invasion has taken place. However, it is likely that invasion is an important event at least in the aggressive form of periodontitis [25, 26].

There are two routes that have been discussed for bacterial invasion in periodontitis [25, 30]. An intercellular route is suggested to take place by motility, e.g., spirochetes [30]. The junctional epithelium is non-keratinized and thin (4–5 cells thick), and in a state of inflammation, the cells are not tightly joined in order to facilitate gingival exudate and migration of PMN cells and macrophages through the gingival barrier. It is hypothesized that this intercellular passage also allows motile bacteria such as *Treponema* and *Campylobacter* species to penetrate the barrier. *Treponema* spp. and *P. gingivalis* produce specific gingipains that can degrade epithelial junctional proteins (E-cadherin and occludin), and this impairs the junction-related structures [31].

The intracellular route has gained more attention since it was noticed that viable and non-keratinized epithelial cells contain bacteria. Buccal epithelial cells regularly contain bacterial cells, mainly streptococci [32]. Interestingly, *P. gingivalis* and other periodontal bacteria have also been shown in such cells [33]. *T. forsythia, Prevotella intermedia*, and *C. rectus* have been identified inside crevicular epithelial cells [34] in vivo, and this uptake is mediated by receptor interaction between the bacteria and the epithelial cells. This is a sophisticated way for bacteria to escape the host defense factors. On the other hand, the desquamation process constantly detaches and removes epithelial cells and their content through the gingival pocket and out into the saliva. Interestingly, *P. gingivalis* has been shown to invade within 15 min, it may replicate within 4 h [35], it may be transferred to underlying epithelial cells, and enter the subepithelial connective tissue [25], before the surface cell becomes detached.

Modulation of the host's response. Most attention for immune modulation has been directed toward the endotoxin/lipopolysaccharides that are released after autolysis of Gram-negative bacteria. The LPS builds up the outer membrane of all Gram-negative bacteria and consists of a toxic Lipid A, a core polysaccharide, and a polysaccharide chain of repeating sugar subunits. The latter is an important antigen (O-antigen) that has been used for classification of several enteric genera. The Lipid A is biologically very active, and it interacts with most humoral systems (complement and coagulation) and with inflammatory B and T lymphocytes [36]. The activation of various inflammatory cells makes the LPS a primary candidate for immune modulation of the gingival inflammation. Since all Gram-negative bacteria release LPS, they may all be involved in the periodontal inflammation process. It is important to emphasize that LPS and the Lipid A structure are strongly hydrophobic and form free membrane structures or outer membrane vesicles [36]. These aggregates may penetrate into tissues and interact with most cells through the interaction of the Lipid A part, which integrates with cell membranes and activates the cell [36]. The toxicity of

LPS varies greatly among various different bacteria. The toxicity is primarily dependent on the Lipid A, but it is modulated by the structure of the core polysaccharide and the length of the O-antigen chain. The impact of the structure for its modulating ability on the host's response is extensively studied for *P. gingivalis* [37]. There is also an increasing interest for outer membrane vesicles formed by *P. gingivalis* and their interaction with the host. Outer membrane vesicles are LPS-membrane structures enriched with proteins, largely gingipains, and those vesicles contribute to *P. gingivalis*–host interaction and pathogenicity [38].

4.4 Transmission Pattern and Colonization

All microorganisms, despite the fact that they may occur as resident, transient, or pathogenic microbiota, are transmitted from the external world. According to Marsh & Devine [15], the establishment of a microbial community passes ecological stages:

Transmission—Acquisition/colonization—Pioneer species—Microbial succession—Increasing species diversity—Climax community. All stages are affected by environmental conditions or modifications. The colonization, succession, and diversity, which lead to a climax community, may differ greatly in composition and microbial activity between sites, individuals, and populations. Factors involved are host receptors for attachments, pH and redox potential, nutrients, and metabolic breakdown products. These factors differ greatly by age, from the newborn child, through childhood, adolescence, and into adulthood.

Streptococci are far and away the most superior microorganisms for colonization of the oral cavity [39]. They are the outstanding pioneer species in all oral niches including mucosal surfaces and tooth surfaces after tooth eruption. They are extremely well adapted to the oral cavity by a multitude of receptor interactions with host cells, salivary glycoproteins, and other salivary and serum components (agglutination). Streptococci bind to other microbial cells (autoaggregation, coaggregation) and to lectins and other food components, e.g., polyglucan production from sugars increases the binding capacity of several streptococci. This exceptionally strong binding capacity is far greater than that of other microorganisms that might be transmitted and incorporated in the microbiota. They have a very broad range of biochemical activity. These activities include both saccharolytic and proteolytic metabolism and the use of glycoproteins from saliva during periods of starvation to produce both acid and alkaline to regulate the ecology. The Streptococci produce bacteriocins, which control the microbiota and block the space and receptors for more pathogenic bacteria. In addition, the Streptococci are generally well adapted and tolerant to environmental changes [15]. The final selection of the pioneer species early in life is primarily determined by the host (the newborn child) through its oral receptor pattern and environment. The best-suited strains for this selection come from the mother and other family members and explain why certain microorganisms occur in some families but not in others. Others that are well adapted to the oral cavity and teeth are *Actinomyces* spp., *Neisseria*, and *Haemophilus* species, while others are outnumbered (coliforms, staphylococci, and more) and are kept in the transient microbiota. Environmental factors modify the prerequisites for acquisition and colonization so that low pH (caused by early sugar intake, e.g., breast-feeding and bottle-feeding) lower the pH to an increasing number of low (compared to streptococci) adherent microorganisms such as acidophilic lactobacilli and *Candida* spp. The comparatively high Eh in newborns and children also gives an advantage to the aerobic, facultative, and oxygen-tolerant microorganisms. The more strict anaerobic bacteria are suppressed even if they are exposed to the child, but they might occasionally find a hidden place to survive. The number of *Fusobacterium, Veillonella,* and *Prevotella* can be frequently found in 3-month-old children although in low number [40, 41]. Although strictly anaerobic, these are also adherent, saccharolytic, acid-tolerant, and can survive in the oral cavity of babies. Other so-called

periodontopathogens such as *P. gingivalis, T. forsythia, Campylobacter*, and *Treponema* species are hardly detected even with very sensitive methods. It is generally believed that they do not occur until later in life (adolescence) when most permanent teeth are fully erupted, some gingivitis is regularly present, and hormonal changes have taken place. These conditions present sufficient pH and Eh environments for survival and growth of these more fastidious proteolytic and non-saccharolytic bacteria [42]. They are low adherent and need mechanical retention, which develops on the dorsum of the tongue and between the teeth and the subgingival dental plaque when teeth are fully erupted and constitute a niche were they reside (non-adherent subgingival plaque) to minimize the risk for elimination. They require hemin or other serum products for growth, which are hardly available until inflammation occurs and they constitute an increasing proportion of the subgingival plaque.

A special attention in the colonization pattern has been paid to the periodontal bacterium, *A. actinomycetemcomitans.* It has been associated with the localized aggressive forms of periodontitis in young individuals (earlier called localized juvenile periodontitis). This bacterium is found to colonize in a family pattern, and the same phenotype is frequently found in mother and child pairs [43]. This bacterium has generally good adherent abilities, but it has strict specificities for host cells (at least humans and old world monkeys) [44]. This bacterial species is facultative anaerobic, tolerant to oxygen stress (catalase positive), moderately saccharolytic, and acid-tolerant, which makes it suitable for early colonization in the oral cavity in children. This bacterium does not seem to be dependent upon other bacteria, and some antagonism between *A. actinomycetemcomitans* and some streptococcal species has been reported [45]. Under most conditions, *A. actinomycetemcomitans,* like most other bacteria, are controlled by the host and the resident microbiota (streptococci). This bacterium occurs in several genetic and phenotypic subgroups (and clones) and aggressive variants (see below) may colonize early in life. If not properly controlled, they increase in number, induce an inflammatory reaction in the gingival tissues, and cause progression of attachment loss and pocketing. The colonization of the gingival area translates the bacteria from a resident stage to infection (see below). The production of specific virulence factors and increased metabolic activity makes the bacteria more aggressive and causes severe environmental changes. The increasing inflammation and exudation promote those bacteria with more proteolytic activity in contrast to those with a saccharolytic metabolism that have no advantage in the subgingival area due to lack of sugars. Facultative bacteria have no advantage of the lowered Eh and a slightly alkaline pH. Other less aggressive subtypes may colonize and compete more efficiently. Few studies have tried to evaluate whether one individual or even one site may harbor several genotypes of the same species. However, in a recent study, up to four different genotypes were found in one deep periodontal site in Thai adults with periodontitis [46]. It is therefore reasonable to argue that there is a dynamic change between genotypes resulting in one dominating genotype at various time points.

Less is known about the *P. gingivalis* genotype distribution, and it is generally believed that *P. gingivalis* has a much more clonal distribution between individuals than *A. actinomycetemcomitans* [47]. Thus, adults living together (periodontitis cases and their spouses) frequently share the same genotype. This indicates that transmission also continues between adults, and the climax community has no upper age limit and may continue throughout the entire life of the host.

4.5 Colonization and Infection

Virulence factors are usually genetically conserved, but it must be emphasized that the variation between genotypes of the same species may be significant [48]. In addition, the phenotypic expression of virulence factors in vivo may strongly differ due to environmental conditions. Even if the genetic basis for the virulence factors is present, their expression and function may be up- or downregulated when they are most/least

needed [49, 50]. The expression of virulence factors in a polymicrobial community or infection is even more complex due to the dependence of microbial interactions, which normally control the microbial homeostasis. Under certain circumstances, the microorganisms may evade the interaction with other microorganisms by activating specific genes. This can suppress the controlling mechanisms in the community, increase their metabolic activity, and cause rapid growth that leads to dysbiosis or overgrowth. This uncontrolled overgrowth of certain microorganisms is the basis for the "Burst theory" proposed by Socransky [51, 52]. Changes in the microbial environment (nutrients, pH, anaerobiosis, bacteriocins, etc.) may result in dysbiosis. Communication occurs between the bacterial cells in the biofilm (quorum sensing) [53]. Small peptides (signaling molecules) are produced to activate certain genes in target bacteria. The role of viruses in the pathology of periodontitis has been little investigated. However, it is clear that viruses such as Herpes simplex virus can directly cause gingival inflammation [54] or can damage the gingiva indirectly by genetic exchange of DNA or plasmids by transduction [55].

From a microbiological point of view, it is pertinent to have a holistic approach to periodontal infections and consider them not essentially different from other polymicrobial infections in the body. Characteristically most polymicrobial infections such as the periodontal, endodontic, and other subepithelial oral infections are predominantly anaerobic [56]. Single anaerobic species may lack essential virulence factors to establish and infect as a monoinfection, and therefore need cooperative mechanisms with other bacteria to formulate a prerequisite environment for survival and growth. Numerous animal experiments have shown the necessity of cooperation with facultatives to reduce the oxygen tension low enough for growth, invasion, and infection [57, 58]. Only few isolates of certain anaerobes have been shown to cause experimental infections in monoculture. The isolates *P. gingivalis* W83 and W50 have such a capacity and thus have been used in numerous experimental in vitro models and in animals [5, 59]. Microbial composition in most anaerobic infections is characterized by its heterogeneity, and the infecting combinations seem to occur at random or as teams (combinations), which include a number of bacteria supporting each other [60, 61]. That does not necessarily exclude that some species more frequently occur in these polymicrobial infections and that some teams have a greater degree of infectiosity. The so-called red complex bacteria (*P. gingivalis, T. forsythia*, and *T. denticola*) are statistically more associated with progressive periodontitis than other bacteria, and the orange complex bacteria occur less frequently in the same sites [13]. A number of studies have reported other bacterial constellations, which also correlate to the deep periodontal pockets and disease progression [62, 63]. A recent review [64] listed 139 species that could be classified as potential periodontopathogens. However, it is unwise to evaluate a single bacterial species independently without concomitantly evaluating other bacteria, their functions in the microbial community, and the infectious process [14, 65]. The etiological role of each single bacterial species in the initiation and progression of periodontitis is still largely a matter of hypotheses [66].

There is one exception to the view of periodontitis as a polymicrobial and predominantly anaerobic infection with a low specificity, and that is *A. actinomycetemcomitans*. This bacterium does not fit into the complex system of the Socransky model [13] and constitutes its own factor using factor analysis [63]. Especially in young individuals, *A. actinomycetemcomitans* occurs independently of other bacterial species. This form of periodontitis resembles a more specific type of infection and thus needs to be described separately (See below).

4.6 The Role of Aggregatibacter Actinomycetemcomitans

A. actinomycetemcomitans (Fig. 4.5) is a facultative anaerobic Gram-negative bacterium associated with periodontitis, and it expresses a number of potential virulence factors [67]. This bacterium is strongly associated with the localized

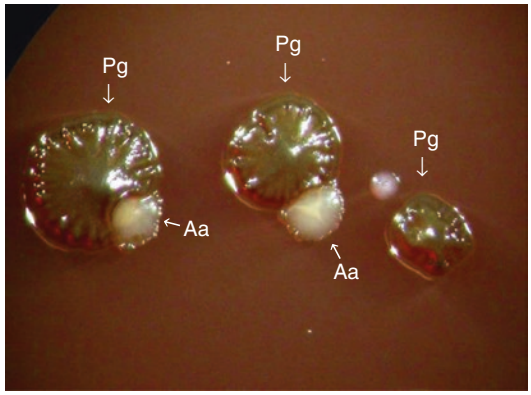

Fig. 4.5 Colonies of *A. actinomycetemcomitans* and *P. gingivalis* from a clinical subgingival sample cultured on a blood agar plate for 5 days under anaerobic condition

aggressive form of periodontitis, but its relation to other forms of periodontitis is still not fully understood [68, 69]. However, instead of connecting the presence of *A. actinomycetemcomitans* with a certain diagnosis, it is obvious that this bacterium plays a role in the early phase of the disease [70, 71]. One hypothesis is that *A. actinomycetemcomitans* colonizes the oral mucosa, and when it is translocated to the gingival margin, it can initiate disease. The presence of this bacterium on an individual basis has been shown to be a strong risk marker for initiation of the disease [70, 72, 73]. Results from a recent study showed that children (6–12 years) of parents with aggressive periodontitis have increased frequencies and quantities of *A. actinomycetemcomitans* as compared with children with periodontally healthy parents [74]. The vertical transmission pattern of this bacterium indicates a strong role as an etiologic factor in this disease, even though none of the children had been diagnosed for aggressive periodontitis already at this early age. The facultative anaerobic property of *A. actinomycetemcomitans* promotes colonization at an early age in individuals with a healthy periodontium and limited accumulation of subgingival microbiota [75]. The importance of interplay between host-genetic factors and the bacteria has been nicely described and named as infectogenomics [76]. In that study it was shown that a significant association between increased presence and concentrations of *A. actinomycetemcomitans* in individuals with an IL-6 polymorphism results in enhanced expression of this cytokine. It was later shown that the growth of *A. actinomycetemcomitans* can be stimulated by ligand binding to a specific cytokine receptor on the outer membrane, the IL-1β-binding protein, which has a high affinity for IL-6 [77]. This discovery might be involved in the findings that show a significantly greater risk for developing periodontitis in individual's positive IL-1 polymorphic sites [78].

A great genetic diversity has been shown within the *A. actinomycetemcomitans* species with six distinct different serotypes and a large proportion of variable genes within the pan genome of this bacterium [79, 80]. Moreover, a number of mutations within the core genome of *A. actinomycetemcomitans* contribute to further differences, where the highly virulent JP2 genotype has attracted most of the attention [73]. This genotype has a 540-base pair deletion in the promoter of the gene operon responsible for expression of a leukotoxin [81]. This genotype of the bacterium is highly leukotoxic and has a certain strong association with disease risk in the carrier, as compared with other genotypes with a full-length leukotoxin promoter [70, 73]. In addition, highly virulent genotypes of *A. actinomycetemcomitans* have also been detected with a 640-base pair deletion or with an insertion in the leukotoxin promoter [82, 83]. The predictable enhanced disease risk in individuals colonized with this bacterium is a unique property among the various periodontopathogens [84].

A. actinomycetemcomitans expresses a number of different virulence factors, including adhesins and exotoxins [67]. The different adhesins of the bacterium allow colonization of the oral mucosa, the tooth surface and the bacterial biofilm, as well as promoting epithelial invasion [85]. Once adhered to the oral surface, the ability to produce virulence factors determines the role of the bacterium in the pathogenesis of periodontitis [86]. Several different virulence factors of *A. actinomycetemcomitans* have been described and have been nicely summarized by Henderson and coworkers [67]. Longitudinal studies have shown the highest odds ratio for disease onset or disease

progression among carriers of the highly leukotoxic JP2 genotype of the bacterium, which indicates an important role of the leukotoxin in the pathogenesis of periodontitis [70, 87]. Recently, a subpopulation within the serotype b of *A. actinomycetemcomitans,* with an intact leukotoxin promoter, but with enhanced leukotoxin production, has been identified [88]. This genotype of the bacterium has also been associated with a significantly increased odds ratio for disease progression, which further strengthens a role of leukotoxin in the disease progression. The leukotoxin promotes resistance to phagocytic killing and affects our defense cells by inducing release of tissue-degrading enzymes and pro-inflammatory cytokines before the challenged cells die [21]. These events are all cellular and molecular mechanisms that are involved in the homeostasis of tissue remodeling [3, 66]. Macrophages exposed to leukotoxin have been shown to release substantial amounts of the pro-inflammatory cytokine interleukin (IL)-1β that activates bone resorption in a mouse calvarial model [89]. The immunogenic properties of the leukotoxin are obvious due to the high concentration of neutralizing antibodies in the peripheral circulation of the *A. actinomycetemcomitans* carriers [90]. The leukotoxin has been shown to be released from the bacterial outer membrane in the presence of serum proteins, and the toxin is protected from degradation by the protease inhibitors present in this mixture [91, 92]. In addition, leukotoxin has also been identified in vesicles secreted from the bacteria, which might promote systemic distribution of the toxin [93]. These properties might contribute to the immunogenic potential of the protein, even though a protective role of specific antibodies in the periodontal pocket is questioned [94]. Leukotoxin is a large pore-forming protein that belongs to the Repeat in ToXin (RTX) family of bacterial proteins that are produced by a number of Gram-negative pathogens [95].

A second exotoxin that is produced by *A. actinomycetemcomitans* is the cytolethal distending toxin (Cdt) [96]. This toxin affects all eukaryotic cells by entering the nuclei of the target cell and causing double stranded DNA cleavage [97]. A subunit of Cdt has homology with human DNaseI, and the Cdt subunit is suggested to be responsible for the Cdt-induced DNA break [98]. This DNA break results in growth arrest of periodontal fibroblasts and lymphocytes [99, 100]. In addition, the Cdt-intoxicated fibroblasts and lymphocytes increase the expression of a receptor activator for NF-kappaB ligand (RANKL), a key component in osteoclast differentiation [101, 102]. About 80% of *A. actinomycetemcomitans* isolates have an intact operon for Cdt expression, which is a prerequisite for expression of an active Cdt toxin [103, 104]. Despite these potent virulence mechanisms of Cdt, results from a longitudinal study, which examined carriers of either Cdt-positive or Cdt-negative bacteria, failed to show a significant difference in disease progression between the two groups [104].

A third exotoxin that is produced by *A. actinomycetemcomitans* is the CagE molecule, a highly conserved protein of 38.6 kDa [105]. Its ability to cause apoptosis in human epithelial cells and its reactivity to serum immunoglobulin from individuals with periodontal disease indicate a possible role in the pathogenicity of the disease [106]. However, a correlation between the ability to express CagE and initiation and progression of periodontitis has not yet been shown.

Lipopolysaccharide (LPS) is a well-characterized pathogen-associated molecular structure, which is found in the outer membrane of most of the Gram-negative bacteria, including *A. actinomycetemcomitans* [107]. It can initiate a strong immune response and serves as an early warning signal of bacterial infection. LPS is initially released from bacterial membranes and outer membrane vesicles that interact with host proteins, and LPS results in increased expression of pro-inflammatory cytokines. Different serotypes of *A. actinomycetemcomitans* have been described based on the LPS-O-polysaccharide antigenicity and host cells, which react differently to LPS from various serotypes [108, 109]. However, independent of serotype, LPS from this bacterium activates expression of pro-inflammatory cytokines that can be activated and released upon exposure to the exotoxins [110, 111]. In addition, the presence

of *A. actinomycetemcomitans* upregulates expression of inflammasome components in human macrophages, which indicates synergistic effects between the two exotoxins on their pro-inflammatory response to their host [112].

4.7 The Role of Porphyromonas Gingivalis

P. gingivalis (Fig. 4.5) is a Gram-negative oral anaerobe that is involved in the pathogenesis of periodontitis [113]. This bacterium is one of the major colonizers in deep periodontal pockets, and it provides a strict anaerobic ecological niche that promotes the bacterium's growth. *P. gingivalis* has been described as a "keystone pathogen" [4]. A microorganism that supports and stabilizes the dysbiotic microbiota associated with a disease state was their criterion for a species to be named as a keystone pathogen. *P. gingivalis* can locally invade periodontal tissues and evade the host defense mechanisms [114]. It utilizes its major virulence factors, lipopolysaccharide, capsule, gingipains, and fimbriae, to establish the infection by interacting with the host. Although it has a high number of potent virulence factors, it has not yet been shown that its presence in a healthy periodontium predicts an increased risk of disease onset. This indicates that this bacterium is probably a late colonizer that invades already diseased tissues and contributes to an increased progression of periodontal breakdown [115].

Early analyses on the restriction fragment polymorphisms of the fimbrillin locus, *fimA*, of *P. gingivalis*, revealed a substantial genetic variation [116]. Later Amano et al. [117] showed that some *fimA* genotypes (*fimA* II and IV) are more prevalent in periodontitis than *fimA* type I, which is most prevalent in healthy periodontal tissue. Fimbriated *P. gingivalis* are more efficient than fimbria-deficient *P. gingivalis* to enter human dendritic cells in vitro [118]. A Type-I fimbriae *P. gingivalis* strain induces more bone loss than Type-II *P. gingivalis* in a mouse model [119], while Type-II fimbriae are associated with increased pro-inflammatory and invasive activities in macrophages.

Six capsular serotypes have been identified among periodontal *P. gingivalis* isolates [120, 121]. The capsule of *P. gingivalis* leads to a reduction in the host inflammatory response, evasion of phagocytosis, and increased virulence [18] as compared with nonencapsulated strains. Two capsular types, K1 and K6, seem to include isolates with a higher virulence than the other capsular types [121]. Non-capsulated strains adhere significantly more than their capsulated variants to pocket epithelial cells [122].

The gingipains are trypsin-like cysteine proteases and are considered to be the major virulence factor of *P. gingivalis* [114]. The direct and indirect activities of gingipains are important in every stage of infection, such as attachment, colonization, invasion, acquisition of nutrients, evasion of host's defenses, and dissemination [123]. The gingipains consist of three enzymes: RgpA and RgpB that degrade proteins with arginine in the p1 position, and Kgp that degrades proteins with lysine in the p1 position [124]. Gingipains also digest a broad spectrum of host proteins, some of which are completely degraded, and this provides peptides for *P. gingivalis* growth and metabolism. Some host proteins are only partially degraded, which might lead to dysregulation of the host's defensive inflammatory reactions and the failure of the host to eliminate *P. gingivalis* [4, 123]. The reduced condition in the periodontal pocket promotes activation of these cysteine proteases [124]. In addition, the gingipains have also been shown to have the capacity to release *A. actinomycetemcomitans* from the microbial biofilm and neutralize its leukotoxin by proteolytic degradation [125, 126]. Gingipain inhibitors have been proposed to be a future tool for therapeutic strategies against periodontal diseases [127].

P. gingivalis expresses a peptidyl-arginine deiminase (PAD), an enzyme that converts

arginine within a peptide (peptidylarginine) into peptidylcitrulline [128]. This bacterium is the only prokaryote that has been reported to possess PAD. Citrullination by human PADs is an important mechanism in normal physiology and inflammation [129]. Systemic autoantibodies to citrulline are strongly associated with the preclinical phase of rheumatoid arthritis [130]. The role of *P. gingivalis* PAD in the pathogenesis of periodontitis is not known, but it may be involved in the association reported between periodontitis and rheumatoid arthritis [131]. However, it has been shown in a mouse model that there is a PAD dependent synergistic effect between these two diseases [132, 133].

In line with reports from other Gram-negative bacteria, *P. gingivalis* expresses LPS that induces a strong pro-inflammatory response [107]. The most well characterized pathway for host-cell activation by LPS is through interaction with the LPS binding protein (LPB) followed by binding to soluble or membrane-bound CD14 molecules that activate the transmembrane toll-like receptor (TLR) 4. These interactions activate intracellular signaling, which results in enhanced expression of proteins involved in the pro-inflammatory response. Interestingly, LPS from *P. gingivalis* has also been shown to act as a TLR 2 agonist [134]. The outcome of TLR2 activation in response to distinct microbial molecules may be influenced by differential TLR2 association with accessory receptors [134]. These activations involve interactions that have been shown to inhibit pro-inflammatory and antimicrobial host responses. The various effects reported by *P. gingivalis* on osteoclast differentiation might be a result of its complex interaction with the TLRs [135, 136].

4.8 Concluding Remarks

We have described the virulence of two major periodontopathogens, *A. actinomycetemcomitans* and *P. gingivalis*, which have different strategies for their pathogenicity in periodontitis in humans (Table 4.1). This is due to completely different spectra of virulence factors expressed during transmission, colonization, growth, and participation of the microbial challenge to the gingival/periodontal tissues and the evasion of host defense factors assembled in the immune-inflammatory host response. It is concluded that *A. actinomycetemcomitans* fulfills the pathogenic role according to the "specific plaque hypothesis," while *P. gingivalis* fulfills its role according to the keystone hypothesis for periodontitis.

Table 4.1 Comparison of virulence pattern for the two periopathogens *A. actinomycetemcomitans* and *P. gingivalis*

	A. actinomycetemcomitans	*P. gingivalis*
Colonization	Vertical transmission from parents and older siblings. Colonizes mucosal and tooth surfaces in early childhood	Horizontal transmission and prefer an established microbial biofilm or gingival pockets, which means a preference of older individuals
Translocation	Colonizes gingival crevice and become a risk marker for tissue degradation	Colonizes gingival pockets and established microbial biofilms
Inflammation	Activate inflammation through LPS and exotoxins	Activate inflammation through LPS and proteolysis
Degradation	Localized, rapid, and deep degradation of the tooth-supporting tissues	General and slow degradation of the tooth-supporting tissues
Stability	Decreases in proportion in an established lesion due to the proteolytic environment that degrades the major virulence factors, adhesins and exotoxins	Increases in proportion due to their ability to detach other bacteria from the biofilm and the advantage of the more anaerobic ecological niche

Clinical Relevance Summary Points

- Periodontitis is a microbe-induced inflammatory disease that affects the tooth-supporting tissues.
- A shift from a symbiotic microbiome to dysbiosis develops concomitantly with disease progression.
- A number of microorganisms have been associated with periodontitis but the role of certain bacteria in the process is essentially unknown.
- *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* have been considered major "periodontopathogens" due to their close association with certain forms of periodontitis. They present unique virulence factors that participate in the survival and growth of the bacteria by their proteolytic activity; by evading the host defense through virulence factors such as leukotoxin and capsule formation; by invading host cells and tissues; and by modulating or dysregulating the host inflammatory immune response.
- While *Aggregatibacter actinomycetemcomitans* seems to play an important role in the early phase of the disease, *Porphyromonas gingivalis* is associated with the anaerobic proteolytic activity in the periodontal pocket once periodontitis has become manifested.
- Both *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* are divided into subtypes and genotypes (clonal types) with quite different pathogenic potential.
- Reduction/elimination of certain marker bacteria such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* can be used as the therapeutic goal in periodontitis treatment.
- The maintenance after periodontal treatment should prevent disease associated bacteria such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* to become reestablished in the periodontal pocket by keeping the gingiva healthy with a minimum of inflammation.

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5 Active Matrix Metalloproteinase-8: Contributor to Periodontitis and a Missing Link Between Genetics, Dentistry, and Medicine

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5.1 Introduction

Periodontitis is one of the most common infection-induced inflammatory tissue destructive diseases globally. According to the epidemiological studies in Western countries, about 30% of populations are affected by periodontitis [1]. The pathogenesis of periodontitis can be briefly described and summarized as follows: the dental plaque, a bacterial biofilm, induces an inflammatory and immune responses in the adjacent gingival and periodontal or peri-implant tissues. The cells of the immune and inflammatory system are together with resident gingival cells (including fibroblasts, cementoblasts, and epithelial cells, bone cells) triggered to express and release pro-inflammatory cytokines, reactive oxygen species, and matrix metalloproteinases (MMPs) [2–5]. MMPs are genetically distinct but structurally related proteinases that can degrade not only almost all extracellular matrix proteins but also non-matrix bioactive molecules such as growth factors, serpins, insulin receptor, apolipoprotein-1, complement components, and pro- and anti-inflammatory cytokines and chemokines [2–5]. Thus, MMPs can modify immune responses [2–5]. The active form of catalytically competent matrix metalloproteinase-8 (aMMP-8; neutrophil

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collagenase or collagenase-2) is the predominant MMP in periodontitis-affected gingiva, gingival crevicular fluid (GCF), peri-implant sulcular fluid (PISF), saliva, and mouthrinse [2–5]. MMP-8 cleaves preferably and efficiently the interstitial collagens, mainly type I fibers of the gingival and periodontal tissues, leading to irreversible soft and hard tissue destruction, i.e., the development of periodontal pockets and attachment loss, and eventually leading to tooth loss [2–5]. Physiological levels of MMP-8 in periodontal tissue also participate to protective and anti-inflammatory resolution of infection-induced tissue destruction [6, 7].

aMMP-8 is released mainly by degranulating neutrophils and lesser extent by resident non-neutrophil lineage mesenchymal cells (fibroblasts, epithelial cells, endothelial cells, plasma cells, cementoblasts, bone cells, etc.) present in the the gingival and periodontal as well as peri-implant tissues of the GCF, PISF, saliva, and mouthrinse [2–5]. Significant and increasing amount of international publications have repeatedly and consistently evidenced that the levels of aMMP-8 in GCF and PISF correlate with the pathology of the periodontium, i.e., with health, gingivitis, and periodontitis as well as peri-implant mucositis and peri-implantitis [2–5]. Moreover, the outcome and reflection of successful periodontal treatment can conveniently be monitored by oral fluid (GCF, PISF, saliva, and mouthrinse) aMMP-8 analysis [2–5]. Overall, these publications demonstrate clearly that (i) oral fluid (GCF, PISF, saliva, and mouthrinse) aMMP-8 values are low in case of a healthy gingiva and peri-implant tissue, (ii) are more increased in gingivitis and peri-mucositis, (iii) are most increased and pathologically elevated in the untreated periodontitis and peri-implantitis, and (iv), in periodontitis and peri-implantitis, reduce to lower values after successful periodontal and peri-implant treatments, for example, after scaling/root planning (SRP), cleaning/depuration, and adjunctive medications, i.e., host-modulating subantimicrobial dose doxycycline or antimicrobial drugs [2–5].

Consequently, aMMP-8 can be utilized as a diagnostic biomarker for tissue-destructive and progressive periodontal and peri-implant inflammation related to active periodotal degeneration (APD), especially reflecting and indicating surrogate and pathologically excessive collagenolysis [2–5]. These outcomes (i)–(iv) of aMMP-8 have not only been evidenced in GCF and PISF but also in saliva and mouthrinse [2–17].

5.2 GCF Monitoring of Periodontitis by aMMP-8

Furthermore, Lee et al. [12] have shown that the GCF collagenolytic activity of MMP-8 is a prognostic parameter; “active collagenase activity” was found to be sixfold elevated in cases with progressive loss of connective tissues [2–5]. In addition, Leppilahti et al. [13–15] have recently and repeatedly demonstrated using MMP-8 immunofluorometric assay (IFMA) that GCF aMMP-8 can predict periodontal treatment and medication outcomes site specifically (Fig. 5.1) [13–15]. Sorsa et al. [10] were able to differentiate between “stable sites” without periodontal breakdown combined with low GCF aMMP-8 levels and “unstable sites” showing tissue breakdown as well as concomitant pathologically elevated GCF aMMP-8. Overall, aMMP-8 in GCF has been shown to be low in periodontal health and increase with advancing and ongoing periodontal inflammation and high oral inflammatory burden associated with APD [5]. These GCF aMMP-8 studies have been described more in details by recent reviews [2–5].

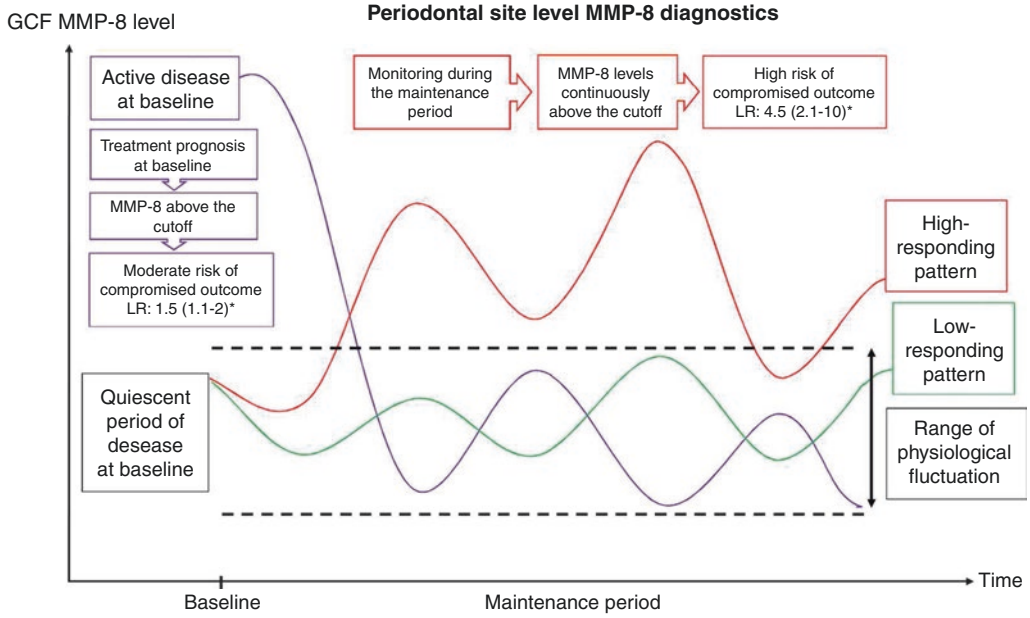

Fig. 5.1 *LR = likelihood ratios (95% CI). Presented likelihood ratios are combined measures of both smokers and nonsmokers reported originally by Leppilahti et al. [13, 14]. Reproduction published with permission of American Academy of Periodontology

5.3 Saliva and Mouthrinse Monitoring of Periodontitis by aMMP-8

aMMP-8 quantifications in addition to GCF and PISF have also been made from salivary or in mouthrinse samples [5]. Various international research groups have published unequivocally and repeatedly that salivary and mouthrinse aMMP-8 is significantly elevated in periodontitis, even in adolescents with initial periodontitis, patients relative to the healthy controls [2–7, 11]. Also a relationship between salivary aMMP-8 concentration and the oral inflammatory status and burden, i.e., active periodotal degeneration (APD), has been established [5–7, 11, 12, 16].

No clinical standard exists for a direct comparison of aMMP-8 data with conventional periodontal parameters. Probing pocket depth (PPD), clinical attachment level (CAL), and X-rays only show the history of the already existing or experienced periodontal pockets and attachment loss [17] and cannot be conveniently used as prognostic factors. Moreover, after successful periodontal treatment, the periodontal pockets can still exist, but they show significantly reduced aMMP-8 concentrations [3–16]. Bleeding on probing (BOP) as well is known to be relevant parameter in some way to predict further periodontal tissue loss [18, 19]. Absence of BOP is regarded to indicate and reflect periodontal health [18, 19]. With this background, we describe the development of the straightforward chairside and quantitative aMMP-8 oral fluid assay technologies to be utilized as periodontal and peri-implant diagnostic tools to distinguish conveniently periodontal and peri-implant health and disease [2–16]. Overall, these salivary and mouthrinse aMMP-8 studies have been described in detail in recent papers [2–17].

5.4 Chairside Monitoring of Periodontitis by aMMP-8

Diagnostic parameters like PPD and CAL, as usually utilized by clinical periodontitis diagnostics, measure mainly the attachment loss that has already occurred [18, 19]. Until now, no prognostic or predictive test and especially no chairside/point-of-care test exists that could predict a future progressive phase during the course of periodontal disease [2–5, 8–16].

There have been attempts to develop chairside systems to be utilized as adjunctive for the quantification to aMMP-8 from GCF, PISF, saliva, and mouthrinse. In this regard, it has been possible to measure and diagnose aMMP-8 to GCF, PISF, saliva, mouthrinse, and/or serum a laboratory test via an aMMP-8-specific immunofluorometric assay (IFMA) or ELISA [2–5]. In fact, such system has recently been commercialized to be used as a diagnostic aid for the dental and medical professionals [4, 5, 16, 20–22].

Recently lateral-flow point-of-care (PoC)/chairside tests (PerioSafe®, ImplantSafe®), discovered in Finland and further developed in Germany, have been developed based on the before-mentioned technologies and monoclonal antibodies [4, 16]. The immunoassay test (PerioSafe®) can be administered to patients according to the manufacturer's instructions, which include a 30 s prerinse with tap water, followed by a 30 s wait, and then rinsing with the test liquid (aqua purificata) for 30 s, and then patients pour the mouthrinse into the a little collection cup accompanied by the test kit. Three milliliters of the rinse is to be drawn up into a syringe, and then a filter is placed on the syringe through which a maximum of four drops are placed in a lateral-flow immunoassay system which showed one single blue line for negative results (no risk) and two blue lines for positive results (increased risk). The test resembles a pregnancy test [2, 5]. The result was read as a color change within 5–6 min. Even a thin second line indicates increased risk for periodontitis and peri-implantitis (Fig. 5.2) [5, 16, 20–22].

Due to the positive results with the chairside test, the clinician can identify and predict an active phase of periodontitis, i.e., active periodotal degeneration (APD), and peri-implantitis sites and patients at risk and intervene therapeutically before the periodontal and peri-implant tissues show further breakdown, i.e., active periodotal degeneration (APD) [5, 16, 20] (Fig. 5.2, Table 5.1). Thus, oral fluid PoC-aMMP-8 chairside test can be utilized in a preventive manner identifying elevated initial and early periodontitis risk, i.e., development of APD, and during maintenance [5, 16, 20–22] (Table 5.1, Fig. 5.2). The PerioSafe® aMMP-8 mouthrinse test has been introduced and validated independently in Nigeria, Finland, the USA, Turkey, Holland, and Germany and repeated and consistently found to exert excellent performance to differentiate periodontal health and disease (Table 5.1) [5, 16, 20–22]. The aMMP-8 test can be used alone and/or in combination with other potential biomarkers such as interleukin-1β (IL-1β) and *Porphyromonas gingivalis* especially in epidemiological studies [23, 24].

The mouthrinse collection is a noninvasive approach and not causing any discomfort for the patient. Due to the simple testing (5–7 min) not necessarily need for dental equipment for knowledge exists (Fig. 5.2, Table 5.1) [5, 16, 20–22]. It is performed with purified water that does not harm the patient. All further and additional processing, analysis, and measurements of potential and other diagnostic components are performed in the laboratory and do not represent an additive burden or discomfort for the patient. The PerioSafe® aMMP-8 test has recently been recommended by the German Society of Periodontology and German Society of Oral and Maxillofacial Surgery.

It can be concluded that no extra risks exist or troubles when the diagnostic tests are performed (Fig. 5.2) [5, 16, 20–22]. Due to the prognostic and predictive chairside aMMP-8 test, patients would benefit from early, fast, exact/precise, convenient, and inexpensive disease diagnosis and detection of current and/or future disease progression [5, 16, 20–22]. Finally, this approach would indicate an enough early therapeutic intervention according to Axelsson and Lindhe [23] and or Reinhardt et al. [24] before an irreversible tissue destruction or APD occurs, respectively

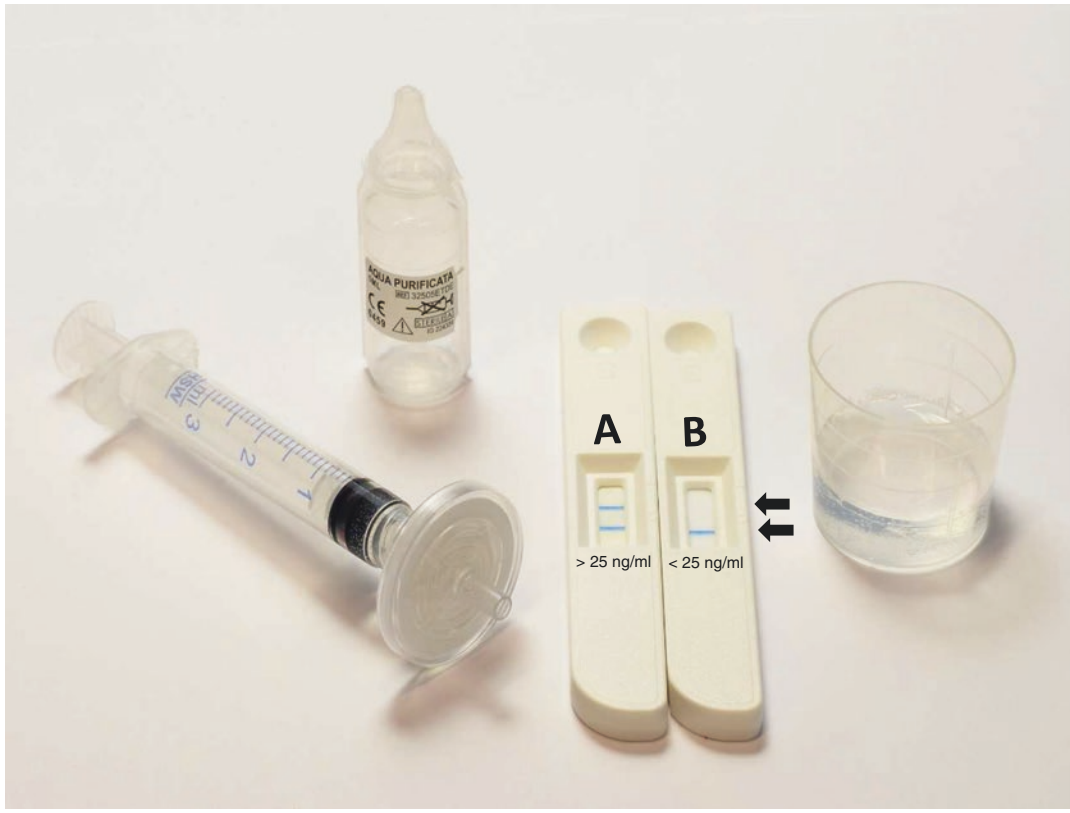

Fig. 5.2 Equipment of PerioSafe® aMMP-8 immunological chairside/point-of-care lateral-flow test, including syringe with filter, test liquid (aqua purificata), lateral-flow immunoassay sticks, test liquid/mouthrinse collection cup. When aMMP-8 in the collected mouthrinse is >25 ng/mL (cutoff), two lines are detected (*A*) indicating that tested patient is positive and at risk for periodontitis. When one line is detected (*B*), patient is negative and under control and out of periodontitis risk as well as under good maintenance. *Arrows* indicate the lines resulting from immunoreactions. In fact, the test resembles closely a typical pregnancy test [2–5]

Table 5.1 The sensitivity and specificity of the PerioSafe® aMMP-8 chairside test according to at least one, two, or more than two ≥4-mm-deep pockets and at least one caries lesion n Finnish adolescents. Original published by Heikkinen et al. [16]. Reproduction published with permission of American Academy of Periodontology

Clinical parameters	At least one ≥4-mm-deep pockets	At least two ≥4-mm-deep pockets	More than two ≥4-mm-deep pockets	At least one caries lesion
The sensitivity of aMMP-8 chairside test	48.3%	63.6%	76.5%	76.5%
The specificity of aMMP-8 chairside test	100%	100%	96.8%	96.8%
p-value	<0.0001	<0.0001	<0.0001	<0.001
False positive	0	0	1	5

[25, 26]. When the test is negative, the patient is under control and out of risk (Fig. 5.2, Table 5.1). The aMMP-8 test (PerioSafe®) can be utilized in diagnostics and maintenance and eventually also in self-diagnostics [5, 6, 20–22].

Undiagnosed and untreated APD, i.e., active progressive periodontitis, gradually leads to missing teeth and tooth loss mainly by the uncontrolled proteolytic action of aMMP-8 [2–5]. Missing teeth or tooth loss has been repeatedly regarded

as surrogated markers of experimented or ongoing periodontitis. Significant causation has been repeatedly presented between missing teeth and circulatory mortality as well as all-cause mortality. In fact, missing teeth predict incident cardiovascular events, diabetes, and death [27].

Regarding interdisciplinary medical and dental cooperation, MMP-8 PerioSafe® test eventually represents the missing link between genetics, medicine, and dentistry [4, 5, 22, 28]. Thus, with PerioSafe®, a MMP-8 test medical professionals (gynecologists, rheumatologists, diabetologists, cardiologists, surgeons, nurses, and other experts) can detect and identify in 5–6 min the patient exposed in the long run to fatal systemic risks from APD and/or peri-implantitis [4, 5, 22]. Patients with repeatedly elevated levels of oral fluid aMMP-8, >25 ng/mL, thus PerioSafe® positive, as indicated by the two lines (Fig. 5.2), should be referred to periodontist or dentist/hygienist for treatments/interventions [2, 26] to reduce the oral fluid aMMP-8 levels to be and stay <25 ng/mL, PerioSafe®, thus negative, as indicated by one line (Fig. 5.2). Furthermore, PerioSafe® identifies genetically predisposed adolescents for initial/early periodontitis [28]. Thus, it can also be regarded as a "gene test" [28].

By these preventive interventions, the fatal related systemic risks (cardiovascular diseases, diabetes, stroke, tooth loss) derived from and affected by APD and peri-implantitis can be avoided [4, 5, 22–27]; in the long run, this is additionally economical for the patients, healthcare systems, and society.

Clinical Implications

Matrix metalloproteinase-8 (MMP-8, neutrophil collagenase, collagenase-2) is a novel candidate biomarker for oral fluid (GCF, PISF, and saliva) and mouthrinse chairside/PoC diagnostics to predict, diagnose, and asses the progressive or active degenerative phases of the episodic periodontitis and peri-implantitis (ADP) as well as to monitor the effects of treatments and medications and maintenance phase of periodontal treatment. Especially active MMP-8 (aMMP-8) is important and useful in these regards. aMMP-8 can be utilized alone or together with other potential biomarkers such as interleukin-1β (IL-1β) and *Porphyromonas gingivalis* to calculate MMP-8 cumulative risk score (CRS) of the subject level as a successful diagnostic tool, especially in large-scale public and/or epidemiological health surveys, in which the complete periodontal examination is not always possible to conduct. PerioSafe test can identify genetically predisposed adolescents for initial/early periodontitis and thus eventually act as a "gene" test. Inexpensive, easy/practical to use, quick (5–7 min), predictive chairside oral fluid PoC aMMP-8 tests, such as PerioSafe® and ImplantSafe®, are currently commercially available for the routine use by dental and medical professionals in fact linking these disciplines; even the self-test done by the patients is possible.

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Inflammatory Pathways of Bone Resorption in Periodontitis

6

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6.1 Introduction

The most common periodontal diseases in humans are periodontitis and gingivitis. These diseases share dental biofilm as their etiologic factor and periodontal inflammation as their main feature. Although the diagnosis, treatment, and prognosis of the vast majority of the periodontitis cases pose no special challenges to the trained periodontist, there are extreme clinical phenotypes at both ends of the susceptibility chain that are disturbingly difficult to identify with the classical periodontal diagnose tools. When a patient presents a differential clinical phenotype, particularly of extreme susceptibility to alveolar bone destruction, the periodontist often found himself/herself lacking of the conceptual framework to determine the best course of action and to provide the best possible care. In spite of the astonishing amount of time and resources devoted to unveil the intricate details of periodontitis' pathogenesis invested in the last four decades, we are still far from a comprehensive explanatory model, and, more importantly, of new therapeutic tools to take better care of the most susceptible patients. That said, new developments in the field of osteoimmunology that will be discussed later in this chapter provide us with an appealing pathway to follow in our search for novel diagnostic tools, therapies, and clinical interventions.

6.2 Generalities of Inflammation in Periodontitis Pathogenesis

Periodontitis' hallmark feature is the inflammatory resorption of tooth-supporting alveolar bone due to uncontrolled host response to periodontal infection. Even though the periodontal infection is essential to trigger the host's immune and inflammatory response, the destructive events that lead to the irreversible disease phenotype of periodontitis are the result of the uncoupling of the soft and mineralized tissue turnover mechanisms caused by the persistence of a chronic and exacerbated inflammatory immune response.

Although the primary role of the immune system is to provide the defense of the host against

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potentially hazardous microorganisms, the persistence of immune responses due to their inability to eliminate the microbial antigens causes the alterations in the tissue's metabolism that eventually results in its irreversible destruction. The anatomic particularities of periodontium and the capacity of periodontal bacteria to form a biofilm structure favor the establishment of a long-lasting and non-resolving chronic inflammation that acquires a destructive nature. The bacterial biofilm adheres to the tooth surface and confers a series of advantages to the microbes, including the isolation and protection from host defensive mechanism, ultimately impairing the eradication of the infectious focus.

It is noteworthy that in periodontal health the immune and inflammatory response is still present. While the exact nature of the host response associated with periodontal health remains to be established, such state is considered an equilibrium state where a low intensity response is enough to limit the invasion and multiplication of periodontal microorganisms without inflicting damage to the host tissues as a collateral damage. This balance is finely tuned and dependent on a series of active modulatory processes, which can be tilted in each direction (exacerbated suppression or activation) by intrinsic or environmental factors. The active suppression of the immune response, without complete abolition of its infection control mechanisms, is supposed to be the cornerstone in the maintenance of stable healthy periodontal tissues.

In this scenario, this chapter focuses in the role of the inflammatory and immunological events that lead to the pathologic destruction of the alveolar bone, and consequently of the periodontal attachment during the progression of periodontitis.

6.2.1 From Health to Inflammatory Immune Response: How Homeostasis Evolves to Disease

After more than four decades of incessant research in the immune aspects of the pathogenesis of periodontitis many lessons have been learned, but we are still only scraping the surface of the pathogenic mechanisms that tilt the balance from health to disease in the periodontal environment. From a clinician's perspective, the understanding of the pathogenic mechanisms of periodontal disease is an invaluable asset in the establishment of a successful treatment plan, general and individual teeth prognosis, and follow-up strategies. In addition, the knowledge of the general mechanisms of periodontitis' pathogenesis helps the clinician to identify putative susceptible subjects and implement individualized therapeutic measures to better their long-term prognosis. Least, but not less important, is the perspective of the development of new therapeutic approaches using the knowledge gained from the applied research, which include the appealing possibility of using immune modulatory drugs. These drugs will be specifically targeted to the periodontal tissues and aimed to abolish the deleterious effects of inflammation in the alveolar bone without dampening the ability of the host to deal with potentially hazardous periodontal microorganisms. As previously stated, although periodontitis is an infection, the pathologic tissue changes characteristic of the disease (particularly bone resorption) are a consequence of the sustained inflammation of the periodontal tissues.

Inflammation can be defined as a protective response intended to eliminate injurious stimuli that causes tissue damage, as well as removing the injured tissue resulting from the original insult. Inflammation can be triggered by physical or chemical trauma or by foreign bodies, including microbes. Even though inflammation is intended to eliminate harmful stimuli, the inflammatory reaction has the potential to cause widespread tissue damage, since the same mechanisms intended to kill infecting microbes and clear the tissue from injured and dead cells have the potential to damage normal tissue.

It is fundamental to understand that the distinction between inflammation and immune response is only didactic and that both processes not only occur simultaneously, but also are functionally integrated. Indeed, the main purpose of inflammation is to facilitate the access of immune cells and defense molecules from the circulation to the

damaged or infected tissues. Thus, most inflammatory events are related to vascular and perivascular events, including increased blood flow, alterations on the vasculature to facilitate immune cells transmigration, and increased extracellular matrix metabolism. These molecular and cellular processes translate into the clinical signs of inflammation (a.k.a. "cardinal signs"), as classically described by Rudolph Carl Virchow in the late nineteenth century: redness, heat, swelling, pain, and loss of function. In the particular case of periodontal diseases, these first inflammatory and immune phenomena translate in the clinical entity known as gingivitis.

In the sequence, we will approach inflammation and immune response as intermingled and mutually dependent occurrences in order to better picture the actual cellular and molecular phenomena that lie beneath the clinical signs and symptoms of periodontitis, which ultimately explain the pathologic irreversible destruction of alveolar bone. We will focus in bone destruction, since it is the defining occurrence of periodontitis, even though the same inflammatory immune events depicted in this chapter could be considered as responsible for the destruction of the remaining of the tooth-support apparatus.

In normal healthy conditions, the gingival tissues are capable of coping with the presence of bacteria due to various innate immune defense mechanisms, among them: the flushing action of gingival crevicular fluid and saliva, the rapid epithelial turnover, the secretion of immune active peptides, a permanent influx of innate immune response cells to the periodontal tissue and the transmigration of neutrophils into the sulcus, and the action of saliva agglutinins and antibodies, which are supposed to limit the potential of the bacterial biofilm to grow and invade [1–4]. Indeed, in a distinctive fashion in comparison with other body tissues, in the healthy periodontium exist a constant subclinical inflammation. When the balance between the infection control mechanism and the subgingival biofilm is lost, the mandatory first step into the pathologic chain of events leading to periodontitis is gingival inflammation, an acute inflammatory response of the gingiva to the microbial insult that is clinically evident [5, 6].

Conceptually, inflammation is categorized in two types: acute and chronic. Acute inflammation is by definition a short duration response, ranging from minutes to a few days, and it is characterized by profuse exudate and the predominance of neutrophilic leukocyte infiltration. With the persistence of the inflammatory stimuli, acute inflammation evolves to chronic inflammation is a long-lasting response, ranging from days to years, characterized by the tissue infiltration of mixed populations of leukocytes and monocytes/macrophages following specific chemotactic gradients, and tissue degeneration including vascular proliferation, parenchymal involution, and fibrosis [7]. The events of acute and chronic inflammation are analog and complementary to events of innate and adaptive response. The first vascular and molecular occurrences of inflammation favor the migration of the cell populations' characteristic of the innate immunity, namely neutrophils and macrophages, which possess very effective but unspecific microbe clearance machineries. When innate immune responses cannot clear the invading microorganisms, the inflammation becomes chronic and the characteristic effector cells of the adaptive immune response invade the tissue, predominantly activated lymphocytes with highly selective and specific microbe killing capabilities [2, 8]. As in any biological process, these rigid definitions do not capture the changing dynamics of inflammation and immune response, where long-lasting chronic inflammatory processes are periodically broken up by acute inflammatory bursts [9, 10].

The inflammatory immune response is triggered by the interaction of resident cells with the bacterial biofilm attached to the tooth surface. The particularities of the periodontal anatomy and periodontal tissue architecture are intimately associated with the natural course and pathophysiology of the inflammatory processes that leads to periodontitis [5, 11]. Bacterial biofilm attaches to the tooth surface, making impossible for the immune system to eradicate the infecting microorganisms efficiently. The attached biofilm acts as a permanent reservoir of microorganism and their products, perpetuating the insult to the periodontal tissues [12].

The junctional epithelium is the first periodontal structure to face the bacterial challenge. This is a highly specialized and unique tissue, characterized by the capability to attach directly to the mineralized surface of the tooth by the formation of an extremely organized structure known as the internal base membrane. The junctional epithelium is more permeable than most epitheliums, mainly because of the incomplete maturation of its supra basal layer, by its unusually high proliferation rate, by the relatively scarce presence of desmosome junctions between cells, and by the absence of keratohyalin [13, 14]. After surpassing the epithelial barrier, infecting microorganisms gain access to the subjacent gingival connective tissue, being gingival fibroblasts the predominant cell type in this compartment. Despite the fact that fibroblast are not professional immune cells and have a limited output of signaling molecules available, such cell type can be also active in the triggering of initial inflammatory events [15].

Extensive evidence indicates that the first immune modulatory events in this process are orchestrated by the keratinocytes of the junctional epithelia and fibroblast of the periodontal connective tissue [16–20]. These cells are capable of "sensing" the environment and differentially react to diverse bacterial antigens and other signals, responding with the secretion of mediators that will mediate the vascular and cellular events of inflammation. Indeed, the resident periodontal cells (epithelial cells and gingival fibroblast) directly interact with the microbes or its products produce and secrete molecular signals to trigger inflammation and chemoattract immune cells [14, 15]. The host cells recognize the microbes by the interaction of pathogen-associated molecular patterns (PAMPs) with PAMP-receptors constitutively expressed in the cell membrane of most cells. Those PAMPs are signature molecules preferentially associated with pathogens and absent from host's cells, such as lipid polysaccharide (LPS), a main component of Gram-negative cell wall, and lipoteichoic acid, a major constituent of the cell wall of Gram-positive bacteria [21]. The archetypal membrane-bound PAMP-receptors are the Toll-like receptor family (TLR), composed of at least nine isoforms named TLR1 to TLR9. The signaling pathway is initiated by the binding of the pathogen-derived ligands to membrane-bounded TLRs, leading to the dimerization of the receptor. The dimerization of the TLRs triggers the recruitment of various protein kinases in the cytoplasmic end of the receptors, ultimately causing the activation of proinflammatory transcription factors (such as NFκB and AP-1) [22, 23].

Following the initial stimuli, classic inflammatory pathways are deflagrated, where the cyclooxygenase pathway oxygenates arachidonic acid producing a variety of proinflammatory molecules, such as prostaglandin E-2 (PGE-2), prostacyclin, and leukotriene A-4. The enzyme cyclooxygenase 1 (COX-1) is constitutively expressed, while COX-2 is induced under proinflammatory conditions and responsible for the amplification of the inflammation [7, 24]. Several in vivo studies have demonstrated that the inhibition of COX-2 activity significantly reduces the alveolar bone loss in experimental periodontitis [25, 26]. In parallel with the activation of classic mediators of the vascular events of inflammation, TLR recognition of microbial ligands results in the secretion of important molecular mediators of inflammation, including inflammatory cytokines (such as TNFα and IL-1β) and chemokines (such as CXCL8/IL-8, CCL2, CCL3, and CCL5), which along the mediators that target the blood vessels, will orchestrate the inflammatory cell influx into the inflaming tissue [1, 27].

At this step, to make room for the profuse inflammatory cell infiltration the perivascular extracellular matrix is degraded by enzymes such as matrix metalloproteinases (MMPs), which also will degrade the extracellular matrix along the way of the cells from the vessel until the inflammatory focus, being this way determined by gradients of chemoattractant molecules [4, 28].

In this environment, it is mandatory to consider that gingival fibroblasts are responsible for the periodontal tissue turnover, doubling as the main source of collagen fibers and of MMPs within the connective tissue. However, under the influence of the copious inflammatory signals, gingival fibroblast and periodontal liga-

ment fibroblasts are directly responsible for the destruction and disorganization of the fibrous component of the extracellular matrix of periodontal tissue by increasing the local production and activity of MMPs [29]. While the extracellular matrix degradation has a physiological value, since it is necessary to facilitate the transmigration and permanence of immune cells within the tissue during the immune response, the problem arises when the inflammatory/immune response turn out to be indefinite and the tissue homeostasis becomes permanently affected, leading to the pathological irreversible destruction of teeth-supportive tissues.

At this stage, the first clinical signs of gingivitis become evident. Namely, redness, swelling and loss of texture of the free gingiva, provoked bleeding (at the clinical examination with a blunt instrument or during normal oral hygiene procedures), and eventual suppuration. When the inflammation persists, the disease progresses into periodontitis, characterized by the widespread destruction of the extracellular matrix and the collagenous fibers that compose the connective attachment apparatus, the apical migration of the junctional epithelium, and the pathological resorption of the alveolar bone and radicular cementum. In the periodontal examination, these histopathological changes are evidenced as increased probing depth usually accompanied by all the clinical signs of gingivitis [30].

6.2.2 The Parallels Between Acute Inflammation and Innate Immune Response

At the early host response phase, the predominant immune cell type in the periodontium is the polymorph nuclear neutrophil [6, 31]. Even in healthy conditions, a stable influx of neutrophils (3×10^5 cells/min) permeates the connective tissue in transit to the sulcus though the junctional epithelium, attracted by a constant gradient of IL-8/CXCL8 permanently secreted from keratinocytes and gingival fibroblast in response to the recognition of PAMPs by TLRs [32–34]. Once inside the tissue, neutrophil leukocytes become activated by the interaction of their own TLRs with PAMPs. After their activation, the neutrophils produce and secrete molecular mediators to amplify the inflammation; also, their phagocytic activity is enhanced, as well as the production of reactive oxygen species (ROS) used to kill the phagocyted microorganism. These ROS affect the oxidative status of the periodontal tissue, activating and sustaining a pervasive inflammatory response that amplifies the initial signaling, and when uncontrolled promotes the spreading of the inflammation to the surrounding tissues [15, 35, 36].

While neutrophils exert a very important anti-infective role in the periodontal environment, it is also important to consider that most of the periodontal microorganisms have evolved complex and elegant strategies of immune evasion and have the capacity to use the immune/inflammatory response to their own benefit, modulating the periodontal microenvironment to adjust it to their specific metabolic needs [37]. For example, the recognized periodontopathogen *Porphyromonas gingivalis* is capable of downregulating the initial immune response of periodontium resident cells, completely abolishing the secretion of the neutrophil-attractant chemokine IL-8/CXCL8 by a gingipain-dependent mechanism [19, 38], facilitating its survival and growth within the periodontal connective tissue. Interestingly, despite the initial dampening of inflammatory cell migration over time will ultimately result in an exacerbated, but inefficient in microbial control terms, host response. Indeed, as previously mentioned, in the development of periodontitis, infecting microorganism resists eradication in part due to the particular anatomy of the periodontal sulcus/pocket and in part due to their proprietary virulence and evasion mechanism along the additional protection conferred by the biofilm structure, eliciting a sustained chronic inflammatory response.

At this point, neutrophils may have or have not succeeded in eliminating the agent that elicited the inflammatory response. If not, additional cell types with different antimicrobial strategies and weapons may be able to achieve the task, but even if neutrophils are successful in eliminating infecting agents, additional cell recruitment is

required to repair the damage (even if minimal) inflicted at the response site. To achieve this objective, a special polarized subpopulation of macrophages with regulatory and reparative functions may be recruited to the damaged tissue. This distinctive subpopulation of macrophages possesses the capacity to potentiate the repair, and in some instances, the fibrosis of the tissue as required [39].

Additionally, monocytes also play a relevant role during the early stages of the acute inflammatory process and initial innate immune response, and readily infiltrate the periodontal tissue in the first hours post infection [12, 40]. Monocytes are attracted from the circulation to the periodontal tissues following gradients of chemokines, such as monocyte chemoattractant protein 1 (MCP-1, a.k.a. CCL2), which is the master regulator of monocyte/macrophage mobilization [41–43]. Some indirect evidence links the infiltration of macrophages into the periodontium with the severity of the periodontitis, since MCP-1/CCL2 levels appear augmented in the gingival crevicular fluid of periodontitis patients and its levels seem to correlate with the severity of the disease [44].

The macrophages are able to phagocyte the invading microorganism into phagocytic vesicles (a.k.a. phagosomes), but require additional cytokine signaling to promote the fusion of phagosomes and lysosomes, where the microbicidal mechanisms take place to kill effectively the ingested microbes. The principal molecular mechanisms of microbe killing and digestion are the conversion of molecular oxygen into reactive oxygen species (ROS), the production of highly reactive nitrogen species, such as nitric oxide (NO), and the action of several proteolytic enzymes [45, 46]. It is noteworthy that all these microbicidal mechanisms, when exacerbated and uncontrolled, are partially responsible for the amplification of the inflammatory response and the degradation of the host's tissues. Indeed, activated macrophages are an important cellular source of MMPs and greatly contribute to the intensification of the degradation of the collagenous matrix in the connective periodontal tissue [47, 48].

In summary, the acute inflammatory response in the early stages of periodontitis development begins when bacteria and their products gain access to the gingival connective tissue throughout the junctional epithelium, whose loose intercellular junctions do not provide an impermeable physical barrier. Proinflammatory molecular signals emanated from the epithelial and connective tissue trigger the first inflammatory events, increasing the blood flow and permeability of the subepithelial gingival plexus and recruiting large amounts of leukocytes to the site, particularly neutrophils and macrophages.

In the coming sections we will describe the chronic inflammatory events that later will be responsible for the bulk of the tissue destruction in periodontitis. Briefly, we can summarize the process as a continued response driven by the impossibility of eliminating the microbes exclusively with the recruitment of neutrophils and macrophages. In turn, other leukocyte subsets are recruited and join the inflammatory infiltrate, and the host response acquires a chronic nature that will result in the dampening of the normal homeostatic balance of the periodontal tissues.

6.2.3 Osteoimmunology and the Molecular Connection

Indeed, when the response becomes chronic, adaptive immune cells invade the tissue and the inflammatory reaction becomes firmly established, flooding the periodontium with additional bioactive proinflammatory molecular signals (cytokines, chemokines, enzymes, ROS, bacterial products and metabolites, etc.) [1, 2, 4, 28]. The accumulation of these molecular signals in the tissue facilitates the spreading of the inflammation to the underlying bone and tamper with the bone homeostasis signaling system, tilting the balance of bone metabolism favoring resorption over formation. More than four decades ago, *Page and Schroeder* postulated that the presence of bacterial plaque closer than 2.5 mm from the alveolar bone could trigger its inflammatory resorption [31, 49]. Nowadays we know that the pathological mechanism underlying this observa-

tion is the disruption of the balance of bone metabolism by inflammatory and immune molecular mediators, which mark the *transition from gingivitis to periodontitis*.

The understanding of the molecular basis of the interplay between inflammatory immune responses and the bone tissue is the keystone of "osteoimmunology," a new and evolving field that studies the shared components and mechanisms between the immune and bone systems. To understand such connection, we must remember that bone is a highly specialized mineralized connective tissue, characterized by constant renewal dependent on the coupling of bone formative and resorptive processes. This dynamic behavior of bone enhances its adaptive capacity to functional demands and increases its healing potential after an injury. The balance between bone resorption and apposition is governed by a unified molecular signaling system and is dependent on the effector functions of specialized bone cells.

The modulatory processes leading to bone resorption in periodontitis are very complex and intricate, and will be described along this entire chapter, including the interaction of different species of bacteria and their subproducts with elements of the innate and adaptive immune systems [50–52]. Fortunately, despite the astonishing complexity of the input signals, the bone has a very straightforward transduction system and a limited number of molecular signals and cells are directly involved in controlling the balance between bone apposition and bone resorption. This is a common finding among complex biological systems, which are capable of "sensing" a vast variety of inputs, but where the effector mechanisms are governed by a much more limited number of "key effector signals." In the specific case of alveolar bone, the system that controls the bone metabolism balance comprises the RANKL/OPG/RANK triad, secreted and recognized by the specialized bone effector cells osteoblasts and osteoclasts [53, 54].

RANKL (receptor activator of nuclear factor κB ligand) is a cytokine, member of the TNF family that can be membrane bounded or secreted, and stimulates osteoclasts' differentiation, cell fusion, and activation leading to bone resorption through calcium-dependent activation of the transcription of *NFATc1* gene [55]. The membrane-bound form is characteristically expressed in the surface of osteoblasts (a.k.a. RANKL isoform 1 and 2) and the soluble form (a.k.a. RANKL isoform 3) is the secreted product of various cell types, including B-cells and T-cells [56, 57]. RANKL is the master activator of osteoclasts and the molecular signal directly responsible for bone resorption. RANKL interacts with its cognate receptor RANK in the surface of osteoclast and osteoclasts' precursors, triggering their recruitment to the bone surface, cell fusion, and activation. The secreted form of RANKL is the molecular signal that couples immune response and bone metabolism. Numerous animal model experiments have demonstrated that alveolar bone resorption could be prevented by the selective inhibition of the RANKL/RANK axis [58, 59]. Osteoprotegerin (OPG) is a soluble protein upregulated in inflammatory conditions, with the capacity to block RANKL's biological functions by competitive inhibition, acting as a decoy receptor, limiting the availability of RANKL able to bind to RANK. The quotient or ratio of RANKL to OPG determines if at any given moment the conditions are favorable for bone apposition or bone resorption. A high ratio of RANKL/OPG creates the conditions favorable to bone resorption, while a low RANKL/OPG ratio favors bone apposition [60, 61]. During bacteria-induced inflammation in experimental periodontitis, it has been demonstrated a net increase in the RANKL/OPG ratio, leading to osteoclast genesis and bone resorption [62]. Analog evidence is also available in human periodontitis [28], and in the closely related condition of pathologic bone resorption of periapical bone as a consequence of endodontic infection [63, 64]. Indeed, the key event in the inflammatory alveolar bone resorption in periodontitis is the manifold increase in the tissue levels of RANKL unaccompanied by an equivalent increase in OPG levels. The resultant augmented RANKL/OPG ratio drives the recruitment of osteoclast's monocyte precursors, their fusion, and later activation. The uncoupling of bone metabolism due to the imbalance between bone apposition and resorption is the result of the

uncontrolled interplay of the immune system and the bone metabolism throughout their common molecular mediators.

Recently, it was reported that RANKL levels in gingival crevicular were increased fourfold in chronic periodontitis patients compared to healthy controls, while OPG levels were equivalent in both groups, causing an increased RANKL/OPG ratio in the patient's group. Interestingly, RANKL/OPG ratios levels were unaffected by the periodontal treatment and remained augmented for at least 6 weeks post-treatment, despite the notorious clinical improvement and normalization of inflammatory parameters of the subjects [65]. This could be the reflection of a long-lasting unbalance of bone metabolism, spanning beyond the resolution of the inflammation in the immediate posttreatment period. Alternatively, this could be the result of an inherent predisposition to increased secretion of RANKL, which could be the underlying molecular basis for increased susceptibility to bone resorption.

6.2.4 Chronic Inflammation and Adaptive Immune Response in Periodontitis

The activation of adaptive immunity has a great influence in the bone loss associated with periodontitis, since numerous evidence points to B and T lymphocytes as the main cellular sources of soluble RANKL during periodontal inflammation [66]. Experimental evidence from SCID mice (Severe Combined Immune Deficient, lacking both T and B lymphocytes), demonstrated the importance of adaptive immunity effector cells in bone loss in periodontitis. When SCID mice where challenged with *Porphyromonas gingivalis*, they demonstrated significantly lesser bone resorption than the control wild-type mice [67].

As previously mentioned, with the impossibility of clearing the insulting microbes by means of the recruitment of neutrophils and macrophages, other leukocyte subsets are subsequently enrolled to join the inflammatory infiltrate, and the host response acquires a chronic nature and mobilizes adaptive immunity cells. Indeed, after the initial acute inflammation and innate immune response had taken place, the selective migration of specific T lymphocyte subsets drives the transition to the adaptive immune response [1, 4]. These two processes are analogous and in a certain way correspondent to the acute and chronic phases of the inflammatory response.

In this second stage of the immune response, T CD4+ lymphocytes (a.k.a. T helper or Th) play a critical role in orchestrating the host's response. T helper lymphocytes secrete bioactive signaling molecules, namely cytokines, as their main effector mechanism, fine-tuning almost every aspect of the inflammatory/immune response. Depending on a series of environmental and host's intrinsic factors, Th lymphocytes could differentiate into distinct subtypes or lineages, each one with a characteristic subset of cytokines in its secretion pattern. The differential lineage commitment depends of the predominant cytokine present in the environment during the process of antigen presentation by antigen-presenting cells (APC) in the regional lymph node. During this process, APC capture antigens in the peripheral tissues and migrate to the regional lymph node, where they encounter a naïve T cell with a specific receptor for the antigen. After the specific interaction of APC-T CD4+ cell, the naïve lymphocyte becomes activated and committed, suffers clonal expansion, and massively migrates into the peripheral tissue to perform their effector functions. It is during this stage that the different lineages of Th lymphocytes emerge and differentiate, dictating the curse of the following steps of the disease [1, 10].

The relative predominance of a subpopulation of Th lymphocytes over the rest determines that the immune response variates from a controlled self-contained process with low potential to destroy the periodontal tissues to an exaggerated reaction with high destructive potential. The knowledge of the existence and regulatory functions of the different Th subpopulations has been one of the most dramatic revolutions in the immunology field of the past three decades and has affected profoundly our understanding of the immune processes underling the clinical course

of periodontal disease beyond the classical pro-versus anti-inflammatory perspective [1, 8, 68].

In the late eighties, the first two Th lymphocyte subtypes where described first in mice and then in humans and named Th1 and Th2 [69, 70].

Th1 cells were characterized as responsible to mediate the immune responses against intracellular pathogens. Naïve Th cells committed to Th1 lineage when the antigen presentation occurred in an IL-12 enriched environment, and the stabilization of the Th1 phenotype was dependent on the transcription of the key transcription factor T-bet [71]. The characteristic cytokine product of Th1 committed lymphocytes is IFN-γ, which is the cytokine responsible for the classical pathway of macrophage's activation, and fundamentally involved in the stimulation of the eradication of phagocyted pathogens [72]. Conversely, Th naïve cells committed to a Th2 phenotype when the antigen presentation occurred in an IL-4 enriched environment, being the stabilization of the phenotype dependent on the transcription of the key transcription factor GATA-3. Th2 cells secrete IL-4, IL-5, IL-9, IL-13, and IL-25, among others cytokines. The committed Th2 cells favor the effector mechanisms involved in the eradication of extracellular pathogens, mainly stimulating the secretion of antibodies by activated plasma cells [1, 73]. As in many biological processes, the lineage commitment to the Th1 or Th2 phenotypes is an excluding and self-amplifying event. Once a naïve Th cell is committed to one of the subtypes, the secreted cytokines will serve as an autocrine stimulus to maintain the commitment, as a positive feedback amplification signal, and as a paracrine stimulus to prevent the commitment of other naïve cells to alternative lineages [74].

The pivotal role of the Th1 subtype in the establishment and progression of periodontitis was supported by extensive evidence indicating increased levels of IFN-γ in the tissues of periodontitis patients [75] and corroborated by in vitro evidence of the augment of IFN-γ protein levels and transcription during the progression of experimental periodontitis [76, 77].

It is noteworthy that the role of the Th1 lineage (and its signature cytokine product: IFN-γ) is of paramount importance in limiting the invasion potential of periodontal pathogens. Experimental evidence in IFN-γ deficient mice demonstrates that although they develop a less severe phenotype of alveolar bone destruction following the infection with *Aggregatibacter actinomycetemcomitans*, they are also more prone to suffer widespread infection, with lethal consequences in some cases. The IFN-γ deficient mice demonstrated reduced levels of many inflammatory cytokines and chemokines, as well as significantly reduced numbers of infiltrating macrophage and markers of macrophage activation in the periodontal tissue [78].

The Th1 lineage is also responsible for the secretion of various cytokines that support, maintain, and amplify the inflammatory response, directly or indirectly favoring the recruitment and permanence of immune cells within the periodontal tissues. Both prototypical inflammatory cytokines, IL-1β and TNFα are characteristic secreted products of Th1 lymphocytes. TNFα and IL-1β produce vasodilatation, stimulate the activation of endothelial cells to increase immune cell recruitment, increase the chemokine production in most cell types, participate in neutrophil activation, and stimulate the secretion and tissue activation of MMPs, among other functions. Even though neither IL-1β nor TNFα is directly involved in the stimulation of bone resorption, they indirectly favor the destruction of bone by stimulating the sustained inflammation of periodontal tissue [79].

Alternatively, some evidence supports the hypothesis that periodontitis pathogenesis is more related to the differentiation and effector functions of the Th2 lymphocyte lineage. This idea is supported by the fact that Th lymphocytes isolated from inflamed gingival tissues predominantly produce IL-4 over IFN-γ after non-antigen-specific stimulation [80], and by the classical histological description of the advanced lesion of periodontitis as a "B-cell type of lesion" [31].

Th2 lymphocytes are the main cellular source of the cytokine IL-4, doubling as an inducer of Th2 polarization, as well as an effector molecular signal. Among its more important functions, it promotes the class switching to IgE secretion in B-cells, and favors the alternative activation of

macrophages in an IFN-γ-independent pathway. It is noteworthy that both effector functions limit the capacity of the immune response to control the periodontal infection, which is predominantly mediated by intracellular pathogens [81, 82]. IgE is the isotype class of antibodies best suited to combat large extracellular parasites (such as helminths) and in not pertinent to fight periodontal pathogens. Further, the alternative activation of macrophages inhibits their microbicide functions, suppresses the production of iNOS and consequently of nitric oxide (NO), and stimulates the secretion of IL-10 and TGFβ, which in turn can downregulate pro-inflammatory and Th1 responses [83]. It is for these reasons that some authors consider that the Th2 polarization in periodontitis may represent an impaired adaptive immune response, in which IL-4 inhibits the more effective Th1 polarization. This may be due to inherent host characteristics, or may be also caused by evasion strategies triggered by highly evolved periodontopathic bacteria [84, 85].

In the beginning of the twenty-first century, a new subset of T helper cells characterized by the secretion of IL-17 was described, and named Th17 accordingly. The polarization of these cells was driven by IL-23 and was dependent on the transcription of the transcription factor RORγT [86]. Along with IL-17, Th17 cells also secrete the cytokines IL-21 and IL-22. Shortly after the discovery of Th17 cells, IL-17 was found to be highly expressed in osteolytic lesions, such as periodontitis and periapical lesions [87].

Cytokines such as IL-23, TGF-β, IL-17, IL-6, and IL-1β are highly expressed in inflamed periodontal tissues, providing the necessary signals to drive the polarization of Th cell to the Th17 lineage. Interestingly, secreted RANKL is a characteristic cytokine product of Th17 cells, which in cooperation with its other proinflammatory cytokine products are capable of tilting the bone metabolism favoring resorption over apposition. Additionally, Th17 cells provide the necessary proinflammatory signals to upregulate the expression, secretion, and activation of MMPs, generating an amplification loop of inflammatory and pro-resorptive mediators [88].

Simultaneous analysis of multiple cytokines in periapical osteolytic lesions demonstrated that a complex network of cytokines drives the evolution of the bone resorption (active lesion) or arrest the progression of the lesion (inactive lesion). Characteristic Th1 and Th17 cytokine products, such as TNF-α, IL-21, IL-17, and IFN-γ, appear strongly associated with the bone resorptive process, while the stabilization of the lesion seems associated with the expression of a different subset of cytokines: IL-10, IL-9, IL-4, and IL-22 [89]. The latter are among the signature cytokine products of a distinct Th lineage with immune suppressive properties, known as T regulatory cells (Tregs).

6.2.5 Adaptive Immune Responses May be Also Protective: A Role for Regulatory T Cells (Tregs)

Tregs are Th lymphocytes associated with the secretion of anti-inflammatory cytokines and reparative molecular signals, such as IL-10 and TGFβ. Their lineage commitment depends on the expression of the transcription factor FoxP3, and they characteristically express the surface markers CTLA4, CD103, and CD45RO. The secretion of IL-10 and the contact inhibition of lymphocyte activation by the CTLA4 co-receptor are the signature effector mechanisms of Tregs, leading to an active suppression of the immune response and a return to tissue homeostasis. Experimental in vivo data associates the presence of Tregs with the attenuation of osteolytic progression in periodontal lesions [90]. Recently, the mechanisms responsible for Tregs' migration to periodontal tissues were unraveled, being the IL-4/CCL22/CCR4 axis responsible for the chemoattraction of these cells to the periodontium. The mechanism can be mimicked by the injection of CCL22-releasing particles, which result in a therapeutic arrest of bone resorptive activity [90–92].

While the mechanisms of Tregs chemoattraction have been discovered, the issue of their origin and generation remains an unsolved question. Interestingly, Tregs have been described as central

elements in the determination of a host–microbe homeostasis in several tissues.

Recently, periodontitis has been characterized as a disease of dysbiosis, where the pathologic process is initiated by the disruption of the normally balanced equilibrium between the host and the resident microbiome. In this context, some keystone pathogens can produce an alteration in the microenvironment that is capable of turning commensal microorganisms into opportunistic pathogens, triggering the disease. This framework requires that in healthy conditions the host must be able to tolerate the presence of microorganism without eliciting robust effector responses. This tolerance is an active process, where the host recognizes harmless microbial antigens and suppresses the development of exacerbate inflammatory immune responses against them to preserve the functional equilibrium. Tregs are at the center of this active tolerance, inducing antigen-specific hyporesponsiveness and suppressing the initiation inflammation by a series of specific mechanisms [8, 93].

Additionally, Tregs can also regulate the duration and intensity of the immune response against pathogens, limiting the destructive potential of uncontrolled reactions. In this sense, it is important to highlight that experimental evidence demonstrates that Treg-mediated immune regulation does not interfere with the capacity of the immune response to fight the infection efficiently, and that the directed recruitment of Tregs into diseased periodontal tissues is capable of restore the homeostatic tissue balance, even in the presence of an infectious burden that usually results in the development of destructive periodontitis [90, 91]. These former properties transform Tregs and their selective recruitment to the periodontium in an attractive therapeutic tool to modulate the immune response, without interfering with the infection control mechanisms while avoiding irreversible tissue damage and promoting repair.

The tissue repair that occurs after the contraction of immune response is an active process regulated and orchestrated by the secretion of specific molecular mediators, such as anti-inflammatory cytokines, endogenous pro-resolving lipid mediators (resolvins), and growth factors.

Resolvins are endogenous lipid mediators that modulate cellular fate and inflammation. They are biosynthesized during the resolution phase of acute inflammation, and are capable of inducing cessation of leukocyte recruitment, reversing of vascular inflammatory phenomena and cause prompt apoptosis of neutrophils [94].

Tregs and mesenchymal stem cells (MSC) play a central role in tissue repair, supporting tissue regeneration by direct control of undesired immune reactivity and by direct interaction with nonimmune tissue cells. Tregs can directly interact with MSC progenitors, favoring their recruitment and differentiation in the required cell types for tissue regeneration [95]. Recent experimental evidence suggests that MSC and Tregs act in coaction, favoring the establishment of an anti-inflammatory environment that promotes the repair of bone defects [96].

Therefore, the sequence of events of adaptive immune response that leads to pathologic alveolar bone resorption can be summarized as follows: after the acute inflammation is firmly established, adaptive immune cells are recruited and infiltrate the periodontium, marking the transition to chronic inflammation. Depending on a series of environmental factors and host's intrinsic characteristic, different lineages of effector T cells might predominate inside the tissue, determining the clinical outcome of the disease. If proinflammatory subtypes predominate (Th1 and Th17), the tissue destruction and bone resorption are favored; conversely, if the anti-inflammatory and pro-reparative lineage predominate (Tregs), the inflammation is halted and the tissue tends to regenerate (Fig. 6.1).

Consequently, the variation in the pattern of Th responses seems to be a critical determinant of periodontal bone loss, since such cell types not only regulate the overall immune response pattern but also may directly interfere in the RANKL/OPG balance [1, 8]. However, we must consider that the inflammatory process is a continuum and frequent acute bursts of inflammation or variations in the pattern of host response occur,

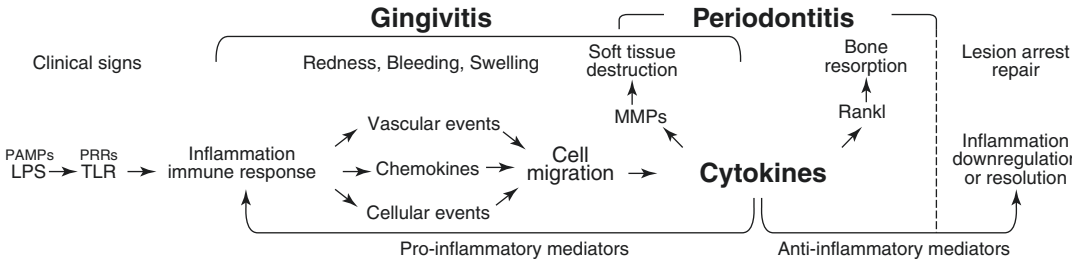

Fig. 6.1 Pathogenic pathway of periodontal diseases. The recognition of PAMPs by PRR triggers the first events of inflammation, which leads to the recruitment of immune cells to the periodontium. The clinical signs are the expression of the underlying inflammatory process. The differential secretion of cytokines by specific subpopulations of infiltrating leukocytes could lead to self-amplification or modulation of the immune/inflammatory response, determining the outcome of the disease

even after the establishment of the chronic phase of inflammation. Indeed, the periodontitis progression is classically described to progress in bursts, where periods of active bone resorption may be followed by periods where the inflammation persists, but the bone resorption seems to be restricted [97, 98]. While this model remains to be confirmed from a molecular point of view, accumulating evidence support the idea that variations in the intensity and nature of the host's inflammatory response may drive the disease evolution via the control of bone resorption.

In this context, virtually any factor or event that could modify the host–microbe interactions and the inflammatory immune response derived from such interactions would influence the evolution and outcome of periodontitis. These complex interactions explain the tremendous variability in susceptibility and clinical phenotype of the disease.

6.3 Risk Determinants to Alveolar Bone Resorption in Periodontitis

Clinicians often face the disconcerting experience of examining a patient with extensive periodontal destruction, but without any obvious clinical finding to explain the severity of the disease. Conversely, the opposed scenario is also possible: a patient with widespread inflammation and abundant presence of dental biofilm, but without any clinical sign of periodontal destruction. These contradictory clinical phenotypes could represent the reflection of the balance and interplay of several underlying risk determinants and environmental risk factors [99]. While in most cases there is a correlation between the amount of dental biofilm and the extent and severity of the periodontitis, patients located at both extremes of the susceptibility spectrum exist and are a routinely encountered in a specialist's dental office. Similarly, patients presenting a lack of response to the classical clinical treatment of periodontitis, without any apparent clinical reason to explain such unresponsiveness, comprise a real and relatively frequent challenge to clinicians.

The factors that contribute to the differential susceptibility to inflammatory destruction of the alveolar bone and other periodontal tissues are not totally understood. Nevertheless, strong evidence points to an increasing number of modifying factors, both innate (e.g., genetic variations) and acquired (e.g., microbial factors, environmental factors, and comorbidities), that can modulate the host–microbe interactions in the periodontal environment, tilting the resistance or susceptibility phenotypes [75, 99–101]. As previously stated, the pathogenic process of alveolar bone destruction is dynamically modulated by the interplay of risk factors affecting the homeostatic balance of the host and his/her capacity to cope with the presence of periodontal microbes (Fig. 6.2).

Indeed, the delicate balance between a protective immune response (without bone loss or periodontal tissue damage) and an exacerbated

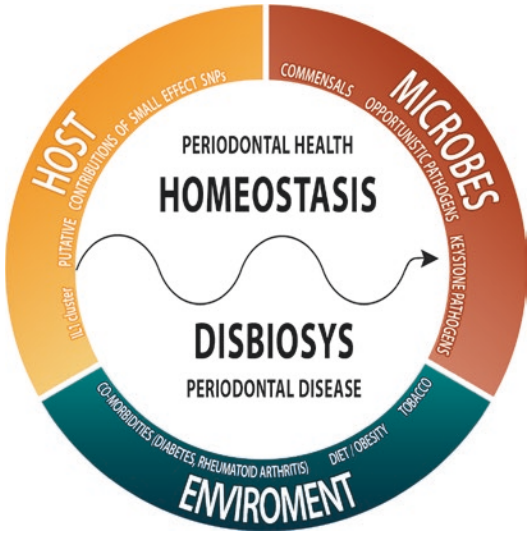

Fig. 6.2 Interaction of risk factors and determinants in the pathogenesis of periodontitis. Environmental, microbiological, and intrinsic host factors determine the balance between the infecting microorganism and the immune response. In homeostatic conditions the host's immune systems are able to tolerate the presence of microorganism without triggering a destructive inflammatory response. The qualitative changes of the oral microbiota (dysbiosis) are a reflection of the loss of balance in the host–microbe interaction leading to disease

immune response (with widespread alveolar bone destruction) can be broken by numerous environmental and host's intrinsic factors. Among them, changes in the oral hygiene habits, placement of deficient dental restorations, antibiotics usage, smoking, nutritional alterations, hormonal changes, metabolic diseases, acquired infections that alters the periodontal microbiota, trauma, corticosteroids usage, stress, and even aging [102, 103]. In addition, there is strong evidence that genetic factors contribute in a significant extent to determine the susceptibility to inflammatory destruction of the tooth-attachment apparatus, determining the quantity and quality of the inflammatory immune response to periodontal infection [104]. Once the balance is tilted, the host's response against the periodontal microbiota can become "destructive" leading to the setting up of the clinical signs of periodontitis: gingival inflammation, increased probing depth of the periodontal sulcus, clinical attachment loss, and alveolar bone resorption.

6.3.1 Acquired Risk Factors

6.3.1.1 Tobacco

The latest consensus report from the 11th European Workshop in Periodontology established that the ask, advise, refer (AAR) approach is the absolute minimum standard of care when dealing with tobacco smokers in the dental clinic. The consensus considered the copious evidence linking tobacco consumption with the occurrence and severity of alveolar bone loss around teeth and dental implants, as well as with negative treatment outcomes [105].

Probably, the most convincing piece of evidence linking tobacco smoking with periodontitis comes from the longitudinal cohort of Dunedin, New Zealand. This birth cohort of 1037 participants has been longitudinally followed since birth in 1972–1973, including a complete dental and periodontal examinations at ages 32 and 36, and tobacco smoking determination at ages 15, 18, 21, 26, 32, and 36. The study demonstrated that smokers had a 23% greater attachment loss than nonsmokers at age 36, confirming the strong association between chronic smoking and periodontal disease [106].

The mechanistic link between tobacco smoking and alveolar bone loss in periodontitis is complex and involves alterations in the microbiome, host's immune response, and metabolism of periodontal tissues. Chronic tobacco consumption leads to profound changes in the periodontal biofilm, dramatically increasing the pathogenicity and disease-initiating capacity of it. Tobacco smoke is responsible for increasing the ability of *P. gingivalis* to form biofilms and decreases its potential to elicit an effective host's immune response, acting in synergy with its proprietary multiple evasion mechanisms [107]. The chronic use of tobacco also generates extensive deleterious effects in the host's immune system, both systemically and locally. At the systemic level, tobacco smoking generates altered tolerance to self-antigens, suppression of the innate immune response, and aberrant adaptive immune responses [108, 109]. At the local level, innate immune cells from smokers (i.e., neutrophils and macrophages) have impaired phagocytic capacity, chemotactic

deficiencies, and increased proinflammatory capacity [110]. Therefore, the clearance of invading microorganism becomes compromised, leading to a sustained chronic and ineffective local inflammation, characterized by the accumulation of neutrophils, lymphocytes, and macrophages within the periodontal connective tissue. Neutrophils can then be activated to degranulate, liberating their rich content of tissue-destructive proteases. Lymphocytes persist within the tissue, liberating proinflammatory mediators that amplify the inflammatory process. Macrophages produce and liberate proteolytic enzymes, reactive oxygen species, and nitrogen species, further contributing to connective tissue degradation [111].

6.3.1.2 Immune Deficiencies

Obviously, any condition affecting the quality and effectiveness of the immune response will have a tremendous impact in the clinical presentation of periodontitis. The most severe forms of periodontitis are those categorized as "periodontitis as a manifestation of a systemic diseases," where the systemic disease is without exception an immune subduing condition, including acquired neutropenia, leukemia, familial and cyclic neutropenia, down syndrome, leukocyte adhesion deficiency syndrome, Papillon-Lefevre syndrome, Chediak-Higashi syndrome, histiocytosis, glycogen storage disease, infantile agranulocytosis, Cohen syndrome, Ehler-Danlos syndrome, and hypophosphatasia [112].

When any of the immune mechanism responsible to withhold the dissemination of the infecting microorganism becomes compromised, the host can develop extensive periodontal destruction and the possibility of suffering the systemic propagation of the infection, with potential severe health outcomes. For example, leukocyte adhesion deficiency type I patients, who suffer from a mutation affecting the CD18 integrin that disrupts the transmigration of neutrophils, often present a very severe periodontitis, with extensive bone loss and a characteristically increased infectious burden within the periodontal tissues [113].

In the case of the acquired conditions, the extensive and rapid alveolar bone loss and periodontal destruction is the common feature, resembling the clinical phenotype of aggressive periodontitis [114]. In the case of the congenital conditions, the periodontal destruction appears very early in life, in some cases affecting both deciduous and permanent dentition and leading to premature exfoliation of all teeth [115].

It is possible to mention the infection by HTLV-1 virus as an additional example of an acquired disturbance on the immune system that impacts periodontitis outcome [116]. HTLV-1 results in an overall deregulation of immune response, being associated with a series of pathological conditions. In periodontitis context, it was demonstrated that even presenting a standard periodontopathogen infection, patients with HTLV-1 demonstrated an exacerbated immune response and increased periodontal destruction.

6.3.1.3 Metabolic Diseases

Some metabolic diseases affect the clinical presentation of periodontitis, correlating with the presence, extension, and severity of the alveolar bone loss [117]. Among them, diabetes mellitus is most widely studied, and a large body of evidence demonstrates that uncontrolled diabetic patients are under increased risk of suffering severe forms of periodontitis, with extensive alveolar bone loss, and with poor response to conventional periodontal treatment [118].

Recent evidence from clinical trials supports the notion that diabetic patients exhibit increased RANKL/OPG ratios, and that the periodontal treatment is capable of diminishing the RANKL/OPG in follow-up periods of 3 months [119]. In this respect, the mechanism behind the increased susceptibility to alveolar bone loss would be the same as in patients without diabetes, and the increased susceptibility would be a consequence of impaired inflammation control mechanisms. Further, evidence from a recent systematic review including 35 parallel randomized clinical trials (2565 participants) demonstrated that periodontal therapy has a measurable effect in the glycemic control of diabetes patients, pointing to a bidirectional association between periodontitis and diabetes mellitus [120]. The linking factor behind the bidirectional relationship of diabetes and periodontitis could be the inflammatory molecular signals, that emanated from the local

environment of the periodontium could act at distant sites affecting the glucose metabolism and modify the course of the disease [121]. A recent systematic review and meta-analysis, including nine clinical trials, demonstrated that periodontal treatment significantly reduced circulating levels of TNFα and C-reactive protein, diminishing the inflammatory burden in type 2 diabetic patients [122]. This evidence supports the mechanistic link of periodontitis and diabetes through inflammatory mediators. It is important to consider that the study of the potential interconnection of multifactorial diseases such as diabetes and periodontitis is complex due the multitude of variables inherent from both conditions. In this scenario, data from experimental models is especially important in the confirmation and understanding of the alleged interaction. Interestingly, experimental models demonstrate that diabetes can in fact disrupt host–microbe homeostasis in periodontal environment, since the spontaneous periodontitis development in diabetic rats involves an unrestricted expression of inflammatory cytokines and tissue destructive factors in the absence of major changes in commensal oral microbiota [123]. In other words, the modifications in host responsiveness caused by diabetes can trigger a destructive inflammatory response against previously well-tolerated microorganism.

Another metabolic disease strongly associated with alveolar bone loss in periodontitis is rheumatoid arthritis (RA). RA patients present the inflammatory degeneration of diarthrodial joints with a profuse infiltration of immune cells into the synovial lining. As in periodontitis, the characteristic bone erosions in inflammatory arthritis are caused by a decontrolled activation of osteoclast due to the inflammatory upregulation of RANKL and the increase of the RANKL/OPG ratio [124]. Epidemiologic data points to a strong correlation between the occurrence of both diseases, suggesting common susceptibility traits and a possible common pathogenesis [125]. The hypothetical pathogenic link could be the capacity of some periodontal pathogens to produce enzymatic changes in structural proteins that trigger a humoral response against self-antigens [126]. Specifically, *P. gingivalis* is the only known bacteria that produce the enzyme peptidyl arginine deiminase (PAD), which catalyzes the conversion of arginine residues to citrulline. The irreversible citrullination of arginine residues changes the structural characteristic of proteins, transforming them in antigens capable of eliciting an immune response [127]. Citrullinated proteins characteristically accumulate in the joints of RA patients, and specific autoantibodies against them are one of the central causes for joint degeneration and bone destruction during the progression of the disease [128, 129]. Additionally, the pro-inflammatory molecular signals liberated in the chronic immune response of periodontitis and RA could reach the circulation, generating the mutual exacerbation and perpetuation of the inflammatory response in distant compartments [130]. In this sense, both diseases will act in synergy in a loop of reciprocal inflammatory amplification. The connection between RA and periodontitis is also supported by experimental model's data, which demonstrate that periodontitis and arthritis interaction in mice involves a shared hyper-inflammatory genotype and functional immunological interferences [131]. In an analog fashion to the scenery described for diabetes, RA also seems capable of disrupting the host–microbe homeostasis, leading to aberrant responses to microorganism previously recognized as commensal and nonhazardous microbiota [131].

Another example of the complexity of the immune mechanisms that drive the inflammatory destruction of host's tissues is the evidence that the high dietary salt intake (characteristic of modern western diet) has the potential to induce enhanced macrophage infiltration, increased inflammatory cytokine secretion, and polarization of immune response towards a Th17 phenotype. In sum, the high dietary salt intake can be regarded as an environmental risk factor for the development of autoimmune diseases [132–134]. Along the same lines, recent evidence points to an association between obesity/metabolic syndrome and exacerbated alveolar bone loss, supported both by epidemiological data and by experimental in vivo evidence [135–137]. Again, the linking factor between both conditions would be the exacerbation of inflammation and the malfunction of inflammation control mechanisms.

6.3.2 Innate Risk Determinants

Genetic susceptibility determinants are among the most studied topics in the periodontal literature. Nevertheless, despite the generalized acceptance of genetic factors as key regulators of the susceptibility to periodontitis, a great controversy still persist regarding the specific contribution of particular mutations to the overall clinical phenotype, and more importantly a model integrating the human genetic diversity into the pathogenesis of periodontitis is still lacking.

The first clear evidence that genetic factors played a central role conferring susceptibility or resistance to periodontal destruction came from the elegant work of Michalowicz et al. with adult twins [138, 139]. Despite later controversies, Michalowicz et al. established that genetic factors accounted for at least a 50% of the clinical variation on the disease phenotype, regulating the susceptibility to suffer bone loss and periodontal destruction. Since that seminal work, many models and hypothesis have been proposed to explain the mechanisms of genetic influence over the phenotype of periodontitis.

The focus of the genetic research in periodontal susceptibility have been the molecular mediators of inflammation, particularly the mutations affecting the levels and expression of cytokines recognized as key regulators of the inflammatory process. The most studied mutations in periodontitis are the polymorphic variations of the IL-1 gene cluster and/or its promoter region. In the late years of the twentieth century, Kornman et al. reported the association between the composite polymorphic genotype for IL-1A (−889) and IL-1B (+3953) genes and the severity of periodontitis in nonsmokers, concluding that the composite polymorphic phenotype contributed significantly to increased IL-1β levels and augmented risk of suffering severe forms of periodontitis [140]. Since then, numerous replication studies in various populations have been conducted with conflicting results. A recent systematic review and meta-analysis of 20 case–control studies in Asian subjects, including in excess of 3000 patients and controls, concluded that the polymorphic form of IL-1B (+3953) was strongly associated with an increased risk of periodontitis in Indians, but not in Chinese [141]. The above example testifies for the difficulty of stablishing associations between genetic variations and the presence and severity of periodontitis, with controversies spanning for decades in the literature and still unresolved.

A major source of bias in periodontal genetic research came from the necessity to select a priori a gene(s) to study in an association research, often based in their putative theoretical involvement in key steps of the immune response or periodontal tissue's metabolism. Nevertheless, this approach has proved inconclusive and most associations between mutations and the occurrence, severity, or extension of periodontitis lack the necessary replication in different populations, as previously exemplified with the case of the IL-1 cluster. Probable causes of these difficulties are multiple, among them: the complexity of the immune regulatory mechanisms, overlap and redundancy in the functions of many mediators of inflammation and bone metabolism, the lack of a linear relationship between gene expression and protein production/cell phenotype, and the lack of a comprehensive understanding of the regulatory pathways that maintain tissue homeostasis. Additionally, the difficulty in the definition of the periodontitis cases and the selection of suitable controls poses another level of complexity over the design of periodontitis genetic association studies [99].

Even with the inherent problems of the classic periodontal genetic studies, the literature consistently demonstrates that mutations altering the host's capacity to mount and efficient yet controlled immune response, or those related to the exacerbation of the bone and connective tissue turnover, are strongly associated with the presence and clinical phenotype of the disease. As examples: the single nucleotide polymorphism (SNP) rs4794067 of the TBX21 gene that increases the transcription of T-bet, leading to an exacerbated inflammatory response, is strongly associated with the occurrence of periodontitis in a Brazilian population [75]. Further, the SNP rs1800872 in the promoter region of the IL-10 that decreases the transcription of IL-10, leading

to impaired immune regulation, is strongly associated with the occurrence and severity of periodontitis in the same population [142]. These two examples show the value of the classical approach to periodontal genetic studies, but also testify for its inherent flaw, where a theoretical framework taking into consideration the functional consequences of the studied mutations is a sine qua non condition. This condition greatly limits the validity of the studies, since many genes are not studied by the lack of a supporting theory linking them to the pathogenesis of periodontitis.

To overcome some of these problems, a new strategy using the results of large genome-wide association (GWA) studies has been recently proposed. The rationale of this approach is to scan the whole genome searching for genes or groups of genes associated with the presence of the disease (or disease subrogate) without any a priori selection bias.

Using the GWA-based selection strategy a completely new set of genes with strong association to the occurrence of periodontitis have been discovered. These previously ignored genes code for neuropeptides, innate immune response receptors, enzymes linked to cytoskeletal rearrangement, intracellular signaling transduction molecules, and proteins directing vesicle fusion during synapsis, and even some noncoding genes or genes with no known function. The diversity of the newly discovered periodontitis-associated genes testifies for the power of the GWA approach, since it allows for the unveiling of disease–gene association without the necessity of a previous theoretical framework [143].

While the real contribution of genetic determinants to periodontitis risk remains to be established, it is important to consider that extensive research efforts in the field aim to provide additional tools to the clinician in the direct risk assessment, which in turn may influence a series of clinical decisions. An interesting example of the clinical value of being able to assign and objective risk value to the presence of certain SNPs, is the algorithm that forms the PRS index (polygenic risk score) for the risk discrimination in breast cancer. The PRS index additively combines the risk effect of 77 SNPs, providing a valuable tool that has predictive value and can be used in the decision-making process during the management of breast cancer cases [144]. An analog tool for the risk assessment of periodontitis, aiding in the long-term clinical management of patients, is an old longing of the practicing periodontist.

6.4 Future Perspectives for the Treatment of Periodontitis: From the Bench to the Dental Chair

The accumulate knowledge of the immune mechanisms driving the progression of bone loss in periodontitis has stimulated the development of new therapeutic approaches to modulate the immune inflammatory response with the intention to limit the adverse consequences of unregulated responses. Theoretically, bioactive molecules with the potential to regulate key points of the immune response could be used as coadjutants in the treatment of periodontitis, enhancing the clinical effectiveness of conventional treatment, preserving the results for longer periods, limiting the progression of periodontal destruction, and modifying the susceptibility to suffer relapses.

Various promising efforts in the development of clinically relevant and safe bioactive molecules are in different stages of experimental testing, with the perspective of reaching to the clinical practice in the near future. The classic approach to coadjuvant pharmacological treatment in periodontitis is the use of anti-infective agents. Although not immune modulatory agents in a strict sense, the use of anti-infective therapy has a pronounced effect in the immune system, decreasing the number of antigens available to trigger an immune response and limiting the extent and duration of it. Characteristically, systemic antibiotics has been used to enhance the results of periodontal treatment in severe or high-risk patients, nevertheless there is a series of problems associated with the use of these agents, including the appearance of bacterial resistance and the occurrence of adverse reactions. Over the years, various controlled-release local anti-infective agents have been developed in an effort to limit the negative

consequences of the use of systemic antibiotics, but providing their putative benefits. In a systematic review and meta-analysis including 32 studies (3705 subjects), the use of minocycline gel, microencapsulated minocycline, chlorhexidine chips, and doxycycline gel during scaling and root planning (SRP) proved marginally better than SRP alone, but not to a clinically significant extent [145]. Another shortcoming of antimicrobial therapy is that after the completion of the treatment the risk factors and susceptibility traits that contributed to the establishment of a pathogenic microbiota (dysbiosis) remain unaltered, leading to a recolonization of periodontal sites by a pathogenic microbiota and to the recurrence of the disease [146, 147].

The new emerging therapeutic approach will use *immune modulatory* drugs, capable of selectively regulating key points of the immune response, specifically limiting the degree of inflammation but without dampening the system's capacity to fight the infection. In an analog fashion to the trend of using controlled-release local anti-infective drugs, the goal is to develop an immune modulatory agent that could be directly targeted to the periodontal tissue, preventing the potential risks of undesirable immune modulation in other body compartments. It is noteworthy that the systemic administration of classic nonsteroid anti-inflammatory drugs (NSAID) as adjunctive treatment in periodontitis, aimed at reducing the levels of arachidonic acid metabolites, has shown no long-term clinically relevant benefits in several clinical trials, pointing to the need of a very specific and focused inhibition of inflammation in order to attain meaningful clinical benefits. Additionally, the potential risks of the chronic use of NSAID, such as gastric ulcer and coagulation impairment, seriously limit their usefulness as adjunctive therapy in periodontitis [148].

As depicted in Figs. 6.3 and 6.4, an interesting approach would be the use of drugs capable of

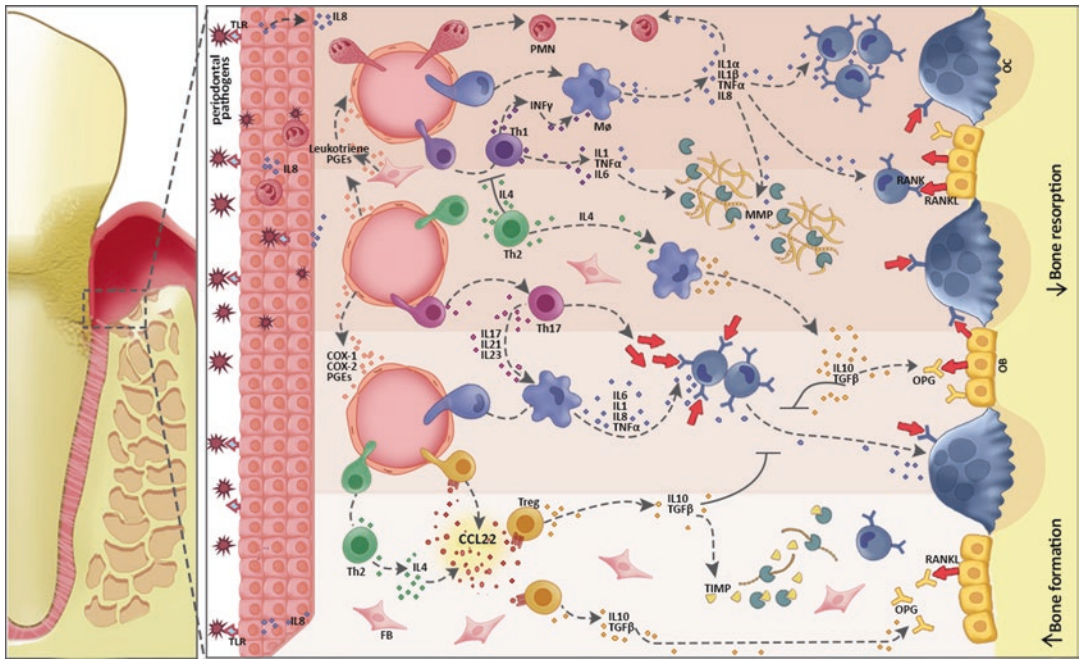

Fig. 6.3 Immune and inflammatory response to periodontal infection. Periodontal pathogens are recognized by the resident cells, which trigger the first inflammatory response mediated by PAMP/PRR interactions. Infiltrating innate immune cells rapidly reach the infection site and amplify the primary response. The adaptive immune cells that appear sequentially determine the outcome of the response. The relative predominance of pro-inflammatory lineages (Th1 and Th17) favors soft tissue destruction and osteoclast genesis. Conversely, when regulatory lineages predominate (Tregs and possibly Th2), the inflammation is halted, osteoclast genesis is inhibited, and the tissue tends to repair

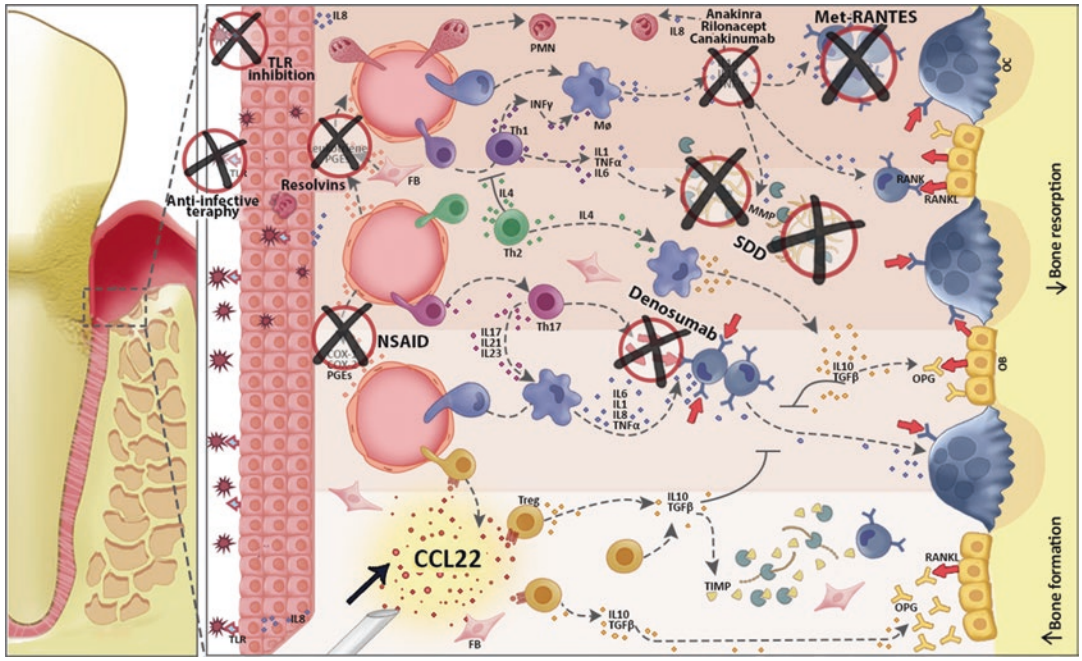

Fig. 6.4 Modulatory strategies to control the inflammatory destruction of alveolar bone. Anti-infective therapy diminishes the presence of the antigen, limiting the response. TLR inhibition offers the possibility of inhibiting the first events of inflammation, but with the danger of facilitating the infiltration of pathogens. Resolvins could exert a modulatory event reversing the vascular events of inflammation and inducing apoptosis of infiltrating leukocytes. NSAIDs inhibit the production of arachidonic acid metabolites, modulating the inflammatory response. Specific antibodies against inflammatory cytokines and receptors (Anakinra, Rilonacept, Canakinumab, met-RANTES, and Denosumab) could inhibit osteoclast genesis directly or indirectly. SDD inhibits soft tissue destruction and the lysis of the demineralized bone matrix. Selective recruitment of Tregs by CCL22 could fine-tune the inflammatory response, leading to an efficient response without tooth-attachment loss

inhibiting the first signaling events in the inflammatory process, but without compromising the later steps of the response. In this sense, Toll-like receptors (TLR) appear as promising therapeutic targets. These receptors are fundamentally involved in the recognition of pathogen-associated molecular patters (PAMPs). TLRs are capable of triggering the first events of the inflammatory cascade, including the upregulation of inflammatory cytokines and MMPs.

Theoretically, the selective inhibition of one or various TLR subtypes could limit and modulate the extent of the initial reaction, thus modifying the nature of the immune response, and preventing the occurrence of an uncontrolled inflammation leading to bone metabolism uncoupling and resorption. Given that TLR are expressed in the surface of every cell type in the periodontal tissue, the immune modulatory potential for a drug targeting these receptors is prodigious. There is a growing body of in vivo evidence demonstrating that the selective inhibition of TLR's activation or signal transduction is protective against periodontitis-induced alveolar bone loss. In a murine model of *Porphyromonas gingivalis*-induced periodontitis, the pharmacological inhibition of GSK3 (a key factor in the signal transduction chain downstream of TLR activation) significantly decreased the systemic levels of TNFα, IL-1β, IL-6, and IL-12 post infection, and suppressed alveolar bone loss [149]. Further, mice genetically deficient (i.e., knock out) for the expression of TLR pathway signaling molecules, such as MyD88, demonstrated resistance to LPS-induced alveolar bone loss, associated with diminished levels of inflammatory cytokines expression and osteoclast differentiation markers [150]. Nevertheless, it is fundamental to bear in mind

that the innate immune events triggered by the activation of TLR are of paramount importance for the control of infection and that any drug targeting this receptors will need to be highly selective and tissue specific to avoid the potential dangers of the spreading of the periodontal infection to other body compartments.

Moving down the inflammatory cascade, the next step susceptible of pharmacological modulation is the selective inhibition of the effector molecules of inflammation. One alternative is the use of nonsteroidal anti-inflammatory drugs (NSAID) to control and modulate the production of pro-inflammatory molecules, such as prostaglandins derived from arachidonic acid. The classical COX-1 and COX-2 inhibitors have the potential to limit the magnitude of the inflammation. Even though they have proven effective in diminishing the alveolar bone loss in experimental models and marginally effective in clinical testing [148, 151, 152], their routine use is unpractical due to the appearance of unwanted side effects in the long-term treatment.

Other emerging therapeutic approach is the use of endogenous pro-resolving lipid mediators (resolvins) to modulate the inflammatory response and protect the alveolar bone from resorption [153]. These lipid mediators are structurally related to prostaglandin and leukotriene, but contrary to the pro-inflammatory activities of the former are capable of inhibit leukocyte recruitment and promote inflammatory resolution. Evidence form *Porphyromonas gingivalis*-induced periodontitis in vivo experiments demonstrated that the administration of Resolvin E1 as monotherapy resulted in complete resolution of the bone lesion and in reduction of systemic markers of inflammation [154].

An additional possible target of modulation are the enzymes responsible for connective tissue degradation, namely MMPs. Collectively, MMPs are responsible for collagen turnover in all periodontal connective tissues, including bone [155]. After bone demineralization, collagenases and gelatinases degrade the organic component of the bone matrix allowing for bone remodeling. Increased collagen lytic activity as a consequence of inflammation is the ultimate responsibility for net bone loss during the progression of periodontitis. Tetracyclines in general and doxycycline in particular have the property of inhibiting the activity of MMPs independently of their antimicrobial action [148, 156, 157]. The inhibition of MMP using systemic subantimicrobial-dose doxycycline (SDD) is a well-established therapeutic approach and there is a three-decade body of evidence supporting its effectivity as an adjunct treatment in chronic periodontitis [158]. The last advance in the use of SDD as MMP inhibitors is the development of sustained release nanostructured films, with the property of delivering a constant dose of doxycycline directly into the periodontal tissue for prolonged periods. Preliminary clinical trials proved that the use of the sustained release films improved the clinical outcome of periodontal treatment after a follow-up period of 2 months [159].

Another alternative is the use of highly specific antibodies against the molecular mediators of inflammation. Although there is not any cytokine inhibitor currently being used for the treatment of periodontitis, several cytokine-inhibiting drugs are routinely used to prevent the inflammatory bone loss in rheumatoid arthritis (RA) [160]. Given the pathogenic similarities of inflammatory bone loss in RA and periodontitis, and the possible common pathogenic mechanisms among them, it is possible to hypothesize that such drugs would be also useful to prevent alveolar bone loss in periodontitis. Three approved drugs for the treatment of RA reduce the activities of IL-1α/β, diminishing the inflammation and preventing bone loss. Anakinra is a recombinant form of the naturally occurring IL-1 receptor antagonist. Anakinra conjugates to the IL-1 receptor, preventing the activity of IL-1α/β [161]. The soluble IL-1β decoy receptor Rilonacept and the neutralizing antibody Canakinumab block the biologic actions of IL-1β [162]. Taking into consideration the link between inflammation and the increasing ratio of RANKL/

OPG previously discussed, the selective down-regulation of IL-1α/β could prove valuable as bone anti-resorptive drugs. Nevertheless, the infectious nature of periodontitis and the risk of impairment of the pathogen clearance mechanisms pose a serious limitation for the use of immune suppressive drugs in the treatment of periodontitis and the potential risks must be carefully weighed.

Yet other possible pharmacological target are the cytokines/receptors responsible for the selective recruitment of osteoclasts precursors to the bone compartment. These chemotactic cytokines (a.k.a. chemokines) and their receptors are responsible for the control of the influx of monocyte/macrophages to the periodontal tissue, and are induced under inflammatory conditions [163]. For example, there is experimental in vivo evidence of the effectiveness of the pharmacologic inhibition of the chemokine receptors CCR1 and CCR5 in reducing the alveolar bone loss in a murine model of *Aggregatibacter actinomycetemcomitans*-induced periodontitis, using the functional competitive inhibitor met-RANTES [163]. In this model, the reduced recruitment of osteoclast precursors and other inflammatory cells caused a protective effect in alveolar bone levels, but proved detrimental to the anti-infective response at higher doses. These results highlight the complexity of immune modulatory therapies, where there is a thin line between bone protective effects and increased risk of systemic spreading of the local periodontal infection.

In an interesting approach and proof-of-principle in vivo experiment, it was demonstrated that harnessing endogenous Tregs and recruiting them to specific sites of periodontal inflammation is effective in limiting bone resorption and promoting the establishment of a regenerative environment, and associated with diminished markers of inflammation [68]. In this experimental setting, endogenous Tregs were recruited to the periodontal tissue using a controlled-release polymeric vehicle designed to create a gradient of CCL22, a known selective chemokine for Tregs. It is noteworthy that the selective recruitment of Tregs to the periodontal tissue did not associate with increased bacterial load or with impairment in the bacteria clearance mechanisms, providing an attractive possibility for safe local immune modulation, which can be explored as a future approach to enhance the clinical results of conventional periodontal therapy [91]. Additionally, approaches based in the induction or chemoattraction of Tregs may restore the local host–microbe homeostasis, potentially resulting in longer-term effects with increased potential impact in avoiding subsequent disease events [8].

Finally, a further possible target of pharmacologic modulation is the RANKL/RANK/OPG axis. As previously stated, the relative abundance and equilibrium among these molecules is largely responsible for the balance between bone apposition and bone resorption. The inflammatory process increases the RANKL/OPG ratio, causing bone resorption. Denosumab is a human monoclonal anti-RANKL antibody approved for use in osteoporosis patients with high risk of fracture [164]. Clinical trials have demonstrated the efficacy and safety of the drug in long-term use, opening the possibility for its use in other bone resorbing pathologies, such as periodontitis [165]. At this time, it is reasonable to suppose that the modulation of the RANKL/RANK/OPG axis to manage periodontitis may become a therapeutic tool available for the clinician in the near future.

Those and other future developments will contribute to the increase and diversification of the therapeutic tools available to the periodontist. The perspective of new therapeutic approaches allowing for the successful treatment of refractory cases is a long-standing desire of the practicing periodontist, but their implementation will require a thoughtful understanding of the underlying cellular and molecular mechanism of periodontitis' pathogenesis, and the accompanying ability of judging the convenience of their use in a particular clinical situation. As the body of knowledge of periodontics swiftly increases, the periodontist must remain up to date to be able to take full advantage of the coming therapeutic advances in benefit of his/her patients.

Clinical Relevance

- Immune response and inflammation protect the host from widespread dissemination of periodontal pathogens.
- Exacerbated or uncontrolled immune responses are responsible for the periodontal tissue destruction.
- A limited subset of signaling molecules link the inflammatory response with the uncoupling of bone metabolism that leads to bone resorption.
- The modulation of these signaling pathways and molecules appears as a concrete possibility to deal with patients unresponsive to traditional therapy.
- For the clinician it is essential to understand the intricate immune pathways involved in periodontitis pathogenesis to be prepared for this next generation of diagnostic and therapeutic tools.

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7 Genetic Influences on the Periodontal Microbial-Host Crosstalk

Luigi Nibali

7.1 Introduction

Previous chapters of this book have discussed the importance of subgingival microbial colonization and of the inflammatory-immune response as triggers of periodontal tissue breakdown. In fact, it appears clear that periodontal health or pathology is the result of the interaction between the human host and its invading microbes. Or perhaps we should not define them as 'invading', since it is well known that microbes not only coexist with their human host, but also provide multiple vital functions for the survival of the host itself [1]. Compelling evidence has now emerged to suggest that host genetic variants have a fundamental effect in regulating the host's relationships with the microbial 'guests' and a better knowledge of how these effects are implemented is crucial in the understanding of disease processes. This chapter will review the evidence on the effect of host genetic factors on periodontal microbial colonization and will provide examples of how this could have an impact in clinical practice.

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7.2 Infectogenomics

In the fourteenth century, one-third to half of the population living in Europe was exterminated by a mysterious disease which was called 'the Black Death'. It is now known that this disease, later named 'plague', was most likely a rodent-associated, flea-borne zoonosis caused by Gram-negative bacterium *Yersinia pestis* [2]. Interestingly, among all people who came into contact with the bacterium, a large proportion became infected and died, some were ill but managed to survive and some had no clinical signs of infection. It appears plausible that each subject responded to infection with *Yersinia pestis* as well as to other potentially fatal infections in a way that was largely determined by his/her genetic make-up [2, 3]. A more up-to-date example is given by the HIV, the virus responsible for AIDS. Following transmission, HIV enters the bloodstream and infects helper T cells, macrophages and dendritic cells, causing killing of T cells (especially CD4 T cells) by CD8 cytotoxic lymphocytes, with reduction in CD4 T cell numbers and loss of cell-mediated immunity [4]. The HIV most commonly uses chemokine receptors CCR5 and/or CXCR4 co-receptor to enter its target cells. The CCR5 receptor is coded for by the CCR5 gene on chromosome 3. A deletion of a 32-bp segment in this gene (named CCR5-Δ32) has been discovered to result in a non-functional receptor which prevents this way of HIV R5 entry

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[4]. This genetic variant is rare in Africans/Asians and more common in North European, possibly due to selective pressure by previous epidemics [3, 5, 6]. Homozygosity to this gene variant is characterized by resistance to infection by the most common strain of HIV, while heterozygosity seems to confer partial resistance with slower progression after onset of AIDS [7]. Based on this principle, CCR5 receptor-antagonist drugs have been experimented for the treatment of AIDS [8].

The evidence described above is in line with a concept defined 'infectogenomics', suggesting that host genetic factors play a major role in determining the response to bacterial colonization [9, 10]. This concept can be extended also to the presence of common 'symbiotic' bacteria and not just pathogens. In other words, host genotypes may influence the composition of human biofilms, including oral biofilms [11]. Therefore, the composition of microbial biofilms in the human body will be dictated by a combination of genetic variants, coupled with environmental factors. As a result of this, a group of human diseases originate from a genetically determined failure to properly recognize or respond to members of the normal human microbiota [11]. This disease-predisposing effect can potentially extend not just to microbial diseases, in the traditional meaning of the term, such as, for example, bacterial vaginosis and periodontitis, but also to diseases not traditionally considered of microbial origin. Among them, rheumatoid arthritis, reactive arthritis and even cancer, which could be influenced by microbial shifts (dysbiosis) even at distant sites. Hence the concept of genetic dysbiosis, which suggests that host genetic variants could be responsible for a range of chronic human diseases through an effect on dysbiosis of microbial biofilms [11].

7.3 Evidence for Genetic Variants Influencing the Response to Microbial Challenge

Where is the evidence for the Infectogenomics principles outlined above? Circumstantial evidence can be derived from studies showing that monozygotic twins frequently have more similar gut microbiomes than non-twin siblings [12]. Furthermore, microbial profiles of faecal samples collected at various times from a given individual are more similar to each other than to the intestinal microbial communities in a different individual [13]. A little dip into the human genome can allow a better understanding of the host-microbial axis. More than 60 million common genetic variants in 19,000–25,000 genes located in 23 pairs of chromosomes are listed in the Single Nucleotide Polymorphism Database (dbSNP) by the National Center for Biotechnology in collaboration with National Human Genome Research Institute [14]. Different individuals are thought to be 99.4% identical in chromosomal structure and 99.9% identical at sequence level [15]. Functional SNPs are located in the gene promoter (affecting gene activity) or in the coding region of the gene (affecting the protein produced). The disease-predisposing effects may be determinant such as for haemophilia A, caused by a specific mutation (single gene defect) in the F8 gene leading to defects in coagulation factor VIII [16]. However, most diseases are characterized by a complex susceptibility profile, where a variety of SNPs contribute to the disease risk. Such SNPs may be involved, for example, in microbial recognition (determining aberrant responses to the normal microbiota), in the inflammatory cascade or in DNA repair (associated with a reduction in the ability to repair damaged DNA).

7.3.1 Microbial Recognition Genes

Following the earlier example of the HIV, it seems reasonable to believe that genetic variants affecting microbial recognition will have a major role in determining the composition of microbial biofilms. The search for the possible 'microbial recognition' gene affecting microbial colonization could focus on pattern-recognition receptors (PRRs), which recognize evolutionarily conserved constituents of microbes called pathogen-associated molecular patterns (PAMPs). PRRs include normally cell-bound proteins such as toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), C-type-lectin like receptors (CLRs), scavenger receptors

(SCs), innate DNA receptor proteins termed AIM2-like receptors (ALRs), members of the complement pathways and peptidoglycan-recognition proteins (PRPs) and soluble PRRs (including collectins, ficolins, pentraxins, galectins, sCD14 and natural IgM). Upon microbial interaction, PRRs activate a series of downstream mechanisms through selective cells signalling, leading to the generation of pro- or anti-inflammatory proteins. Mutations in the coding or promoter regions of PRR genes could result in an altered ability to recognize microbial patterns, affecting the 'binding/recognition' process and its downstream pathways, leading to aberrant response to microbial challenge and shifts in the normal biofilm composition [17].

The circumstantial evidence for a role of host genetic variants in determining microbial colonization is strengthened by observations on inflammatory bowel disease, encompassing Crohn's disease (CD) and ulcerative colitis (UC). Genetic variants in the NOD2 gene, coding for an intracellular pattern recognition receptor able to recognize molecules containing bacterial muramyl dipeptide [18], are now recognized as increasing the risk of CD [19]. In an experimental ileal inflammation model in mice [20], when NOD2-CD-susceptible animals were subjected to *Toxoplasma gondii*-induced ileitis, an increase in inflammation and dysbiosis was noticed (shift from mainly Gram-positive to Gram-negative bacteria, associated with invasive *E. coli*) compared to non-genetically susceptible animals. Furthermore, genetic variants in the NOD2 and autophagy-related 16-like 1 protein (ATG16L1) have been associated with gut microbiota structure alterations, including decreased *Faecalibacterium* levels and increased *Escherichia* levels [21]. These results are likely due to an alteration of the inflammatory cascade resulting from an aberrant response upon microbial recognition through the NOD2 receptor.

Some evidence exists also for the effect of microbial recognition genes on microbial presence in vaginal biofilms. In a study on the vaginal microbiota of 144 pregnant women, detection of *A. vaginae* and *G. vaginalis* by PCR was studied in relation to 34 single nucleotide polymorphisms pertaining to 9 genes involved with Toll-like receptor-mediated pathogen recognition and/or regulation. While no association between these SNPs and presence of bacterial vaginosis was detected, some of the studied SNPs were associated with carriage of *A. vaginae* and *G. vaginalis* during early pregnancy. The authors suggested that some degree of genetic susceptibility involving pathogen recognition may occur, which influences vaginal presence of potential pathogenic microorganism [22]. In a separate study on 238 pregnant women, TLR4 genotypes were associated with increases in vaginal pH and in vaginal detection of *Gardnerella vaginalis*, *Prevotella*, *Bacteroides* and *Porphyromonas*, suggesting that genetic variants may drive a change in the vaginal environment which favours the growth of pathogenic bacteria [23].

7.3.2 Genes Involved in Inflammatory Pathways

It is now becoming clear that inflammation can have profound effects on microbial communities, causing a progressive decrease in the microbial diversity through an increased availability of substrates for growth of Gram-negative bacteria (e.g. iron and serum, dead or dying cells) and loss of niche and substrates for Gram-positive flora (e.g. mucus, goblet cells) [24]. Therefore, it is conceivable that variants in genes directly involved in inflammatory pathways, could impact the threshold for dysbiosis and the ability to resolve the dysbiosis-inflammation cycle generated by an acute trigger [20]. Based on this concept, genetic variants affecting inflammatory responses may be major candidates for an effect on microbial biofilm composition. The evidence for this comes mainly from studies on periodontal disease which will be discussed in the next section.

7.4 Genetic Effects on Microbial Colonization: Studies in Periodontal Disease

Previous chapters of this book described how pathogenic pathways leading to periodontal breakdown involve the role of subgingival

microbes, host response and environmental factors and how important the crosstalk between host and bacteria is. This chapter introduced the concept of 'Infectogenomics' to mean the effect of host genetic background on the colonizing microbes, and examples relative to inflammatory bowel disease and bacterial vaginosis have been provided. In the last 10–15 years, evidence for periodontal infectogenomics [25] has also emerged. In particular, it is striking how the JP2 leukotoxic strain of *A. actinomycetemcomitans* has a strong tropism of for subjects of mainly North African and West African descent, increasing the risk of development of Localised Aggressive Periodontitis (LAgP) [26]. Since carriage of this strain does not seem to depend on geographic location but rather on ancestry, it is likely to be linked with heritability and with the host genetic make-up. Figures 7.1 and 7.2 show a typical clinical and radiographic presentation of a case of Localized Aggressive Periodontitis (LAgP), characterized by a molar-incisor pattern of bone and periodontal attachment loss.

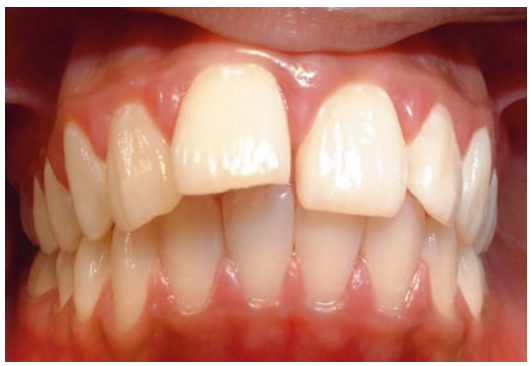

Fig. 7.1 Clinical photograph of 15-year-old non-smoker LAgP patient of Afro-Caribbean origin, showing buccal migration of the upper right central incisor and generally good oral hygiene

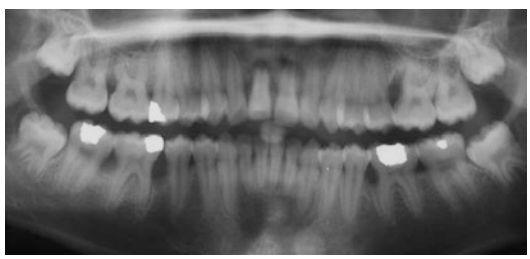

Fig. 7.2 Panoramic radiograph of patient shown in Fig. 7.1. Please note localized alveolar bone loss affecting mainly upper right central incisor and first molars

The understanding that heritability accounts for about half of the risk of developing periodontitis [27] has led to a flourish of studies trying to identify inflammatory, metabolic or structural gene polymorphisms which could predispose to periodontal diseases [28]. Most studies focused on selected candidate SNPs, starting from the case-control association study suggesting an effect of the Interleukin-1 (IL-1) 'composite genotype' on disease predisposition [29]. More recent studies are often using an explorative genome-wide approach [30, 31]. However, studies so far failed to reach a consensus after analyses in different populations and settings, with promising studies pointing towards the role of SNPs in ANRIL (antisense non-coding RNA in the INK4 locus), COX2 (cyclooxygenase 2), IL-10 (Interleukin-10) and DEFB1 (β-defensin-1) and possibly others in disease predisposition [31, 32]. These genes are involved, respectively, in glucose and fatty acid metabolism regulation (ANRIL gene) [33], coding for antimicrobial peptides involved in the epithelial response to microbial invasion (DEFDB1 gene) [34] and in the periodontal inflammatory response (COX2 and IL-10 genes) [35, 36].

Sigmund Socransky and Anne Haffajee were probably the first to investigate the relationships between SNPs supposed to affect the periodontitis trait and presence of subgingival microbes. In their 2000 paper, they observed an association between IL-1 genotypes and presence of subgingival microbes [37]. In particular, more IL-1 'genotype positive' subjects [29] exhibited high mean counts of 'red' and 'orange' subgingival species than 'genotype negative' subjects. Bacteria found at higher levels in IL-1 genotype positive subjects were *Bacteroides forsythus*, *Treponema denticola*, the *Fusobacterium nucleatum* subspecies, *Fusobacterium periodonticum*, *Campylobacter gracilis*, *Campylobacter showae*, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus gordonii* and 3 *Capnocytophaga* species. The differences in bacterial colonization by genotype were mainly visible in deep peri-

odontal pockets (>6 mm). Based on these results, the authors postulated that genetic variants might either directly affect bacterial growth and virulence or alter the inflammatory milieu, favouring the growth of specific bacteria. In contrast with these findings, no associations between IL-1 composite genotypes and subgingival bacteria analysed by PCR were detected in a similar study published shortly afterwards [38].

Our group has extensively investigated the associations between candidate genetic variants affecting the inflammatory response (e.g. interleukin-1 and interleukin-6 genes) and subgingival detection of periodontopathogenic bacteria by culture and polymerase chain reaction (PCR). In 45 untreated aggressive periodontitis (AgP) patients from London, IL6 and Fc-γ polymorphisms were both associated with increased odds of detecting *A. actinomycetemcomitans*, *P. gingivalis* and *T. forsythensis* after adjustment for age, ethnicity, smoking and disease severity [39]. In particular, subjects with supposedly pro-inflammatory IL6 genotypes [40, 41] had increased detection of *A. actinomycetemcomitans* and *P. gingivalis*. The study was repeated in a rural population living in Andhra Pradesh, India [42]. Subjects had subgingival plaque samples taken and analysed by checkerboard DNA-DNA analysis for 40 periodontal taxa and had their DNA extracted for IL6 SNP analyses. In this population not exposed to regular dental care and to use of antibiotics, most subjects harboured *A. actinomycetemcomitans* and *P. gingivalis* subgingivally, which did not allow any analysis on bacterial detection by genotype. However, associations between IL6 genotypes and elevated counts of *A. actinomycetemcomitans* and *Capnocytophaga sputigena* were observed, strengthening the previous report. This was further confirmed when a population of 267 chronic and aggressive periodontitis patients was studied. Host DNA samples were extracted from blood samples and analysed for five IL6 SNPs, while subgingival plaque samples were analysed by PCR for the presence of *A. actinomycetemcomitans* and *P. gingivalis*. The study confirmed again the association between IL6 supposedly 'pro-inflammatory' genetic variants and presence of *A. actinomycetemcomitans* and of both bacteria concomitantly [43]. To explore this concept further, we conducted a pilot treatment study on 12 AgP patients selected based on their IL6 genotypes ('pro-inflammatory IL6 haplotype positive' vs. 'IL6 haplotype negative'). In this population, higher *A. actinomycetemcomitans* counts were detected subgingivally in IL6 'haplotype positive' subjects before treatment. Despite a reduction after non-surgical and surgical treatment, these subjects showed a sharp increase in counts of *A. actinomycetemcomitans* again 3 months after periodontal treatment, suggesting a strong genetic influence on gingival pocket re-colonization, which was not observed in IL6 'haplotype negative' subjects [44].

A larger study used a genome-wide approach in 1020 subjects participating in the Atherosclerosis Risk In Communities (ARIC) study to investigate the relationship between host genotypes and eight periodontal pathogens analysed by checkerboard DNA-DNA hybridization [45]. They detected no genome-wide significant signals, but suggestive evidence ($p < 5 \times 10^{-6}$) of association for 13 genetic loci and 'red' and 'orange' complex microbiota. The same effect direction was detected in a second sample of 123 African-American participants. Interestingly, these authors confirmed the moderate association our group previously reported between IL6 SNPs and high 'red complex' colonization. No association was detected between any of the identified SNPs with CP diagnosis, suggesting once more the examination of bacterial colonization as a distinct trait to 'presence of disease' [45]. Recently, in a case-control study analysing polymorphism TBX21-1993T/C (rs4794067) in healthy ($n = 218$), chronic periodontitis ($n = 197$) and gingivitis patients ($n = 193$), no associations were detected between genotypes and presence of 'red complex' bacteria [46]. A summary of genetic variants shown to be associated to detection of subgingival periodontal bacteria is provided in Table 7.1. A more systematic and comprehensive review of the literature on periodontal infectogenomics has been recently published [47].

Table 7.1 Summary of genetic variants shown to be associated with detection of subgingival periodontal bacteria in some of the studies reviewed in this chapter

Study	Population	Study design	Genetic variant-microbial association
[37]	U.S. University-based	CP case-control candidate gene association study with checkerboard DNA-DNA microbial analysis	IL-1 genotypes: counts of *B. forsythus*, *T. denticola*, *F. nucleatum*, *F. periodonticum*, *C. gracilis*, *C. showae*, *S. constellatus*, *S. intermedius*, *S. gordonii*, 3 *Capnocytophaga* species
[39]	UK University-based	AgP case-control candidate gene association study with microbial culture analysis	IL6 and Fc-γ R genotypes: *A. actinomycetemcomitans, P. gingivalis* detection
[42]	Indian rural village	Cross-sectional candidate gene association study with checkerboard DNA-DNA microbial analysis	IL6 genotypes: *A. actinomycetemcomitans*, *C. sputigena* counts
[43]	UK University-based	Mixed CP and AgP case-control candidate gene association study with microbial PCR analysis	IL6 genotypes: *A. actinomycetemcomitans, P. gingivalis* detection
[45]	U.S. University-based	GWAS with checkerboard DNA-DNA microbial analysis	13 loci (including KCNK1, FBXO38, UHRF2, IL33, RUNX2, TRPS1, CAMTA1 and VAMP3): suggestive evidence of associations with 'red' and 'orange' complex/*A. actinomycetemcomitans*

CP chronic periodontitis, *AgP* aggressive periodontitis

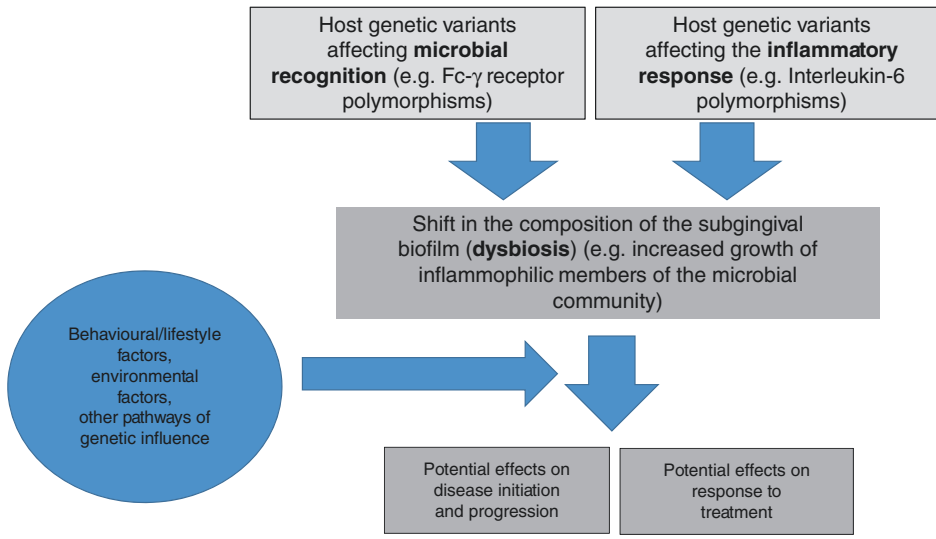

Fig. 7.3 Schematic representation of periodontal infectogenomics

Summarizing the findings above, there is now increasing evidence that host genetic variants can have an effect on periodontal pathology by influencing the subgingival bacteria composition. The shifts in *A. actinomycetemcomitans*, *P. gingivalis* and more in general 'red complex' bacteria may be a small representation of changes in the subgingival biofilm ('dysbiosis') (see Fig. 7.3). There is still a lack of studies investigating whether health-associated bacteria may be affected by specific

genetic variants. The supposed shift towards a more pathogenic microbiota may occur through the effects on microbial recognition and inflammation discussed above. It is conceivable that a more inflamed milieu, characteristic of patients with 'pro-inflammatory' genetic profiles, may favour the growth of bacteria which grow well in inflamed environments, then shifting the whole microbiota towards a disease-predisposing one.

7.5 Challenges and Future Directions

Periodontal genetic research still has a long way to go before it identifies clear predisposing host gene variants in different populations. This is complicated by issues such as sample size, definition of health and disease, genetic methodology, difficulty at controlling for other predisposing factors and epigenetic influences. Recently, gene variants in the IRF5 gene have been associated with IBD [48], while SNPs in the DEFDB1 gene and PRDM1 gene have been associated with chronic and aggressive periodontitis, respectively ([31, 49]). These gene polymorphisms appear to be able to contribute to a disturbance of the immunological barrier, thus promoting dysbiosis of the local microflora, potentially predisposing to disease. Hence, it would be interesting to focus periodontal infectogenomics research on a variety of genes with an effect on microbial recognition and host response. The availability of new metagenomics techniques gives the possibility to explore the associations with health-associated as well as with pathogenic bacteria. Furthermore, there is a lack of studies in periodontally healthy populations, which could give some insights on the genetic influence on the host-microbe crosstalk in health-associated biofilms. Among subjects with healthy periodontia living in a rural population in India, the association between IL6 genetic variants and *A. actinomycetemcomitans* was confirmed (as in periodontitis patients) [42]. However, a paucity of data exists on healthy subjects in other settings. A better knowledge of how gene variants affect the composition of the subgingival biofilm could shed light into potential pathogenic pathways and could open new management avenues. The above-mentioned pilot study in AgP patients selected based on their IL6 haplotypes [44] could, for example, suggest that 'IL6 positive' subjects may benefit from adjunctive antimicrobial therapy, as they might be more likely to have a tendency to re-developing dysbiotic, disease-associated biofilms also after treatment. Studying mechanisms of association between the subgingival biofilm and other biofilms elsewhere in the body, such as in gastrointestinal tract, vagina and skin, could shed light into mechanisms of host-bacteria crosstalk.

Clinical Relevance

- Host genetic variants seem to play an important role in determining the composition of microbial biofilms in the human body, including the dental biofilm.
- Some subjects may be more predisposed to periodontal disease onset and progression through the activity of host genetic variants in the response to the microbial challenge.
- Knowing which subjects are more predisposed to colonization by specific microbes could affect the clinical management of periodontitis cases.

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Antimicrobial Peptides: Roles in Periodontal Health and Disease

8

Daniel Jönsson

8.1 Antimicrobial Peptides

Why do cockroaches and rats survive in sewers and other extremely challenging conditions? The answer is that through evolution they have been equipped with antimicrobial peptides (AMPs) that protect them from microbiological insult. In the case of cockroaches and rats, the AMPs are very potent, allowing for these challenging environments [1–3]. AMPs are the most ancient and primitive arm of the human immune system and are expressed in mammals, insects, fungus, trees—virtually every multicellular organism that coexist with bacteria, including bacteria. AMPs cover the outer barriers of our body, such as epithelium and skin, enabling us to live in coexistence with what some consider a complex organ—the microbiome [4]. As we now, through the technological advancements in microbiology, start to comprehend the complexity of the microbiome, we can also appreciate the complexity of the AMP-profile.

AMPs and agents mimicking AMP molecular properties are widely used in the everyday dental clinic that practice periodontal treatment and care, namely penicillin and chlorhexidine, respectively. The endogenous cationic AMPs, like cathelicidin LL-37 and defensins, exert a similar mechanism on prokaryotic cell walls as chlorhexidine, indicating an importance of maintaining a healthy homeostasis between the oral AMP-profile and the oral microbiome to promote and sustain periodontal health.

The online AMP database APD3 (http://aps.unmc.edu/AP/) includes 1007 AMP, out of which 112 are human AMPs. It is not within the scope this chapter to discuss all 112 peptides, but rather introduce the reader to human AMPs, particularly in the context of the oral milieu and periodontal disease.

8.2 Introduction

AMPs were first described more than 90 years ago [5]. We now know that this diverse group of peptides display their antimicrobial effects through different mechanisms. AMPs are often referred to as the endogenous antibiotics, although they are actually more efficient than antibiotics, as they clean up after themselves! One of the problems with antibiotics, especially when treating sepsis, is that although the bacteria get lysed the cell wall can still cause pro-inflammatory and proapoptotic signaling [6]. AMPs, on the other hand, do not only lyse bacteria, but also neutralize endotoxins, including lipopolysaccharides (LPS) from gram-negative bacteria.

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Perhaps because of the antibiotics reference, there has been a focus on the cell lytic activity of AMPs. The cell lytic action of AMPs are definitely important in the phagosomes of leukocytes and in the core of inflammation, e.g., in the periodontal lesion. However, when it comes to the complex and intricate homeostasis between the microbiome and AMPs in the oral cavity, the neutralizing effects of AMPs on bacterial endotoxins may be more important, as it occurs at lower concentrations of AMPs typically associated with health. The concentration of the AMP LL-37 found in GCF in periodontal health is sufficient to reverse LPS-induced production of pro-inflammatory cytokines [7]. The homeostasis between bacteria and the inflammatory response in the gingival sulcus is always ongoing, including in clinically as well as histologically healthy tissues.

8.3 History of AMPs

Sir Alexander Fleming, Nobel Prize awardee in 1945, discovered AMPs in 1922. He published his findings in the paper "On remarkable bacteriolytic element found in tissues and secretions" [5], in which he recognized the antimicrobial capacity of endogenous nasal secretions. He named the activity lysozyme, as it lysed the bacterial lawns on a culture-dish. Six years later, Sir Fleming made the discovery that would start a new era in medicine and save billions of lives when he described the antimicrobial capacity of *Penicillium notatum* [8].

Insects, like moths and flies, have a much less developed inflammatory response that consist of hemocytes (fagocyting cells) and an AMP humoral defense. Therefore, AMPs are a more crucial segment of the inflammatory response in insects than in vertebrates [9]. In 1962 Stephens and Marshall [10] discovered that heat stable relatively small (compared to lysozyme) AMPs were produced by wax moth larvae when they were exposed to *Pseudomonas aeruginosa*. Interestingly, the protective effect of the AMPs could transfer to another moth by hemolymph transfusion, showing that these innate protective peptides are soluble.

The history of research on AMPs has also been the story of the research on innate immune response. Hans Boman studies dating from the 50s to his death (passed away 2009) is an expose of the progress of research in the field of innate immune response and AMPs in particular. Starting with a continuation on Stephens' and Marshall's work were Boman and colleagues vaccinated flies by an injection of harmless bacteria which protected against *Pseudomonas aeruginosa*, indicating a cell-free defense [11]. Due to the work of Boman's group we now have a much more full picture of the innate immune response, particularly the cell-free epithelial response [12, 13]. Boman's group also published an important paper on the LL-37 and periodontal disease, reporting that lack of the cathelicidin LL-37 may be the reason why Kostmann patients get periodontal disease [14].

The arginine-rich cationic peptides that later were named defensins and cathelicidin were first described to possess antibacterial properties against both gram-positive and gram-negative bacteria by Zeya and Spitznagel in 1963. The molecules extracted were from guinea pigs polymorphonuclear leukocytes. In 1984 Selsted et al. purified the molecules from rabbit granulocytes and named them defensins [15]. The same research group later identified defensins in humans [16].

A recent game changer in AMP-research is that beta-defensins can unmask potent antimicrobial activity by structural changes [17]. This suggests that AMPs that have been doomed to be relatively less potent and consequently less important have to all be re-evaluated.

8.4 AMPs Grouping

Some reviews on AMPs focus on defensins and cathelicidin [13, 18], some have more inclusive approach, and include metal ion chelators, proteinase inhibitors, peroxidases, and agglutinating peptides [19, 20]. This is mainly because defensins and cathelicidin have been more extensively researched than any other AMPs. The grouping here is inclusive and thusly a bore detailed

Table 8.1 Categorization of AMP based on their antimicrobial action

Group	Antimicrobial action	Members
Cationic peptides	To destabilize cell membrane and to neutralize LPS	Alpha- and beta-defensins, LL-37, azurocidin, CCL-28, heparin-binding growth factor, histatins, statherin, adrenomedullin, calcitonin gene-related peptide, neuropeptide Y, substance P, and vasoactive intestinal peptide
Lipid-binding peptides	Also a cationic peptide, but the lipid-binding action intensifies the binding to cell membrane and LPS	Bactericidal/permeability increasing protein, parotid secretory protein and palate lung and nasal epithelial clone family
Lysomzyme	Damages surface exposed peptidoglycans	Lysozyme
Agglutinating and adhesive peptides and proteins	Agglutinate bacteria and prevents them from attaching to surfaces	Mucin 7, β-2-microglobulin, fibronectin, surfactant protein A, proline-rich proteins, prolactin-inducible protein
Ion chelators	Disturb intracellular signaling pathways of bacteria, and thereby restrict its growth and vitality	Lactoferrin, S100 proteins, and ATP
Protease inhibitors	Inactivate proteases from bacteria	Cystatins, trappin gene family members secretory leukoprotease inhibitor protein and elafin
Peroxidases	Cell degradation and loss of action through the peroxidase system	Lactoperoxidase and myeloperoxidase

description of all AMPs would be too comprehensive. For further, more detailed description, please see the references. Table 8.1 summarizes the categorization of AMPs.

Cationic peptides—Cationic peptides are small (< 10 kDa) positively charged molecules that can perforate the cellular membrane of both gram-positive and -negative bacteria and interact with the LPS/CD14-signaling cascade that initiates production of pro-inflammatory cytokines. The human cathelicidin LL-37, beta- and alpha-defensins dominate the group. LL-37 is expressed in neutrophils, macrophages, and epithelial cells. LL-37 has a broad-spectrum antimicrobial effect and many immunomodulatory effects [12, 18]. LL-37 is also important in wound healing, often lacking in chronic ulcers [21].

In general, alpha-defensins are the defensins of neutrophils (and also Paneth cells), and beta-defensins the epithelial defensins. Both LL-37 and alpha-defensins are an important part of the intracellular phagocytosis mechanism of neutrophils, and released into the extracellular space when the cells burst. Beta-defensins are released through LPS-mediated signaling [18, 22]. Cationic AMPs also include: azurocidin [23], CCL28 [24, 25], heparin-binding growth factor [26], histatin 1, 3, and 5 [27, 28], statherin [29], and the neuropeptides adrenomedullin [30, 31], calcitonin gene-related peptide [31], neuropeptide Y [31], substance P [31], and vasoactive intestinal peptide [31].

Lipid-binding AMPs—This group includes bactericidal/permeability increasing protein (BPI) and its homologs; BPI-like proteins (including parotid secretory protein (PSP)) and PLUNC (palate lung and nasal epithelial clone family). PLUNC can further be categorized into short and long PLUNC, also known as BPI fold containing family A and B, respectively. BPI is a boomerang-shaped cationic molecule that exerts bactericidal activity by perforating the cell membrane (preferably gram-negative bacteria) and binds endotoxins, including LPS which it binds with high affinity [32]. Neutrophils and epithelial cells, including salivary duct cells, express the BPI-proteins [33]. PLUNC-proteins are multifaceted and not only possess strong LPS-neutralizing capacity, but also inhibit dendritic cell growth, act as chemoattractant and opsonization agent [34]. BPIs are actually upregulated in mucosa by resolvins [35], which may offer a pharmacological

approach to treating periodontal disease [36]. Decreased PLUNC expression in nasal polyps has been associated with multibacterial colonization in chronic rhinosinusitis [37].

Lysozyme—The first AMP to be categorized was lysozyme [5]. It is a small protein (145 kDa) present in body fluid, including saliva, but also in neutrophils. Lysozyme mainly exerts its activity against cell membrane integrity of gram-positive cells by damaging surface exposed peptidoglycans [38].

Agglutinating and adhesive peptides and proteins—Agglutinating bacteria inactivates them and prevents them from attaching to surfaces. This group consist of peptides, like mucin 7 [39–41] and β-2-microglobulin [42], but also big molecules that are technically proteins rather than peptides, like fibronectin [43–46], surfactant protein A [47], proline-rich proteins [48], and prolactin-inducible proteins [49].

Ion chelators interact with and disturb vital intracellular signaling pathways of the bacteria, and thereby restrict its growth and vitality. Examples of AMP chelators are lactoferrin [50], S100 proteins [51, 52], and, according to a newly published paper, also ATP [53]. Lactoferrin single-nucleotide polymorphisms (SNPs) are associated with aggressive periodontitis [54] and have broad antibacterial, antiviral, and antifungal properties [55]. Lactoferrin knockout mice are more susceptible to *A. actinomycetemcomitans*-induced periodontitis [56]. Lactoferrin reportedly inhibit *P. gingivalis* proteases by its ion chelator mechanism [57]. This also inhibited the biofilm formation capacity of *P. gingivalis.*

Protease inhibitors inactivate proteases from bacteria, but also through other mechanisms [58, 59]. The group includes cystatins [58–61], the trappin gene family members secretory leukoprotease inhibitor protein and elafin [62]. Cystatins reportedly exert antibacterial effect, specifically on *P. gingivalis* [61] and *A. actinomycetemcomitans* [59]. Ganeshnarayan et al. [59] investigated the affinity of salivary peptides to *A. actinomycetemcomitans* in subjects with high salivary anti-*A. actinomycetemcomitans* activity and found that lactoferrin, immunoglobulin A, kallikrein, and cystatin SA bind to *A. actinomycetemcomitans*. Cystatin SA demonstrated an antimicrobial activity on *A. actinomycetemcomitans* in vivo, which was reversed by cystatin SA antibodies. Interestingly, the antimicrobial effect was independent of protease inhibitory function. Cystatin 9 also exerts an immunomodulatory function, increasing the efficiency of macrophage phagocytosis and promoting an upregulation of macrophage proteins involved in anti-inflammation and anti-apoptosis while restraining pro-inflammatory associated proteins [58].

Peroxidases [63] in the oral cavity are lactoperoxidase [64] and myeloperoxidase [63] which form the peroxidase system of saliva. The reaction products are active against several bacteria associated both with dental caries and periodontal disease [63].

Importantly, the oral AMPs are not separate entities, but complement each other. Choi et al. allowed *P. gingivalis* LPS to interact with pooled saliva and then investigated what AMPs were attached to the LPS molecule. They found interaction with alpha-amylase, cystatin, prolactin-inducible protein, lysozyme C, immunoglobulin components, serum albumin, lipocalin-1, and submaxillary gland androgen regulated protein 3B [65]. This indicates that the saliva, and probably to a similar extent GCF, is to be considered as a pot of AMPs that share the ability to interact with bacteria and to neutralize their endotoxins. Indeed, there is a synergistic relationship between AMPs [66].

8.5 AMPs in the Periodontium

In gingiva and oral mucosa, the inflammatory responses face unique challenges confronting an immense quantity of bacteria. The mucosal response can first be broken down into the innate and adaptive immune response, and the innate inflammatory response can be further categorized into the acellular and cellular response. The acellular response comprises AMPs, which is the very first line of defense. The AMPs are mainly found in saliva and pellicle as well as the epithelium. If or when the microbes cross the epithelium through the epithelial barrier, they are exposed to the phagocytic cells, like stationed

macrophages and Langerhans cells. In the gingival sulcus there is also a high presence of the phagocytic neutrophils. In a prolonged exposure of microbes to the antigen-presenting cells, T-cells will be recruited and activated, and B-cells will differentiate into plasma cells.

The oral epithelium is highly specific, and the AMP expression is different between the different mucosal sites, and in response to different infections. When comparing the expression of defensins in healthy oral tissue samples from gingiva, tongue, buccal mucosa, labial mucosa, submandibular glands, small labial glands, and dental pulp, the expression is higher in gingiva and in the submandibular gland [67]. In addition, the tissue response of AMP-expression in periodontitis causes a higher expression of defensins than candidosis-infections [67].

The gingival sulcus has a challenging mission—to maintain the epithelial barrier around the tooth, which penetrates the mucosa. To hinder the down-growth of bacteria and to sustain the junctional epithelial barrier there is a high presence of AMPs in the sulcus, due to the high inflammatory activity, even at clinically healthy sites [68–70]. Particularly, the high density of neutrophils in the periodontium causes a high concentration of AMPs, as several important AMPs, such as LL-37 and alpha-defensins, are abundant in neutrophils [69, 71]. The high bacterial load in sulcus in itself also induces AMP-expression through toll-like receptor (TLR) and nucleotide oligomerization domain (NOD) signaling, causing a feedback loop [72]. In periodontal disease, the epithelial barrier in sulcus is lost, and the only remaining barrier between the bacteria and the alveolar bone is a strong inflammatory response together with neutrophil extracellular traps (NETs).

When neutrophils in the periodontium undergo cell death, they release a whole range of AMPs that end up in gingival crevicular fluid of the periodontal pocket. Importantly, neutrophils also form NETs [73]. NETs are DNA strings from neutrophils that compose the extracellular matrix of the innate immune response of the periodontal pocket that capture bacteria, degrade their virulence factors, and kill them [74]. The NETs contain large quantities of AMPs, mostly (70%) are different histones [75], but there is also neutrophil elastase [75], S100 A8 and A9 [75], azurocidin [75], cathepsin [75], lactotransferrin [75], calprotectin [75], cathelicidin LL-37 [76], and several others [75]. For more detailed description of NETs, please see Cooper et al. [74].

Due to the high presence of neutrophils in the periodontal lesion, the AMP levels become so high that they do not only lyse bacteria, but also resident cells, like periodontal fibroblasts and osteoblasts [7, 77]. This may be due to the need of more space to allow the periodontal lesion to expand.

When the inflammatory response is suppressed, due to, e.g., severe immunosuppressive therapy, bacteria may reach the alveolar bone, which potentially can cause osteonecrosis [78]. It could be that the threat of deadly osteonecrosis (prior to Fleming's discovery of penicillin [8]) is the evolutionary incentive of the strong periodontal inflammatory response that unfortunately causes tissue breakdown and tooth loss.

8.6 Double Edge Swards

Many of the AMPs display a large array of functions; LL-37 can affect apoptosis (both pro- and anti-) [7, 77, 79–81] and chemoattractant [82], fibronectin can agglutinate bacteria [43–45], but is obviously also a key component in extracellular matrix. The functional duality of AMPs does in some instances cause a paradox. This can be exemplified by LL-37, which is important in wound healing [21] and has well-known antimicrobial features, but in psoriatic lesions LL-37 is found to reach concentrations exceeding that of healthy skin [83, 84]. Because of the high concentrations in psoriatic lesions and the capacity to activate the innate immune response when released extracellularly, they are often termed alarmins (for an excellent review on alarmins [85]). As visualized in Fig. 8.1, the concentration of LL-37 found in healthy GCF is primarily anti-inflammatory, and the concentrations found in GCF in periodontitis reaches the concentrations proapoptotic in periodontal ligament cells [7].

The duality aspect of AMPs is important to bear in mind, as focusing on the antimicrobial

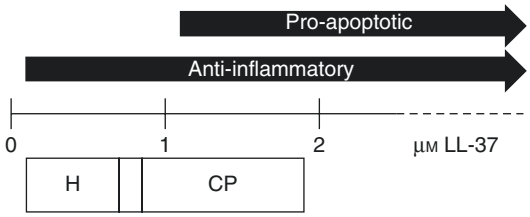

Fig. 8.1 The antimicrobial peptide LL-37 is anti-inflammatory in concentrations associated with periodontal health (*H*) but proapoptotic in concentrations associated with chronic periodontal disease (*C P*). The width of the boxes represents the upper and lower quartile of LL-37 in GCF in health and chronic periodontitis [68]

capacity may attract ideas on increasing the levels of these peptides pharmacologically to intervene infections, such as periodontitis. On the contrary, focusing on the pro-inflammatory and proapoptotic effects may generate thoughts on suppressing them, which would probably cause severe microbiological dysbiosis. One way to brake this catch 22 may be to utilize agents that increase AMP-levels to healthy physiological concentrations. Vitamin D deficiency causes LL-37 levels to drop, reportedly increasing susceptibility to infections in the lung; however vitamin D supplementation can increase the LL-37 levels revering the risk of lung infection [86].

Considering that the antimicrobial capacity of AMPs have been known for about 90 years and that the concept of alarmins was introduced about a decade ago [87], our knowledge of AMPs in the oral cavity mainly focuses on the antimicrobial capacity.

8.7 The Homeostasis Between AMPs and the Microbiota

From the day we are born, until that final day there is a constant homeostasis between the inflammatory defense and the commensal microbiota. The moment we die microbiota in the oral cavity, in the gut, on the skin, and elsewhere no longer has AMPs and cell mediated inflammatory response as counterpart, and consequently our bodies start to molder. During our lifetime, disruption of this complex homeostasis causes diseases, including periodontal disease. In one situation, a disruption of homeostasis causes an overgrowth of a specific microbe, as in the case of *Helicobacter pylori* in peptic ulcers [88]. In another situation, the commensal microbiota can be disturbed and become pathogenic from changes in the inflammatory response or milieu and therefore display a nonspecific microflora, as in Crohn's disease [89] and periodontal disease in which the composition of the microbiota can differ even between sites in the same mouth [90]. Unfortunately, we know less about the changes in AMP-profile during these diseases than we know about the changes of the microbiota.

As illustrated in Fig. 8.2, AMPs can change the microbiota, through neutralizing endotoxins and disintegrating cell membrane integrity. Reversely, the oral microbiota can alter the AMP-profile by LPS-TLR/CD14 cell signaling. For obvious reasons studies proving causal changes in the microbiota in response to alterations of specific AMPs is primarily performed in mice models and human in vitro benchtop models. There are however case presentations of diseases and single mutations in humans that also add to the knowledge of the importance of AMP-microbiota homeostasis.

One example of mouse model are the transgenic mice models. To show the impact and magnitude of effect from human alpha-defensin 5 (HD-5), Salzman et al. [91] developed a transgenic mouse model expressing HD-5. Mice lack defensin 5 and the transgenic mouse expression of HD-5 in their Paneth cell epithelium. Interestingly the transgenic mice were resistant to *Salmonella typhimurium*, compared to wild-type mice, indicating the importance of HD-5 in defense against Salmonella infections. In another example Wehkamp et al. [92] report a lower expression of HD 5 and 6 in ilium Paneth cells and intestinal mucosal extract from subjects with Crohn's disease compared to healthy controls. Using a similar model of transgenic mice as Salzman et al. [91], they found a difference in the luminal microbiota when comparing HD-5-positive and -negative mice.

There are mouse models that show importance of the adaptive immune system in turning a commensal microbiota into a pathogenic. In the models

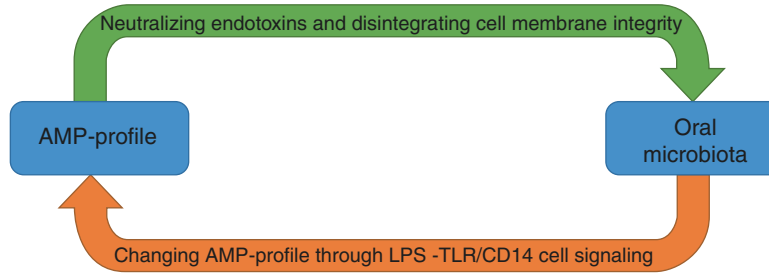

Fig. 8.2 The reciprocal relationship between the AMP-profile and the oral microbiota

the microbiota is disturbed by changes in the innate immune response, including AMPs, which causes a dysbiotic/pathogenic microbiota, that can then be transferred to wild-type mice in which dysbiosis is sustained. One example is a study by Garrett et al., where transgenic T-bet (transcription factor) knockout mice developed ulcerative colitis, causing a dysbiotic microbiota. When the dysbiotic microbiota was transferred to genetically intact wild-type mice, they too developed ulcerative colitis. Similarly, mice that lack the bacterial flagella sensitive toll-like receptor 5 (TLR5) express metabolic syndrome features and insulin resistance and also a dysbiotic gut microbiota. Transferring this gut microbiota to wild-type mice caused similar metabolic syndrome and insulin resistance in wild-type mice [93]. What these studies show is that even though it may be tempting to "prove" microbiological causality by transferring dysbiotic microbiota from a diseased animal to a healthy that then gets the same disease/condition, the inflammatory response may very well still be of key importance in creating this dysbiotic microbiota.

8.8 AMP Cell Membrane Interaction

Because the cell wall lipid bilayer is negatively charged, it attracts cationic peptides, including both cationic peptides, like LL-37 and defensins, but also the lipid-binding AMPs and lysozyme. Cationic AMPs reduce the cell membrane integrity through membrane perturbation. At a certain peptide/lipid ratio on the cell surface the peptides orient in a perpendicular manner and insert into the bilayer, forming transmembrane pores [94–96]. At a low concentration the peptides are bound parallel to the lipid bilayer, but as the concentration of peptides increase, the peptides begin to orient in a perpendicular orientation and form "barrel-stave" shaped pores in the lipid membrane [95]. This research is based on lipid bilayer models, and although it is a possible model, it may be simplistic since different membrane proteins constitute about 50% of the microbial membranes [96]. Zwitterionic phospholipids and cholesterol are prominent constituents of eukaryotic cell membranes, and they will strongly reduce the interaction of cationic AMPs and the cell membrane by changing the membrane net charge to less anionic [96]. Therefore, there needs to be higher cationic AMP levels to form pores in cell membranes of eukaryotic cells than in bacteria.

The first step of the AMP-lysis of bacterial membrane is attraction of AMPs to the bacteria. The obvious mechanism for AMP attraction is through electrostatic bonding between the cationic AMPs and the anionic LPS and teichoic acid on gram-negative bacteria and gram-positive bacteria, respectively. For the AMPs to reach the lipid bilayer, the AMPs have to penetrate the LPS and peptidoglycans. This may be through a process termed "self-promoted uptake," in which AMPs first bind to LPS, causing destabilization, which then allows excess to the lipid bilayer [97]. Microbiome specific lipid receptors may also be involved in the AMP antimicrobicidal action [98].

To disrupt the integrity of eukaryotic cell membranes much higher levels are required than for prokaryotic cell, that high levels are reached in some pathologic conditions, such as in the gingival sulcus during periodontal disease and in psoriatic lesions [7, 68, 83, 84]. The effect

of cationic AMPs on eukaryotic cell vitality is cell specific and may be cytotoxic in some cells types, proapoptotic in others, and anti-apoptotic yet another [79–81, 99–105]. Part of the explanation of this cell specificity may be endogenous peptides and proteins that can reverse the action of cationic peptides, including mucin [106, 107] and the membrane protein p33 [105, 106].

Lysozyme exerts its anti-microbiological activity preferentially against gram-positive cells, because of its peptidoglycan-degrading property. Lysozyme hydrolyzes the bond between *N*-acetyl glucosamine and *N*-muramic acid leading to degradation of peptidoglycan in the gram-positive cell wall, and thereby access to the lipid bilayer. This catalytic process on the peptidoglycan layer described is termed muramidase activity; however, this was recently challenged by Nash et al. [108]. They reported antibacterial activity also from muramidase deficient recombinant lysosome in vitro, and in muramidase deficient mice in vivo. Nevertheless, lysozyme is an important AMP against gram-positive cells.

To underline the importance of the whole AMP-profile, a study investigating the effect of BPI and alpha-defensins on *Escherichia coli* cell growth found a synergistic effect between the AMPs [109]. Other groups have confirmed synergy between AMPs [66], which indicates an importance of the heterogeneity of the AMP-profile.

8.9 AMPs and Endotoxins

The most important pathway for triggering dysbiosis through an inflammatory response is when LPS, a gram-negative bacteria cell wall segment, binds to LPS-binding protein (LBP) at macrophage surfaces. When CD14 and toll-like receptor 4 (TLR4) recognize the LPS/LBP complex, a signaling cascade is initiated through MyD88 and TRIF that activate the transcription factors like NF-κB and AP-1, which subsequently activates the transcriptional activity of genes of pro-inflammatory cytokines [110–113]. The LPS/CD14 signaling cascade is not macrophage or even leukocyte exclusive, but can occur in most cells, including periodontal fibroblasts [114]. As iAMPs have the ability to interrupt the initiation of this signaling cascade through several different mechanisms—(a) neutralizing LPS by binding to the molecule, (b) disrupting aggregates of LPS, (c) binding of AMPs to CD14, and (d) by scavenging LPS (Fig. 8.3).

LPS is an anionic molecule and is build up by a hydrophilic O-antigen, a polysaccharide core with a negative charge and a glycophospholipid, lipid A. Lipid A is the active segment expressing the endotoxic activity. The interaction between LPS and AMPs depends on the net charge of the AMP and hydrophobicity [115]. Electrostatic binding prefers binding to the polysaccharide

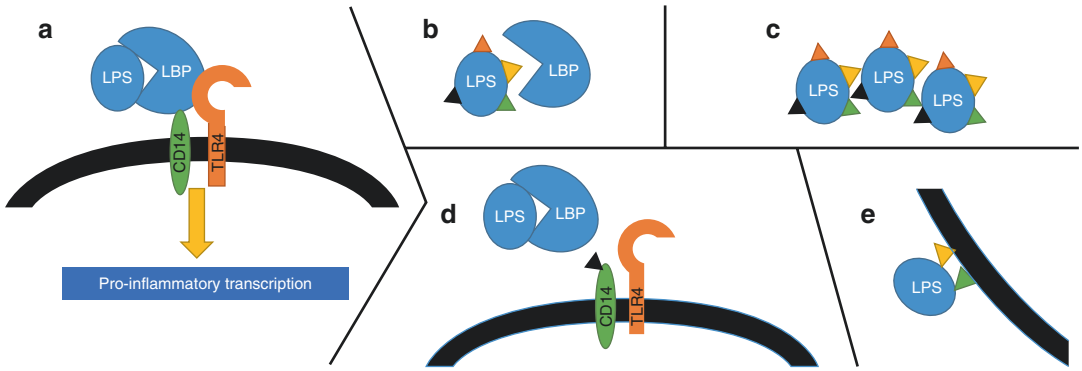

Fig. 8.3 Panel (**a**) illustrates LPS/LBD/CD14/TLR4 transcription, which is an important pathway for pro-inflammatory transcription. This pathway of pro-inflammatory transcription can be disrupted by AMPs through (panel **b**) AMPs binding LPS disabling the bonding of LPS and LBP, (panel **c**) AMPs disintegrating LPS aggregation, (panel **d**) AMPs attaching to CD14 and thereby disrupting the subsequent cell signaling. Lastly, (panel **e**) AMPs can attach to cell membrane and attract scavenged LPS

segment of LPS and hydrophobic interaction dominates binding to lipid A [116], however small cationic peptides are able to penetrate the LPS layers and bind to lipid A [117]. Binding of AMPs to lipid A segment of LPS neutralizes the endotoxic properties and prevents the LPS/LBP complex to form and downstream transcription of pro-inflammatory cytokines to take place [118–120]. BPIs have a high affinity to lipid A due to hydrophobicity and are cationic. Due to the high affinity of BPI to LPS, LPS favors BPI-mediated endotoxin neutralization to binding to LBP, resulting in an attenuation of the pro-inflammatory cellular response at neutrophil-rich inflammatory sites, such as the periodontal lesion [121–123]. The cationic peptides LL-37 and several defensins also bind and neutralize LPS [18].

LPS aggregation is an important event, as monomer endotoxins exert less endotoxic activity [124]. LL-37 as well as other AMPs can disintegrate LPS aggregation and thereby exert an anti-inflammatory effect [118, 125].

AMPs also have the ability to bind directly to CD14 without activating the receptor and by doing so blocking LPS [126, 127].

Finally, incorporation of cationic AMPs in human cell membranes induce a net positive charge, which offer anionic LPS to bind elsewhere than to LBP and CD14 and thereby causing scavenging of LPS and a net anti-inflammatory effect [128].

Although the scavenging hypothesis is relatively novel, the most important aspect is that all four pathways in which cationic AMPs can interact with LPS coexist and adding, e.g., LL-37 to a LPS in an in vitro model causes a substantial anti-inflammatory effect, both in leukocytes and other cell types [7, 118, 129].

Conclusion

AMPs are already being used in the everyday clinic when treating periodontal disease, the challenge is to make them more efficient and tailor them according to the patients' need. The Kostmann patients with LL-37 deficiency would benefit from LL-37 mimicking drug. Patients with aggressive periodontitis and dysfunctional lactoferrin may benefit from supplementation with recombinant lactoferrin. Perhaps a salivary test checking the AMP-profile of saliva from subjects with periodontal disease could be a first step along that line. The next step, producing the drug may be more challenging due to the functional duality of many AMPs. With continued research in this field, we may however be able to pinpoint the separate functional entities of the different peptides.

The intricate homeostasis between AMPs and the oral microbiome works perfectly well in most people and may very well be the answer to the question the general dentists asks themselves after seeing an older patient with less impressive oral hygiene and no periodontal disease. Therefore, it may be a poor strategy to use AMPs on a larger population in toothpastes, and rather have tailor made supplementation. As mentioned in this chapter changing the AMP-profile can alter the microbiome and thereby disrupt this homeostasis.

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Periodontal Pathogenesis: Conclusions and Future Directions

9

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This textbook on periodontal pathogenesis aspired to provide an overview of our current understanding of biological processes underlying the disease, and discuss their clinical relevance and applicability. We now realize that periodontal disease affects 10% population in its severe form, and that it is primarily a disease of aging. Despite advances in prevention measures over the past decades, the prevalence of the severe form of disease has not decreased. This highlights periodontitis as an existing health problem, unlikely to be completely resolved with our present therapeutic approaches. Continuous enrichment of our biological knowledge of the disease and continuous efforts in bridging this knowledge to the clinical application will hopefully lead to more holistic approaches for diagnosis, evaluation of patient susceptibility, or selection of efficient treatment options. This conclusive chapter summarizes updates and take-home messages from previous chapters, to consider further.

The microorganisms that reside in the oral cavity, or the "oral microbiome" are highly diverse. The number of the different known types is likely to increase, as the technologies to discover those have become more sensitive and sophisticated. Microbes may just be transient travelers in the oral cavity, or find favorable conditions for their growth and survival. The stable composition of the oral microbiome is a result of the dynamic, yet balanced, interaction of the microbes with each other, with their host, and with their local environment in general. Microbiome stability is indeed a requirement for oral health because it favors the survival and persistence of health-associated species. These species not only prevent the overgrowth of endogenous opportunistic pathogens or the invasion of exogenous pathogens, but they also prime the immune system to be at readiness level. Changes in microbial composition are perceived by the immune system as deviations of the normal, which then strives to correct them via inflammatory responses. Changes in microbial composition can be induced by variations of the oral milieu. Hence, unfavorable conditions for the survival of commensals may lead to overgrowth of opportunistic disease-associated species, a concept that has led to the ecological plaque hypothesis on how oral microbiota can cause periodontal disease [1]. Although several of those species are usually referred to as "periodontal pathogens," they are suited for the definition "opportunistic pathogens"; due to that they are frequently also detected in health, albeit at low numbers. This leads us to postulate that no single bacterial species alone is responsible for the disease, but groups of them, under certain conditions, may act together to cause disease.

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Bacteria are prone to grow on tooth surfaces, in the form of organized biofilm communities. This preferential polymicrobial lifestyle provides to the individual species robustness and further survival possibilities. The microbial composition of biofilms is very complex and can vary not only between individuals, but also between sites of the same individual. Bacterial survival and growth depends on their adaptability to the microenvironmental conditions at a given niche of the oral cavity, such as availability of suitable nutrients, oxygen, and other physicochemical parameters. Importantly, biofilms can form both under aerobic and anaerobic conditions. Subgingival biofilms are formed by bacteria that can colonize the periodontal pocket and grow under its anaerobic conditions, during the progression of periodontal disease. No one of the individual species populating a subgingival biofilm can single-handedly cause the disease. Instead, groups of them can synergize and establish a dysbiotic relationship with the host, eliciting a chronic inflammatory response [2, 3]. Opportunistic pathogens in the subgingival biofilm, such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, produce a number of virulence factors that can contribute to this dysbiotic relationship with the host and elicit a disadvantageous host response.

In periodontal health, a low grade of harmless immune response is elicited by the periodontal tissues. The purpose of this is to control microbial colonization and to "guard" the tissues against invasion and multiplication of oral microorganisms, at the same time avoiding to inflict collateral damage to the tissues. Hence, the control of the immune response without its complete abolition is a key to maintenance of periodontal health. Yet, an uncontrolled immune response will cause a chronic inflammatory tissue destruction, which will clinically manifest as periodontitis. Subsets of cells of the immune system (initially neutrophils and later on lymphocytes) and inflammatory signaling molecules are orchestrating this destructive process. The net result is the production and release of proteolytic enzymes, such as matrix metalloproteinases (MMPs), within the gingival tissue. The catalytically active MMP-8, which degrades interstitial collagens, is a predominant MMP in periodontitis-affected gingiva [4]. A large body of scientific literature evidence demonstrates that the levels of aMMP-8 in gingival crevicular fluid and saliva correlate with the occurrence of periodontal disease, and that periodontal treatment is commensurate with the reduction of its levels in these fluids [5]. Hence, aMMP-8 can constitute a biological indicator for excessive collagen tissue breakdown and thus a diagnostic biomarker for progressive periodontal disease. Accordingly, a limited number of molecules of the immune response can tip the balance between bone formation and resorption towards of the latter [6]. In particular, the RANKL-OPG axis is a key in this process, as RANKL stimulates osteoclast formation and bone resorption, whereas OPG inhibits this action [7]. An increase in the RANKL/OPG ratio in gingival crevicular fluid indicates the occurrence of periodontal disease [8], but treatment of the disease by conventional means does not affect this, thus indicating that pharmacological modulation of this set of molecules is a path to explore in future treatment modalities [9, 10].

While it is clear that the dysbiotic interaction between the host tissues and the microbes of the biofilm community is accountable for inflammatory periodontal tissue destruction, it is also clear that genetic susceptibility somehow holds a fundamental role in this process. Genetic factors of the host, or gene variants in different populations, can influence microbial colonization and composition of the biofilms, and can reduce the biological threshold for triggering an inflammatory response. This genetically driven modulation of the interaction between the host and its colonizing microbes may underline the susceptibility to the disease processes by accelerating dysbiosis [11]. In other terms, some individuals may be genetically more predisposed than others for developing periodontal disease, or may exhibit higher progression rate of the disease. The antimicrobial peptides present in saliva and oral cavity in general may also play a role in the genetic susceptibility to periodontal disease. They establish an intricate homeostatic relationship with the

oral microbiome, and changes in one's antimicrobial peptide profile may also lead to dysbiosis.

9.1 Future Directions in Diagnosis and Treatment of Periodontal Disease

In terms of microbial etiology of periodontal disease, it is becoming evident that shifts in the composition of the subgingival microbiota are more crucial than the mere presence of a single or a handful of pathogens for the progression of periodontal disease. Reduction or elimination of certain "marker" species such as *A. actinomycetemcomitans* and *P. gingivalis* could serve as indicative measures of the efficiency of the periodontal treatment, yet monitoring the stability of the pocket microbiome on a full scale, and in a fast and cost-efficient manner, during the maintenance would be ideal. In monitoring the disease, there is accumulated evidence over the years to suggest that measuring aMMP-8 in oral biofluids holds strong promise for the prediction, diagnosis and progression of the disease, and is already applied in chair-side/point-of-care applications. This could be used alone, or in combination with other host inflammatory or microbial biomarkers. Development of point-of-care devices for microbiological and immunological detection that would assist clinicians in the diagnostic, monitoring, and selection of treatment aspects are underway [12].

Biofilms are difficult microbiological targets to hit, and the "one size fits all" chemo-mechanical disruption approach that we follow today cannot be perfectly achieved on a full mouth scale. Thinking in a more "ecological" manner, periodontal disease occurs due to dysbiosis between these microbial communities and the host, impacted by genetic and environmental pressures. In this light, treating the disease entails not only elimination of the opportunistic pathogens, but also of the conditions that lead to their establishment. Hence, "correction" of the oral environmental conditions that can cause dysbiosis (e.g., smoking, hormonal deregulation, hyperglycemia, dietary factors) may improve the prognosis of the chosen therapeutic intervention. Understanding genetic predisposition to periodontal disease, and how this may affect the microbial colonization patterns could facilitate prevention or clinical management, particularly in susceptible populations. In addition, proactive modulation of signaling pathways and molecules that are involved in periodontal connective tissue and bone breakdown, such as aMMP-8 and RANKL/OPG, are warranted. Such interventions would help arrest periodontal disease on the molecular level, and could thus be included in the growing list of "adjunctive" periodontal treatment modalities used in handling patients unresponsive to traditional therapy. Hence, as molecular modulatory therapies are becoming more readily available and applicable, they are expected to lead to more predictive treatment outcomes.

Steering towards a better understanding of biological mechanisms underlying periodontal disease is the preamble for implementing this knowledge in particular clinical situations, and a drive for personalized dental medicine [13]. Therefore, it is of great significance that the clinical audience receives an updated understanding of the intricate biological mechanisms of periodontal pathogenesis, in order to be in readiness level for the next generation of diagnostic and treatment options. Reciprocally, it is important that biological researchers remain receptive to feedback from their clinical counterparts in seeking what would be a meaningful and feasible application for clinical reality and patient handling.

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