Emerging Therapies in Periodontics

Sinem Esra Sahingur
Editor

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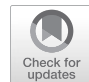
Part I

Periodontal Therapies Targeting Host Immune and Inflammatory System



1 Evolving Paradigms in the Pathogenesis and Management of Periodontitis

Sinem Esra Sahingur

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1.1 Introduction

Periodontal diseases are complex diseases of multifactorial origin which are initiated by a diverse group of dysbiotic oral microbial species, yet the disease progression, clinical presentation, and response to therapy are driven by the deregulated and non-resolving inflammatory responses due to host genetic and epigenetic factors, environmental, and systemic perturbations (e.g., improperly designed restorations, obesity, stress, aging, uncontrolled diabetes, immune disorders, and use of certain medications) and lifestyle preferences (e.g., smoking, eating habits) [1–7]. If not treated or properly managed, periodontal disease results in loss of periodontal tissue architecture, creates esthetic and functional complications and eventually tooth loss. Persistent and severe forms of the disease are also associated with numerous systemic complications [8].

Despite many advances in the field, periodontitis continues to be a substantial medical, psychological, and financial burden worldwide affecting more than 50% of the adult population [9]. The prevalence of periodontitis increases with age and considering the increased lifespan, it is imperative to understand the biology of aging periodontal tissues and incorporate the needs of these patients in clinical management [10, 11]. Similarly, with the growing number of implant-supported restorations in clinical practice, the clinicians and researchers are now faced with the challenge of increased prevalence of peri-implantitis, which is a disease affecting the tissues surrounding implants [12, 13].

The ultimate goal of periodontal research is the development of effective preventive and

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therapeutic strategies to improve clinical outcomes and achieve pristine oral and systemic health. As we enter the era of precision medicine which aims to personalize therapies, research efforts are directed to fully characterize the key cellular and molecular factors that regulate periodontal tissue homeostasis, understand the interactions between host and the microbiome, and identify disease susceptibility markers and risk modifiers for more targeted approaches. Integration of biological sciences, engineering, and omics data with the rapidly emerging technologies in clinical, basic, and regenerative medicine will be at the center of personalized periodontics. Future periodontal practice will be shaped by genetic, molecular, cellular and physiological analyses and assessment of environmental exposures and lifestyle preferences and incorporation of biological data and technical advances to plan individualized preventive and treatment strategies [14–18].

Many reports are already available in the literature, which discuss various non-surgical and surgical therapies with and without adjunct antimicrobial and/or anti-inflammatory agents to manage periodontitis and peri-implantitis [15, 19–21]. Most of these therapeutic modalities are considered as the standard mode of clinical practice and effective in the majority of the cases exhibiting mild to moderate forms of the diseases. Yet, the research also indicates that anti-infective therapy using non-specific mechanical approaches and antimicrobials alone does not provide long-term stability and fails to effectively manage almost 25% of periodontitis patients. There are also growing concerns about the development of antibiotic resistance. Moreover, the currently available host modulation therapies exhibit various side effects. Hence, successful management of periodontitis requires alternative modes of preventive and therapeutic strategies assessing individual risk and causation and targeting multiple disease predisposing factors [22].

In this book, we aimed to take our thinking further and provide a glimpse of emerging strategies and concepts which are currently being tested in preclinical and/or clinical settings and show promise as future novel therapies in the era of precision oral care. An up-to-date information is provided in each chapter about novel concepts and new studies toward better management of periodontitis and peri-implantitis. We focus on therapeutics targeting immune response and microbiome as well as recent advances and technologies in laser therapy and regenerative medicine including protein and cell-based approaches and 3D printing. The key biological processes in health and disease in aging periodontal tissues and implications for patient management are also discussed.

Following paragraphs provide a brief overview of the evolving paradigm in the pathogenesis of periodontal diseases and patient management and share perspectives for precision oral health care. More detailed discussions about the critical host immune and inflammatory pathways, oral cavity microbiome in health and disease, and novel periodontal regenerative strategies are highlighted in each subsequent chapter.

1.2 The Pathophysiological Stages of Periodontal Lesion

The periodontium consists of various cells of myeloid and non-myeloid origin distributed within four components including gingiva, periodontal ligament, cementum, and alveolar bone. Even though each periodontal division is distinct in its location and composition, they all function as one unit and the cellular activities occurring in one site can affect the others. The periodontal tissues are constantly exposed to a diverse group of microbiome including commensal and external bacteria, viruses, and fungi as well as stress originating from chemical and mechanical trauma, which trigger activation of immune and inflammatory pathways in an attempt to protect the host and maintain health. The periodontal tissue homeostasis and architecture are preserved by the balance between host destructive and repair processes to this constant stress. Thus, multiple cells with different functions are involved in various stages of periodontal pathology and the interplay between microbial components, the cells of

the periodontal tissues, and the constant influx of inflammatory mediators and tissue-derived enzymes determine the transition from health to disease [23].

The initial inflammatory response in the periodontium is triggered by the sensing of microbial-associated molecular patterns (MAMPs) (e.g., lipopolysaccharide [LPS], lipoproteins) and/or damage/danger-associated molecular patterns (DAMPs) (e.g., nucleic acids, fibrinogen, heat-shock proteins) through specialized pattern-recognition receptors (PRRs) (e.g., Toll-like receptors [TLRs], complement receptors, and NOD-like receptors [NLRs]). These interactions mainly activate nuclear factor kappaB (NF-kB) and mitogen-activated protein kinase (MAPK) signaling pathways, leading to the production of proinflammatory cytokines and chemokines that aid in the development of an efficient innate immune response to eliminate the insult and coordinate development of an adaptive immune response. Although activation of the immune system is crucial to combat infections, it is equally important to have properly functioning regulatory pathways to facilitate timely termination of inflammation, allow resolution and tissue healing thereby prevent collateral tissue damage [8, 24–26].

The periodontal disease progression follows four classical histological stages: *initial*, *early*, *established*, and *advanced* lesions [27, 28] (Fig. 1.1). These definitions are based on the distinctive histological features of the developing lesion with regard to the presence of specific cells and the extent of tissue destruction, yet the disease progression is a highly interactive and dynamic process where there is commonality between each stage, with similar cells and inflammatory mediators, histological characteristics, and clinical symptoms. Further expanding onto these classical definitions of periodontal lesion, the transition from health to disease state was categorized in four schemes as follows: (1) *"acute bacterial challenge phase"* which refers to the initial response to the commensal microbiome (*initial lesion*), (2) "*acute inflammatory phase"* which defines the initial inflammatory reaction (*initial* and *early lesions*); (3) "*immune response phase"* which defines the progression of immune and inflammatory responses involving activation of numerous types of mononuclear cells (*early and established lesions* or gingivitis), and (4) "*regulation and resolution phase*" which defines the stage where a normal protective host response may either terminate (health) or deviate toward a more prolong and destructive chronic immunoinflammatory state in susceptible individuals (*advanced lesion* or periodontitis) [29] (Fig. 1.1). While these histological/clinical phases provide an overview of the events occurring between host and microbiome within the oral cavity leading to health or disease states, the biological processes are not linear and independent from each other and there is significant interplay between phases.

The *initial* sequelae in gingival inflammation is a subclinical acute inflammatory lesion which is characterized with increased vascular dilation and blood flow accompanied by the migration of neutrophils toward the lesion due to chemotactic stimuli originating both from microbial cells and host-derived inflammatory mediators such as IL-8 (CXCL8), complement components C5a and C3, and leukotrienes. There is also activation of complement and kinin systems and arachidonic acid pathways and possible collagen loss. The *initial* lesion is typically response to continuing presence of acute microbial challenge and if the acute inflammation fails to resolve rapidly, it may evolve into a chronic inflammatory lesion [30, 31].

Early lesions display accentuation of the features of the *initial* lesion with increased vascularization, accumulation of more PMNs and lymphocytes (mainly T-cells) as well as continuous activation of complement and arachidonic acid pathways. Macrophages, plasma cells, and mast cells start to appear at the site of acute inflammation. These cells produce pro-inflammatory mediators including TNF-α, IL-1β, IL-6, IL-8, and IL-17, which may exacerbate the inflammatory response and promote progression to more advanced stages of the disease. There is further loss of the collagen fiber network around the inflammatory infiltrate due to the activation of the local immune system. Clinical features may include bleeding, edema,

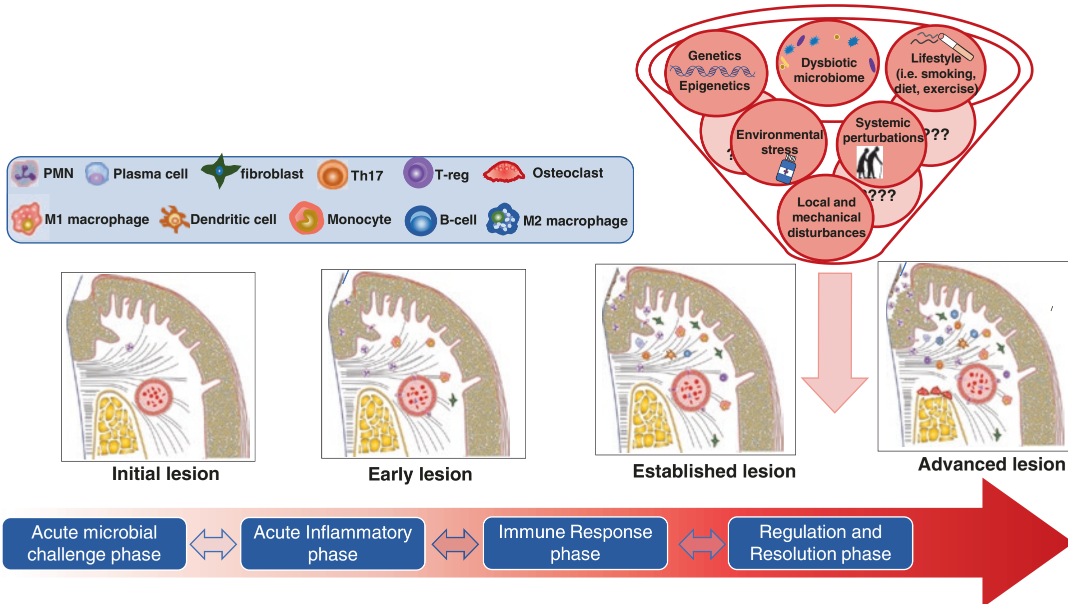

Fig. 1.1 The schematic model of periodontal disease progression. During the initial response, *acute microbial challenge phase,* microbial components (e.g., lipoproteins, LPS, nucleic and fatty acids, and FMLP) trigger the release of inflammatory mediators (e.g., IL-1, IL-8, TNF, prostaglandins, and matrix metalloproteinases) from the epithelial cells. Further release of histamine from mast cells and activation of endothelial cells drive the migration of neutrophils to the site. *The acute inflammatory phase* (early lesion) is characterized by the augmentation of the local inflammatory responses, activation of serum protein systems (e.g., complement), additional leukocyte recruitment, activation of macrophages, and release of more inflammatory and immune mediators and chemotactic substances. *Immune and inflammatory phase* (established lesion) depicts a further increase in inflammatory and cellular activity and transition from an acute lesion to a chronic state. In addition to the continuous activation of macrophages and serum proteins, T cells, B cells, and plasma cells appear in the tissue. During the *regulation and resolution phase*, there is orchestrated activity of all systems to control microbial insult and balance the tissue destructive and healing phases of the immune response. If tissue homeostasis is sustained, the established lesion does not further progress and remains stable with periods of activity and resolution for years. Failure of timely termination of inflammation alters microbial community, and generates a non-resolving inflammatory state which is further perpetuated by microbial dysbiosis eventually leading to the destruction of extracellular matrix and loss of alveolar bone. The advanced lesion of periodontitis is characterized by the initial loss of attachment and alveolar bone. There is increased cellular activity and production of more inflammatory mediators and tissue-derived enzymes by activated PMNs, macrophages, monocytes, lymphocytes, fibroblasts, osteoblasts, and osteoclasts. It is imperative to fully understand the factors which increase susceptibility and molecular and cellular events occurring in each phase to manage the disease

and erythema. Both initial and early lesions constitute the acute inflammatory reactions to oral commensals [30, 31].

As the disease progresses into the *established* or the *immune response phase*, there is a transition from an acute lesion to a chronic state. The manifestations of *early* and *initial* changes still persist, along with the appearance of B-lymphocytes and continuingly increased numbers of PMNs, macrophages, monocytes, and T-cells. There is also the presence of extravascular immunoglobulins in the connective tissue and in the junctional epithelium. In the *established* lesion, which is clinically diagnosed as *gingivitis*, there is a substantial loss of gingival extracellular matrix due to increased collagenase activity and activation of the local immune system, but without bone loss. It is now well recognized that gingivitis is reversible and can remain stable with periods of exacerbation and remission indefinitely and only progresses to periodontitis (*advanced lesion*) in susceptible individuals [30, 31].

The *regulation and resolution phase* determines the final stage of the transition to periodontitis or *advanced lesion*. At this stage, the symptoms of *established lesion* and *immune response phase* persist with the progression of inflammation to involve the alveolar bone. Production of more inflammatory mediators such as cytokines, chemokines, arachidonic acid metabolites (prostaglandins), and complement proteins by activated PMNs, macrophages, monocytes, lymphocytes, fibroblasts, and other host cells can cause oxidative damage by promoting the release of tissue-derived enzymes such as matrix metalloproteinases (MMPs). Furthermore, cytokines can act on stromal and non-stromal cells causing increased expression of receptor activator of nuclear factor kappa-B ligand (RANKL) while decreasing osteoprotegerin (OPG) production. If the inflammation is not resolved, destruction of extracellular matrix and irreversible alveolar bone loss occur (*advanced lesion* or periodontitis) [31] (Fig. 1.1).

Substantial progress has been made in defining the cellular and molecular mechanisms which participate in the pathophysiology of periodontal inflammation. It is revealed that periodontal disease progression does not follow a linear pattern and involves a highly coordinated and interconnected array of biological and cellular events that are shaped by multiple host and environment-specific factors contributing to the susceptibility of the disease (Fig. 1.2). We now know that innate immunity does not simply represent different forms of physical and chemical barriers to

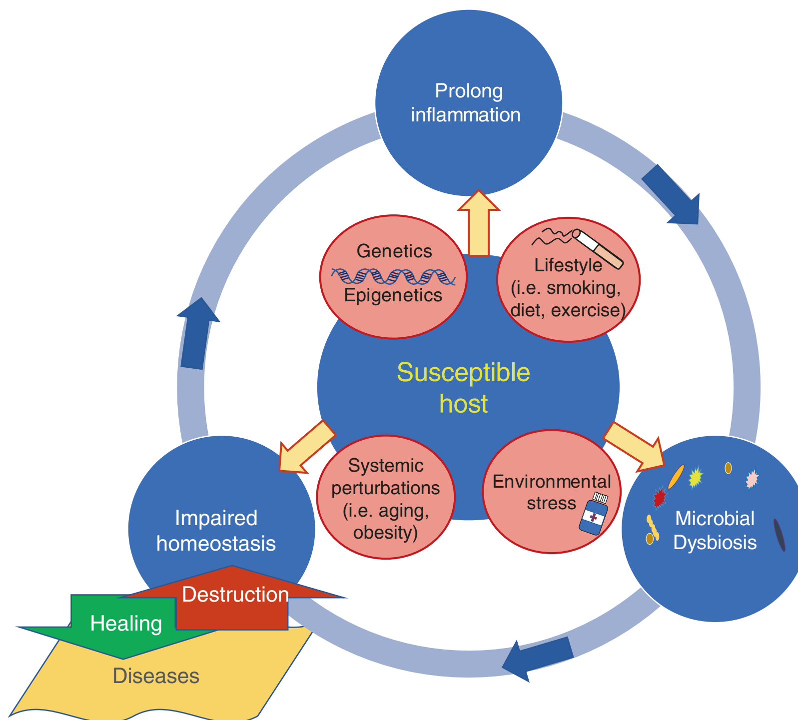

Fig. 1.2 The schematic model of non-resolving and self-perpetuating periodontal inflammation and predisposing factors. Periodontitis is not solely a bacterial infection or a linear host response to a dysbiotic microbiome but rather characterized by a self-perpetuating state of prolonged inflammatory response and impaired tissue homeostasis involving highly coordinated and interconnected array of biological and cellular events which are driven by multiple host and environment-specific factors. Factors which increase susceptibility for periodontitis are still under extensive investigation and include host genetic and epigenetic alterations, systemic perturbations (e.g., obesity, stress, aging, uncontrolled diabetes, immune disorders, and use of certain medications), environmental and local disturbances (e.g., microbial dysbiosis, improperly designed restorations, imbalanced masticatory forces), and lifestyle preferences (e.g., hygiene, smoking, eating/drinking habits, exercise)

microbial insult, but constitutes a highly specialized and organized network of cellular and molecular pathways which interact closely with each other as well as the adaptive arms of the immune response to balance the tissue homeostasis within the oral cavity [31–33]. It has been revealed that local tissues can play a critical role in sustaining homeostasis through local production of homeostatic molecules or endogenous regulators and that cells are trained or programmed based on their specialized niches in the body [34]. The discovery of the dynamic reciprocal interactions between the host immune and inflammatory responses and bone tissue started an emerging field of study named osteoimmunology [35]. While the research over the last decade uncovered the biological pathways and host-pathogen interactions during the course of the initial inflammatory response, we still do not know much about the regulatory pathways that drive the timely termination of inflammation and promote resolution and healing. Defining key regulators of inflammation at the cellular and molecular levels in each stage of the progressing periodontal lesion and understanding the individual factors (e.g., genetic, systemic, and local) that modulate these processes will allow us to predict the disease susceptibility and develop personalized strategies to improve clinical outcomes. In addition, understanding the downstream regulatory factors which play role in maintaining tissue homeostasis will be critical to develop therapeutics without off-target effects.

1.3 Evolving Paradigms in the Pathogenesis of Periodontitis

There has been a significant amount of research over the last 60 years in clinical and basic sciences combined with technological advances which led to distinct “eras” in our understanding of periodontal disease pathogenesis and patient management (Fig. 1.3). The concepts defining the periodontal microbiology have evolved from the “*nonspecific plaque hypothesis*” in the 1960s which associated the amount of the entire plaque microbiota with the disease status [36] to the “*specific plaque hypothesis*” in the 1980s which proposed a direct role of specific bacteria in the etiology of periodontitis [37]. In the 1990s, these two models were modified as the “*ecological plaque hypothesis*” which suggested that molecular and physiological changes within the tissues, such as variations in pH and amount of certain host proteins, could promote the growth of Gram-negative species and trigger more inflammation

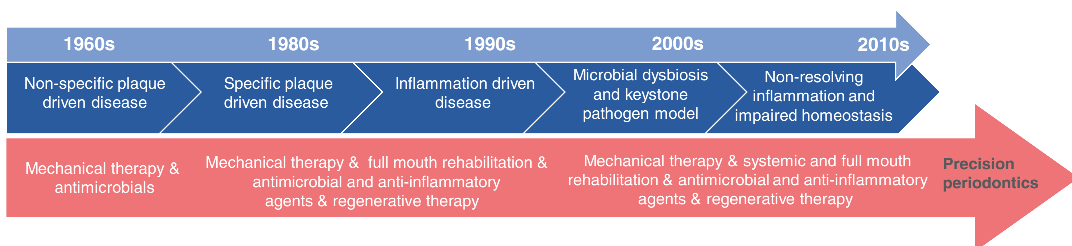

Fig. 1.3 Evolving paradigms in the pathogenesis and management of periodontitis. In the mid-twentieth century, periodontitis was recognized as an infectious disease of multibacterial origin and managed either through extraction or non-specific mechanical therapy with and without antimicrobials and oral hygiene instructions. Over the years, rigorous research in microbiology, immunology and regenerative medicine and the realization that the oral cavity is tightly connected to the rest of the body led to the reappraisal of the pathogenesis and clinical management of the disease. Today, periodontitis is characterized as a non-resolving inflammatory disease to a dysbiotic microbiome and incorporation of host modulation therapy in clinical practice is revealed to improve clinical outcomes. Future periodontal practice will adapt tools of precision medicine to prevent and treat the disease on a personalized level and address the multifactorial nature of the disease and create tailored strategies for each individual’s unique biological profile and habits with the premise of achieving better oral and systemic health, decreased financial burden, and improved quality of life

and tissue loss [38]. Around same years, research efforts extended beyond microbiology in an attempt to define the host immune and inflammatory responses during the course of periodontal disease. Several reports highlighted the significance of host-derived factors in the disease pathophysiology and noted that individual variations in host immune response to oral microbiome are the main drivers of the susceptibility of periodontitis [39, 40] (Fig. 1.3).

The awareness of the significance of the inflammatory component of the disease led to the reappraisal of the definition of periodontitis from a classical infectious disease, one being solely of bacterial origin, to an "*inflammatory disease*" [39, 41]. In this new era, periodontitis was characterized by a non-resolving inflammation not fully capable of eliminating the initiating pathogens. Most recently, "*polymicrobial synergy and dysbiosis*" and "*keystone bacteria*" models have been proposed which are consistent with the notion that periodontal bacterial composition is much more diverse than previously predicted and that host response to these distinctive communities vary greatly between individuals which eventually determine the susceptibility for diseases [42, 43]. In the *"dysbiosis theory"*, it is hypothesized that the influence of individual species in a polymicrobial community could alter host-microbial interactions leading to destructive inflammation. It is argued that gingival inflammation, in response to early colonizing bacteria, changes the subgingival environment and promotes the growth of certain endogenous accessory bacteria which then assist in the colonization and metabolic activities of other pathogens until they are outgrown by the more potent pathobionts. This synergistic and symbiotic polymicrobial environment initiated and fueled by the deregulated inflammation further enhances inflammation and tissue destruction [43] (Fig. 1.3).

Today, it is revealed that periodontitis is not solely a bacterial infection or a linear host response to a dysbiotic microbiome but rather characterized by a self-perpetuating state of prolonged inflammation due to various factors involving host and environment subsequently leading to microbial dysbiosis, unsustainable inflammation, and tissue destruction (Fig. 1.2) [44]. Consistently, the research also indicates that anti-infective therapy using mechanical approaches and antimicrobials often fails to effectively manage the disease in the long term [22]. As we continue to investigate the oral cavity microbial communities and characterize their pathogenicity within the changing microenvironments, current paradigm indicates that therapeutics targeting inflammation should be integrated to the periodontal practice to restore tissue homeostasis and microbial dysbiosis. Therefore, continuous research is needed to identify the key biological pathways and downstream regulatory molecules at the interface of host and pathogen interactions to develop more targeted therapies. Subsequent chapters will discuss some of these novel studies.

1.4 Evolving Paradigms in Periodontitis Patient Management

Not many years ago, the goals of traditional periodontal therapy have been to remove deep pockets, control gingival bleeding, and achieve proper oral hygiene in all patients through mechanical therapy with and without antibiotics and continuous supportive periodontal care (Fig. 1.3). In clinical practice, this goal is seldom accomplished and tissue breakdown continues in the majority of cases. In addition, growing concerns about the global antibiotic resistance fueled research efforts toward the identification of more selective modes to manage the disease. Remarkable progress has been made in the understanding of the molecular basis and susceptibility factors of periodontal disease and in the development of new biomaterials and advanced surgical techniques and diagnostic tools [15, 17, 18, 32, 45]. Current periodontal practice has become more focused to individual needs and incorporated full mouth and systemic rehabilitation and addressing predisposing factors such as smoking cessation and diabetes control in patient management. Yet, even this mode of therapy fails to

achieve desired clinical outcomes in certain groups of patients, and periodontitis continues to be the primary cause of tooth loss in industrialized countries predominantly affecting people older than 40 years of age. Thus, the development of better approaches to prevent and successfully treat periodontitis is vital to general and oral health. It became also clear that the host response to the microbiome is under the influence of many factors including genetics, systemic and local factors, and lifestyle choices (Fig. 1.2). Hence, the development of predictable preventive and therapeutic strategies to diminish destructive inflammation and regenerate tissues will depend on taking into consideration individual differences and predisposing factors in clinical decision making.

Strategies that are currently being tested to treat the disease include targeting key inflammatory pathways or microbial components. Periodontal microbial dysbiosis can be targeted by anti-bacterial drugs, probiotic intake, and mechanical debridement [46, 47]. As a non-invasive therapeutic approach for periodontitis, host modulation therapy has been investigated targeting cytokine/chemokine network, MMPs, arachidonic acid metabolites, and more recently endogenous regulators of inflammation and modifying nutrition and lifestyle choices [32, 48–52]. Yet, side effects are of concern for some of the clinically available host modulation therapies including GI complications and impaired wound healing [53]. While the use of low-dose doxycycline has been considered to be safe, concerns with antibiotic resistance limit the use of antibiotics in general [47]. Therefore, recent investigations focus on targeting downstream molecules and regulatory pathways and/or unique microbial components as therapeutics with the premise of eliminating side effects.

Genetics can explain a considerable amount of variation in the clinical presentation of periodontitis and effect of specific genotypes on the key biological events and disease phenotypes are under investigation [54, 55]. Another emerging field in periodontology is epigenetics which investigates the dynamic interactions between genes, environment and human behavior and how they lead to genome modifications, influence gene expression, and subsequently modify susceptibility for periodontitis [56]. With increased lifespan, the influence of aging on disease phenotype should also not be overlooked [10, 57].

The "*precision periodontics*" un-doubtfully is the next phase for our profession which will consider individual inflammatory response, microbial composition and predisposing factors and adapt 4Ps of precision medicine (denoting predictive, preventive, personalized, and participatory) in clinical practice [16, 22] (Fig. 1.3). In this new era, patient management will include personalized strategies for the prevention of periodontal and peri-implant diseases and tailored therapies toward individuals' unique biology and risk profiles, which will take into consideration many elements in clinical decision making including cellular and molecular analyses, genetic and epigenetic factors, environmental and systemic perturbations (e.g., improperly designed restorations, obesity, stress, aging, uncontrolled diabetes, immune disorders, and use of certain medications), and life style preferences (e.g., smoking, diet) [14, 16].

In the following chapters, novel concepts and strategies targeted to complement, cytokines and endogenous regulators of inflammation, lipid mediators, and genetic and epigenetic approaches are discussed in the management of periodontitis and peri-implantitis. Insights are provided about the microbiome in health and disease and new-generation antimicrobials and probiotics. Emerging paradigm in regenerative medicine using cell and protein-based approaches, 3D printing, and laser technology is highlighted. As the population is progressively getting older, the biology of aging and its effect on the periodontium and patient management are reviewed.

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2 Complement C3 as a Target of Host Modulation in Periodontitis

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2.1 Introduction

Periodontitis is a common inflammatory disease that is induced by tooth-associated biofilms and leads to the destruction of the periodontium, that is, the tissues that surround and support the teeth (gingiva, periodontal ligament, cementum, and the underlying alveolar bone) [1]. Periodontitis is also associated with increased risk for certain systemic conditions, such as atherosclerosis, rheumatoid arthritis, and diabetes [2–4]. If not properly treated, this oral inflammatory disease may lead to tooth loss and possibly impaired mastication. Effective treatment of periodontitis contributes to better metabolic control in type 2 diabetes and improvement of endothelial function associated with the reduction of systemic inflammatory markers [5, 6]. Severe periodontitis afflicts approximately 10% of adults worldwide [7, 8]. Current therapies are not always effective and periodontitis continues to be a significant public health and economic burden [8–10].

Recent human microbiome studies and animal model-based mechanistic investigations together indicate that the pathogenesis of periodontal disease is not mediated by a few specific bacteria ("periopathogens") but actually involves polymicrobial synergy and dysbiosis [11, 12]. In the context of periodontitis, "dysbiosis" denotes an alteration in the relative abundance and/or influence of individual members of a polymicrobial community (as compared to their abundance and/or influence in periodontal health) leading to imbalanced host-microbial interactions that precipitate destructive inflammation and bone loss [12].

Although necessary for the initiation and progression of periodontitis, the polymicrobial communities do not constitute a sufficient cause for this oral disease. Indeed, it is the host inflammatory response to the polymicrobial dysbiotic challenge that ultimately inflicts damage to the periodontal tissues [13, 14]. Moreover, the dysregulated and destructive inflammatory response not only fails to control the dysbiotic challenge but also supports further microbial growth by providing nutrients in the form of tissue breakdown products (e.g., degraded collagen as a source of amino acids and heme-containing compounds as a source of iron) [11, 15]. These considerations justify the rationale for host-modulation approaches to the treatment of periodontitis. Host-modulation strategies may be used as adjuncts to improve current therapies (e.g., mechanical debridement to remove the pathogenic biofilm), which, as mentioned earlier, are often inadequate to control periodontal disease.

In this review, we summarize and discuss studies in humans and animal models that implicate the complement system as a driver of periodontal disease pathogenesis, thus leading to the development of rational complement-targeted therapies for treating periodontitis.

2.2 Complement and Regulation of the Host Response

Before discussing the involvement of complement in periodontitis, it would be instructive to give a background on the complement system and how it regulates immunity and inflammation. In addition to the classic group of serum proteins (C1-9), the integrated complement system comprises some 50 proteins, including pattern-recognition molecules, convertases and other proteases, receptors that interact with different immune mediators, and cell surface-associated or fluid-phase regulatory proteins [16]. The complement cascade can be initiated by distinct mechanisms (classical, lectin, or alternative) that converge at the third component (C3). The triggering of the classical pathway is initiated by antigen-antibody complexes recognized by the C1q subunit of the C1 complex ($C1qr_2s_2$) which is thereby activated. The lectin pathway is induced through interaction of secreted pattern-recognition molecules (such as the mannose-binding lectin [MBL], ficolins, and collectins) with specific carbohydrate moieties on microbial or damaged host cell surfaces. Subsequently, both the classical and the lectin pathways proceed through C4 and C2 cleavage for the generation of the classical/lectin C3 convertase, C4b2b (Fig. 2.1). The alternative pathway is initiated by

low-level, spontaneous hydrolysis of C3 to C3(H_2O) (also known as “tick-over” mechanism) or by properdin (factor P; FP)-mediated binding of C3b, leading to the formation of the initial alternative pathway C3 convertases in the presence of factors B (FB) and D (FD). As long as there is no sufficient negative regulation (e.g., as is normally the case with bacteria or other non-self surfaces), these initial activation events are followed by rapid activation of the alternative pathway. The alternative pathway can be divided into two arms: the one mentioned above which initiates complement activation (through “tick-over” or by FP) and a second one that mediates amplification independently of the initiating mechanism. FP is involved also in the amplification loop as it binds to and stabilizes the C3 convertase (C3bBb), thereby boosting the overall complement response. Complement can also be activated independently of the initiation mechanisms by “extrinsic” proteases (such as thrombin and plasmin from the coagulation cascade) that can directly cleave and activate C3 or C5 (Fig. 2.1).

Complement activation, as described above, leads to the generation of effectors that “complement” the ability of antibodies and phagocytes to clear microbial pathogens (via C3b opsonization), promote inflammation (via the C3a and C5a anaphylatoxins that activate specific G-protein-coupled receptors, C3aR and C5aR1 (CD88), respectively), and lyse susceptible pathogens. The latter function involves the terminal pathway, to which all three initiation pathways converge. Here, C5-derived C5b binds to C6 and the resulting C5b-C6 complex is bound by C7. The newly formed C5b-C7 complex subsequently inserts into the target membrane followed by C8 binding to the C5b-C7 complex which causes a small pore in the membrane. The membrane attack complex (MAC) is formed by the binding of C9 molecules to the C5b-C8 complex [16, 17] (Fig. 2.1). In sublytic amounts, however, the MAC influences cell signaling pathways and promotes inflammation [18].

The activation of the complement system is regulated by several fluid-phase and cell surface-associated proteins [16]. The regulators functioning in the fluid phase are C1 inhibitor (C1-INH), factor I (FI), factor H (FH), and C4b-binding protein (C4BP), whereas the cell surface-associated regulatory molecules include complement receptor 1 (CR1; CD35), membrane cofactor protein (MCP; CD46), decay accelerating factor (DAF; CD55), and CD59 [16]. Optimal regulation by these proteins prevents uncontrolled activation and amplification of the complement system to preserve the integrity of host cells and tissues, while at the same time allowing the induction of immune responses against pathogenic challenges.

Complement is not a closed system working simply to tag and kill microbes but can shape the host immune response also by cross-talk interactions with other immune and physiological systems (e.g., Toll-like receptors [TLRs], coagulation system, adaptive immune cells) [19, 20]. Through these interactions, complement integrates innate and adaptive immunity, mediates the clearance of immune complexes, cellular debris and apoptotic cells, contributes to normal tissue and organ development, and promotes tissue repair after injury [16, 21]. Regarding its influence on the adaptive immune response, complement can stimulate B cells via C3d, which engages the B-cell co-receptor complex (CR2–CD19–CD81) [22], but also guides the quality and magnitude of T-cell activation [23]. The stimulatory effects of complement on T-cell activation could, at least in part, be mediated by the anaphylatoxins C3a or C5a which regulate antigen-presenting cell (APC) maturation and function but also appear to exert direct effects on the functional co-stimulation and differentiation of naive CD4+ T cells [24]. In this regard, C3a and C5a can be generated locally within the APC–T-cell interface, where reciprocal cognate interactions induce the expression of C3a and C5a receptors (C3aR and C5aR1) in both APCs and T cells. Genetic absence or pharmacological blockade of C3aR and C5aR1 signaling on human or mouse CD4+ T cells suppresses AKT-mTOR and enhances TGFβ1 signaling, which facilitates the induction of CD4 Foxp3+ regulatory T cells [25]. Conversely, induction of C3aR and C5aR1 signaling in mouse FoxP3+ regulatory T cells causes

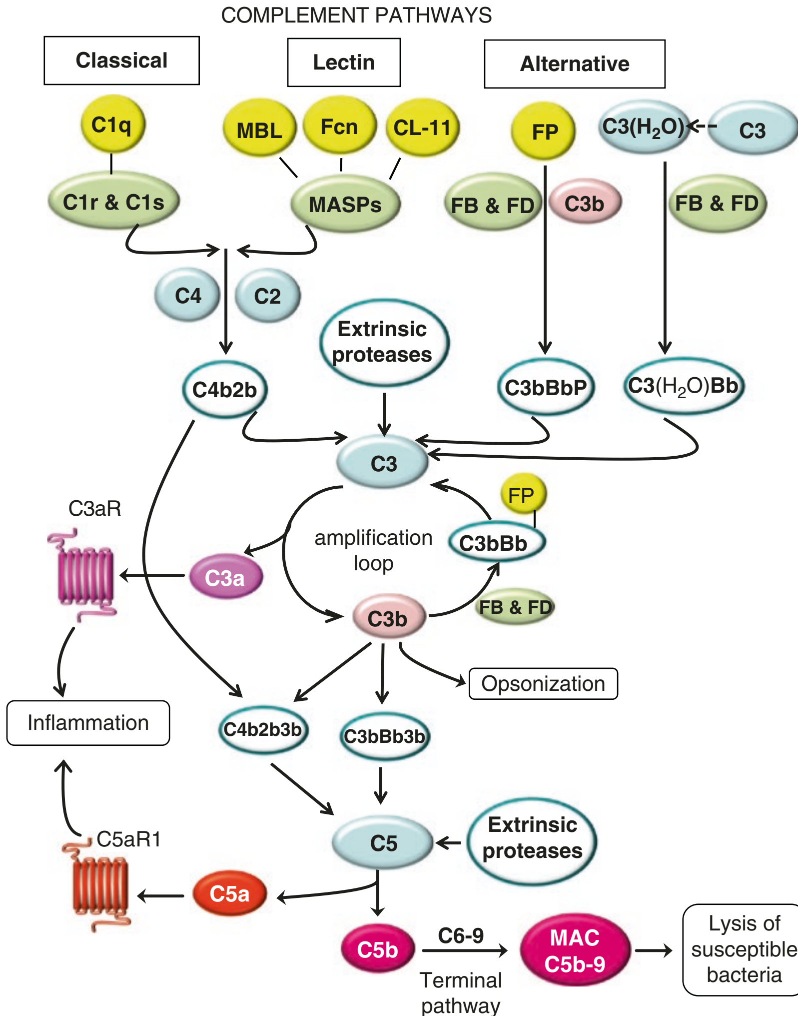

Fig. 2.1 Complement initiation pathways. The complement cascade can be triggered by distinct mechanisms (classical, lectin, or alternative) that converge at C3. The activation of the classical pathway is initiated by antibody-mediated activation of the C1 complex (C1q, C1r, and C1s). The lectin pathway is triggered through the binding of secreted pattern-recognition molecules (such as the mannose-binding lectin [MBL], ficolins [Fcn], and collectin 11 [CL-11]) to microbial surfaces. Subsequently, both the classical and the lectin pathways proceed through C4 and C2 cleavage for the generation of the classical/lectin C3 convertase (C4b2b). The activation of the alternative pathway can be divided into two arms: In the first, the alternative pathway is initiated either by a "tick-over" mechanism involving spontaneous hydrolysis of C3 or by properdin (FP)-mediated binding of C3b, leading to the formation of the initial alternative pathway C3 convertases in the presence of factors B (FB) and D (FD). The second arm, known as the "amplification loop," involves amplification of complement activation independently of the initiating mechanism. FP is also involved in the amplification loop as it binds to and stabilizes the C3 convertase, thereby enhancing the overall complement response. Complement can also be activated by "extrinsic" proteases (such as thrombin and plasmin) that can directly cleave and activate C3 or C5. Complement activation leads to the generation of effectors that promote phagocytosis (via C3b opsonization), inflammation (via anaphylatoxins C3a and C5a), and lysis of susceptible pathogens (via the formation of C5b-C9 membrane attack complex [MAC])

AKT-dependent phosphorylation of the transcription factor FoxO1, which in turn suppresses FoxP3 expression and, consequently, the suppressive function of regulatory T cells [26].

Studies in the 1980s showed that human lymphocytes produce various complement components, regulators, and receptors, implying the presence of a cell-intrinsic complement system [27, 28]. In recent years, the notion for a functionally significant intracellular complement system has been strengthened [29]. Thus, it is thought that the activation of C3 and C5 may not necessarily occur extracellularly. In this regard, there is evidence for the existence of intracellular stores of complement components and receptors (e.g., anaphylatoxin receptors and their ligands; C3b, factor B, factor H), which were shown to perform housekeeping and surveillance functions within human T cells [30–32]. These include regulation of T-cell homeostatic survival as well as effector T-cell responses, such as induction of IFNγ-expressing Th1 cells [23, 30].

Furthermore, complement was shown to regulate inflammasome activation in different cell types. In human monocytes, activation of C3aR triggers the release of ATP into the extracellular space, thereby promoting P2X7 activation and synergizing with TLR signaling to trigger NLRP3 inflammasome and secretion of IL-1β [33]. In human epithelial cells, the formation of sublytic MAC (C5b-8, C5b-9) causes intracellular Ca^{2+} fluxes resulting in the activation of the NLRP3 inflammasome and IL-1β release [34]. On the other hand, C1q, which binds apoptotic cells and facilitates their clearance by tissue macrophages (efferocytosis), suppresses NLRP3 inflammasome activation during efferocytosis by human macrophages [35].

Components of the complement system are increasingly being implicated in hitherto unanticipated intracellular immune and homeostatic functions. For instance, recent studies have revealed new C3-dependent antimicrobial mechanisms that may interfere with the ability of intracellular pathogens to replicate in the cytosol of non-immune cells after escaping from phagosomes [36, 37]. Nonenveloped viruses and bacteria opsonized with C3 cleavage fragments in the extracellular space can be sensed in the cytosol in a C3-dependent manner and trigger mitochondrial antiviral signaling (MAVS), which induces the production of pro-inflammatory cytokines. C3-coated viruses can moreover be restricted via proteasome-mediated degradation [37]. C3-opsonized microbes that are sensed in the cytosol in a C3-dependent manner can also interact with autophagy-related protein 16-1 (ATG16L1), thereby triggering ATG16L1-dependent autophagy and resulting in the targeting of the cytosolic microbes to autophago-lysosomes for degradation [36]. More recently, C4 (specifically C4b) was shown to inhibit viral infection through capsid inactivation in a manner independent of C3 activation [38].

2.3 Complement Involvement in Periodontal Disease

Despite its significance in host immunity, complement can become dysregulated or excessively activated due to host genetic or microbial virulence factors. When that happens, complement can switch from a homeostatic to a pathological effector that drives a wide range of inflammatory disorders, including periodontitis [39, 40]. Ample evidence from clinical and histological studies indicates that complement is involved in periodontitis. Biopsies of chronically inflamed gingiva or samples of gingival crevicular fluid (GCF) obtained from periodontitis patients exhibit significantly increased abundance of activated complement fragments relative to control samples from healthy individuals [41–47]. Complement can be present in GCF at up to 70–80% of its concentration in serum, although certain activated fragments can be found at much higher levels in GCF attributed to local generation in the periodontium [47–49]. Induction of experimental gingivitis in human volunteers was shown to lead to progressive elevation of complement cleavage products correlating with increased clinical periodontal indices of inflammation [45]. Conversely and consistently, periodontal therapy, which successfully reduced clinical indices measuring periodontal inflammation and tissue destruction,

resulted in decreased activation of C3 as shown by analysis of GCF samples [50]. Consistent with this, a study using integrative gene prioritization and databases from GWAS and microarray experiments proposed C3 as a likely candidate gene in periodontal disease pathogenesis [51]. Moreover, the expression of C3 is among the top 5% genes that is most strongly downregulated following periodontal therapy [41]. A single nucleotide polymorphism of C5 (rs17611), which is associated with increased serum C5 levels and susceptibility to the complement-associated disease liver fibrosis [52], was shown to be more prevalent in periodontitis patients than in healthy individuals [53]. These correlative studies suggested that complement may be involved in the pathogenesis of periodontitis.

Studies in appropriate animal models are necessary to test causation and thus link a disease to candidate mechanisms, thus confirming therapeutic targets and paving the way to human clinical trials [54]. Studies in rodents, including complement-deficient mice, have demonstrated a cause-and-effect relationship between complement activation and periodontitis and offered insights into the underlying mechanisms [55–58]. Indeed, these studies showed that complement is involved in both the dysbiotic transformation of the periodontal microbiota and the inflammatory process that leads to the destruction of periodontal bone [55, 57] (Fig. 2.2). For example, the ability of the keystone periodontal pathogen *P. gingivalis* to subvert the host immune response and promote dysbiosis requires its ability to instigate subversive C5aR1-TLR2 crosstalk, and is therefore counteracted in C5aR1-deficient mice [55, 57]. Furthermore, experiments in wild-type and C3-deficient mice established that C3-mediated inflammation is required for the long-term sustenance of the dysbiotic microbial

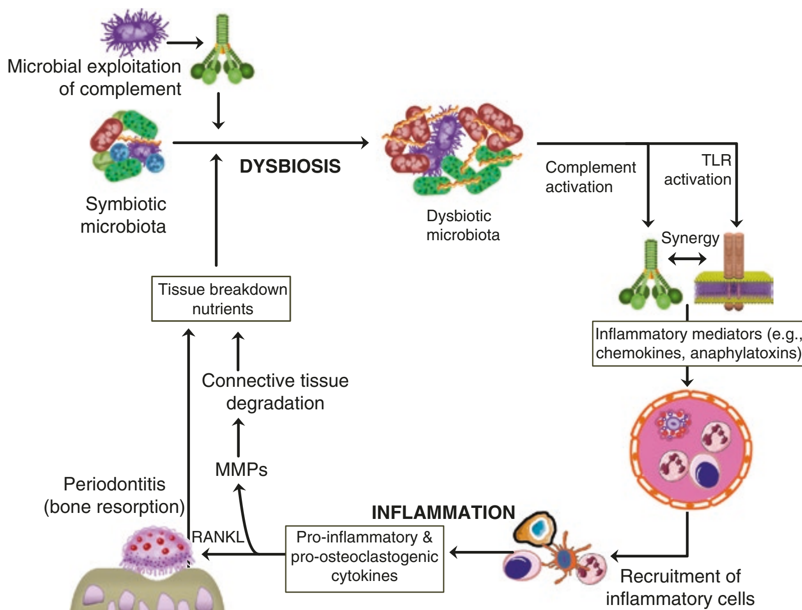

Fig. 2.2 Complement involvement in periodontitis. Periodontitis results from the disruption of host-microbe homeostasis leading to dysbiosis and inflammation that is in great part dependent on complement and its interactions with other signaling pathways, such as those activated by Toll-like receptors. In part, dysbiosis is mediated by pathogen subversion of complement pathways (details in text). Moreover, complement-dependent inflammation not only causes degradation of connective tissue and bone loss but also provides nutrients (tissue breakdown products) that foster further growth and persistence of the dysbiotic microbiota. Therefore, complement inhibition can, at least in principle, inhibit both dysbiosis and destructive inflammation, a notion that is supported by findings from animal studies (details in text). *MMPs* metalloproteinases, *OPG* osteoprotegerin, *RANKL* receptor activator of nuclear factor-κB ligand, *TLR* Toll-like receptor

community and for maximal induction of inflammatory bone loss [58].

C3aR or C5aR1 signaling pathways cross-talk with and amplify TLR-dependent inflammatory responses in both the circulation and peripheral tissues including the periodontium [59–61]. Importantly, complement inhibition alone is adequate to block complement-TLR crosstalk signaling regardless of the presence of uninhibited TLRs, thereby suppressing periodontal dysbiosis and destructive periodontal inflammation [55, 57]. As TLR activation can also be triggered by endogenous TLR ligands (e.g., biglycan, hyaluronan fragments, and heparan sulfate fragments) that are released upon tissue injury [62, 63] (i.e., during active periodontal disease), inhibition of the complement-TLR crosstalk may also contribute to controlling the progression of periodontitis.

2.4 Periodontal Bacteria and Inflammation

Although periodontitis is associated with strong complement activation, complement may not provide effective host immunity in the periodontal pockets as periodontal bacteria have a number of protective mechanisms against complement-mediated killing. For example, *Porphyromonas gingivalis* and *Prevotella intermedia* can co-opt physiological soluble regulators (inhibitors) of the complement cascade, such as C4BP which the bacteria can capture on their cell surface and thus protect themselves against complement-dependent opsonization and killing [64, 65]. In the same context, *Aggregatibacter actinomycetemcomitans* uses its outer membrane protein-100 to bind the alternative pathway inhibitor FH and acquire resistance to complement killing in serum [66]. Similarly, *Treponema denticola* can capture FH by means of an 11.4-kDa cell-surface lipoprotein (thus designated factor H-binding protein) [67], whereas *P. intermedia* binds the serine protease FI, a major inhibitor of complement that degrades C3b and C4b in the presence of cofactors such as C4BP and FH [64]. Whereas bacterial proteases, such as the Arg-specific gingipains of *P. gingivalis* and the karilysin of *Tannerella forsythia* cleave C5 to release biologically active C5a, the same proteases rapidly degrade the C5b fragment, thereby blocking the generation of the MAC [48, 68]. The serum resistance of *P. gingivalis* was additionally attributed to the presence of a surface anionic polysaccharide (APS) designated A-LPS (LPS with APS repeating units) [69]. Mutants of *P. gingivalis* lacking A-LPS are sensitive to complement killing in 20% normal serum [70].

In general, however, *P. gingivalis*, *P. intermedia*, *T. forsythia*, and *T. denticola* interact with complement in complex ways involving both inhibitory and stimulatory effects [71–75]. This seemingly paradoxical microbial behavior may be attributed to the dynamics of survival tactics of periodontal bacteria: on the one hand, the bacteria need to escape immune clearance, and on the other to stimulate inflammation and thus the flow of GCF as a source of nutrients [15]. This paradox may be exemplified by *T. denticola* which, as mentioned above, expresses an FH-binding protein [67]. Interestingly, however, *T. denticola* can also use a serine protease termed dentilisin to cleave FH that is attached on its surface [67, 76]. Thus, the function of dentilisin appears to offset the action of the FH-binding protein. An interesting hypothesis is that *T. denticola* may regulate the activity of FH depending on context, either to acquire protection against complement or to inactivate FH to promote local inflammation and hence the flow of GCF-derived nutrients. The *T. denticola* dentilisin may also hydrolyze the α-chain of C3 and generate iC3b [77], which opsonizes *T. denticola* [78]. Interestingly, iC3b-mediated phagocytosis is often associated with inadequate killing mechanisms or even immunosuppressive signaling [79–82] and is consequently exploited by pathogens to promote their adaptive fitness. Indeed, not only periodontal bacteria such as *P. gingivalis* but also *Mycobacterium tuberculosis*, *Bordetella pertussis, Francisella tularensis,* as well as HIV-1 promote their intracellular survival by exploiting complement receptor-3 (CR3; CD11b/CD18)-mediated internalization, either by direct interaction with CR3 or following iC3b opsonization

[83–88]. Moreover, *P. gingivalis* can selectively inhibit the killing capacity of phagocytes without suppressing their inflammatory response, which, as pointed out earlier, serves the nutritional needs of the entire dysbiotic microbial community. Specifically, *P. gingivalis* can uncouple a host-protective TLR2–MyD88 pathway from a TLR2–MyD88-adaptor-like (Mal)–PI(3)K pathway, which enhances the fitness of *P. gingivalis* and bystander bacteria by blocking phagocytosis and promoting inflammation [57, 89].

The above-discussed studies provide further rationale that complement inhibition is a promising host-modulation approach since it can potentially counteract microbial immune subversion and thus suppress the dysbiosis of the periodontal microbial community.

2.5 Translational Studies for Safety and Efficacy of C3 Inhibition in Periodontitis

The immune system and the anatomy of the periodontium in non-human primates (NHPs) are similar to those of humans, and periodontitis in NHPs exhibits clinical, microbiological, and immunohistological features that are similar to those seen in human periodontal disease [90–92]. Therefore, the cynomolgus monkey model is more predictive of drug efficacy in humans as compared to widely used animal models (rodents, rabbits, or dogs). NHPs, specifically cynomolgus monkeys, were used to determine the suitability of C3 as a therapeutic target in periodontitis. The inhibitor used was Cp40, a third-generation analog of the compstatin family of compounds, which are small peptidic inhibitors with an exquisite specificity for human and non-human primate C3 [93–95]. The original compstatin was discovered after screening a phage-displayed peptide library and is a cyclic peptide of 13 residues (I[CVVQDWGHHRC]T-NH2) [96]. Cp40 has a subnanomolar affinity for C3 (KD = 0.5 nM; 6000-fold greater than that of the original compstatin) and a human plasma half-life (48 h) that exceeds expectations for most peptidic drugs. Cp40 is being clinically developed for human use by Amyndas Pharmaceuticals and is designated AMY-101. AMY-101 and earlier compstatin analogs bind to C3 and prevent the cleavage of this complement protein by C3 convertases (Fig. 2.3). Therefore, AMY-101 can directly block the release of the anaphylatoxin C3a and the generation and surface deposition of C3b, regardless of the initiation pathway of complement activation. Moreover, the decrease of C3b resulting from the action of AMY-101 impairs the amplification of the complement response through the alternative pathway of complement activation. By blocking

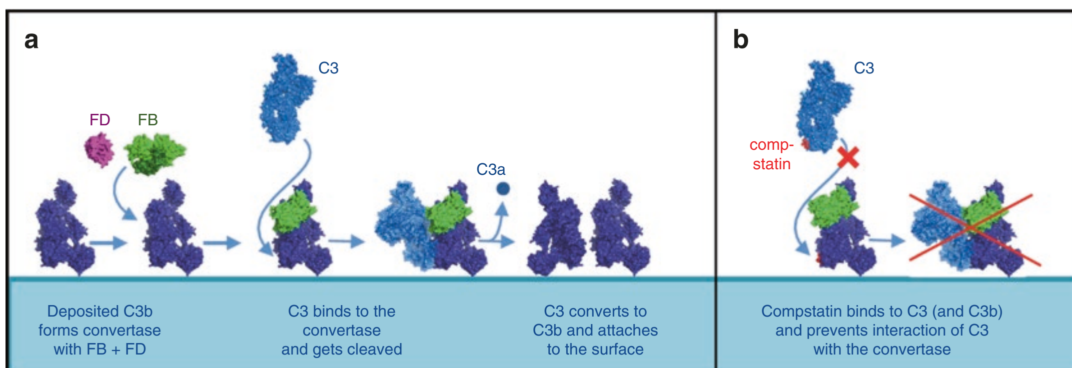

Fig. 2.3 Model of C3 activation and its inhibition by compstatin (**a**) Depiction of key protein interactions resulting in the formation of C3 convertases on a target surface (e.g., a microbial cell surface). Native C3 binds to the nascent convertase and is cleaved into its active fragments, C3a and C3b. (**b**) Compstatin acts by blocking protein-protein interactions; specifically, it binds both native C3 and C3b and sterically hinders the binding of native C3 by C3 convertases, hence preventing C3 cleavage into its active fragments. *FB* factor B, *FD* factor D. From [93]. Used by Permission

C3 activation and the assembly of C3b-containing convertases, the drug also inhibits the generation of downstream effector responses, such as the generation of the anaphylatoxin C5a and the formation of MAC [93, 95].

Consistent with the above-discussed findings from C3-deficient mice [58], local C3 inhibition by AMY-101 in a model of ligature-induced periodontitis in adult cynomolgus monkeys prevented the development of gingival inflammation and alveolar bone loss, as evidenced radiographically [58]. Specifically, AMY-101 caused a significant decrease of gingival index and clinical attachment loss, which correlated with reduction in the GCF levels of pro-inflammatory and osteoclastogenic cytokines (TNF, IL-1β, IL-17, and RANKL) and with diminished osteoclast numbers in bone biopsy specimens [58]. On the other hand, the GCF content of osteoprotegerin (OPG), a natural inhibitor of RANKL, was maintained at higher amounts in AMY-101-treated sites than in control sites, thus favorably reversing the RANKL/OPG ratio, which is a potential indicator of periodontitis [97].

AMY-101 was also tested under more rigorous conditions. Specifically, it was determined whether local administration of AMY-101 could inhibit periodontitis in a therapeutic (as opposed to preventive) setting, that is, to suppress pre-existing, naturally occurring severe periodontitis in aged cynomolgus monkeys [98]. This study involved a 6-week treatment period with AMY-101 and a 6-week follow-up period without AMY-101 treatment. Whether administered once or three times weekly, AMY-101 caused a significant reduction in clinical indices that measure periodontal inflammation (gingival index and bleeding on probing), tissue destruction (probing pocket depth and clinical attachment level), or tooth mobility. These clinical changes were associated with significantly reduced levels of pro-inflammatory mediators and decreased numbers of osteoclasts in bone biopsies. The protective effects of AMY-101 persisted, although at reduced efficacy, for at least 6 weeks following drug discontinuation. Therefore, AMY-101 can reverse pre-existing chronic periodontal inflammation in the absence of additional treatments such as scaling and root planing. Moreover, these studies confirmed the causative role of C3 in periodontitis using NHPs as the closest model to human periodontitis.

GCF samples obtained from the animals used in the above-discussed study [98] were additionally used for proteomics analysis [99]. Specifically, the samples were processed for filter-aided sample preparation (FASP) digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The human orthologs were subjected to gene ontology analyses using protein analysis through evolutionary relationships (PANTHER) and the data became available via ProteomeXchange (PXD009502). Gene ontology analysis revealed the involvement of both the alternative and classical pathways of complement activation in naturally occurring NHP periodontitis, although the former pathway was the most enriched of all biological pathways identified by this analysis [99]. These findings are consistent with early clinical periodontal studies showing activation of both the alternative and classical pathways of complement as determined by analyses of GCF samples from periodontitis patients [47, 50, 100]. Another important target identified by the proteomic analysis of GCF samples from AMY-101-treated NHPs was leukocyte degranulation. The ability of AMY-101 to suppress exocytosis likely represents a host protective mechanism given that neutrophils are responsible for significant tissue damage in human periodontitis, in large part through degranulation of cytotoxic molecules and tissue-degrading metalloproteinases [101–104].

Further studies were undertaken to determine the local safety of the AMY-101 drug. To this end, a therapeutic dose of AMY-101 (50 μL of 2 mg/mL solution corresponding to 0.1 mg/site) was injected in healthy gingiva of posterior teeth in five NHPs. Each animal received a total of four injections, one per quadrant; two injections were with AMY-101 and the other two injections involved water for injection containing 5% dextrose (control). In each animal, AMY-101 was administered on both maxillary and mandibular quadrants (2 sites total), whereas the control solution was injected on the two contralateral

sites. AMY-101 and control solution were injected a total of three times, at days 0, 7, and 14, followed by a 2-week observation period without further injections. Careful daily clinical examinations revealed no signs of irritation after the injection of AMY-101 or control solution throughout the observation period [105]. It should be noted that systemic exposure with AMY-101 following local injection into the gingiva should be negligible, hence not affecting complement functions in circulation or other tissues. In the treatment regimen used in the above-described NHP study [98], a total of 1.5 mg AMY-101 was injected (15 sites at 0.1 mg/site). Even if the full local dose were injected systemically, this would lead to an AMY-101 amount of 0.2–0.3 mg/kg bodyweight in NHPs (or, in case of humans, to 0.02–0.03 mg/kg bodyweight). On the other hand, a much higher systemic AMY-101 dose is required (specifically 1–2 mg/kg bodyweight) to achieve target-exceeding drug levels in NHPs [106]. In conclusion, small amounts of locally injected AMY-101 that might end up in the blood should be readily bound by excess circulating C3 (1.0–1.5 mg/mL).

As discussed earlier, weekly intragingival injections of AMY-101 (0.1 mg/site) improved the periodontal condition of NHPs with natural chronic periodontitis [98]. A less frequent but yet successful regimen would facilitate the application of AMY-101 for human use. To explore this possibility, it was examined whether AMY-101 (0.1 mg/site) can be effective also when administered once every 2 weeks or once every 3 weeks [105]. Specifically, AMY-101 was injected locally into the gingiva of anterior and posterior teeth on both sides of the maxilla. Clinical examinations were performed at baseline and at weekly or biweekly intervals thereafter throughout the study to determine the progression of the disease and the potential beneficial effects of AMY-101. Clinical readings made before AMY-101 injection served as baseline controls. The mandible was not treated but was monitored by clinical periodontal examination during the entire study for comparative purposes. The study consisted of 6 weeks of AMY-101 treatment (treatment period), followed by 6 weeks without AMY-101 treatment (follow-up period). Regardless of the frequency of administration, AMY-101 caused a significant reduction in clinical indices that measure periodontal inflammation (gingival index and bleeding on probing) or tissue destruction (probing pocket depth and clinical attachment level). Interestingly, differences between baseline and subsequent readings reached statistical significance at or after 6 weeks, i.e., at the time-point when the treatment with AMY-101 was discontinued. Many of the differences observed at 6 weeks remained statistically significant even at 12 weeks (bleeding on probing, probing pocket depth and clinical attachment level). The aforementioned clinical indices were also monitored in the untreated jaw (mandible) during the same 12-week interval. In contrast to the improved clinical condition in the AMY-101-treated maxillae, the clinical indices in the untreated mandibles did not show significant differences in the course of the study as compared to their baseline values [105]. In conclusion, AMY-101 can induce a long-lasting clinical anti-inflammatory effect in periodontal disease.

Given that AMY-101 is also being considered for systemic disorders and periodontitis is a highly prevalent disease [107], it was tested whether AMY-101 can also protect against periodontitis when administered systemically. Using the same animal model of naturally occurring NHP periodontitis, AMY-101 was thus administered in 10 animals via subcutaneous injection at a concentration of 4 mg/kg bodyweight, once per 24 h for a total of 28 days [105]. To determine the progression of the disease and the potential beneficial effects of AMY-101, clinical examinations were performed at baseline (week 0) and throughout the study (at 1-, 2-, 3-, 4-, and 11-week time-points). Additionally, biopsies were taken from the gingiva and bone at baseline, 4 and 11 weeks. Systemically administered AMY-101 caused a significant and long-lasting reduction in probing pocket depth, an index that measures tissue destruction. The protective effect was first observed at week 4. Importantly, the protective effect persisted without waning for at least another 7 weeks (week 11), despite the fact that the drug was discontinued after week 4.

Improvement of bleeding on probing, which assesses periodontal inflammation, was also observed at weeks 2 and 3. Histological observations at 4 weeks showed that AMY-101 inhibited the expression of pro-inflammatory and pro-osteoclastogenic cytokines (IL-17 and RANKL) and elevated the expression of OPG in the connective tissue adjacent to the alveolar bone, as compared to their baseline expression. Moreover, AMY-101 treatment caused a decrease in the complement cleavage fragments C3d and C5a, thus confirming its capacity to inhibit complement activation in the periodontium. In conclusion, systemic AMY-101 improves the periodontal condition of NHPs, which is stably maintained for at least 7 weeks after drug withdrawal [105]. These data further suggest that patients treated for systemic disorders (e.g., paroxysmal nocturnal hemoglobinuria) can additionally benefit in terms of improved periodontal condition. In this context, it should be noted that the monitoring of NHPs under prolonged systemic exposure to an inhibitory concentration AMY-101 (up to 3 months) showed no significant alterations with regards to biochemical, hematological, or immunological parameters in their blood or tissues relative to control animals treated with vehicle alone [108]. Moreover, despite complete inhibition of C3 in the plasma, skin wounds inflicted on the AMY-101-treated animals did not exhibit signs of infection but rather displayed a trend for accelerated wound healing relative to the vehicle-treated controls [108], in line with earlier findings in C3-deficient mice which had faster skin wound healing as compared to C3-sufficient controls [109].

2.6 Concluding Remarks and Outlook

New functions attributed to complement have transformed our perception of it from a blood-based antimicrobial system to a global regulator of innate and adaptive immunity and inflammation [21]. The multifaceted nature of complement interactions with other systems is reflected in the range of inflammatory disorders that are driven by complement dysregulation or overactivation, such as in periodontal disease. Thus, C3-targeted inhibition of complement in periodontitis is expected to modulate more than the complement cascade per se, thus influencing systems and functions that are interconnected with complement. These include, but are not limited to, neutrophil recruitment and function, T-cell-mediated inflammation, and osteoclast activation [58, 99, 110–114].

The capacity of the C3 inhibitor AMY-101 to block periodontal inflammation and bone loss in cynomolgus monkeys provides strong support for the appropriateness of C3 as a treatment target for human periodontitis. Both the classical and alternative pathways of complement activation were implicated in human periodontitis [47, 50, 100]. Assuming that carbohydrate or glycoprotein components of periodontal bacteria can activate the lectin pathway, the concomitant inhibition of all three pathways by AMY-101 is likely to provide increased protection against periodontitis as compared to inhibition of each individual pathway alone. Consistent with this notion, C3-deficient mice display enhanced protection against periodontal inflammation as compared to FD-deficient mice (our unpublished observations). In this regard, it should be noted that a recent study demonstrated activation of the alternative pathway in FD-deficient mice in a model of glomerulonephritis, suggesting the presence of an FD bypass mechanism for the assembly and activity of the alternative pathway convertase [115]. Such bypass mechanisms have been described, for instance, kallikrein can substitute for FD to activate the alternative pathway C3 convertase [116]. Pharmacological blockade of downstream pathways, such as C5aR1 signaling, may not be as protective as blocking C3 since C3aR signaling also contributes to inflammatory bone loss in experimental periodontitis [55]. In 2017, AMY-101 successfully completed a phase I safety trial in human volunteers, showing a very good safety profile (ClinicalTrials.gov Identifier: NCT03316521) [40] and in 2019, the drug received Investigational New Drug (IND) approval by the U.S. Food and Drug Administration for the conduct of the first

clinical study to evaluate its efficacy in adults with gingivitis (ClinicalTrials.gov Identifier: NCT03694444).

Although AMY-101 was successfully applied as a stand-alone treatment for both induced and naturally-occurring periodontitis in cynomolgus monkeys, the drug is intended to be developed as an adjunctive therapy to the treatment of human periodontitis, that is, to significantly enhance the current standard therapy, which is not effective for all patients [117]. Ideally, host-modulation interventions should not be implemented only in a therapeutic setting but also on a preventive basis to high-risk individuals, if these could be identified prior to the onset of periodontitis. Individuals who are likely at high risk to develop periodontitis include cigarette smokers and diabetic patients [118–120]. Clinical studies have shown that gingivitis (reversible periodontal inflammation that remains contained within the gingival epithelium and the underlying connective tissue without affecting the alveolar bone) precedes the onset of chronic periodontitis and, moreover, the absence (or treatment) of gingivitis represents a good indicator for long-term maintenance of periodontal health and prevention of chronic periodontitis [121–123]. Moreover, longitudinal studies demonstrated that sites that do not progress to gingival attachment loss are characterized by less gingival inflammation over time, whereas those sites that do progress have persistently greater levels of gingival inflammation [123–126]. Given that gingivitis is a major risk factor and a necessary pre-requisite for periodontitis, AMY-101 could also be used for the treatment of gingivitis. Finally, since severe periodontitis may adversely affect systemic health by enhancing the risk for conditions such as atherosclerosis, diabetes, and rheumatoid arthritis [2–4], it is possible that complement-targeted therapy in periodontitis may exert a beneficial effect on concurrent systemic inflammatory conditions.

In conclusion, the safety of AMY-101 and its protective effects in a highly relevant preclinical model of periodontitis indicate that it is a promising candidate drug for the treatment of periodontitis, a notion that merits investigation in human clinical trials.

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Conflict of Interest Statement: J.D.L. is the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors (including third-generation compstatin analogs such as AMY-101). J.D.L. and G.H are inventors of patents or patent applications that describe the use of complement inhibitors for therapeutic purposes, some of which are developed by Amyndas Pharmaceuticals. J.D.L. is also the inventor of the compstatin technology licensed to Apellis Pharmaceuticals (i.e., 4(1MeW)7W/POT-4/APL-1 and PEGylated derivatives such as APL-2/pegcetacoplan). The other authors declare no competing interest.

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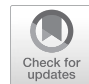

Resolvins in Periodontal Tissue Homeostasis (Emerging Therapies)

3

Audrey Rakian, Rubie Rakian, Nada Alshehri, and Thomas E. Van Dyke

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3.1 Introduction

The periodontium consists of the hard and soft tissues that surround and support the teeth, including the gingiva, alveolar bone, periodontal ligament, and cementum (cellular and acellular). Periodontal disease is initiated by the formation of dental plaque or biofilm on the surface of the tooth, subgingival or supragingival. In health, the biofilm bacteria and host maintain a symbiotic host-microbe relationship. However, when the complexity of the biofilm increases, resultant inflammation tips the symbiotic host-microbe relationship and the biofilm becomes dysbiotic. The dysbiotic microbiota tips the balance from homeostasis to disease by further amplifying inflammation. This results in over-activation of the host inflammatory response that does not resolve, and leads to host-mediated destruction of the periodontal tissues that result

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in serious functional and esthetic problems. Therefore, understanding the resolution of inflammation mediators and pathways is essential to the design of rational periodontal treatment paradigms and regeneration of periodontal tissues.

In the first stage of periodontal disease, gingivitis, the presence of bacteria causes an inflammatory response and the gums become swollen and red. At this stage of the disease, with plaque removal by daily brushing and flossing and regular professional cleanings, the disease is reversible, and the gums return to a healthy state. Gingivitis can become an established, but stable inflammatory lesion [1]. The trigger that takes a stable gingivitis lesion to a destructive, active periodontitis lesion remains elusive, but it is almost certainly related to the degree of inflammation [2]. In periodontitis, uncontrolled inflammation destroys the supporting structures of the teeth, including bone. If left untreated, bone loss progresses, teeth become loose and eventually fall out. Using current therapies, the disease is largely irreversible and can only be controlled with surgical debridement and at times in conjunction with antibiotics.

Recent studies suggest that in periodontitis, the uncontrolled inflammation and tissue destruction is due to a failure of resolution of inflammation pathways [3–7]. To prevent progression from acute inflammation to persistent, chronic inflammation, the inflammatory response must be tightly regulated to prevent additional tissue damage. More recent discoveries have revealed a new class of molecules that naturally provide this tight regulation. These are called specialized pro-resolving lipid mediators (SPMs) that promote the resolution of inflammation, clearance of infection, reduction of pain, and promote tissue regeneration via novel mechanisms [8].

3.2 Inflammatory Pathways

Inflammation is the body's natural defense mechanism in response to harmful stimuli. Inflammation is a complex biochemical pathway initiated when the innate immune system detects an infection, toxin, or tissue injury. This leads to the production of chemical mediators that physiologically induce the cardinal signs of inflammation. In acute inflammation, the host intercepts and destroys the insult, resolves the inflammation, and restores tissue homeostasis [8, 9]. When an acute inflammatory response does not resolve, the inflammation becomes chronic and can lead to pathogenesis of many chronic inflammatory diseases, such as periodontitis [2, 8, 10]. Therefore, an appropriate and timely host response is crucial to halt the progression of an acute inflammatory response to a chronic inflammatory disease, and induce the return of tissue homeostasis.

Arachidonic acid is a polyunsaturated fatty acid ubiquitous in the plasma membrane bound to the second carbon of the glycerol backbone. The arachidonic acid pathway is a central regulator of the inflammatory response, and is initiated when phospholipase A_2 (PLA_2) cleaves arachidonate creating free arachidonic acid. There is also a secondary, two-step pathway for arachidonate production involving phospholipase C and diglycerol lipase. Arachidonic acid is then metabolized by two major types of enzymes: (1) lipoxygenases, which lead to the synthesis of hydroxy-acids (hydroxy-tetraenoic acids, HETEs) that are further metabolized to leukotrienes and (2) cyclooxygenases (COX1 and COX2), which lead to the synthesis of prostaglandins, prostacyclins, and thromboxanes (Fig. 3.1). These lipid mediators of inflammation play an important role in the regulation of signal transduction implicated in pain and inflammatory responses [9].

Control of inflammation can be attempted with pharmacological agents such as non-steroidal anti-inflammatory drugs (NSAIDS) or corticosteroids that suppress, inhibit, or block pro-inflammatory signaling pathways [8]. All NSAIDs inhibit COX activity, whereas corticosteroids inhibit PLA_2 activity. However, they have many undesirable side effects and can cause immunosuppressive opportunities for infection [9]. In addition, the long-term use of corticosteroids can cause serious and irreversible problems [8]. Therefore, new therapeutic

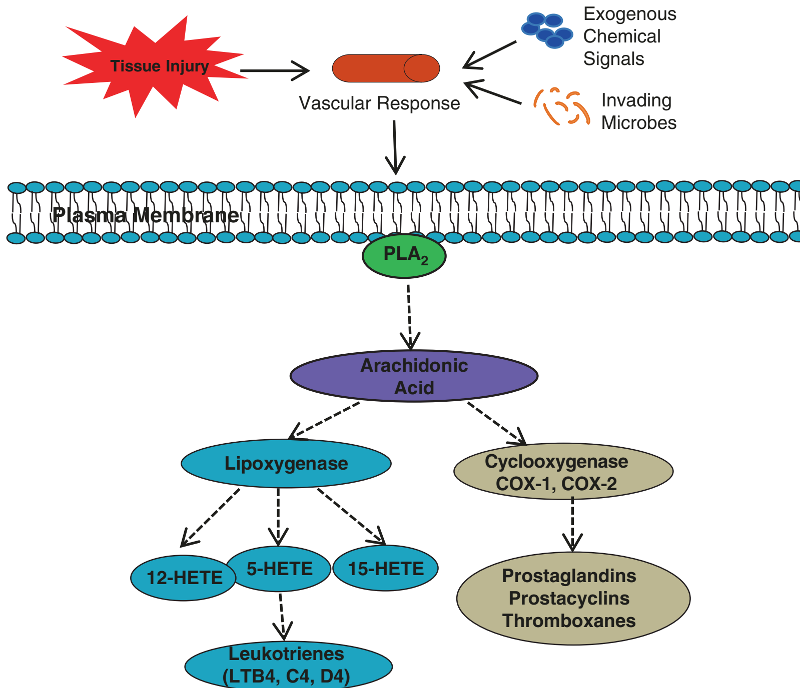

Fig. 3.1 Pro-inflammatory mediators are produced by the metabolism of arachidonic acid. In response to injury or infection, phospholipase A_2 (PLA_2) cleaves arachidonic acid (AA) from the two positions of membrane phospholipid diglycerides. AA acts as a substrate for COX activity to produce prostaglandins, thromboxanes, and prostacyclin, whereas 12-HETE, 5-HETE, and 15-HETE are generated by the lipoxygenase pathway, with 12-HETE and 15-HETE being end products, while 5-HETE is further metabolized to leukotrienes B_4, C_4, and D_4. When the concentration of cells containing lipoxygenases and their pro-inflammatory products is high in the exudate, a "class switch" occurs within neutrophils to express 15-LO, which along with excess 12- and 15-HETEs results in the double substitution of AA (5,12-HETE, 5, 15-HETE) leading to the synthesis of pro-resolution molecules that stimulate resolution of inflammation and promote restoration of tissue homeostasis

interventions need to be developed for the treatment of chronic inflammatory diseases.

3.3 Natural, Endogenous Resolution of Inflammation (Lipoxins)

The ideal outcome of the acute inflammatory response is the removal of the harmful stimuli followed by inflammation resolution and return of tissue homeostasis. Resolution of inflammation is an actively regulated biochemical process, not simply a passive decay of pro-inflammatory mediators, mediated by newly synthesized chemical compounds that stimulate the resolution response. These specialized pro-resolving lipid mediators (SPMs) resolve inflammation, clear infection, and stimulate tissue regeneration [9]. SPMs include the ω-6 polyunsaturated fatty acids (PUFA) (arachidonic acid)-derived lipoxins (LXs) and ω-3 PUFA (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA))-derived family of resolvins (Rvs), protectins, and maresins [8] (Fig. 3.2). LXs are synthesized from endogenous fatty acids (arachidonic acid), whereas Rvs are synthesized from dietary fatty acids (EPA and DHA), from sources such as fish oil, as they are not efficiently produced in the body [11, 12] (Fig. 3.2).

Lipoxins mediate the main events in the resolution of inflammation, including cessation of polymorphonuclear neutrophils (PMN) influx and macrophage clearance of debris [13]. The resolution of inflammation is initiated by an active class switch in the mediators prostaglan-

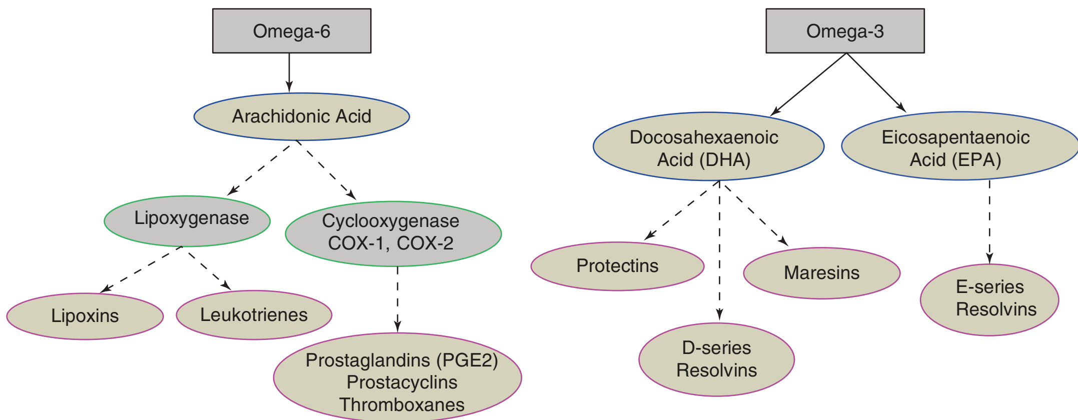

Fig. 3.2 Polyunsaturated fatty acid (PUFA)-derived lipid mediators. The pro-resolving mediators include omega-6 PUFA (arachidonic acid)-derived lipoxins, and omega-3 PUFA (EPA and DHA)-derived resolvins of the E- and D-series, respectively. Protectins and maresins are also DHA-derived SPMs. These mediators are involved in the initiation of the resolution response that results from "class switching" from pro-inflammatory dominated to pro-resolution dominated inflammatory exudates

dins and leukotrienes to the production of lipoxins [11]. Lipoxins stimulate endothelial nitric oxide and PGI_2 release, enhance macrophage phagocytosis of apoptotic neutrophils, phagocytic microbial clearance, and eventual efflux of macrophages to lymph nodes (efferocytosis) [13]. PGI_2 along with PGE_2 synthesized by leukocytes allows neutrophils to transmigrate across endothelial cells and move along a chemotactic gradient of leukotriene B_4 (LTB_4) [14, 15], a potent chemoattractant, at which time the lipid mediator class switching occurs and neutrophils congregate in purulent exudates [13, 16]. Failure of these resolution events leads to enhanced prostaglandin and leukotriene production, chronic inflammation, dysregulation of tissue healing and can result in fibrosis [17]. Lipoxins, when in sufficient quantity, reduce inflammation, stimulate re-epithelialization and wound healing, as well as tissue regeneration by counter-regulating the pro-inflammatory chemical mediators [13].

3.4 Resolvins

Resolvins (Rvs) are potent autacoids derived from omega-3 (n-3) PUFA that regulate the resolution phase of the acute inflammatory response [18]. These bioactive lipids induce the main functions of resolution of inflammation, including phagocytosis of apoptotic neutrophils by macrophages, halting further recruitment of PMNs, enhancing the clearance of all inflammatory cells and promotion of tissue regeneration [19–21]. Rvs stimulate anti-inflammatory and pro-resolving pathways similar to LXs, but with a different mechanism of action and through different receptors on inflammatory and stromal cells. Rvs consist of two major groups with distinct chemical structures. The resolvin E-series (RvE1, RvE2, and RvE3) are metabolized from EPA, and the resolvin D-series (RvD1, RvD2, RvD3, RvD4 RvD5, and RvD6, protectins and maresins) are transformed from DHA [22].

The interaction between Rvs and specific receptors modulates the fate of innate immune cells and counter-regulates active inflammation. Rvs act as agonists and signal through selective G-protein-coupled receptors (GPCR) [11, 23, 24]. RvE1 is the best characterized resolving in periodontal disease. There are two main receptors for RvE1 identified on inflammatory cells. The active receptor on macrophages is ERV1 (aka chemokine-like receptor 1 and chemR23) and on neutrophils and osteoclasts it is leukotriene B4 receptor type 1 (BLT1). Upon selective binding to its receptor, RvE1 attenuates nuclear factor-kappa B signaling and reduces the

production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα). The binding of RvE1 to ERV1 or BLT1 has been shown to have a positive influence on cell fate in inflammation. The Rvs of the D-series target a different set of receptors, including G-protein coupled receptor (GPCR) 32 (RvD2) and LXA_4 receptor/formyl peptide receptor 2 (FPR2) RvD1. Specifically, RvD1 interacts with both LXA_4 receptor and GPCR 32 on phagocytes, suggesting that each plays a role in resolving acute inflammation [11, 24].

3.5 Resolution of Inflammation as a Therapeutic Modality

Resolvins have emerged as a novel class of potent molecules that counteract excessive inflammatory responses and stimulate pro-resolving mechanisms [25]. The use of resolvins as a novel therapeutic approach to prevent and treat chronic inflammatory diseases, including periodontitis, has been tested in several animal models [20, 26–28], and is in the early phases of human clinical trials.

In a rabbit periodontitis model, the role of inflammation in periodontitis pathogenesis and the therapeutic potential of RvE1 was evaluated. Experiments were carried out to evaluate the impact of RvE1 in rabbit periodontitis initiated by the human pathogen *Porphyromonas gingivalis* (*P.g.*). The established rabbit model showed that the ligature of the lower second premolars with silk sutures and topical application of *P.g.* to the ligature three times per week induced severe periodontitis within 6 weeks. The treatment consisted of topical application of RvE1 to the ligature three times per week for 6 weeks at the same time as the *P.g.* The results showed that topical application of RvE1 prevented the initiation and progression of periodontitis and host-mediated destruction of alveolar bone, by inhibiting osteoclast-mediated bone resorption [27].

In a second study, the potential of resolvins and lipoxins for treating existing, established periodontitis was first established in the same rabbit model of ligature and *P.g.*-induced periodontitis. The only difference was that after the establishment of disease at 6 weeks, *P.g.* application was stopped, and the rabbits were treated with RvE1. Controls included placebo and comparison to the treatment with the structurally related lipids PGE_2 and leukotriene B_4. The PGE_2 and leukotriene B_4 each enhanced the development of periodontitis and worsened the severity of the disease. However, treatment with RvE1 promoted the resolution of inflammation, reduced the systemic inflammatory markers C-reactive protein and IL-1β, and resulted in the reversal of the disease with complete regeneration of tissues lost to disease [20].

In a study conducted by Lee et al. [26], the temporal dynamics of inflammation-induced dysbiosis of the periodontal microbiota and the impact of RvE1 were examined in a ligature-induced periodontitis rat model. Periodontitis was induced in rats using ligature placement alone without adding a human pathogen to induce disease in order to study naturally occurring dysbiosis of the rat microbiome associated with progressive periodontitis. Global differential gene expression in periodontal tissue was determined in health, periodontitis, and periodontitis treated with topical RvE1 to obtain an unbiased assessment of inflammatory changes. To determine the impact of inflammatory status on the composition of the microbiome, 16S rDNA sequencing was performed in parallel. As shown in previous studies conducted in rabbits and mice, prophylactic treatment with topical RvE1 prevented the disease-associated changes in the gene expression profile of periodontal tissues and significantly prevented alveolar bone loss. In addition, the topical application of RvE1 successfully treated established ligature-induced periodontitis in the rat with significant regeneration of lost periodontal soft tissues and bone. At the same time, shifts in the local microbiota induced by inflammation were markedly reversed by the control of inflammation with RvE1, and treatment of established periodontitis with RvE1 significantly shifted the rat subgingival microbiota associated with disease progression back toward homeostasis [26].

Localized aggressive periodontitis (LAP) is an early onset, rapidly progressing form of inflammatory periodontal disease. In a clinical study conducted on patients with LAP, there was an increase in surface P-selectin on circulating platelets, and elevation in β2 integrin expression on neutrophils and monocytes of LAP patients compared to healthy, asymptomatic controls, indicating chronic upregulation of the inflammatory response in these patients [5]. There was also a significant increase in platelet-neutrophil and platelet-monocyte aggregates in circulating whole blood of LAP patients compared to asymptomatic controls. In addition, whole blood from LAP patients generated an increase in pro-inflammatory LTB4 and a significantly less 15-HETE, 12-HETE, 14-HDHA (precursors of SPMs), and lipoxin A_4. Macrophages from LAP subjects exhibited reduced phagocytosis. In in vitro studies with LAP macrophages, RvE1 (0.1–100 nM) rescued the impaired phagocytic activity in LAP macrophages [5].

In a mouse model of ligature and *P. gingivalis (P.g.)*-induced periodontitis, the impact of RvD2 in modulating the immune response was investigated. Mice were infected with *P.g.* and RvD2 was administered intraperitoneally during and after *P.g.* infection. Treatment with RvD2 prevented ligature and *P.g.*-induced alveolar bone loss by upregulation of osteoprotegerin (OPG) levels and downregulation of receptor-activator of nuclear factor κB ligand (RANKL) expression, suggesting that RvD2 prevented osteoblast-mediated and T-cell-mediated signaling of osteoclast formation by RANKL leading to alveolar bone loss [29].

Most recently, the use of RvD2 as an intracanal medicament in the treatment of apical periodontitis was tested in rats in vivo. In this study, Siddiqui et al. [30] induced periapical lesions by exposing the pulp of rat mandibular first molars. The exposed pulps were left open to the oral environment for 3 weeks to ensure bacterial contamination. The mandibular first molars were then cleaned with 70% ethanol, followed by pulpal debridement and irrigation with 2.5% sodium hypochlorite. The canals were then dried with paper points and filled with 20 μL RvD2. The control group received the same treatment without the RvD2. The results demonstrated that RvD2 treatment of infected root canals in rat molars reduced the number of bacteria and inflammatory cell infiltrate in the pulp. In addition, the swelling decreased and periapical lesions were reduced. The pulp began to regenerate and fostering the growth of pulp-like tissue and the apexification of periapical bone lesions. In the control group, pus formed at the root of the tooth, bacteria flourished, infection persisted, and no pulp regeneration occurred [30].

3.6 Inflammation, Periodontitis, and Systemic Inflammatory Diseases

Atherosclerosis is a cardiovascular disease (CVD) that can lead to ischemic complications, including myocardial infarction and stroke, which have the highest rate of morbidity and mortality in the United States [31]. In atherosclerosis, inflammation plays a fundamental role in mediating all stages of the disease, from initiation through progression and ultimately, the thrombotic complications [32]. Patients with periodontitis share many risk factors with patients who have systemic inflammatory diseases, such as atherosclerosis [33]; they are older, predominantly male, and exhibit similar stress and smoking behaviors [33, 34]. In one study, the New Zealand White rabbit model was utilized as a model for periodontitis with bacterial challenge and cardiovascular disease was induced with a high cholesterol diet with similar characteristics to humans to assess the impact of periodontal disease on atherosclerotic lesion development. It was found that periodontal inflammation promoted atherosclerotic plaque inflammation and destabilization [35]. The direct impact of the treatment of periodontitis with RvE1 was demonstrated. The preventive use of oral-topical RvE1 protected hypercholesterolemic rabbits against aortic atherosclerotic plaque formation whether there was periodontal disease or not. Local, oral-topical RvE1 treatment significantly prevented

extensive atheromatous plaque formation induced by periodontal disease [35].

The role of Rvs has also been assessed in other chronic inflammatory diseases, and has been associated with resolution of acute kidney injury [36], acute lung injury, the micro- and macrovascular response to injury [28, 37, 38], and inhibition of microglia-activated inflammation in neurodegenerative disorders [38]. In a rabbit model of arterial injury induced with a balloon catheter, local delivery of RvD2 was employed to examine vascular injury in vivo. Following balloon angioplasty, rabbit femoral arteries were immediately exposed to and incubated with RvD2 (10 nM vs. vehicle) for 20-min. Results showed that the local delivery of RvD2 reduced vascular smooth muscle cell responses to vascular injury, and subsequent neointimal hyperplasia [37]. Short-term effects on the local tissue response were also evaluated 3 days post-injury and showed that cell proliferation was decreased in RvD2-treated vessels compared to control, and leukocyte infiltration after injury was inhibited. There was also a significant reduction in inflammatory gene expression of TNF-α, MCP-1, and IL-1α in the RvD2-treated arteries vs. vehicle-treated controls [37].

In type 2 diabetes mellitus, another chronic inflammatory disease, obesity, and metabolic syndrome-related chronic inflammation lead to micro- and macro-vascular damage, disruption of lipid metabolism, and abnormalities of neutrophil-mediated events [37]. Periodontal disease and diabetes have a reciprocal relationship. Diabetes is a significant risk factor for the development and severity of periodontal disease, and periodontal infections have a significant impact on diabetic control [39, 40]. In periodontitis and type 2 diabetes, unresolved inflammation is a critical underlying factor that is sustained by a deficiency of lipoxins and resolvins [5, 41–44]. In a type 2 diabetes transgenic mouse model overexpressing the human RvE1 receptor, ERV1, the impact of RvE1 on the phagocytosis of *Porphyromonas gingivalis* was investigated [28]. ERV1 transgenic mice are resistant to periodontitis and exhibit a reduced inflammatory phenotype and respond to RvE1 better than wild type animals. Diabetic mice (*db/db* leptin receptor-deficient strain) are significantly more susceptible to periodontitis than wild type. In addition, ERV1 transgenic mice with diabetes are protected from periodontitis and topical exogenous RvE1 enhances the response. Another interesting finding at first glance seems contradictory. RvE1 decreased neutrophil counts, but increased phagocytosis and clearance of *Porphyromonas gingivalis*. These experiments emphasize the principle that more inflammation is not always better and that controlled and tightly regulated inflammation is necessary for optimal outcomes of the acute inflammatory response. Excess inflammation can be harmful and actually inhibit bacterial clearance and enhance tissue damage [28].

3.7 Periodontal Regeneration

The actions of lipoxins and resolvins are not limited to inflammatory cells. Human mesenchymal stem cells (MSCs) modulate immune-inflammatory responses and their modulation can provide a unique therapeutic approach to treat chronic inflammatory diseases, including periodontal disease. SPMs have been reported to be produced by stem cells and their production is altered in chronic inflammatory diseases [45–48]. It has been proposed that the beneficial actions of SPMs are attributed to their ability to directly enhance stem cell proliferation and differentiation and indirectly modulate the actions of local factors such as cytokines, hormones, growth factors, free radicals, carbon monoxide, and interactions among them [49, 50].

In one study, mesenchymal stromal cells were assessed for their ability to produce specialized pro-resolving lipid mediators when exposed to carbon monoxide (CO) and in the presence of docosahexaenoic acid (DHA) or arachidonic acid (AA). The results showed that SPMs were greater in DHA exposed cells, compared with AA exposed cells, particularly RvDs. The study then took a step further to assess the role of the RvD1, RvD2, and aspirin-triggered [AT]-RvD3 in the interaction of MSCs with neutrophils for their

ability to promote bacterial phagocytosis by neutrophils. The results showed that MSCs exposed to either RvD1 or RvD2, and then incubated with neutrophils had robustly increased phagocytosis compared with neutrophils exposed to RvDs alone or MSCs alone. However, exposure of MSCs to AT-RvD3 did not promote neutrophil phagocytosis to a level significantly greater than neutrophils exposed to MSCs alone. In a mouse sepsis model, the administration of CO preconditioned MSCs to mice 6-h after the onset of cecal-ligation and puncture (CLP)-induced sepsis led to significantly improved survival when the cells were exposed to DHA (92% survival), compared with cells exposed to AA (58% survival). Taken together, these data suggest that the production of SPMs contributes to improved mesenchymal stromal cell efficacy when exposed to carbon monoxide, resulting in the resolution of inflammation and improved therapeutic response during sepsis [50].

3.8 Dietary Supplementation of Omega-3 Polyunsaturated Fatty Acids in Periodontal Disease

Many studies over the past few years have focused on the benefits of dietary supplementation of omega-3 PUFAs, such as fish oil, in patients with chronic inflammatory diseases [51–59]. In periodontal disease, the combination of scaling and root planning (SRP) with dietary supplementation of omega-3 fatty acids significantly reduced the gingival index, probing depth, and clinical attachment gain compared to the control (SRP with placebo) [55]. In a separate study conducted by El-Sharkawy et al. [54], the host modulatory adjunctive therapy of omega-3 PUFAs combined with low dose aspirin in the treatment of periodontal disease was tested in a double-blinded clinical study. The experimental group consisted of 40 healthy individuals with chronic periodontitis who received dietary supplementation of fish oil (900 mg of EPA + DHA) and 81 mg of aspirin in combination with SRP, whereas the control group, which also consisted of 40 healthy individuals, were treated with SRP and placebo. There was a significant reduction in mean probing depths and increased attachment gain after 3 and 6 months in the experimental group compared to the control suggesting that omega-3 PUFAs combined with low dose aspirin may provide a sustainable, low-cost intervention to augment periodontal therapy.

Naqvi et al. [56] conducted a double-blind randomized controlled trial to evaluate the influence of the adjunctive therapy of DHA + aspirin on specific bacterial burden. The effect of daily 2 g DHA supplementation and low-dose aspirin was compared to the daily consumption of placebo in patients with moderate periodontitis. The results indicated that there were broad improvements in clinical parameters including significant reduction in mean pocket depth and gingival index. More importantly, the authors concluded that the adjunctive therapy of DHA + aspirin improved periodontitis by modulating the host inflammatory response.

3.9 Conclusion

Periodontal disease represents a highly complex interaction between host and microbial dental plaque. Although the etiology is bacterial, recent discoveries have clarified that the pathogenesis is due to a failure of endogenous resolution of inflammation pathways and an excessive inflammatory response. Rvs have proven to have potent pro-resolving actions in different disease models. The potential for local delivery of resolvin analogs offers a new approach for therapy in periodontal disease. Human clinical trials involving omega-3 PUFA dietary supplementation combined with low dose aspirin as an adjunct treatment for periodontal disease are promising, and suggest the possible use of omega-3 PUFA derivatives, such as Rvs or LXs, in conjunction with periodontal therapy for the treatment of periodontal disease.

Increasing evidence from in vitro and in vivo animal studies and results from early phases of human clinical trials suggest that Rvs and LXs hold great potential to resolve excessive inflammation and restore periodontal tissue

homeostasis in periodontal disease. Although promising results have been obtained in animal models, additional preclinical studies are necessary to unravel the signaling pathways and molecular mechanisms underlying the resolution of periodontal inflammation, and elucidate the role of stem cell modification by SPMs. Further clinical trials are warranted to test the safety and efficacy of SPMs for drug development.

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4 Salicylic Acid Polymers in Periodontal Tissue Healing

Dana Graves, Yue Cao, Paulo Coelho, Lukasz Witek, and Kathryn Uhrich

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As a bioactive small molecule, salicylic acid (SA) is known for its various therapeutic effects, including antipyretic, anti-inflammatory, and antimicrobial capabilities [1, 2]. For bone regeneration applications, the concentration of SA is crucial. As a non-steroidal anti-inflammatory drug (NSAID), SA inhibits cyclooxygenase (COX) activity, reduce inflammation, and impair bone regeneration [3–5]. Compared to other NSAIDs, SA only inhibits COX activity at relatively high concentrations (>5 μg/mL in cell-based assays) [6, 7]. To address the short half-life [8, 9] in vivo and achieve controlled sustained release, SA was incorporated into a polymer backbone with linker molecules to yield the biocompatible and biodegradable salicylic

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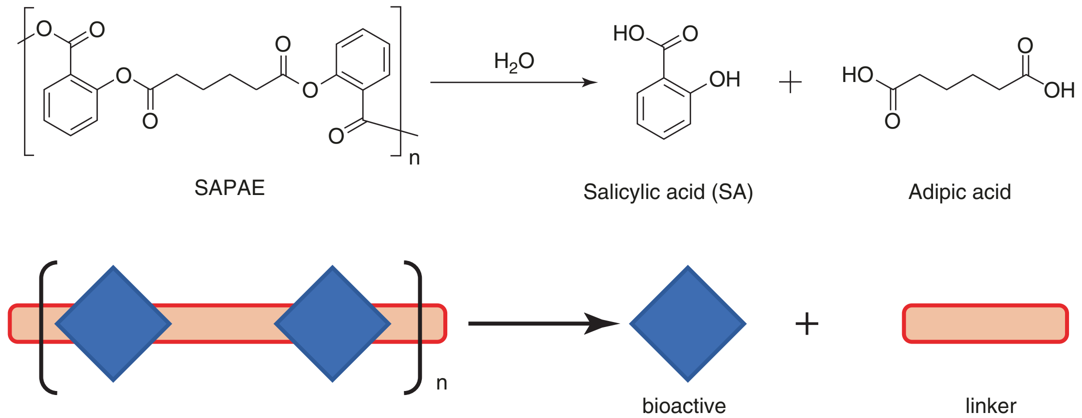

Fig. 4.1 Hydrolytic degradation of salicylic acid-based poly(anhydride-esters) (SAPAE)

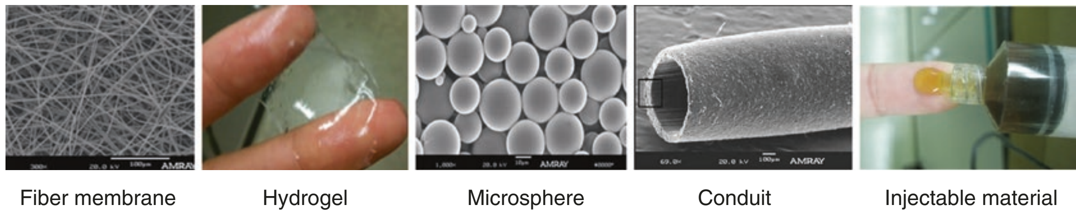

Fig. 4.2 Versatile formulations of salicylic acid-based poly(anhydride-esters) (SAPAE)

acid-based poly(anhydride-esters) (SAPAE) [10] (Fig. 4.1).

SAPAE production is well suited to industrial-level scale-up and has low costs [11]. For controlled release applications, the SAPAE exhibits significant advantages including high SA load, surface erosion, and synthetic modifications. High loading of SA can be achieved in SAPAE which ranges from 60 to 80 weight percent SA. SAPAE undergoes surface erosion, resulting in a near zero-order SA release. The release time could be tuned from days to months by varying the linker groups [10]. The thermal and mechanical properties can be modified by choosing different linker groups for specific applications [12, 13].

4.1 Formulations: Fibers, Gels, and Microparticles

Bioactive SAPAE polymers exhibited controlled release properties in various formulations including fibers [13–15], hydrogels [16–19], microspheres [20–23], coatings [24], and injectable materials (Fig. 4.2).

Biodegradable polymer membranes have been recognized as important candidates for physical barriers in guided bone regeneration (GBR) procedures, which create a secluded space for bone growth and prevents connective tissue from growing into the space [25]. As an ideal material for physical barrier, SAPAE membranes have many advantages including biocompatibility and biodegradability, good processability and manageability, adequate mechanical and physical properties. Moreover, SAPAE membranes demonstrate the localized release of nonsteroidal anti-inflammatory drugs (NSAIDs) SA, which can delay or prevent bone formation by inhibiting cyclooxygenase-2 (COX-2) [26]. As a result, the SAPAE membranes significantly reduce ectopic bone formation outside the defect [15]. The porous flexible SAPAE membranes can be formulated by electrospun SAPAE with polycaprolactone (PCL) together with SAPAE as the SA

delivery system and PCL as a mechanical modifier [15].

Hydrogels are three-dimensional (3D) crosslinked networks of polymers, capable of imbibing large quantities of water or biological fluids [27]. Such polymeric networks are formed by chemical crosslinks (covalent bonding) or physical crosslinks (intermolecular interactions and entanglements) [28–31]. To develop SA-release hydrogels, the SAPAE polymer was blended with another water-soluble polymer then swollen in water. Poly(*N*-vinyl-2-pyrrolidone (PVP), poly(acrylic acid) (PAA), and poly (*N*-isopropylacrylamide-*co*-acrylic acid) (PNIPAM-*co*-AA)) have been used in hydrogel preparation with SAPAE [16–19]. The SAPAE enables high loading and sustained release of SA, whereas the other polymer provides the mechanical properties by forming a soft material with increased plasticity and hydrophilicity. Generally, the SA-release hydrogels with higher content of SAPAE and chemically crosslinked gels exhibit stiffer, higher viscoelastic behavior, which is useful for applications such as drug delivery implants or patches, as they are less susceptible to displacement or site removal by biological fluids [18].

Biodegradable polymer microspheres are widely used as delivery systems. Unlike fibers or matrix formulations, microspheres can be injected rather than surgically implanted [32]. Polymer accumulation in vivo is negligible because it is biodegradable, the release rate can be accurately controlled, and the degradation products are readily cleared. Formulation of SAPAE into microspheres is of particular interest because the degradation process is dominantly controlled by surface erosion [33]. Moreover, SA is released from the polymer as an NSAID, which can be combined with another bioactive molecule that is physically encapsulated. The strategy of "dual delivery" has attracted interest because this combined therapy through the simultaneous administration of two or more drugs may have greater therapeutic efficacy compared to each drug individually [34]. The SAPAE microspheres can be produced with protein-loaded microspheres [20, 21].

Bacterial contamination, especially in the form of biofilms, represents a major concern [35–38]. Bacterial colonization and biofilm formation on any substratum proceeds by a series of complex physical and biological processes [39, 40]. The antimicrobial substances that specifically act against biofilms could be realized by sustained release antimicrobials to reduce microbial contamination. Salicylates and other NSAIDs are known to prevent bacterial adhesion to medical devices [41], although the mechanism is not clear. Biofilm formation on SAPAE disks and SAPAE-coated surfaces were monitored to evaluate their antibacterial property. As shown in Fig. 4.3, *Pseudomonas aeruginosa* biofilm formation on SAPAE surface was hindered by the released SA, preventing cell accumulation by five orders of magnitude compared to an inactive control [42]. Similarly, SAPAE also hindered the formation of *Salmonella* biofilms effectively [43].

4.2 Localized SA Release Influences Inflammation with Bone

Inflammation has been shown to cause the formation of osteolytic lesions by stimulating osteoclast-mediated bone resorption and interfering with bone coupling. SA is a potent inhibitor of prostaglandins (PGE) [44], that are involved in bone resorption. The effect of localized release of SA from SAPAE on healthy bone and tissue was evaluated in vivo using a mouse model [45]. SAPAE polymers were compression-molded to produce membranes with thickness ranging from 0.1 to 0.3 mm. In the mouse, the palatal and alveolar bone were exposed after reflection. SAPAE membrane was placed on the bone, which was also adjacent to the maxillary first molar. Visual and histopathological observations show that in tissue adjacent to the SAPAE membrane, the density of inflammatory cells and swelling were decreased (Fig. 4.4). After 20 days, greater thickness of new palatal bone was observed with less bone resorption in the region near the

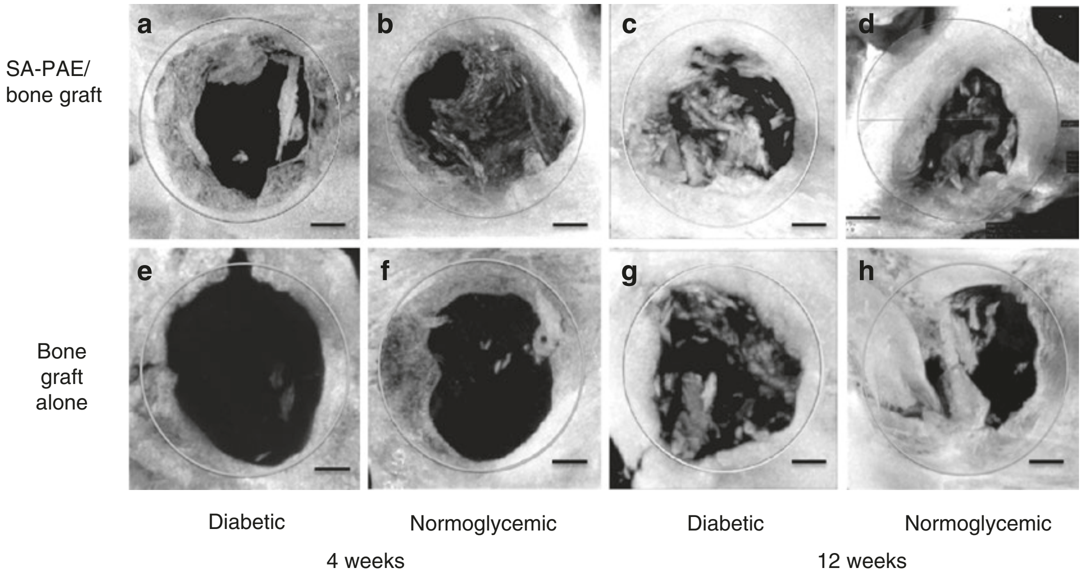

Fig. 4.3 SAPAE enhances bone formation in a critical size mandibular bone defect. Critical size defects were created in normoglycemic and streptozotocin-induced type 1 diabetic rats and osseous healing was visualized by microCT. Panels (**a**–**d**) represent animals treated with SAPAE/graft and (**e**–**h**) treated with graft alone. Reprinted from J Control Release 171: 33–37 (see [55])

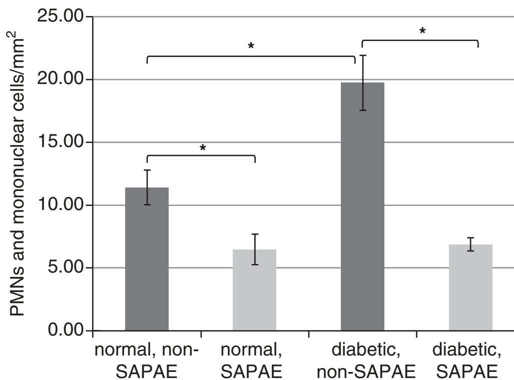

Fig. 4.4 SAPAE reduces inflammation in diabetic and normal healing osseous wounds. PMNs and mononuclear cells per mm^2 were measured in osseous defects at 4 weeks. Reprinted from Journal of biomedical materials research. Part A 104: 2595–2603 (see [56])

SAPAE membranes suggesting enhanced new bone formation.

4.3 Bone Regeneration with SAPAE

To evaluate the efficacy of SAPAE as GBR barrier materials, SAPAE-modified 3-D osteoconductive ceramic scaffolds were evaluated as a combined GBR system for concurrent control of inflammation, soft tissue ingrowth, and bone repair in a rabbit cranial defect model [46]. The SAPAE polymer in the system suppressed inflammation and displayed no deleterious effect on bone formation, indicating the concentration of SA released by the SAPAE is in the appropriate range to suppress the host inflammation response while still allowing bone formation. The SAPAE polymers in conjunction with the osteogenic scaffold can be a useful GBR device.

To further explore the potential utility of SAPAE polymers for GBR, porous PCL scaffolds containing BMP-2 and SAPAE were fabricated to treat 1-cm-diameter defects in rabbit parietal bones [47]. The SAPAE polymer affected the resorption of calcium sulfate within the scaffold and prolonged the release of BMP-2. At the same time, SAPAE did not impair bone formation in the defect. These observations indicate that SAPAE paired with BMP-2 can be optimized for use in GBR to help repair large bone defects.

It is also possible to develop a scaffold that incorporates a bioactive molecule into polymer backbone prior to scaffold formation. Copolymers

of the SA-adipic (SAA) moiety that releases SA upon hydrolysis, and the SA-α, α'-bis(*o*-carboxyphenoxy)-oxylene (OCOX) moiety that provides physical integrity were developed. The copolymers were formulated into microspheres and then sintered in a mold to yield an interconnected porous scaffold. The chemical incorporation of SA allows very high drug loading up to 45 wt% and displays sustained drug release (30–50 days) that is comparable to the duration of bone regeneration [48, 49]. In addition, the scaffolds have suitable modulus (10–30 MPa) and porosity (60%) for bone regeneration [50–52].

4.4 SAPAE for Treatment of Osseous Defects in Diabetic Conditions

Periodontal and craniofacial defects are often treated with bone grafting. Poorly controlled diabetics have highly variable outcomes [53, 54]. Proof-of-concept studies were performed with SAPAE in diabetic rats [55]. In these studies, SAPAE was ground into a fine powder and mixed with freeze-dried bone allograft and mineral oil where the control group received allograft/oil alone. Two groups of rats were examined. One had type 1 diabetes induced by streptozotocin injection and the normal group received citrate buffer alone. A rat mandibular bone defect model was used in which a critical size defect was created and examined at 4 and 12 weeks. An initial microcomputed tomography (microCT) analysis was performed at 4 weeks to measure initial bone formation. SAPAE-treated bone grafts had significantly more bone fill compared to bone graft alone in both diabetic and normal animals, demonstrating accelerated healing in both groups. At 12 weeks, when considerable bone remodeling has already occurred, SAPAE treatment in diabetic rats increased the amount of new bone by 43% compared to graft alone. No significant effect was observed with SAPAE in the normal animals at the remodeling stage. The results with microCT were confirmed by histologic analysis. Treatment of diabetic rats with SAPAE/bone graft induced 115% more bone fill compared to graft alone, whereas SAPAE in normoglycemic rats did not significantly increase bone fill.

Mechanistic studies were initiated to explore how SAPAE promoted healing [56]. The number of osteoblasts, osteoclasts, and inflammation in the area of healing bone was assessed. Since inflammation is elevated in diabetic wounds and may suppress bone formation [57–59], the impact of SAPAE on inflammation was assessed by measuring polymorphonuclear and mononuclear leukocyte infiltration at 4-weeks, when initial bone formation is clearly evident [58, 60]. Diabetic osseous wounds had significantly more polymorphonuclear neutrophils (PMNs) and mononuclear leukocytes. SAPAE reduced the inflammatory infiltrate in the diabetic animals as well as normoglycemics. Inflammation was also examined by measuring IL-1α, which was increased in diabetic osseous defects [56]. SAPAE treatment significantly rescued the high levels of IL-1α in the diabetic rats and returned them to normal levels. Thus, at both the cellular level (leukocyte counts) and the molecular level (IL-1α), SAPAE reduced inflammation particularly in diabetic rats such that the effect of SAPAE was more prominent in diabetic compared to normal rats.

One of the effects of inflammation on bone is to stimulate osteoclastogenesis. This effect is significant as the amount of bone formed reflects both the activity of osteoblasts and removal by osteoclasts. Diabetes significantly increased the number of osteoclasts and SAPAE treatment reduced osteoclasts in diabetic groups at both 4 and 12 weeks. This result is significant as osteoclastogenesis and osteoclast activities are exacerbated by diabetes-enhanced inflammation [61]. Thus, one of the effects of reduced inflammation due to SAPAE treatment was lower osteoclasts numbers and activity compared to their non-SAPAE counterparts. The ultimate effect of reduced bone resorption in inflammatory conditions is to preserve the amount of bone, especially since bone coupling is reduced in the face of an inflammatory microenvironment [60]. The latter is supported by the examination of osteoblast density (Fig. 4.5). Bone-lining

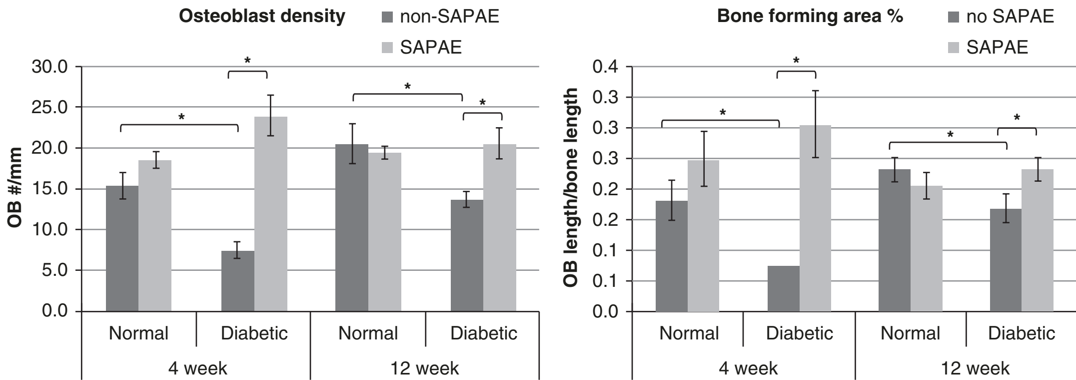

Fig. 4.5 SAPAE increases osteoblast numbers and promotes new bone formation in diabetic osseous defects. Left: Bone-lining osteoblast density. Right: Percent area of new bone compared to the total defect area. Reprinted from Journal of biomedical materials research. Part A 104: 2595–2603 (see [56])

osteoblast numbers were reduced by ~50% in the diabetic animals compared to normal controls. This finding is consistent with previous results demonstrating that diabetes causes an increase in apoptosis in mesenchymal stem cells that can differentiate into osteoblasts, induces a reduction in osteoblast proliferation, and causes an increase in osteoblast cells death mediated by caspase-3 in vivo, all of which can contribute to reduced osteoblast numbers [61, 62]. Treatment of diabetic animals with SAPAE blocked the negative effect of diabetes on osteoblast numbers so that they were returned to normal levels in the 4-week experiments. At 12 weeks, diabetic mice also had fewer osteoblasts. Similar to results at 4 weeks, the application of SAPAE with the grafting material preserved the osteoblast numbers so that there was no decrease in the diabetic animals.

Taken together, the above results with SAPAE indicate that slow release of SA suppresses prolonged inflammation found in diabetic osseous healing. It normalizes this deficit in diabetic bone healing by reducing inflammation, osteoclasts, and restoring osteoblast density to the normal levels (Fig. 4.5). By affecting all three parameters, we postulate that the effect of SAPAE remains prominent in diabetic animals at 12 weeks (Fig. 4.5).

In vitro studies were carried out to describe the SAPAE pharmacokinetics using a novel agar-based system to realistically mimic the release of SAPAE in vivo. The initial low release of SAPAE observed in the first few days may allow the early events of inflammation to proceed normally after injury to initiate the healing cascade [63]. At days 3–6, higher levels of localized SA were detected, which can initiate resolution of inflammation that has been shown to persist in diabetic animals and may hinder bone formation [64]. After 1 week in vitro, the level of SAPAE (~0.5 mg/mL) was higher than the IC50 (5–20 mg/mL) [65], which is expected to be therapeutically effective [19]. The bulk SA release profile was similar to a previous study [55], in which there was an initial lag period followed by sustained SA release. However, the agar-based system showed a much longer release duration (60% cumulative release at day 3 1) compared to the previous study (100% release at day 16) [55]. The effects of SAPAE treatment on inflammation are clearly evident at 28 days in vivo [55]. This study investigated the local pharmacokinetics of SAPAE using a novel agar-based system and evaluated the cellular and molecular mechanisms of localized and sustained SA release on diabetic bone regeneration. With the agar system, a continuous and slow localized release of SA was observed for more than a month; these data correlate with the prolonged local anti-inflammatory effect observed in vivo, as demonstrated by histological analysis. The prolonged inflammation mitigation down-regulated osteoclast density and activity, and increased osteoblastogenesis, particularly in diabetic animals [66, 67]. As a result, SAPAE

significantly enhanced bone formation, especially in diabetic animals [55].

4.5 Potential Treatment of Peri-implantitis (Peri-implantitis) with SAPAE

Implant therapy is a well-documented approach to maximize dental rehabilitation in response to tooth loss [68]. Peri-implant disease has similarities to periodontitis. Both are bacteria-initiated and the loss of bone requires a host-mediated complex inflammatory process, which can impact tooth and implant survival [69, 70]. Up to half of the patients with dental implants will develop peri-implantitis, which poses a significant oral health problem [71, 72]. Peri-implantitis is characterized with an excessive and prolonged inflammation that compromises bone [67]. Bacteria-induced inflammation around dental implants plays a dominant role into promoting osteoclast-mediated bone resorption and inhibiting bone formation leading to net bone loss around implants, similar to the loss of bone and bone uncoupling that occurs in periodontitis [73]. Peri-implant infection is an unresolved oral health condition that once established may lead to implant loss.

4.6 Diabetes and Peri-implantitis

Approximately 300 million individuals (~8.5% of the world's population) are currently diagnosed with type 2 diabetes mellitus (DM) and is projected to surpass 500 million by 2035 [74–76]. Although implant placement is often initially stable in diabetic individuals, several reports indicate that osseointegration is reduced (less bone formed around the implant) [77–80] and are often at greater risk for peri-implantitis [81–84], similar to the risk of more severe periodontal disease [85, 86]. Moreover, DM has been shown to affect bone formation in steady-state conditions and contribute to osteoporosis and increased fracture risk as well as reduced bone formation in response to osseous injury or following periodontal bone resorption [60, 75, 80, 87–89]. Hyperglycemic conditions are known to cause a shift to a more pathogenic microbiome [90–94] that potentially accelerate peri-implantitis-related bone loss [90, 95].

4.7 Potential Role of SAPAE in Peri-implantitis in Normal or Diabetic Situations

Current methods to arrest and treat peri-implantitis are largely unpredictable and often ineffective. Treatment strategies for peri-implantitis have focused on access flap procedures and implant surface decontamination, local and systemic administration of antimicrobial agents, as well as bone grafting procedures [96, 97]. There are two major issues: (1) How to stop the progression of peri-implantitis and (2) once peri-implantitis has progressed, how to effectively induce bone regeneration around the implant. Both are challenging in healthy patients since no effective therapy currently exists [72]. Furthermore, the local and systemic inflammatory conditions presented by diabetes are thought to present additional challenges to overcome. While surgical debridement and surface decontamination/detoxification have been widely utilized to treat peri-implantitis, the sustained local delivery of antibacterial agents that mitigate peri-implantitis' prolonged inflammation leading to bone healing and reattachment to the implant surface has been regarded as the ideal treatment.

Local and systemic conditions potentially converge to make treatment of peri-implantitis difficult [82, 96, 98, 99]. Sustained controlled delivery can be achieved with polymeric biodegradable devices such as polymers of SAPAEs. As described above, the chemical incorporation yields a high drug loading of ~70% that releases SA and biocompatible adipic acid in a sustained manner upon hydrolysis [100]. SA is an NSAID and suppresses activation of nuclear factor kappa-B (NF-κB) and inhibits the production of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 [101]. Reduced inflammation also improves

the survival of osteoblasts and reduces osteoclastogenesis [102–105]. Additionally, the SAPAE polymer is antibacterial [42] due to its ability to inhibit bacterial adhesion and other mechanisms such as reduced quorum sensing [42, 106, 107]. SA release begins after a lag time (e.g., ~3 days) that allows initial inflammation to occur and initiate healing events [56]. Given peri-implantitis microbial etiology [108] and related inflammation, SAPAE presents essential properties for treating patients with advanced peri-implantitis, and further work is warranted to optimize treatment regimens. Current studies in a large animal model are underway to determine whether the local application of SAPAE can inhibit the inflammation and progression of peri-implantitis. Another avenue that may be fruitful is the use of SAPAE to promote bone regeneration after peri-implantitis has caused the loss of bone surrounding an implant. Based on its properties both are potential treatment approaches.

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5 Targeting MAPK/MKP Signaling as a Therapeutic Axis in Periodontal Disease

Keith L. Kirkwood

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5.1 Overview of Osteoimmunology During Periodontal Disease Progression

Periodontal disease manifests as an intimate combination of inflammation and bone resorption, eventually leading to tooth loss. Alveolar bone is a highly dynamic tissue that constantly undergoes a remodeling process in which bone resorption and bone deposition are balanced in the presence of a commensal oral microbiome. When chronic inflammation in the overlying soft tissues reaches a certain threshold at a critical distance from the bone surface, bone resorption pathways are activated, resulting in a disruption of this balance to favor net bone loss [1].

The receptor activator of nuclear factor kappa-B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) system, which controls osteoclast development, differentiation, activation, and function, is a key mediator of bone loss in periodontal disease. RANKL is an essential cytokine in osteoclast formation and resorptive function contributing to bone loss. This mechanism is regulated by the RANKL decoy receptor osteoprotegerin (OPG), which blocks RANKL signaling through its cognate receptor, RANK. The imbalance of the RANKL/OPG ratio is thought to deregulate bone remodeling, driving bone loss when RANKL concentrations exceed OPG relative to normal physiology [2]. Through interactions with its associated receptor RANK on the cell

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surface on various osteoclast precursor populations, RANKL stimulates differentiation and maturation of cells from the monocyte/macrophage lineage to form functional osteoclasts resulting in ensuing bone resorption. Thus, the RANKL/OPG ratio eventually determines bone turnover [3–5].

RANKL expression requires different signaling pathways depending on the nature of extracellular stimulation, cell type, and even cell differentiation state. Indeed, RANKL expression has been shown to increase inflamed periodontal tissues from various cell types, including osteoblastic cells, bone marrow stromal cells, endothelial cells, mononuclear cells, and periodontal ligament fibroblasts [6–10]. Importantly, many secreted pro-inflammatory cytokines and other mediators have been shown to converge and stimulate RANKL in these cell types, including IL-1β, TNF-α, IL-6, IL-8, IL-11, IL-17, MMPs, and PGE_2, all of which are upregulated in periodontal tissues (reviewed in [11].)

Oral bacterial periodontal pathogens can directly or indirectly regulate osteoclast formation. Direct activation of osteoclast progenitors can occur in the presence of RANKL or indirectly through inducing RANKL expression by non-osteoclastic cells (osteoblasts, periodontal ligament fibroblasts, etc.), thereby stimulating RANK on defined osteoclast precursor populations to form mature osteoclasts. RANKL binding activates NFATc1, which is a key transcription factor required for osteoclastogenesis [12]. However, inflammatory chemokines and cytokines are also known to directly induce osteoclast formation independent of RANKL [13–15]. Thus, RANKL-RANK signaling and pro-inflammatory cytokines drive osteoclastogenesis in the context of periodontal disease and associated alveolar bone resorption (Fig. 5.1). This chapter will focus on crucial intracellular signaling pathways that lead toward osteoclast differentiation in response to periodontal environmental cues from a basic and translational science perspective that can be manipulated for therapeutic intervention to aid in the management of periodontal disease progression.

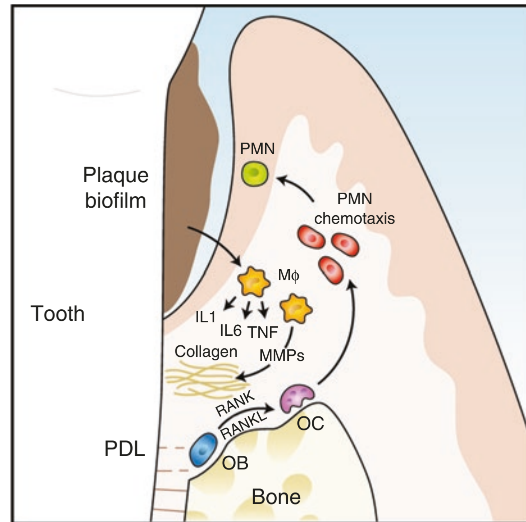

Fig. 5.1 Anatomy of periodontal tissues and cellular biology of inflammatory bone loss. Illustration depicts cell responses to bacterial plaque biofilm on the root surface within the periodontium. Polymorphonuclear lymphocytes (PMNs) and macrophages (Mϕ) secrete inflammatory cytokines and matrix metalloproteinases (MMPs) to increase the immune response and degrade connective tissue matrix. Osteoclastogenesis is induced to these stimuli to increase bone resorption through RANKL/RANK system. Adapted from [3]

5.2 General Aspects of the MAPK/MKP Signaling Axis

The innate immune system serves as the first of defense against periodontal pathogenic organisms and its interaction with microbial components activates multiple signaling cascades, including mitogen-activated protein (MAP) kinase pathways. Among the various signaling molecules that regulate inflammatory pathways, MAP kinases are critical since their activation significantly contributes to the regulation of inflammatory diseases, including periodontal diseases.

MAP kinases are highly conserved serine/threonine protein kinases in eukaryotes. The MAPKs are organized in modules (MAPKKK→MAPKK→MAPK) sequentially activated by a cascade of dual phosphorylation events at tyrosine/threonine residues. Beginning with the activation of upstream MAP kinase

kinase kinase (MKKK), MAP kinase kinase (MKK) is further activated by MKKK at two serine residues. MKK in turn activates MAP kinase by phosphorylating the MAPKs at the adjacent threonine and tyrosine residues localized within a conserved activation loop motif [16]. Three MAP kinases, p38, c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (ERK), are the best studied [17].

Individual extracellular stimuli preferentially activate distinct MAP kinases to activate different effector molecules. Many growth factor and G-protein linked receptors, cell adhesion, phorbol esters, and some oncogenes are linked to activation of the ERK MAP kinases, which are involved in cellular chemotaxis, cell cycle progression and mitogenesis, oncogenic transformation and metastasis, neuronal differentiation and survival, and in processes underlying memory and learning. Inflammatory cytokines (such as IL-1 and TNFα) and a number of cell stress-inducing factors (such as heat shock, osmotic shock, ultraviolet radiation, and oxygen radicals) preferentially lead to activation of JNK and p38 MAP kinases. In all, MAPK signal transduction cascades play a pivotal regulatory role in the biosynthesis of numerous cytokines, chemokines, and other inflammatory mediators that are necessary for the immune system to fight pathogenic infections.

Once activated, the MAPKs can target an array of downstream substrate proteins for phosphorylation, including downstream serine/threonine kinases, cytoskeletal elements, cell death regulators, and many nuclear receptors and transcription factors (including AP-1, NF-κB, or CAAT-enhancer-binding protein) which activate specific gene promoters (illustrated in Fig. 5.2). For example, NF-κB can bind to the promoter regions of many pro-inflammatory cytokine and chemokine genes and activate their transcription. In addition to the regulation of the expression of inflammatory mediators, MAPKs are also implicated in the regulation of reactive oxygen and nitrogen species, which are critical for killing microbes engulfed by phagocytes. MAPKs also regulate gene expression through promoting chromatin remodeling [17] and participate in the transport, stabilization, and translation of cytokine mRNA transcripts that contain specific AU-rich elements [18]. It is well established that p38 MAPK activates MAP kinase-activated protein kinase (MK)-2 through phosphorylation. MK2, in turn, inactivates trans-acting pro-decay RNA-binding protein, including tristetraprolin (TTP), by phosphorylation. Phosphorylated TTP is then sequestered via 14:3:3 protein as a chaperone to prevent the binding of TTP with ARE mRNA motif within the 3′-untranslated region (UTR) of the mRNA. Thus, ARE mRNAs are spared from TTP shuttling to degradation machinery, and TTP-mediated de-adenylation and destabilization of ARE-containing transcripts are inhibited, providing specific mRNA translation [19–21].

The MAP kinase phosphatase (MKP) family of proteins has the ability to negatively regulate MAP kinase activity by dephosphorylating MAPK proteins, and 11 such family members have been identified to date with the most widely studied, archetype member being MKP-1. MKPs have been classified according to their substrate specificity and their primary cellular location. The ERK-selective MKP proteins exist in the cytoplasm, including MKP-3, MKP-X, and MKP-4, the inducible MKP proteins are nuclear, including MKP-1 and MKP-2, whereas the JNK/p38-selective MKPs are both nuclear and cytoplasmic and include MKP-5 and MKP-7 [22]. Many processes regulated by MAPKs have been shown to be regulated by MKPs. MKP-1 is a phosphatase that negatively regulates the innate immune response through dephosphorylation of p38, ERK, and JNK in response to various stimuli [23–26].

5.3 MAPK/MKP Signaling in Osteoclastogenesis

In the oral microbial environment, bacterial constituents including gram-negative-derived lipopolysaccharide (LPS) can initiate inflammatory bone loss as seen in periodontal diseases. Bacterial LPS can stimulate the expression of IL-1β, TNFα, IL-6, and RANKL by activating

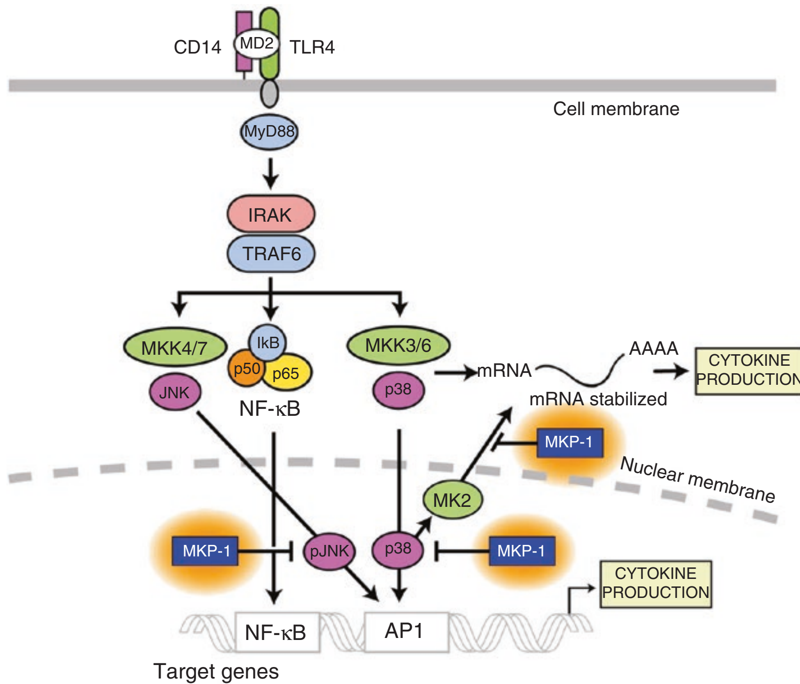

Fig. 5.2 MAPK/MKP innate immune signaling. TLR-2 and TLR-4 are depicted as examples of TLR receptors expressed in the cells of periodontal tissues. Upon ligand binding, these TLRs recruit adaptor protein MyD88 and activate common upstream activator (IRAK/TRAF6 and TAK1) of NF-kB and MAP kinases. TLR-4 may also activate NF-kB independent of MyD88 and with delayed kinetics (red dotted line). Activated NF-kB and MAP kinases translocate to the nucleus and bind to their motifs (NF-kB, AP-1, respectively) in the promoter of target genes (including early-response and inflammatory genes) and induce their transcription into mRNA which will ultimately lead to increased cytokine production. p38 MAP kinase is also involved post-transcriptional regulation of pro-inflammatory genes (e.g., IL-6, Cox-2) by modulation of mRNA stability in the cytoplasm. MAP kinase phosphatase-1 (MKP-1) negatively regulates MAPK activation via dephosphorylation of target kinases at multiple points of kinase activation. (Legend: *TLR* toll-like receptor, *CD14* cluster of differentiation 14 molecule, *MD2* myeloid differentiation protein 2, *MyD88* myeloid differentiation primary response gene 88, *IRAK* interleukin-1 receptor-associated kinase, *TRAF6* TNF receptor-associated factor 6, *TAK1* TGF-beta-activated kinase 1, *MKK* mitogen-activated protein kinase kinase, *ERK* extracellular signal-regulated kinase, *JNK* c-Jun N-terminal kinase, *AP-1* activator protein-1)

the innate immune responses, as well as non-immune cells such as osteoblasts [5, 27, 28]. The production of inflammatory cytokines results from the activation of kinase-induced signaling cascades and transcriptional factors. LPS initiates this cascade by binding CD14 as well as toll-like receptors (TLRs), mainly TLR-2 and TLR-4 [29–31]. Regardless of which TLR is engaged, LPS increases expression of RANKL, IL-1, PGE_2 and TNFα, each known to induce osteoclast activity, viability, and differentiation [32, 33]. In addition, activated monocytes, macrophages, and fibroblasts all produce cytokines, such as TNFα, IL-1β, and IL-6, within periodontal lesions [34, 35] and have all been found to be significantly elevated in diseased periodontal sites compared to healthy or inactive sites [36–40]. These cytokines orchestrate the cascade of destructive events that occur in the periodontal tissues, and trigger the production of an array of inflammatory enzymes and mediators including matrix metalloproteinases (MMPs) and prostaglandins. Moreover, pro-inflammatory cytokines directly or indirectly recruit and activate osteoclasts through RANKL-dependent and independent pathways, resulting in irreversible bone destruction [41, 42]. This information indicated several years ago that understanding inflammatory

cytokine expression mechanisms in macrophages may be important in the management of periodontal diseases. Indeed, blocking TNFα has been proven to effectively inhibit osteoclast formation [43] and blockade of TNF has been used as a probe to understand the molecular basis of osteoclastogenesis and also as a target for therapeutic agent development in rheumatoid arthritis and periodontal diseases [44, 45].

RANKL, and its ability to regulate osteoclastogenesis, is well established [46, 47], which is highlighted by the finding that the deletion of RANKL or its receptor RANK results in an osteoclast-deficient state of osteopetrosis [48]. RANKL signaling in osteoclastogenesis activates six key signaling pathways in osteoclasts: nuclear factor of activated T cells (NFAT) c1, nuclear factor kappa B (NF-kB), Akt/protein kinase B (PKB), JNK, ERK, and p38 [49]. NFAT proteins, which are expressed in most immune-system cells, play pivotal roles in the transcription of cytokine genes and other genes critical for the immune response. The transcriptional activity of NFAT is regulated by its intracellular localization. NFAT is heavily phosphorylated in its regulatory domain. Phosphorylation of NFAT results in masking of the nuclear localization signals, with consequent cytoplasmic sequestration of the transcription factor. Several protein kinases including p38 [50], JNK [50], and GSK3β priming kinase protein kinase A [51] can rephosphorylate NFAT, causing the transcription factor to exit the nucleus and relocate to the cytoplasm. Thus, phosphorylation of NFAT through the MAPK/MKP axis is critical for the master osteoclast transcription factor, NFATc1, to translocate into the nucleus of osteoclast progenitor populations to activate the osteoclastogenic machinery.

It is clear that MAPK and NF-κB signaling are key signaling pathways engaged following *A. actinomycetemcomitans* exposure [52–54]. In murine-derived macrophages, the cytoplasmic TLR adaptor protein, MyD88, was essential for *A. actinomycetemcomitans*-induced phosphorylation of IkB-α, which promotes NF-kB activation, and all three MAPKs: p38, JNK, and ERK [55]. *A. actinomycetemcomitans* LPS also led to phosphorylation of NF-κB subunits (p65 and p105) JNK, p38, and ERK MAPKs in rat macrophages [52, 54]. LPS and TNF-α activated p38/JNK MAPKs and directly induced osteoclast formation from the mouse macrophage/monocyte cells [15]. In the *A. actinomycetemcomitans* LPS rat model of experimental periodontitis, an increase in osteoclast formation was observed along with the increased expression of pro-inflammatory cytokines IL-1β, TNFα, and IL-6 [56]. Furthermore, in a rat calvarial model, *A. actinomycetemcomitans* induced osteoclast and resorption pit formation [53]. These studies illustrate that cytokines, including RANKL, are expanded by *A. actinomycetemcomitans* and result in oral bone loss subsequent to osteoclast formation and require specific intracellular signaling pathways to accomplish these goals.

The relevance of p38 MAPK in the regulation of expression of IL-6, MMP-13, and RANKL in periodontal ligament fibroblasts and osteoblasts has been throughly investigated [6, 57–59]. Here, using classic small molecule inhibitors and genetic constructs, p38 MAPK was shown to be a key signaling pathway maximal expression of these inflammatory and osteoclastogenic mediators in vitro. More mechanistic studies followed showing different mechanisms that p38 MAPK signaling used to control target gene expression. For example, IL-6 was regulated at the level of mRNA stability via p38, whereas MMP-13 was largely controlled at the gene promoter level [57, 60]. Interestingly, p38 regulated RANKL expression in bone marrow stromal cell populations through far distal gene promoter elements located over 120 kb upstream of the transcriptional start site [6]. These studies highlight the fact that although p38 MAPK signaling is involved in key pro-osteoclastogenesis factors, the mechanisms vary dramatically.

In addition to p38 MAPK, the involvement of MAPK cascade proteins, MAPK-activated protein kinase 2 (MK2), and MAPK-phosphatase (MKP)-1 in osteoclastogenesis has been addressed. MK2 is a phosphorylation substrate of p38α/β MAPK that is activated by *A. actinomycetemcomitans* LPS in macrophages [52, 53]. Like p38 MAPK, MK2 has also been shown to play a key

role in osteoclastogenesis. MK2 deficiency regulates osteoclast function in vitro and in mice [61]. MK2 signaling positively regulated osteoclast-specific gene expression of *Acp5*, *Rank*, *Oscar*, and *Mmp9* in osteoclast differentiation assays and bone resorption on cortical bone slices. Mechanistically, MK2 deficiency attenuated binding of NFATc1 to the promoter regions of *Calcr*, which encodes calcitonin receptor, and *Acp5*. Our group demonstrated MK2 signaling-regulated pathogenic and physiological bone turnover [62]. Using a murine calvarial model, *A. actinomycetemcomitans* was used to study the influences on bone remodeling, since both calvarial bone and alveolar bone are formed by intramembranous ossification. Osteoclast formation indeed increased from days 3 to 5 during *A. actinomycetemcomitans* exposure in $Mk2^{+/+}$, but not in $Mk2^{-/-}$ mice. Osteoclast levels correlated with resorption pits observed from microcomputed tomography (μCT). In the absence of MK2, bone loss was significantly reduced further confirming the positive regulatory role in bone resorption. This study also demonstrated that MK2 signaling differentially inhibits osteoclast differentiation by sex.

Defined osteoclast progenitors (dOCP), described by the immunophenotype $CD45R^{neg}GR\text{-}1^{neg/lo}CD11b^{lo}$, appear to have the most osteoclastogenic potential [63]. MK2 deficiency downregulated $dOCP^{lo}$ osteoclastogenesis, but not other dOCP populations in osteoclastogenesis assays. In addition, sex seemed to be different in MK2-deficient mice. Osteoclasts derived from male and female $dOCP^{lo}$ cells formed the most osteoclasts within 3 days when compared to $Mk2^{+/+}$ $dOCP^{-}$ and $dOCP^{hi}$, consistent with the report that $dOCP^{lo/-}$ are the most osteoclastogenic [64]. We also demonstrated that MK2 signaling regulates mRNA expression of both osteoclast fusion genes, *Oc-stamp* and *Tm7sf4* during osteoclastogenesis and MK2 deficiency also led to a reduction in *Ctsk*, a transcript critical for osteoclast function. Thus, MK2 signaling is critical for osteoclastogenesis from the male $dOCP^{lo}$ cells and regulates osteoclast fusion genes *Oc-stamp* and *Tm7sf4*.

On the opposing side of p38/MK2 signaling, MKP-1 signaling in osteoclastogenesis has been investigated. As described above, MKP-1 is primarily responsible for dephosphorylation of phospho(p)-p38 and p-JNK, thus contributing toward downregulation of cytokine production including IL-6, TNF-α, IL-10, and CXCL1 in macrophages. Using the dOCP populations, initial studies addressed the role of MKP-1 in *Dusp1*-deficient mice. As expected, LPS and bacterial-induced systems indicated that in the absence of MKP-1, when more p38/JNK signaling is prolonged, there is more bone loss and associated osteoclastogenesis [53, 65]. Importantly, MKP-1 signaling is actually required for RANKL-induced osteoclastogenesis while MKP-1 signaling is a negative regulator of LPS-induced osteoclastogenesis [14, 66]. In defined $Dusp1^{-/-}$ dOCP populations, there were fewer and smaller osteoclasts formed in response to RANKL through a mechanism whereby NFATc1 nuclear translocation is impaired [66]. Additionally, chemical inhibition of either p38 or JNK reversed NFATc1 nuclear translocation in $Dusp1^{-/-}$ dOCP populations. Previous work established that *Dusp1*-deficient mice have a smaller skeletal phenotype compared to matched WT littermates [67]. Interestingly, our group has also shown that the deletion of *Dusp1* resulted in less osteoclasts as a result of reduced Vitamin D-induced RANKL expression in bone marrow stromal populations due to impaired RXR/VDR heterodimer nuclear translocation [68]. Thus, there appears to be a mechanistic basis for therapeutics that would induce MKP-1 expression/activity since this pathway is required for osteoclastogenesis induced by physiological cues, such as RANKL and Vitamin D, but restrains bacterial or inflammatory-induced osteoclastogenesis, as observed in periodontal diseases.

5.4 Therapeutic Targeting of p38/MK2 Signaling in Periodontal Disease

Several therapeutic approaches to block the progression of inflammatory bone loss observed in periodontitis include host modulation of MMPs, COX2, and arachidonic acid metabolites.

However, these therapies target singular mechanisms of alveolar bone destruction. Cytokines are well known to compensate for one another, thereby limiting the effect of cytokine-specific inhibitors. Alternatively, targeting a common regulatory mechanism for multiple cytokines may repress periodontal disease progression and improve treatment response. Also, inflammatory cell signaling pathways that generate inflammatory and tissue destruction proteins have become promising therapeutic targets. Therapeutic modulation of signaling pathways can affect various genes, depending not only on the pathway but also on the relative position targeted for inhibition in the signaling cascade. Because the p38MAPK/MK2 pathways phosphorylate downstream targets, primarily RNA binding proteins that regulates pro-inflammatory cytokines, including IL-6, TNFα, GM-CSF, IL-8, and iNOS, through mRNA stability, their pathway components could be excellent targets for therapeutic designs.

The translational significance of p38 MAPK signaling in periodontal disease progression was observed when an orally active specific p38 inhibitor reduced periopathogenic LPS-induced bone destruction in a rat model [69]. To study the preventive function of p38 inhibitors in periopathogenic LPS-induced experimental alveolar bone loss using a rat model, two simultaneous doses of SD-282 (15 or 45 mg/kg), a small molecule inhibitor of p38α, were administered twice daily by oral gavage for 8 weeks. Bone area and volumetric analysis by μCT indicated significant bone volume loss with LPS treatment, but these endpoints were partially inhibited with both doses of the p38 inhibitor (see Fig. 5.3). Histological examination indicated significantly fewer osteoclasts adjacent to the areas of active bone resorption, including the periodontal ligament area, and a significant decrease in IL-6, IL-1β, and TNF-α expression in p38 inhibitor-treated groups compared with LPS groups by immunostaining. This proof-of-principle study supports the role of an orally active p38α MAPK inhibitor (SD-282) to potentially benefit LPS-induced alveolar bone loss.

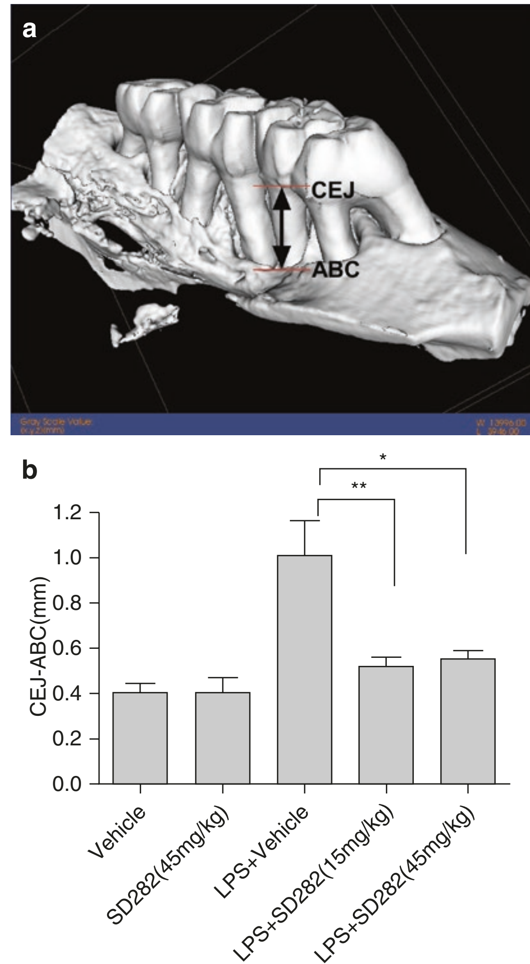

Fig. 5.3 *A. actinomycetemcomitans* LPS induces significant linear bone loss which is blocked by a p38 MAPK inhibitor. (**a**) Reformatted μCT isoform display from 8 weeks *A. actinomycetemcomitans* LPS-injected rat maxillae exhibits dramatic palatal and interproximal bone loss. Landmarks used for linear measurements were the cementoenamel junction (CEJ) to the alveolar bone crest (ABC). Differences between these anatomical locations using defined locations of 2-D displays determined alveolar bone loss. (**b**) Linear bone loss as measured from the CEF to AB (Mean ± SEM). Significant bone loss ($p < 0.01$) was observed between control ($n = 6$) and *A. actinomycetemcomitans* LPS-injected rats ($n = 12$). Significant reduction of LPS-induced periodontal bone loss ($**p < 0.01$ for SD-282, a p38 inhibitor, [15 mg/kg; $n = 8$] and $*p < 0.05$ for SD-282 [45 mg/kg; $n = 8$]) [69]. Reproduced with permission [69]

Clinically, one primary therapeutic goal is to prevent further advancement of alveolar bone loss. Thus, in a follow-up study with the orally

active p38 inhibitor, a different model was used once experimental periodontitis was established [70]. The periodontal disease state was established by LPS injections to the palatal molar gingiva three times per week for 4 weeks. The p38α MAPK inhibitor SD282 (45 mg/kg) was administrated from weeks 5 through 8 via oral gavage with the continuation of the LPS injections. The data from this study revealed that treatment with an orally active p38 MAPK inhibitor stopped the established periodontal disease progression in vivo and decreased inflammatory cytokine (IL-1β, TNF-α) expression and osteoclastogenesis. Interestingly, in this study, p38 MAPK inhibitor had a slight anabolic effect on the alveolar bone. The reasons for this are unclear, and may be due to a relatively high suppression of osteoclastogenesis without compensatory cessation of osteoblastic differentiation. Conceptually, this makes p38 inhibitor strategies appealing as a host-modulating agent for the treatment of periodontitis because physiologic bone turnover would occur, but inflammatory bone loss (induced by LPS, IL-1β, and TNFα) would be pharmacologically antagonized.

Collectively, these data highlight the therapeutic potential of this novel class of inhibitors in bacterial-induced alveolar bone loss—the hallmark of periodontitis, but developing p38 inhibitors as a therapeutics in clinical settings have failed due to unacceptable safety profiles, central role for activation of various downstream kinases and transcription factors, ubiquitous expression, toxicity, significant off-target effects, and lack of oral bioavailability [71]. Preclinical and clinical side effects include hepatotoxicity, cardiotoxicity, light-headedness, central nervous system toxicities, skin rash, gastrointestinal tract symptoms, and bacterial infections. To date, no p38 inhibitors have been approved by the Food and Drug Administration for any clinical usage.

Due to the concerns about p38 inhibitors indicated above, targeting downstream substrates of p38 MAPK and factors that regulate transcription, nuclear export, mRNA stability, and translation could be a promising therapeutic alternative for inhibiting inflammatory gene expression to treat various inflammatory diseases. As a direct substrate of the stress-activated MAPK p38α and β [72], MAPK-activated protein kinase 2 (MAPKAPK-2, MK2) is regulated exclusively by p38α/β [72].

Targeting MK2 should be a more specific target than p38, with potentially fewer side effects, because MK2 acts on a more limited downstream substrate repertoire compared to p38. Importantly, MK2-deficient mice are viable with a normal phenotype [73, 74]. Therefore, there has been much research exploiting MK2 as a molecular target for the development of experimental therapeutics for the number of conditions such as RA, Alzheimer's disease, atherosclerosis, and cancer. As periodontal disease has remarkably similar inflammatory pathways and mediator profiles with other inflammatory diseases [3, 75, 76], it is reasonable to anticipate that MK2 would be an attractive and potentially selective target for the treatment of periodontitis. However, targeting MK2 with small molecular inhibitors is complex and difficult because of the relatively planar ATP binding site of this critical MAPK.

In our study, we hypothesized that silencing MK2 through an RNAi strategy would provide a novel anti-inflammatory target that selectively blocks signaling mechanisms needed for enhanced cytokine mRNA stability/translation in periodontitis progression. First, we validated MK2 silencing in cytokine production in vitro to evaluate the feasibility of choosing MK2, instead of p38, as a highly specific and potent drug candidate. Our data clearly showed that LPS-induced IL-6 expression was significantly attenuated, both at the mRNA and protein levels, a result consistent with previous observations in $MK2^{-/-}$ mice [73, 77]. We observed that MK2 siRNA delivery significantly reduced TNFα mRNA and protein expression. The role of MK2 in the regulation of LPS-induced inflammatory cytokine gene expression is further confirmed by significant reductions of mRNA expression for COX-2, IL-1β, and the chemokine CXCL1 in cells transfected with MK2 siRNA. MK2 siRNA gene knockdown changed the activation of JNK and ERK MAPKs without obvious phospho-p38 expression variation, suggesting the existence of crosstalk and compensatory mechanisms and

underscoring the complexities of Toll-like receptor signaling pathways. Secondly, in vivo studies employed the rat LPS-induced experimental periodontitis model to further elucidate the role of MK2 in the pathogenesis of periodontitis and evaluate the therapeutic potential by targeting MK2 employing an RNAi strategy in periodontal disease. The protection of MK2 siRNA from alveolar bone loss in LPS-induced periodontitis model was further verified by μCT analysis (see Fig. 5.4; [52]). Histological examination displayed MK2 siRNA in vivo delivery attenuated the inflammatory infiltrate associated with *A. actinomycetemcomitans* LPS-induced bone loss. This is consistent with the decrease of osteoclast formation after MK2 silencing. In conclusion, with an RNAi strategy, our recent work validated that MK2 plays a role in a preventive model of experimental periodontitis, suggesting a novel target for controlling periodontal inflammation.

With regards to infection, although MK2 deficiency attenuated *A. actinomycetimcomitans*-induced calvarial inflammation and bone loss, it did not enhance growth or colonization of *A. actinomycetemcomitans*. Additional studies from our group have shown that although macrophage trafficking to the site of infection is diminished through local chemokine expression alterations, the ability to kill bacteria in an experimental calvarial infection model was evident in MK2-deficient mice [78]. It should be noted that during the management of our mouse colony we observed that MK2-deficient mice were no more susceptible to natural death than WT mice living in the same specific pathogen-free environment. Taken together, targeted inhibition of MK2 in disease pathogenesis could partially downregulate RANKL, osteoclast formation, bone loss, and inflammation without promoting susceptibility of the host.

5.5 Therapeutic Targeting of MKP-1 Signaling in Periodontal Disease

As MAPKs are activated by phosphorylation of critical tyrosine, serine or threonine residues, negative regulation of MAPK activity is mediated by the MAPK phosphatases (MKPs) that dephosphorylate these functional residues [79]. Early studies conducted using cultured immortalized macrophages provided compelling evidence that MKP-1 attenuates TNF-α and IL-6 after LPS stimulation [80–82]. MKP-1 functions as a feedback control mechanism, which governs the production of pro-inflammatory cytokines by deactivating p38 and JNK, thereby limiting proinflammatory cytokine biosynthesis in innate immune cells exposed to microbial components [80, 81, 83]. Consistent with in vitro data, *Dusp1*/MKP-1 null mice had markedly more production of pro-inflammatory cytokine TNFα, IL-6, and an anti-inflammatory cytokine IL-10 compared with wild-type animals. Sustained p38 and JNK activity in response to stress support the central role of MKP-1 in the restraint of the innate immune response and in the prevention of endotoxemia, experimentally induced autoimmune arthritis, septic shock syndrome, and multiorgan dysfunction during pathogenic microbial infection [82–86].

The critical role of the p38/MKP-1 axis of regulation on the innate immune response and in maintaining bone homeostasis has been clearly demonstrated. Moreover, as MKP-1 not only regulates p38 MAPK, but also JNK and ERK activities, overexpression of MKP-1 has potent capacity to prevent an exuberate immune response and osteoclastogenesis in response to stimuli compared with p38 MAPK inhibitors. Using gain- and loss-of-function approaches, the role and potential therapeutic target of MKP-1 in inflammatory bone loss was explored.

First, decreased IL-6 expression was observed in murine macrophage cell line RAW264.7 transfected with expression vector containing MKP-1 cDNA in pSRII-Flag. These data provided in macrophages support the role of MKP-1 in the negative regulation of *A. actinomycetemcomitans* LPS-induced p38 activation and IL-6 production [65]. In the experimental periodontitis model, wild type and *Dusp1* null mice received *A. actinomycetemcomitans* LPS injection in the palatal region or PBS control 3 times/week for 30 days. Results indicated that, in LPS-injected *Dusp1*$^{-/-}$ mice, significantly greater bone loss occurred

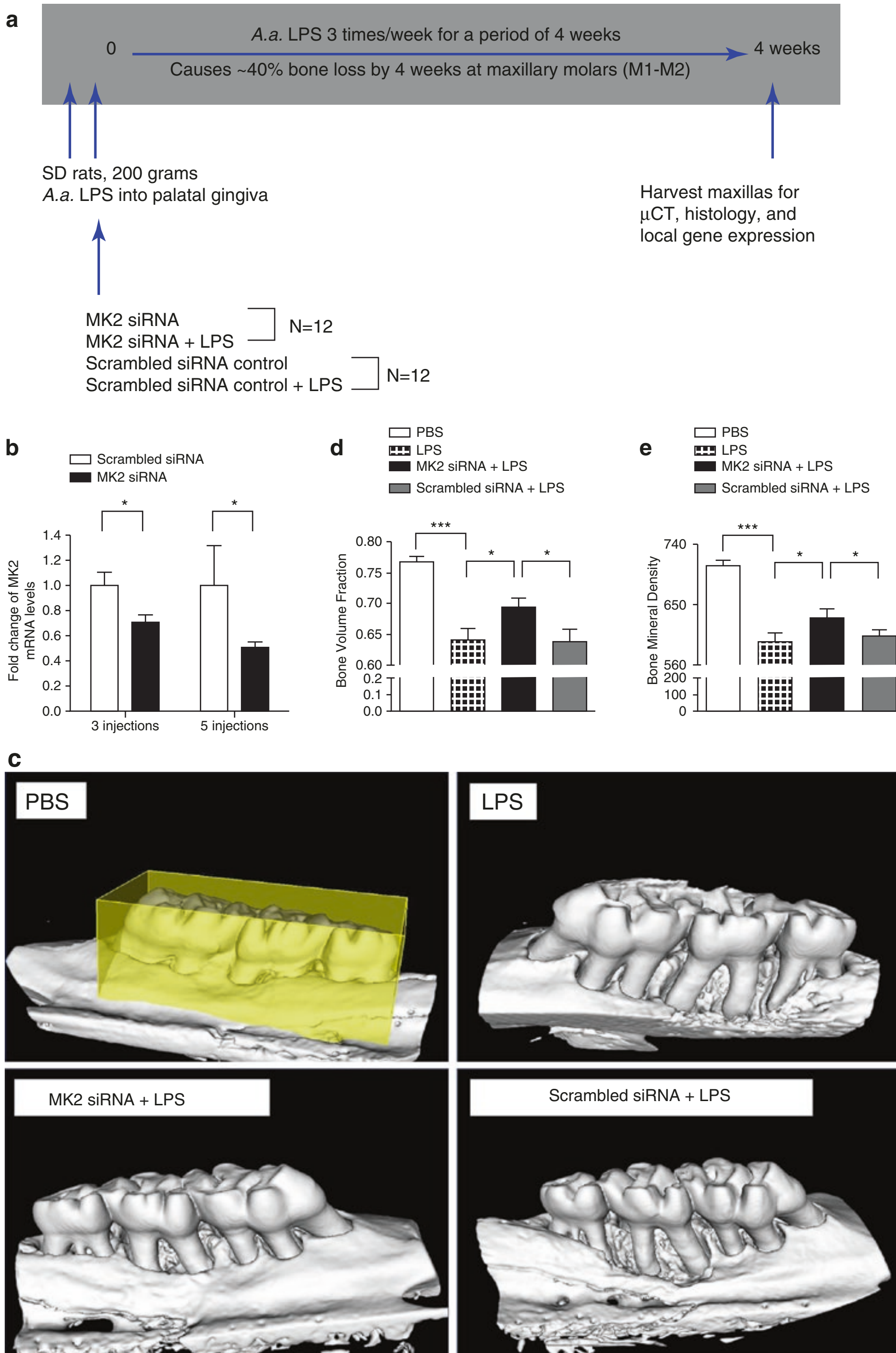

Fig. 5.4 Specific MK2 siRNA in vivo delivery silenced target gene expression and reduced LPS-induced bone loss. (**a**) A schematic showing overall experimental protocol. (**b**) MK2 mRNA expression in palate gingiva after 3 and 5 times siRNA in vivo delivery. Results are expressed as mean ± SE (n = 5 or 6 rats/group, $*P < 0.05$). (**c**) Representative µCT images of rat maxillae from indicated treatment groups. ROI for quantitative analysis is highlighted. (**d**) Volumetric analysis of bone loss levels. (**e**) Bone mineral density (BMD) analysis of bone loss levels. ($*P < 0.05$, $***P < 0.001$) [52]. Reproduced with permission [52]

with more inflammatory infiltrate in the periodontal areas injected with LPS and a significant increase in osteoclastogenesis compared with *Dusp1*$^{-/-}$ control sites or either wild-type littermates. Thus, MKP-1 displayed a protective response in a chronic model of inflammation and bone loss [65].

In gain-of-function experiments, MKP-1 was able to dephosphorylation all three MAPKs via MKP-1 gene transfer with recombinant adenovirus MKP-1 in rat macrophages. Ex vivo data indicated that in bone marrow macrophages transduced with an adenovirus containing the *Dusp1*/MKP-1 gene, both wild-type and *Dusp1* null mice had a significant decrease of IL-6, IL-10, TNFα, and select chemokine levels compared to LPS cultures [54]. In addition, bone marrow cultures from *Dusp1* null mice exhibited significantly more osteoclastogenesis induced by LPS than when compared with WT mice. This observation correlated with more osteoclasts seen in bone marrow cells of MKP-1 KO mice compared with osteoclasts from WT mice in response to LPS stimuli. Furthermore, in vivo MKP-1 gene transfer in an experimental periodontal disease model attenuated bone resorption induced by LPS (see Fig. 5.5; [54]). Histological analysis confirmed that periodontal tissues transduced with Ad. MKP-1 exhibited less infiltrated inflammatory cells, less osteoclasts, and less IL-6 than compared with rats of control groups. Together, our studies indicate the importance of MKP-1 in the development of immune responses that contribute to LPS-induced alveolar bone loss. It can be used as a key therapeutic target to control inflammation-induced bone loss associated with increased MAPK activation.

Since no MAPK inhibitor is currently Food and Drug Administration (FDA)-approved, exploration toward the development of innovative agents that would function to increase endogenous MKP-1 expression/function to act as a novel anti-inflammatory agent to reduce periodontal bone loss was initiated. While MKP-1 agonists have not been developed into

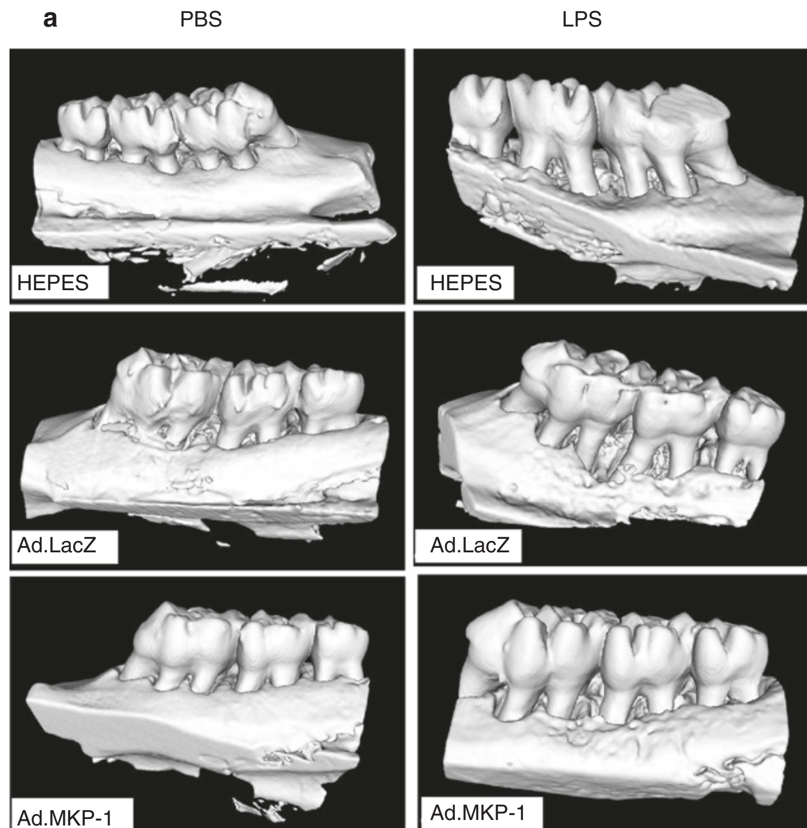

Fig. 5.5 MKP-1 gene transfer alleviated bone resorption in rats after LPS challenge. Eight-week-old male Sprague-Dawley rats (17 rats/group) were injected either Ad.MKP-1 or Ad. LacZ (1×10^9 pfu in 4 μL), or HEPES buffered saline (4 μL). 48 h after the adenovirus injection, the rats were injected with 2 μL of either 20 μg of LPS (from *A. actinomycetemcomitans*) or PBS 3 times a week for 4 weeks. (**a**) Representative microcomputed tomography images of rat maxillae from indicated treatment groups. (**b**) Volumetric analysis of bone loss levels ($n = 7$ for PBS groups, $n = 10$ for LPS groups, $*p < 0.05$); [54]. Reproduced with permission [54]

Fig. 5.5 (continued)

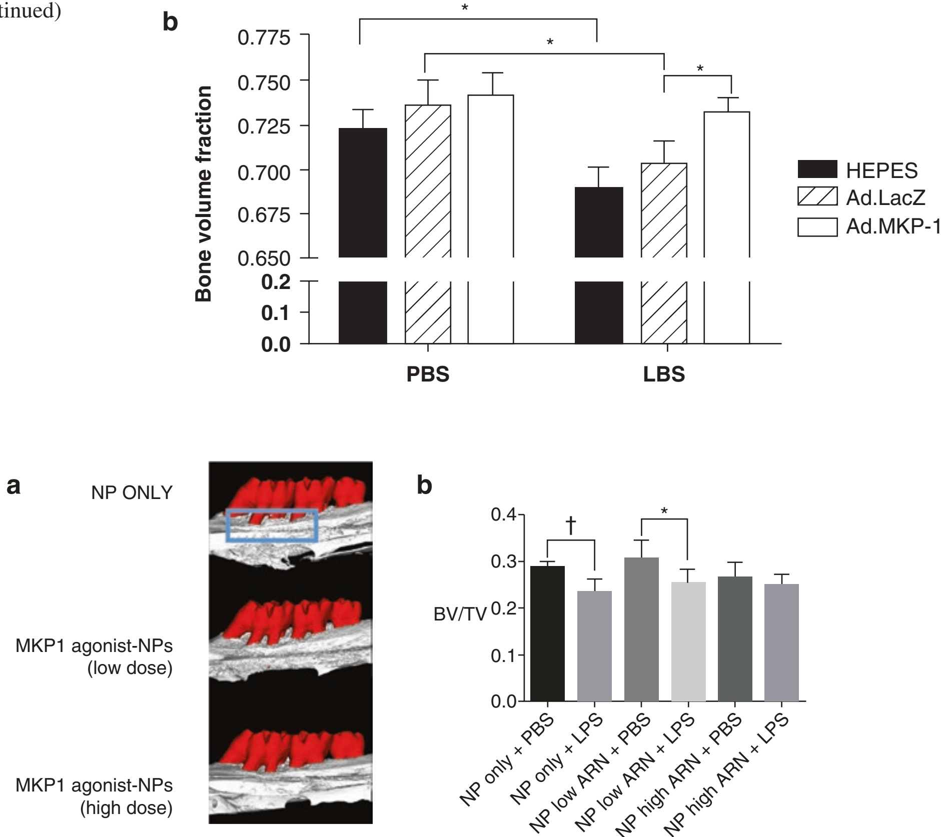

Fig. 5.6 ARN-NPs alleviated bone resorption in a preclinical periodontitis model. Eight-week-old male Sprague-Dawley rats were injected PBS alone, NP only (no ARN), NP-ARN high (10 μM), or NP-ARN low (1 μM) for 14-day treatment groups. After 4 h, rats were injected with control (PBS, 2 μL) on the right side or LPS (10 μg/2 μL) on the left side. Treatments and stimulation were administered every other day for 14 days. (**a**) Representative microcomputed tomography images of rat maxillae from indicated treatment groups of the region of interest (ROI) defined by the boxed area. (**b**) Volumetric analysis of bone loss levels ($n = 7$ for PBS groups, $n = 10$ for LPS groups, $*p < 0.05$, $^{\dagger}p < 0.01$). Reproduced with permission [99]

FDA-approved therapeutics, there are a few agents that are FDA-approved that harbor MKP-1 agonist properties [87]. Auranofin is a well-established disease-modifying antirheumatic drug (DMARD) that never had a known mechanism of action until more recently when it was shown to induce MKP-1 expression [87]. Nanomedicine in dentistry has utilized many materials (e.g., polymeric, ceramic and metallic, lipids, liposomes, dendrimers, and hybrid systems with coatings) to deliver numerous therapeutics, including those locally into the periodontal sulcus [88–97]. Our innovative approach uses an encapsulation strategy to package Auranofin into a nanoparticle (NP) drug delivery system. NPs used in this system selectively target phagocytic cells [98] and thus will have a better potential to reduce cytokine inflammation in the periodontal tissues (Fig. 5.6). Thus, nanotechnology-driven therapeutic delivery strategies were explored to reintegrate Auranofin into a locally delivered agent for the adjuvant treatment of chronic periodontitis [99]. The data show that Auranofin, a well-established DMARD agent, can be functionally loaded into PLA-PEG nanoparticles to control periodontal inflammation and prevent experimental periodontal bone

loss. Optimization of these types of reservoir delivery systems, including drug-loaded nanoparticles, may provide a highly important treatment modality which can be tailored to suit the needs of this periodontal treatment. Using this type of nanoparticle delivery system, it is speculated that clinical use of ARN-NPs would potentially be able to help control periodontal inflammation clinically to reduce inflammation-related alveolar bone loss.

5.6 Conclusion

Adjuvant therapies for periodontitis that modulate the immune response have been around for decades. Many of these therapies target host enzymes that destroy tissues, such as doxycycline inhibition of matrix metalloproteinases, or nonsteroidal anti-inflammatory drugs (NSAIDs) that target cyclooxygenase (COX)-2 [100]. Other agents that would be predicted to be potent anti-resorptive agents, including bisphosphonates or others that target receptor activator of nuclear factor-κB-ligand (RANKL), have potential therapeutic application in the treatment of periodontal diseases, but carry oral side effects including osteonecrosis of the jaw. Thus, there is a need for the development of new adjuvants that would target multiple cytokines, chemokines, and enzymes locally to manage chronic periodontitis without debilitating side effects. This chapter describes new emerging translational knowledge suggesting that agents targeting the MAPK/MKP signaling axis, common to multiple inflammatory targets, represent the next generation of rationally designed professionally applied therapeutics to manage chronic periodontitis.

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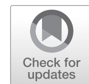

6 The IL-17/Th17 Axis as a Therapeutic Target in Periodontitis

Nicolas Dutzan, Loreto Abusleme, and Niki Moutsopoulos

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6.1 Introduction

Periodontitis is one of the most prevalent inflammatory diseases worldwide [1]. It involves an imbalanced relationship between local microbial communities and the host immune response resulting in the destruction of tooth supportive structures [2]. This inflammatory disease might lead to tooth loss, masticatory dysfunction, and potentially modify the course of systemic diseases, leading to a decrease in the quality of life of patients [2–5]. This common pathology and its consequences have become a considerable economic burden and a public health problem [5, 6].

The treatment of periodontitis has been mainly focused on decreasing the tooth adherent bacterial load and surgically modifying local factors that contribute to the increase of the microbial burden. However, this treatment does not directly tackle the dysregulated inflammatory host response to that microbial challenge

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[7, 8]. These standard therapeutic approaches are not useful for all patients, and approximately 20–25% of subjects with advanced stages of periodontitis continue to have disease progression and tooth loss after appropriate treatment and supportive therapy [8–13]. This evidence has reinforced the need for further interrogation of periodontitis pathogenesis with the intent to identify disease-driving mechanisms and potentially reveal biologically supported therapeutic targets.

The investigation of periodontitis pathogenesis has revealed that the immune response and inflammation play a vital role in the destructive events that characterize this disease [14, 15]. Indeed, immune cells, such as CD4+ T cells, have been described as a significant component of the inflammatory infiltrate in the periodontitis lesion [16–20]. Furthermore, mechanistic studies have suggested that CD4+ T cells are necessary for the progression of alveolar bone destruction during periodontal disease [21–23]. Among the CD4+ T helper (Th) cell subsets, Th17 cells and their signature cytokine interleukin-17A (IL-17A or IL-17) have been recently identified as drivers of periodontitis immunopathology becoming a plausible therapeutic target for periodontal immunopathology modulation [24, 25].

6.2 Interleukin-17: A Cytokine with Critical Functions in Mucosal Immunity and Inflammation

IL-17A is the first described member of the six cytokines (IL-17A to IL-17F) that compose the IL-17 family [26]. This cytokine has been positively correlated with clinical parameters of periodontal tissue destruction and, in more recent work, it has been shown to play a crucial role in inflammatory alveolar bone loss [27–29].

IL-17 receptor (IL-17R) family contains five members (from IL-17RA to IL-17RE) with IL-17RA and IL-17RC being necessary for IL-17A signaling. Activation of IL-17R triggers intracellular signals that elicit gene transcription and will initiate distinct biologic functions depending on the cell type, with different responses seen in stromal compared to hematopoietic cells [26, 30].

IL-17A is not a potent inducer of inflammation by itself, but it can act in synergy with several pro-inflammatory cytokines. It is well established that IL-17A stabilizes tumor necrosis factor-α (TNF-α) mRNA transcripts and cooperates with lymphotoxin, interferon-γ (IFN-γ), and IL-22 [26, 30–32]. Furthermore, IL-17A promotes the secretion of cytokines that help to maintain and increase the numbers of IL-17-producing cells, such as IL-1β, IL-6, IL-23, and TNF-α, creating a cytokine environment that helps to maintain a robust and sustained IL-17 response [31, 32].

The primary physiologic role of IL-17A is to mediate host defense against extracellular bacteria and fungi [33–35] and to regulate barrier integrity [36]. Studies in patients with genetic defects in the Th17/IL-17 axis have demonstrated severe dysbiosis of oral fungal and bacterial communities and recurrent oral *Candida* infections, indicating a critical role of this axis for oral immunity [34, 37–39]. In this homeostatic and protective context, IL-17A activates the expression of antimicrobial-proteins like β-defensins, S100 proteins, and lipocalin-2 [26]. Also, IL-17A promotes epithelial integrity in the mucosa of the lower gastrointestinal tract, regulating the production and cellular localization of tight-junction proteins claudin and occludin, stabilizing epithelial cell connections and limiting permeability during epithelial injuries [26, 31, 32, 36]. Other protective functions of IL-17A include the recruitment of immune cells to control invading microbes, with the induction of neutrophil recruitment as the hallmark role for this cytokine. To mediate this function, IL-17A stimulates the production of granulopoietic factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) and chemokines such as CCL2, CXCL1, CXCL5, and CXCL8 which attract neutrophils to the infection site [31, 40]. Additionally,

IL-17A promotes CCL-20 expression which recruits IL-17-producing immune cells such as innate lymphoid cells (ILCs) and Th17 cells. IL-17-mediated cell recruitment is also enhanced by the induction of several matrix metalloproteinases (MMPs) including MMP-1, -2, -3, -8, -9, and -13 and by the stimulation of cyclooxygenase COX-2 which enhances cell migration through PGE2 production [41–43]. It is this ability to recruit immune cells that make the IL-17-mediated response not only beneficial but potentially detrimental during pathology.

Inflammatory and autoimmune diseases such as rheumatoid arthritis (RA), type 1 diabetes, multiple sclerosis (MS), inflammatory bowel diseases (IBD), psoriasis, ankylosis spondylitis (AS), and periodontitis have been associated with increased levels of IL-17A and accumulation of IL-17A-secreting cells [26, 30, 31]. Likewise, genome-wide association studies (GWAS) have linked single nucleotide polymorphisms (SNPs) in genes of the IL-17A pathway with autoimmune diseases such as psoriasis and Crohn's disease (CD) [44, 45]. Inhibition of the cytokine IL-17A and its upstream mediator IL-23 are now the first-line treatment for chronic autoimmune/inflammatory diseases such as psoriasis, psoriatic arthritis, ankylosis spondylitis, and CD as we further discuss below.

6.3 Th17 Cell Differentiation

The original description of Th cell functionality involved two main effector subsets, the Th1 and Th2 cells. Th1 were characterized by IFN-γ production and have a prominent role in the eradication of intracellular pathogens. On the other hand, Th2 secrete IL-4, IL-5, and IL-13 and have evolved to contribute to the elimination of parasitic infections [46]. In this dichotomic description of effector Th cells, the function of $CD4^+$ T cells in infections caused by extracellular pathogens and fungi was not fully explained. This knowledge gap was filled 20 years later when Th17 cells were described and added to the $CD4^+$ T effector cells repertoire [47].

Th17 cells are characterized by secretion of IL-17A, IL-17F, IL-22, and GM-CSF, targeting epithelial and other stromal cells as well as inducing the recruitment of granulocytes and enhancing the activation of monocytes [48, 49]. As one of the primary sources of IL-17A, the Th17 cell function is tightly associated with this cytokine. Immune regulation at barrier sites, neutrophil and other immune cell recruitment and promotion of epithelial integrity are also part of the Th17 physiologic function. Likewise, the pathologic function of these cells has been associated and implicated in the pathogenesis of inflammatory diseases such as RA, psoriasis, IBD, Sjögren's syndrome, and periodontitis [24, 25, 35, 47–50].

Differentiation of Th17 cells from a naïve T cell involves T-cell receptor (TCR) activation, co-stimulatory molecules, and the appropriate surrounding cytokine milieu. This pro-Th17 cytokine environment can be composed of transforming growth factor-β (TGF-β), IL-1β, IL-6, and IL-23 [47–50]. Indeed, studies suggest that Th17 cells differentiated under a different cytokine milieu (i.e., TGF-β + IL-6 or IL-1 + IL-6 + IL-23) appear to be functionally and transcriptionally distinct. These differences in the cytokine microenvironment might also explain whether Th17 cells are pathogenic or non-pathogenic in terms of inducing disease [49, 51].

Regulation of Th17 differentiation is to a great extent driven by the transcription factors signal transducer and activator of transcription-3 (STAT3) and the orphan nuclear receptor RORγt [52, 53]. Patients with mutations in STAT3 or RORγt genes have impaired Th17 differentiation and disrupted immune responses against fungi and extracellular pathogens [34, 37, 54, 55].

The implication of Th17 cells in the pathology of devastating inflammatory diseases together with their tightly regulated differentiation process has prompted the study of Th17 cell differentiation mechanisms aiming to develop and then offer therapeutic alternatives to patients affected with these often-refractory inflammatory disorders.

6.4 The Th17/IL-17 Axis in Periodontitis

As a barrier site, the oral cavity is exposed to a rich and diverse community of microorganisms as well as antigens from food and airborne particles [56–58]. How these diverse signals may modulate the Th17/IL-17 pathway in health and disease was not well understood until very recently [24, 35, 37, 56, 59].

Studies in our laboratory have revealed that the homeostatic regulation of Th17 cells at the oral/gingival barrier occurs in a commensal-independent manner. Germ-free mice have an undisturbed Th17 response, suggesting the participation of microbiome-independent factors in the induction of this immune network. Furthermore, we determined that a critical trigger of this response is the damage that occurs physiologically upon mastication [28]. In response to this gingival damage, epithelial cells produce IL-6 which is vital for Th17 generation at this site. This commensal-independent mechanism starkly contrasts with what has been observed in other barrier sites such as the lower gastrointestinal (GI) tract and skin, where steady-state Th17 cell development has been tied to colonization by commensal bacteria and dependent to other cytokine signals such as IL-1β [60–62].

Unlike homeostatic oral Th17 cells, periodontitis-associated Th17 cell expansion relies on the local dysbiotic microbiome [24]. Indeed, our recent work has determined that changes in the balance of bacterial communities during periodontitis lead to Th17 cell expansion, which in turn triggers periodontal immunopathology. This Th17 cell accumulation observed during periodontitis necessitates not only the cytokine IL-6 but also IL-23, underlining a distinct regulation of Th17 cells during health and periodontitis [24].

The fact that periodontitis and RA have similar pathological features and the crucial role of Th17/IL-17 in RA immunopathology sparked interest in the early 2000s in the study of this axis in periodontitis. Since then, the connection between IL-17/Th17 cells and periodontitis has been widely studied [35, 59, 63, 64]. Th17 cell accumulation and IL-17A overproduction have been reported in lesions of human periodontitis [24, 65, 66]. This cytokine and signature T-cell subset has been shown to positively correlate with clinical parameters of periodontal inflammation and destruction [24]. In addition, an increase in IL-23 and other pro-inflammatory and osteoclastogenic mediators related with Th17, such as IL-6 and the receptor activator of nuclear factor κB ligand (RANKL), together with RORC gene (which encodes RORγt, "master switch" of Th17 differentiation), have also been linked with periodontal lesions [67–70]. Furthermore, *Porphyromonas gingivalis*, an oral bacteria associated with dysbiotic microbial changes during periodontitis [71], has been shown to stimulate myeloid antigen-presenting cells to drive Th17 polarization by the production of Th17-polarizing cytokines such as IL-1β, IL-6, and IL-23 [66]. Importantly, inflammatory resolution following the treatment of periodontal disease leads to a significant reduction of tissue levels of IL-17A and Th17-related cytokines compared to pretreated tissues [72].

Work from our laboratory and colleagues on patients with genetic defects that predispose to periodontitis reveals additional mechanisms by which the IL-17/Th17 axis can become amplified in periodontitis. Studies in leukocyte adhesion deficiency-I (LAD-I), a rare genetic disorder due to mutations in CD18 (integrin β chain-2) which affects the adhesion and transmigration of neutrophils into tissues, have revealed the essential role of tissue neutrophils in the regulation of IL-23/Th17 axis [73, 74]. Patients with LAD-I typically present with aggressive and severe periodontitis at an early age and often lose their entire dentition during teenage years, despite antibiotic treatment and standard of care dental cleanings [75, 76]. In the lesions of LAD-I periodontitis, we have documented increased levels of IL-23 and IL-17A as well as IL-17A-dependent neutrophil factors and chemo-attractants [75]. It is proposed that apoptosis of tissue neutrophils is essential for the downregulation of IL-23 responses. Therefore, in LAD-I, IL-23/IL-17 responses become unleashed in the absence of tissue neutrophils and cause significant

immunopathology [74, 77]. Indeed, experimental models have demonstrated that aberrant IL-17 responses are drivers of immunopathology in LAD-I [75].

Recently, the role of Th17 cells and IL-17A as drivers of periodontitis pathogenesis has also been established for common chronic forms of periodontitis. Studies led by our group and others have revealed that IL-17A protein neutralization and notably, genetic ablation or pharmacological inhibition of Th17 cells, diminishes bone loss in murine models of periodontitis [24, 25, 27]. Similar results were observed in a human setting. Our studies in a unique patient cohort with a genetic defect in Th17 cell differentiation (autosomal-dominant hyper IgE syndrome patients) reveal decreased periodontal inflammation and bone loss in the setting of natural Th17 deficiency [24]. Collectively, these results underscore the crucial role of Th17 and IL-17A in periodontitis pathogenesis and provide compelling evidence for a targeted therapeutic approach in periodontitis.

6.5 How Do IL-17A and Th17 Cells Mediate Periodontitis Immunopathology?

There is limited mechanistic evidence of how the Th17/IL-17 axis drives inflammatory bone loss in periodontitis. However, hypotheses have been proposed based on the study of Th17/IL-17 axis in vitro and in other disease models.

It is well known that IL-17A induces the expression of MMPs in fibroblasts, epithelial cells, and endothelial cells, as well as RANKL expression in osteoblasts, a key factor for osteoclast differentiation and function [35, 43, 59, 78, 79]. Indeed, the induction of RANKL on osteoblasts is necessary for IL-17A-mediated periodontal bone loss [25]. All these mediators have also been associated with periodontitis, making the IL-17-RANKL and IL-17-MMPs relation a plausible hypothetical mechanism of tissue destruction mediated by the Th17/IL-17 axis during periodontitis (Fig. 6.1).

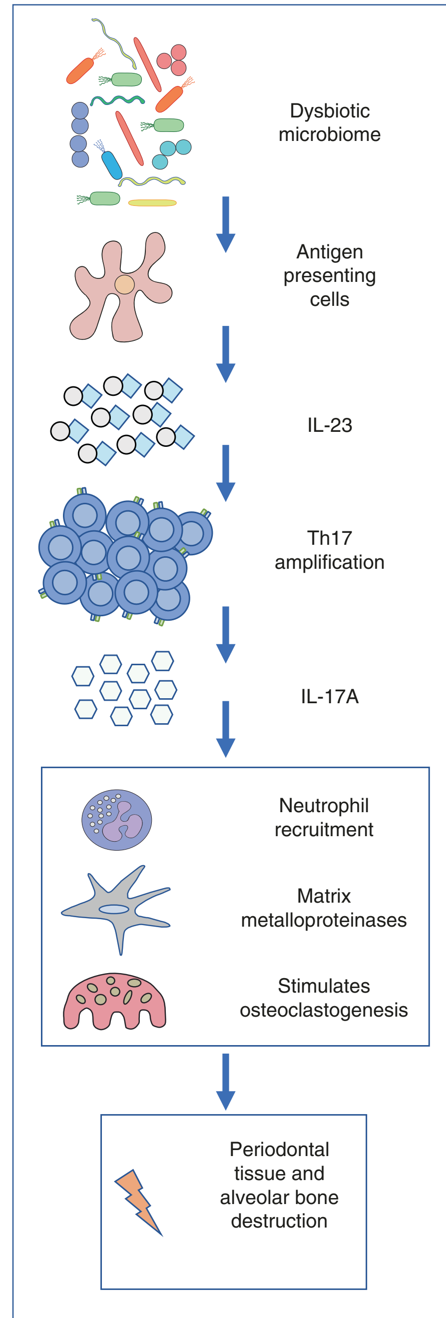

Fig. 6.1 Th17-IL-17-mediated periodontal immunopathology (current thinking). Changes in the tooth-associated microbiome elicit IL-23 production by antigen-presenting cells (APC). IL-23 stimulates local amplification of Th17 cells in gingival tissues. Overproduction of IL-17A by Th17 leads to immunopathology and periodontal bone loss via mechanisms which may involve neutrophil recruitment, macrophage activation, and upregulation of RANKL

The second pathway by which IL-17/Th17 may drive pathology is through neutrophil recruitment. As it was stated above, one of the critical functions of IL-17A is immune cell recruitment, particularly neutrophils [31]. These granulocytes are one of the dominant immune cell populations in the gingiva and play important antimicrobial roles at this site [56, 65]. These same mechanisms which are essential for homeostasis, when exaggerated might also be the culprits of damage and tissue destruction (Fig. 6.1) [74]. Associated with the increase in IL-17A and Th17 cells in periodontitis, neutrophils also accumulate in inflamed gingival tissues, posing a consistent threat to tissue integrity [24, 65, 74]. Indeed, we and others have demonstrated that a decrease in neutrophil numbers protects from bone loss in the inflammatory setting of experimental periodontitis [24, 27].

Evidence from the experimental animal models has revealed the existence of pathogenic subtypes of Th17 cells in the setting of inflammation and autoimmunity [80]. These pathogenic Th17 cells are induced via a specific cytokine network which includes IL-23 (discussed above). They acquire additional characteristics and become pathogenic in the setting of disease. The cytokine IL-23 has been uniquely connected to the induction of pathogenic Th17 cells in various settings [49, 80–83]. This cytokine is necessary for inflammation and autoimmunity development, and its therapeutic targeting can inhibit the development and relapse of these conditions [83, 84]. IL-23 neutralization targets pathogenic sources of IL-17A, minimizes tissue inflammation, and leaves the protective function of IL-17A-producing cells intact [36]. Studies have demonstrated that IL-23-driven Th17 cells acquire a unique transcriptional program and co-produce cytokines that aid in their pathogenicity such as GM-CSF, IFN-γ, and TGF-β among others [80, 85]. In periodontitis, a subset of Th17 cells has been shown to co-produce GM-CSF and IFN-γ; however, the pathogenic potential of this subpopulation has not been established [24].

6.6 Therapeutic Targeting of the Th17/IL-17 Axis

Advances in molecular biology and biochemistry have given us a detailed understanding of crucial immune receptors and cell activation. New technological tools have allowed for the production of therapeutic agents targeting cytokines, their receptors, and signaling pathways. Antibodies and small molecules against many immune factors have been approved for the treatment of diseases such as RA and psoriasis. These agents have revolutionized the treatment of immune-mediated diseases, providing disease alleviation for numerous patients [86, 87].

The understanding of Th17 and IL-17A role in autoimmune diseases has ignited the development of biological agents targeting that pathway. Antibodies against IL-17A, IL-17RA, and IL-23 have been approved in the U.S. Food and Drug Administration (FDA) for the treatment of plaque psoriasis, psoriatic arthritis, and Crohn’s disease [51, 88]. Additional efforts have also concentrated on the study of low molecular weight compounds targeting intracellular factors key for Th17 differentiation and expansion [49, 50].

Monoclonal antibodies directed against the IL-17A cytokine itself (secukinumab and ixekizumab) and its receptor IL-17RA (brodalumab) have demonstrated to be effective in plaque psoriasis treatment (Fig. 6.2). Indeed, 30–60% of patients treated with these agents showed 100% disease clearance in the Psoriasis Activity and Severity Index (PASI) [89, 90]. Secukinumab and ixekizumab have been FDA-approved for the treatment of psoriatic arthritis, and brodalumab is currently under evaluation [89]. Additionally, secukinumab has demonstrated significant reductions in the clinical signs and symptoms of ankylosing spondylitis [91]. In contrast with those results, mixed responses to IL-17A treatment in RA patients have been reported [92, 93] and in patients with IBD the administration of anti-IL-17A has not only failed to improve disease symptoms, but it has also increased disease activity [94]. The most common adverse effects of these treatments include nasopharyngitis, upper

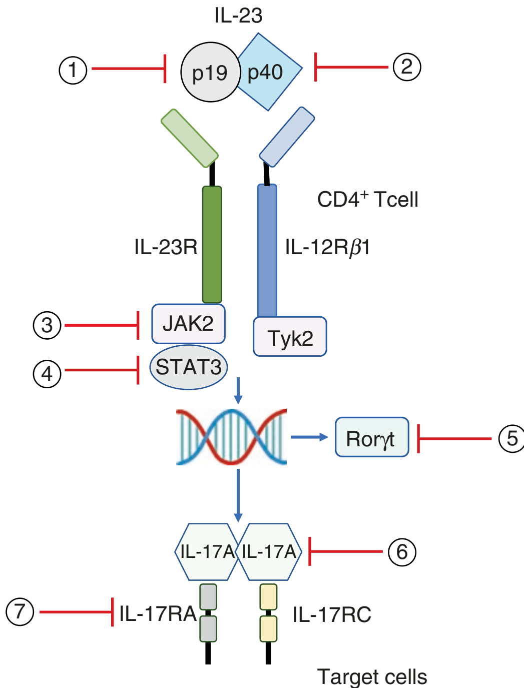

Fig. 6.2 Therapeutic targeting of the Th17/IL-17 axis. Schematic diagram depicting different targets and pharmacologic inhibitors for the Th17/IL-17 axis. IL-23 engagement with its receptor activates intracellular signaling in CD4+ T cells contributing to Th17 differentiation and expression of RORγt and IL-17A production. IL-17A signals through its receptor inducing different responses (previously explained in Fig. 6.1) in target cells. Each number represents different biologics and small molecules targeting the Th17/IL-17 axis. (1) p19 subunit inhibitors guselkumab and tildrakizumab. (2) p40 subunit inhibitor ustekinumab. (3) JAK inhibitors such as tofacitinib and ruxolitinib. (4) STAT3 inhibitors. (5) RORγt inhibitors such as digoxin and GSK805. (6) IL-17A inhibitors secukinumab and ixekizumab. (7) IL-17RA inhibitor brodalumab. *JAK* Janus kinase, *STAT3* signal transducer and activator of transcription-3

respiratory tract infections, mucocutaneous candidiasis, transient neutropenia, and injection site reactions [89]. These adverse effects, while mild do emphasize the dichotomous role of IL-17A in the human immune response: on one side driving inflammation and the other providing protection against infection and integrity to barrier sites [30].

Based on the concept that there are diverse cellular sources of IL-17A, both homeostatic and pathogenic, and that distinct cytokine signals will support the induction of pathogenic Th17 cells, it has been suggested that targeting the Th17 cell subset itself might provide better clinical efficacy compared to targeting the IL-17A cytokine [50]. Consistent with this idea, ustekinumab, an inhibitor of the p40 subunit that is shared by IL-23 and IL-12, is currently FDA-approved for the treatment of psoriasis and psoriatic arthritis and has shown to have efficacy in the treatment for Crohn's disease (Fig. 6.2) [95]. Additionally, several antagonists against the IL-23p19 subunit (which inhibits only IL-23 without disrupting IL-12) are being studied for the treatment of psoriasis and other diseases [88, 89]. Recently, the two IL-23p19 antibodies guselkumab and tildrakizumab received FDA approval for the treatment of moderate to severe plaque psoriasis (Fig. 6.2). Head-to-head trials of guselkumab versus ustekinumab and tildrakizumab versus etanercept (which targets TNF-α) demonstrated the superiority of these two anti-p19 biologics for psoriasis treatment [96, 97]. The adverse effects associated with the use of anti-p19 treatment include upper respiratory tract infections, nasopharyngitis, and headaches. Studies also demonstrated that in contrast with what was observed upon anti- IL-17A treatment, mucocutaneous candidiasis was infrequent with IL-23 blockade [89]. This clinical observation could be explained by the fact that at least in oral mucosa, innate T-17-producing cells may be crucial homeostatic IL-17A producers at steady state which help to restrain *Candida* infection [24, 98].

In recent years, small molecules that target key intracellular molecules have also been studied for the treatment of autoimmune diseases. Compared to monoclonal antibody therapy, small-molecule treatment has a lower cost, oral bioavailability, and treatment discontinuation results in a rapid reversibility of its immunomodulatory effects [86, 99]. Small molecules that target the Janus kinase (JAK) pathway have been FDA-approved for the treatment of RA and are under study for the treatment of other diseases like IBD and psoriasis [100, 101]. Several cytokines employ JAKs to exert their effect; thus tar-

geting these molecules inhibits a broad spectrum of cytokines, some of them critical for the Th17 amplification observed during periodontitis such as IL-6 and IL-23 [100, 101]. Other cytokines, also associated with periodontal destruction such as IFN-γ and IL-21 [102, 103], utilize JAK molecules for their signaling, transforming JAK inhibition in a plausible method to restrain Th17/IL-17 overexpression and inflammatory pathology observed in periodontitis (Fig. 6.2).

The inhibition of transcription factors key for Th17 development (RORγt and STAT3) by small molecules is also currently under study (Fig. 6.2) [49, 50, 104]. A RORγt inverse agonist has been used in preclinical models of psoriasis, IBD, arthritis, and EAE with favorable results [105]. From these compounds, digoxin which is used to treat various heart conditions was among the firsts described. This drug has been shown to inhibit Th17 differentiation in mice, ameliorating EAE and also delaying arthritis onset and severity [106, 107]. A modified digoxin molecule, not toxic for human T cells, has been shown to block human Th17 cell differentiation [106].

On the other hand, transient chemical inhibition of RORγt (using the GSK805 compound) has been effective in reducing IL-17A production by Th17 cells providing therapeutic benefit in murine models of intestinal inflammation. Also, this compound has also been found to be effective in reducing the frequency of Th17 cells isolated from tissues of patients with IBD [108]. Two clinical trials for psoriasis treatment have been completed (NCT02548052 and NCT02555709); however, at the submission of this manuscript, the results of these trials have not been made public. Recently, our work has demonstrated that pharmacological inhibition of RORγt in murine models of periodontitis diminished alveolar bone destruction, illustrating that this mechanism is a plausible method to inhibit the accumulation of Th17 in gingival tissues and to restrain the damage produced by periodontitis immunopathology [24]. However, more evidence would be needed before establishing the use of these small molecule compounds by clinicians as another complementary therapeutic tool for periodontal treatment.

6.7 Therapeutic Targeting of the Th17 Axis in Rare-Monogenic Forms of Periodontitis

To date, the Th17 axis has been therapeutically targeted in periodontitis, in a proof-of-concept study of a single patient with leukocyte adhesion deficiency-I-associated periodontitis. As discussed above, patients with LAD-I present with an aggressive form of periodontitis at an early age attributed to an excessive amplification of the IL-23/Th17 response [75, 76].

Based on critical observations in humans and preclinical studies in murine models, our group and collaborators considered the treatment of a patient with LAD-I periodontitis, via inhibition of the IL-23/Th17 axis. This single LAD-I patient was treated with ustekinumab, an antibody that blocks the activity of both IL-23 and IL-12 (by targeting the common chain of these two cytokines, p40) [76, 95]. In this study, a 19-year-old man with severe periodontitis, unresponsive to standard care and antibiotics, was treated with ustekinumab using the approved dose of this antibody for psoriasis (45 mg subcutaneously at baseline, at week 4, and every 12 weeks after that). This treatment resulted in a dramatic reduction of gingival inflammation. 3 weeks after the first injection, bleeding on probing was reduced to only 40% of the sites, as compared to 90% before ustekinumab treatment. Besides, gingival levels of IL-17A and IL-23 decreased to levels found in healthy gingiva. The inflammation and inflammatory-mediator levels remained low the entire time reported (14 months) and importantly the patient experienced no adverse events throughout this period [76].

This work suggested IL-23 mediated inflammation as the driving force of LAD-I periodontitis and became the impetus for further exploration of IL-23 blockade and Th17 cell modulation for the treatment of IL-17 overproduction in the oral mucosa and, particularly, at the gingival tissues. Based on this work, an interventional study has been initiated at the NIH hospital to treat LAD-1 patients with ustekinumab (NCT03366142).

However, it is essential to be cautious and not to make overreaching conclusions from a single case. Although clinical results from this case were promising both at the level of safety and efficacy, results from the clinical trial are necessary to fully assess the utility of IL-23 blockade for LAD-I related immunopathology. Furthermore, the safety and efficacy of Th17 blockade are undetermined in common forms of periodontitis, yet the prospects of such treatments in humans now can be based on substantial biological evidence.

6.8 Conclusion

The evidence that standard therapeutic approaches do not resolve all clinical cases of periodontal disease [8–11] has highlighted the need for further interrogation of periodontitis pathogenesis with the intent to identify disease-driving mechanisms and potentially reveal biologically supported therapeutic targets. The extensively documented expansion of IL-23/IL-17 responses during periodontitis, the mechanistic evidence for a pathogenic role of IL-17/Th17 axis in experimental periodontitis, the tremendous development of pharmacological targets of Th17 responses, and the preliminary evidence that Th17 inhibition is beneficial in rare forms of periodontitis [24, 27, 28], provide a compelling basis to consider Th17 inhibition as a promising therapeutic strategy in common forms of periodontitis.

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7 Epigenetic and Gene Modification Precision Medicine Approaches for the "Chronic Destructive Perio-Diseases": Periodontitis and Peri-implantitis

Denis F. Kinane

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7.1 Introduction

There is a large body of classical periodontal literature that expounds the utility of non-surgical and surgical therapy in the treatment of periodontal and now peri-implant diseases, the "perio-diseases." They focus on the removal of the plaque biofilm and adjunctive tissue correction aimed at creating a stable periodontium that is more resistant to future disease progression. Multiple approaches have demonstrable success, non-surgical root planing, ultrasonic debridement, surgical access flaps and full mouth debridement and all rely on follow-up appointments and adequate subsequent patient homecare to stabilize the health of the periodontium. Removal of predisposing factors such as amalgam overhangs, implant cement excess or poor crown margins, or cessation of risk contributing behaviors such as smoking or poor diabetic control are all needed. This review should be taken in the context of the full acceptance of all of these established methods of improving periodontal health and creating resistance to new disease: but this review seeks to take our thinking further and utilize current accepted paradigms regarding the etiology of the disease, the contributing host factors and using precision medicine-like approaches using our vast laboratory and animal-based knowledge of systems biology, molecular,

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and –omics knowledge, to fashion new approaches. These new approaches need to be considered, tested and decisions on their utility reached, while maintaining the current proven techniques for the good of the patient. The new approaches considered here relate to the new paradigm that susceptibility to periodontal disease is more dependent on variations in host response rather than changes in the composition of the plaque biofilm. A relatively new approach is the reduction in inflammation using the resolvins which we will not address here as there already exist multiple thorough descriptions in the literature. Similarly, we will not address changing the oral microbiome using immunization or replacement microbiome techniques, probiotics as these have similarly been addressed previously. We will address via examples, three techniques involving epigenetics, modifying the genome by changing genetic haplotypes or SNPs, and using CRISPR approaches to delete genes involved in the inflammatory response.

Microbial biofilm, quantity and composition and duration of exposure, in balance with a host response that is either resistant or over–sensitive, will determine the susceptibility to periodontitis and peri-implantitis. These are essentially chronic inflammatory reactions that are destructive to the host tissues. The term dysbiosis is currently in use and is defined here as the change in microbial flora from one that does not cause the host to over-react, to a flora that the host mounts a disproportionate challenge to, and thus a destructive inflammatory host response ensues. There is interplay between the host and the microbial plaque biofilm and certain host's genetic makeup invokes dysbiosis readily whereas other individual's host defenses may not result in the same chronic inflammatory destruction seen with the same microbial challenge. It is these hyper-inflammatory responders and their etiopathogenic make-up that we are considering modifying therapeutically in this review. Thus changing the innate or adaptive immune response could change the inflammatory response and thus change the course of the disease. We will present data and discussion on therapies: (1) that could enhance inflammation-related netosis; (2) reduce hyper-inflammation related to epigenetic modifications of innate immune receptors of periodontal tissues; and (3) reduce hyper-inflammatory states caused by an IL-8 haplotype.

7.2 Modification of Netosis in Periodontal Disease as a Potential Therapy

Neutrophil extracellular trap (NET) formation is one of the key elements in many recently discovered innate immune functions that have gained much appreciation due to its link to various types of pathogenic interactions and chronic inflammatory and infective diseases. Periodontitis is one of the most prevalent chronic diseases in humans [1–4] and this disease serves as a model testing ground for discovering the molecular triggers for netosis and the design of therapies to either enhance or reduce netosis.

Numerous bacterial species have been reported to induce and be trapped by NETs, including periodontal pathogens [5–7]. *F. nucleatum* is one of the most abundant species in the oral cavity of healthy and diseased individuals [8]. *F. nucleatum* has a role in the periodontitis-related biofilm, due to its remarkable adhesive and adaptative properties [9]. Also, it has an essential role in supporting the growth of various bacterial species [9–11]. Being a bridge bacterium in the interaction between early and late oral colonizing bacteria, it is a key pathogen in the development of the dental plaque biofilm [11]. Thus, it is linked to various forms of periodontal diseases starting from mild gingivitis to advanced periodontitis [8, 12–14]. Consistent with previous observations, *F. nucleatum* (MOI 1:10) was able to form NETs which were clearly visible by fluorescence microscopy and SEM and thus we chose *F. nucleatum* as a model organism to further investigate NET production and mechanisms governing NET formation in vitro.

While the list of microbes and molecules capable of stimulating NET release is increasing, their induced response is not identical. Bacterial-host interaction elects different immune responses via a varied group of receptors and cytokines

[15]. By using qPCR arrays, custom designed to detect innate immune, apoptosis, and GPCR signaling pathways, we sought to investigate which pathway is most likely related to *F. nucleatum*-induced NETosis. Interestingly, our data showed that *F. nucleatum* markedly upregulated NOD1 and NOD2 in PMN during netosis, suggesting a key role for these receptors in NETosis. Furthermore, to determine if the upregulation of NOD1 and NOD1 receptors occurs with other bacterial stimulation, we challenged neutrophils with *P. gingivalis* and *A. actinomycetemcomitans*. We found that between all groups, *F. nucleatum* caused the most significant upregulation in NOD1 and NOD2. NOD1 and NOD2 receptors are the first NLRs reported as direct intracellular pattern-recognition receptors (PRRs) [16]. It has been shown that NLRs are necessary sensors of specific PAMPs. However, the mechanism by which NLRs detect the PAMPs remains poorly understood, and it is still unclear if they directly bind to PAMPs, or through adaptor proteins [17, 18]. Given that NOD1 and NOD2 were significantly increased when challenged with *F. nucleatum*, we further analyzed the role of NOD1 and NOD2 receptors in NETosis.

The HL-60 cell line is commonly used for neutrophil studies. Using the CRISPR-Cas9 gene editing system we developed NOD1 and NOD2 knockout HL-60 cell lines, for our experiments. When challenging NOD1 and NOD2 knockout HL-60 with *F. nucleatum*, the NET release was significantly reduced when compared to control cells. Quantification of histone-associated DNA fragments (H1, H2A, H2B, H3, and H4) in the cytoplasm of NOD1 knockout cells was significantly reduced, but NOD2 knockout cells showed no significant changes compared to control. This indicates that NOD1 but not NOD2 is associated with histones release. In addition, staining with neutrophil elastase (NE) revealed that while *F. nucleatum* successfully induced NET formation in HL-60 wild-type cells, NOD-1 knockout HL-60 cells formed significantly less NETs. Thus, confirming that NOD1 and NOD2 receptors play a role in the NET formation, with NOD1 being more associated with histone release. Knowing the importance of NOD1 and NOD2 receptors in NET formation, we further investigated the downstream activation of these receptors and its link to other essential NET-related proteins such as PAD4, MPO, and NE. Peptidylarginine deiminase enzymes (PAD4), catalyze protein transformation into peptidyl-citrulline in a Ca^{2+}-dependent manner [19]. PAD4 citrullination of histones is essential for chromatin decondensation as a crucial step for NETs formation [20]. Thus, we investigated the link between the PAD4 enzyme and NOD-like receptors. *F. nucleatum* successfully induced PAD4 activation and citrullination of histones as detected by immunostaining of Cit-H3 confirming the role for NOD1 and NOD2 in NETosis.

Treating neutrophils with the MDP ligand strongly upregulated NOD2 receptor transcription, while C12-iEDAP ligand was insignificant in NOD1 upregulation. Both NOD1 and NOD2 ligands significantly upregulated IL8 expression, indicating that despite the insignificant upregulation of NOD1, its receptor activity was increased. Moreover, each bacterial species has a different combination of surface antigens that result in variations in the stimulation of host cells. The fact that *F. nucleatum* highly upregulates NOD1 in neutrophils illustrates that there might be a peptide other than that present in C12-iEDAP that is more specific to the peptidoglycan of *F. nucleatum* resulting in NOD1 activation. NOD1-specific inhibitor (ML130) significantly downregulate PAD4 activity while NOD2 inhibitor (GSK717) had no significant results, confirming that only NOD1 is related to histones released via PAD4 activation. These data coincide with our findings in HL-60 knockout histones quantification. The citrullination of proteins by PAD enzymes is regulated at a transcriptional, translational, and activation levels [21]. We found that PAD4 activity significantly increased with NOD1 ligand stimulation. Moreover, this increase in PAD4 activity was reduced when pretreated with NOD1 inhibitor. On the other hand, NOD2 stimulation and inhibition had no significant effect on PAD4 enzymatic activity. Thus, confirming that NOD1 but not NOD2 regulated PAD4 at both transcriptional and translational levels in our experiments.

By understanding the processes that governs the NET pathway, we can further understand their role in periodontal infection and disease. Furthermore, studying the pathways of NET formation related to both oral and systemic health helps in understanding and targeting the proteins, such as PAD4 and NOD-1 and NOD-2, leading to NET-related diseases. In addition to NET's role in restricting the spread of infection, there is a growing body of evidence that links NETosis and other various systemic disorders elucidating the importance of an efficient, non-invasive therapeutic modality.

7.3 Possible Novel Therapeutic Intervention Utilizing Netosis Manipulation

A drug therapy that controls the initiation of the NET pathway, focused on the NOD receptors or PAD4, could be exploited in treating NET-related diseases such as chronic inflammatory periodontal diseases. The periodontal pocket provides an ideal niche to test such therapeutics given its accessibility, its relationship to inflamed tissue and the offending microbial biofilm and the availability of local delivery systems.

7.4 Epigenetic-Related Therapeutic Approaches and Future Perspectives

Evolutionarily epigenetic events provide homeostatic control of gene regulation. However, in dysbiosis these changes might pose significant problems in inflammatory diseases. The studies that govern epigenetic changes may provide answers to periodontal disease onset and progression from the host perspective. Because of the onset, nature, and accessibility of this disease, periodontitis serves as an excellent inflammatory disease model to study epigenetics. The main caveat is that presently the epigenetic reprogramming drugs are not target and site-specific; nonetheless, demethylating drugs such as 5-Aza-2′-deoxycytidine (decitabine) could offer the reversal of methylated genes in the inflammatory network. Further research is needed to identify drugs that could specifically demethylate DNA at a given loci. The clinically approved HDAC inhibitor, suberoylanili dehydroxamic acid (SAHA), suppresses class 1 and 2 HDACs. The inhibitor increases the acetylation of histone tails that unwinds the chromatin and induce gene expression. Histone methyltransferase inhibitor (HMTase) inhibits histone methyltransferase EZH2 and activates tumor suppressor gene expression including certain inflammatory genes. However, the systemic toxicity of HMTase is yet to be evaluated. Although many questions remain, DNA methylation and histone modifications are important targets for new drug development against periodontitis. Epidemiological studies and in vivo modeling of experimental periodontitis can offer clues in understanding bacterial-induced disease-specific epigenetic changes in DNA methylation and/or histone modifications that could point toward novel therapeutic targets.

7.5 Epigenetic Reprogramming of Epithelial Cell TLR2 Signaling by *Porphyromonas gingivalis*

Pathogen-mediated gain of epigenetic reprogramming in humans is recently being implicated in cancer and immune-mediated diseases. Periodontitis is an immune-mediated disease instigated by multiple biofilm pathogens including *Porphyromonas gingivalis* that significantly possesses numerous virulence factors. The host innate immune system recognizes *P. gingivalis* mainly through TLR2 and TLR4. Nevertheless, *P. gingivalis*-induced epigenetic reprogramming remains to be elucidated. With this background, we sought to characterize *P. gingivalis*-induced epigenetic reprogramming in human gingival epithelial cells (HGECs), hypothesizing that epigenetic modifications by this bacterium at the chromatin level may induce periodontal disease susceptibility in humans. To perform this study we isolated HGECs from patients and characterized them based on their inflammatory responses

to *P. gingivalis* as "normal" (normal cytokine responders) "hypo-responsive" (diminished cytokine responders). DNA was extracted from these cells and subjected to methylation-specific qPCR array for the TLR-related gene network. The "normal" cells were stimulated with *P. gingivalis* (MOI:5) for 30 min at 0, 4, 8, and 16 h time intervals. After 48 h of last stimulation, the cells were split and repeated *P. gingivalis* stimulation cycle with or without 1 μM of 5-Aza-2′-deoxycytidine also known as decitabine (DNA methyltransferase inhibitor). After which, TLR2 promoter CpG methylation was determined by methylation-specific qPCR assay. DNA from healthy and periodontitis-affected tissue was subjected bisulfite sequencing to determine the extent of TLR2 promoter CpG methylation. Time course *P. gingivalis* stimulation was done and the protein was subjected to immunoblot against various histone antibodies.

We were able to identify TLR2 promoter CpG hypermethylation in "hypo-responsive" cells. This methylation status was reversed by the use of decitabine restoring the TLR2 and pro-inflammatory cytokine expression. Interestingly, we observed the induction of TLR2 promoter CpG methylation by *P. gingivalis* in HGECs. Moreover, the tissue from periodontal sites showed sporadic TLR2 CpG methylation compared to healthy sites as revealed by bisulfite sequencing. We also observed rapid histone modifications upon *P. gingivalis* stimulation in HGECs.

Our conclusions were that we were able to show previously unknown specific epigenetic reprogramming of epithelial chromatin by *P. gingivalis* that may contribute to periodontal disease susceptibility. Furthermore, we were able through the therapeutic use of decitabine, to remove the methyl groups (albeit not precisely and specifically), to restore cellular TLR2 function, and thus hypothetically to reduce susceptibility to periodontal disease [22, 23]. Clearly, this is early work but the promise in inflammation, infection, and cancer therapeutics of epigenetic correction needs to be thoroughly investigated particularly as a cancer preventive measure in sites with precancerous lesions or suspected tendencies to develop cancer [23].

7.6 Modifying IL-8 Using CRISPR: Therapeutic Possibilities in Periodontal Disease

Interleukin-8 (IL-8) is a pro-inflammatory chemokine produced by cells such as epithelial cells, fibroblasts, endothelial cells, macrophages, lymphocytes, and mast cells upon exposure to the inflammatory milieu. IL-8 secretion leads to activation and migration of neutrophils from the peripheral blood to sites of infection that manifest in the clearance of pathogens. Controlled induction of IL-8 is crucial in the maintenance of homeostatic balance. For example, elevated IL-8 induction can lead to exacerbated inflammation in chronic inflammatory diseases [24]. On the contrary, inhibition of IL-8 secretion may delay neutrophil influx creating an advantage for pathogen survival leading to chronic infection. Elevated IL-8 expression has been attributed to a number of diseases such as chronic obstructive pulmonary disease [24], hypertension [25], carcinogenesis [26]), idiopathic pulmonary fibrosis [27], and chronic periodontitis [28].

Previous studies have investigated the association of single nucleotide polymorphisms (SNPs) with the level of IL-8 gene expression [29]. The SNP rs4073 (alias −251) in the IL-8 gene has been considered functional, since the −251A allele was related to higher levels of IL-8 production in vitro, after stimulation with lipopolysaccharide and cytokines [30]. This agrees with the finding that the AA genotype of −251 SNP in the IL-8 gene was associated with greater IL-8 mRNA expression [31]. However, another study demonstrated that the TA genotype in this −251 SNP was associated with increased IL-8 mRNA levels [32].

Interleukin-8 (IL-8) gene polymorphisms have been considered as susceptibility factors in periodontal disease [33, 34]. We have used the CRISPR/Cas9 system to engineer the IL-8 gene, and tested the functionality of different haplotypes. Two sgRNA vectors targeting the IL-8

gene and the naked homologous repair DNA carrying different haplotypes were used to successfully generate HEK293T cells carrying the AT genotype at the first SNP—rs4073 (alias −251), TT genotype at the second SNP—rs2227307 (alias +396), TC or CC genotypes at the third SNP—rs2227306 (alias +781) at the IL-8 locus. When stimulated with Poly I:C, ATC/TTC haplotype, cells significantly upregulated the IL-8 at both transcriptional and translational levels. To test whether ATC/TTC haplotype is functional, we used a trans-well assay to measure the transmigration of primary neutrophils incubated with supernatants from the Poly I:C stimulation experiment. ATC/TTC haplotype cells significantly increased the transmigration of neutrophils confirming the functional role for this IL-8 haplotype.

Interleukin-8 (IL-8) is considered an important chemokine in periodontal disease. This cytokine is produced by a variety of cells and may function in concert with other members of the cytokine family to regulate the host's innate responses [1, 35]. Specifically, this cytokine attracts leukocytes from the periphery to the sites of infection and activates them to become phagocytes. Intra-cutaneous administration of IL-8 in vivo induced local exudation and long-lasting accumulation of neutrophils [36]. Although there are other chemokines involved in neutrophil recruitment to the site of infection, IL-8 receptor knock-out mice showed delayed neutrophil influx into the kidneys and bladder and were unable to eliminate bacteria from the tissues [37]. This suggests that IL-8 is indispensable for neutrophil migration and function. Neutrophil function is not only important in acute infections but also plays a major role in chronic inflammatory disorders such as periodontitis, atherosclerosis, psoriasis, rheumatoid arthritis, inflammatory bowel disease, diabetes, and cancer [38].

Recent meta-analyses showed a positive association of −251(T/A) polymorphism on IL-8 gene to chronic periodontitis [39]. Few case-control studies have investigated different haplotypes in the IL-8 gene that were found to be associated with periodontitis [40], such as the ATC/TTC haplotype whose carriers of this particular haplotype had two times higher disease susceptibility [41, 42]. In spite of this, the IL-8 protein levels in the GCF of patients were not correlated to the carriage of ATC/TTC haplotype [43]. This absence of correlation could be attributed to the limited sample size in that study, besides the individuals with chronic periodontitis enrolled in that study were not affected by the severe and generalized disease forms.

Faced with the lack of functional assays [44], we hypothesized that an in vitro study with more controlled conditions could be able to detect the potential influence of different haplotypes in the IL-8 mRNA and protein levels. This present study demonstrated that the presence of ATC/TTC haplotype can upregulate the IL-8 in both mRNA and protein levels. These higher IL-8 mRNA and protein levels coupled with neutrophil migration could explain the lower periodontopathogens levels found in patients carrying the ATC/TTC haplotype [41, 42]. Also, high levels of IL-8 may increase the inflammatory and immune response and subsequent damage to the integrity of the periodontium. We think that the present findings could explain why the periodontal destruction may occur in patients who were considered to be genetically susceptible to chronic periodontitis with a lower microbial challenge because of the presence of the IL-8 ATC/TTC haplotype than in patients without it [40–42].

The same ATC/TTC haplotype was also shown to be associated with bronchial asthma, while the rs4073T > A SNP when tested alone did not show significant association [45]. Other studies reported a similar lack of association with chronic periodontitis when an SNP was analyzed individually, but when the haplotype was considered in the analysis, the association with the disease was revealed [46]. Therefore, these studies corroborate the idea that haplotypes are more powerful for the detection of disease association than individual polymorphisms and they may give more information on the basis of disease [46]. Despite the previously reported functional role of rs4073T > A SNP, a study by Hacking et al. [47] failed to confirm this association. Hacking et al. and others suggested that the existence of another SNP (closer to rs4073T > A)

could play a role in modulating IL-8 gene expression [40].

Hence, we noticed in our study that the +781(C/T) SNP rs2227306 in the IL-8 gene seems to influence the IL-8 at both mRNA and protein levels, considering that the difference in the haplotypes analyzed here was related to the alleles in that position.

A study by Ahn et al. [48], in patients with idiopathic pulmonary fibrosis (IPF), showed increased IL-8 levels in patients carrying the A allele at the rs4073T > A SNP. The authors used a luciferase assay to measure the activity and determined the level of IL-8 in the presence or absence of promoter SNP. They found increased luciferase activity in the presence of rs4073T > A SNP on the promoter of IL-8 gene. The authors concluded that IL-8 promoter SNP may increase susceptibility to the development of IPF via the upregulation of IL-8 [48]. By using a similar reporter vector system, Meade et al. [49], investigated bovine IL-8 promoter haplotypes in vitro. The authors found that the luciferase promoter carrying one of the haplotype IL-8-h2 (C–GTAC) highly upregulated luciferase activity upon LPS and TNF stimulation confirming SNP functionality and suggesting a differential transcriptional factor binding to IL-8-h2 promoter (such as C/EBP, Oct-1, NFκ B, and NFAT) [49]. Hacking et al. [47] observed that C/EBPb (CCAAT/enhancer binding protein-beta) bound to the transcriptional complex in the presence of rs4073T > A allele in respiratory epithelial cells but not in primary lymphocyte cells suggesting cell-type specificity in transcriptional regulation [47]. Although the luciferase reporter assays is an accepted technique that can be used to test the functionality of SNP, we believe that it is an artificial system that does not contain complex regulatory elements found on the chromatin. To overcome this gap in the functional analysis of haplotypes and test our hypothesis, we adopted a novel technique called clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided Cas9 nuclease system [50] to edit the IL-8 gene in human embryonic kidney cell line (HEK293T). This technology is currently a burning topic in the field of genome editing and an invaluable tool to engineer genomes of choice [51]. For generating IL-8 editing cell lines, we choose HEK293T cells as the model system as this cell line is commonly used with CRISPR/Cas9 technology, making them a well-established model system to test the efficacy of RNA-guided endonucleases in gene editing [52]. With this technique, we were able to successfully edit the IL-8 gene within the HEK293T genome to carry different haplotypes with unprecedented precision and ease. Further, we were able to test the effect of different haplotypes in the IL-8 gene transcription, protein and also functionally evaluate their ability in modulating neutrophil transmigration. The methodology used for this purpose offers a simple and applicable framework for generating validated edited cell lines but also could be utilized therapeutically. Thus modifying the IL-8 haplotype by CRISPR, or other gene modifying techniques, could be performed locally to change susceptibility to periodontal disease and create a novel permanent therapeutic approach.

Taken together, our data provide evidence that carriage of the ATC/TTC haplotype in itself may increase the influx of neutrophils in inflammatory lesions and influence disease susceptibility. Thus using CRISPR or some other gene modifying technique, we can reduce the hyper-inflammatory effect of the IL-8 haplotypes associated with susceptibility to periodontal disease. In summary, our data demonstrate that the ATC/TTC in the IL-8 gene can have a positive outcome on the transcriptional and translational levels of the IL-8 gene and thus may modulate neutrophil recruitment at the site of infection. Because of the critical role neutrophils have in periodontal disease, it is plausible that IL-8 haplotypes could contribute to periodontal disease susceptibility and thus CRISPR could be utilized in periodontal therapy.

7.7 Conclusion

The backbone of periodontal therapy will always be the mechanical and chemical treatment of the dental plaque biofilm, profession-

ally and through home care preventive practices. Multiple advances have been made in both areas, whether it be the guided biofilm therapy that incorporates a system of ultrasonics and air polishing to remove offending biofilm and calculus as needed, or the administration of chemical antiseptic mouthwashes or antibiotics. Despite these advances and their wide adoption, periodontitis and peri-implantitis are still highly prevalent and novel therapeutics focusing on individual susceptibility have a place. This review has considered three such individual host response-related therapies that aim at modifying the host in a precision medicine like approach.

These new approaches need to be considered, tested and decisions on their utility reached, while maintaining the current proven techniques for the good of the patient. The new approaches considered here relate to the new paradigm that susceptibility to periodontal disease is more dependent on variations in host response rather than changes in the composition of the plaque biofilm. Thus we have introduced three examples covering epigenetic modifications and using CRISPR approaches to delete genes and or modify haplotypes involved in the inflammatory response.

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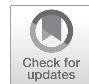

8 Cellular Senescence in Aging Mucosal Tissues Is Accentuated by Periodontitis

J. L. Ebersole and O. A. Gonzalez

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8.1 Cellular Senescence and Aging

Understanding the mechanisms that cause the progressive decline of cellular and tissue function is a clear strategy to enhance the quality of the aging process, as well as having the potential to extend the lifespan. Normal cells do not divide indefinitely due to replicative senescence [1–3]. Telomeres are the DNA and proteins that cap and stabilize the ends of linear chromosomes, preventing their degradation, and the erosion of telomeres is a crucial mechanism responsible for the replicative senescence of mammalian cells [4, 5]. Multiple base pairs of telomeric DNA are lost with each cell cycle, and proliferating cells experience progressive telomere shortening. These eroded telomeres generate a persistent DNA damage response that initiates and maintains the senescence growth arrest [6–8]. Once the telomeres become critically short, they lose their function and signal normal cells to cease proliferation with a characteristic senescent phenotype [4] (Fig. 8.1).

While cell division is essential for the survival of multicellular organisms that contain renewable tissues, replicative senescence is an example of a more general process, termed cellular senescence, which arrests the growth of cells in response to many stimuli. Genomes are continually damaged by environmental insults, oxidative metabolism, and, in dividing cells,

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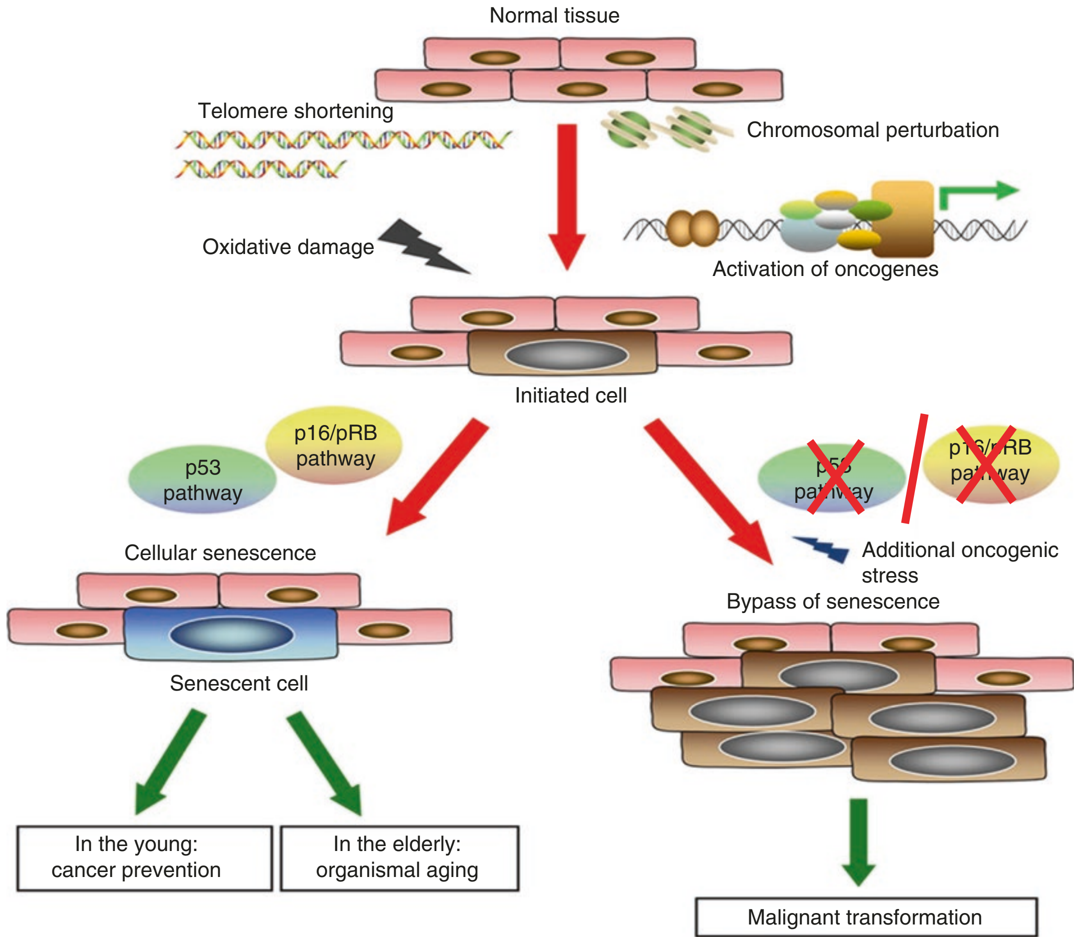

Fig. 8.1 Schematic of cellular senescent pathway expressing in neoplastic development and aging. (Adapted from https://www.nestacertified.com/hallmarks-of-aging-role-of-glycation-part-7-cellular-senescence/)

errors in DNA replication and mitosis. Depending on the level and type of damage, cells can attempt repair, or die [9, 10]. However, complex organisms have evolved at least two cellular mechanisms, apoptosis and cellular senescence, to suppress the proliferation of defective cells, often at risk for oncogenic transformation (Fig. 8.1). Whereas apoptosis kills and eliminates defective cells, cellular senescence irreversibly arrests their growth. Recent findings suggest a molecular complexity leading to the expression of a senescent phenotype and, as such, have suggested other consequences of cellular senescence for multicellular organisms. Cellular senescence has a major role in various complex biological processes, including tumor suppression/promotion, normal tissue repair, and altered cell and tissue functions in aging [11]. The majority of cell types that have the capacity to divide can undergo senescence when appropriately stimulated [12, 13], as a physiological process that can be related to changes occurring at the cellular level of aging. Thus, cellular senescence, although useful in preventing malignancy, appears to also promote aging processes.

It is now well recognized that the biology of aging is impacted by both environmental and genetic factors with accumulated DNA damage causing declines in function, and genetic makeup pre-disposing to variation in the rate of damage accumulation and rapidity of functional decline [14, 15]. Senescence-inducing stimuli are represented

by a variety of stressors, including strong mitogenic signals, such as those delivered by certain oncogenes or highly expressed pro-proliferative genes [16–19], DNA damage, and non-genotoxic chromatin perturbations that lead to a state of permanent cell cycle arrest [13]. Senescence can, thus, occur following a period of cellular proliferation or with a more rapid expression in response to acute stress. Once cells have entered senescence, they cease to divide and undergo a series of morphologic and metabolic changes. While cellular senescence is considered to be important in pathophysiologic control of cellular functions, detailed information on in vivo expression in various tissues remains to be elucidated. Recent studies have provided important insights regarding the manner by which different stressors/stimuli activate signaling pathways leading to senescence. Stress-induced senescence causes cells to initiate senescence prematurely and results from DNA damage, oxidative stress, interferon (IFN)-related responses, with intracellular signaling through insulin growth factor (IGF) or mitogen-activated protein kinases (MAPK) [10, 20]. Various exogenous agents, such as histone deacetylase inhibitors, relax chromatin and can activate DNA damage response proteins, including ataxia telangiectasia mutated (ATM) and the p53 tumor suppressor [21], resulting in a senescence responses [22, 23]. These reports also indicated that growing cells appear to react to a combination of different physiologic stresses acting simultaneously, and subsequent signaling pathways activated by these stresses engage both p53 and retinoblastoma (Rb) proteins. The combined levels of activity of these two tumor suppressor proteins appear to determine whether cells enter senescence [16, 17, 19]. The senescence program activates p53 and Rb signaling, redirecting the cells away from the normal cell cycle. Thus, senescence-inducing stimuli cause epigenomic disruption and genomic damage.

8.2 Aging and Periodontitis

Epidemiological data demonstrates clearly that periodontitis increases significantly in prevalence and severity with aging [24–26] and is disproportionately exhibited in racial/ethnic minority individuals and males, in individuals with various systemic diseases, related to socioeconomic status, and even geographic location [27]. These findings are consistent with broader health data demonstrating that aging is accompanied by increased susceptibility to autoimmune, infectious, and inflammatory diseases, including periodontitis [28–32]. A recent review by Lamster [33] emphasizes the breadth of noncommunicable diseases (NCD) across aging global populations with adverse impacts on oral health. Oral health management for improved population metrics will require a better integration of emerging knowledge on aging processes and therapies, and clinical decisions within the context of the breadth of the altered biophysiology of aging patients [34].

A paradigm in periodontal disease is the requirement for the subgingival microbial ecology to trigger a chronic immunoinflammatory lesion with a range of host biomolecules contributing to the tissue destruction that hallmarks the disease [35, 36]. This alteration in the character of the host responses has been suggested to result from changes in the quantity and quality of the microbial challenge that minimize protective responses and enhance the production of mediators that can contribute to collateral tissue damage in response to the microbial changes. Thus, decreased immunity in aging accompanied by increased chronic inflammation is thought to underpin the perception of periodontitis as a “disease of aging” suggesting that it represents an inexorable process with general population susceptibility [33, 37–39]. However, data are available that demonstrates a subset of the aging population that appear resistant to this disease and an elevated prevalence of the elderly maintaining an essentially healthy oral cavity throughout the lifespan. These observations indicate that the clinical manisfestation of periodontitis appear similar in paitnets whether the are 40 or 80 years of age. Thus, the underlying biologic processes leading to the disease that have been generally defined in a normal adult patient cohort, might also be reflected in disease in the aged patieint (Fig. 8.2).

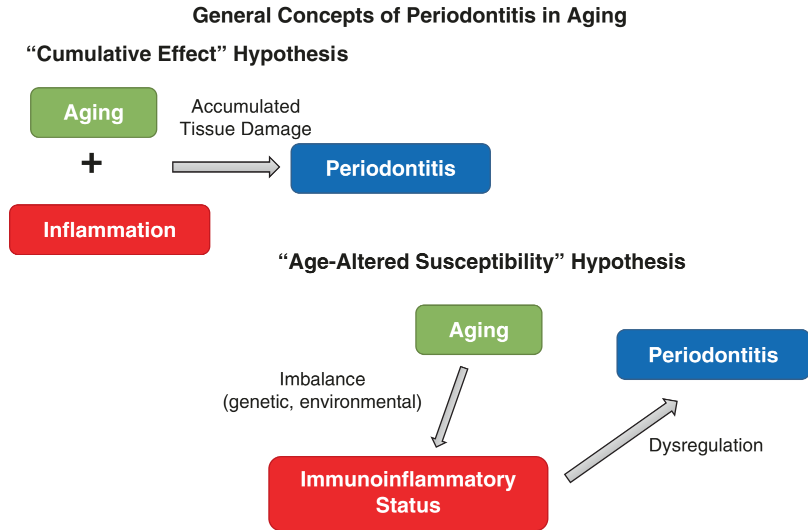

Fig. 8.2 Models of potential interactions of aging processes, inflammation, and periodontitis comparing a cumulative effect interpretation versus periodontitis being a reflection of patient-specific altered susceptibility occurring via combinations of genetics and environmental stressors. Adapted from Hajishengallis [120]

To more fully elucidate the effects of aging on the mucosal responses and periodontal milieu related to oral health and periodontitis, we conducted an investigation using a nonhuman primate model of periodontitis that has extensive similarities in clinical, microbiological, and immunological features to human periodontitis [40–43]. This nonhuman primate (rhesus) model of periodontal infection and inflammation [40, 43–45] is being used to document aging-associated gingival transcriptome and microbiome changes to elucidate altered molecular pathways that could enhance risk for disease and/or correlate with the disease initiation and progression. These studies are documenting the transcriptome in gingival tissues, as a representative mucosal tissue, obtained from animals representing young individuals (approximately 10 year old humans) to aged individuals (approximately 70–80-year-old humans). The transcriptome of aged gingival tissues in nonhuman primates exhibited significant differences in various innate and adaptive immune pathways that are critical for maintaining a symbiotic relationship with the oral microbiome and could predispose to microbial dysbiosis and periodontitis (Fig. 8.3). We have identified significant differences in apoptosis pathway, inflammasome components, pattern recognition receptors, hypoxia responses, antigen processing, osteoclast differentiation, and B cell, T cell, and macrophage features of gene expression profiles associated with aging, often even in healthy aged gingival tissues [46–57]. In particular, aging seems to enhance an anaerobic gingival environment coupled with an ability of gingival tissues/cells to sense and respond to microbes (in particular invasive pathogens) that appears to be critically compromised with aging. It is unclear at this point if these changes are related with specific cell types (epithelial cells, fibroblasts, immune cells) or is a general characteristic for the entire periodontium. Thus, a story is evolving suggesting that gingival tissue characteristics in aging are significantly different even in health and that these tissue alterations may presage the aging individual to an enhanced risk of destruction from the chronic periodontal infections (Age-altered susceptibility hypothesis, Fig. 8.2).

The analysis presented here hypothesized that the mucosal gingival tissues in aging animals demonstrated enhanced expression of genes reflective of cellular senescence in clinically healthy tissues, reflecting an increased risk for infection and alterations in function accompanying inflammation. Moreover, genes representing this cellular pathway would be significantly enhanced in periodontitis tissues resulting from the chronic stressors of infection and the local immunoinflammatory lesion of the disease. Individual samples were used for gene expression analyses, and Table 8.1 pro-

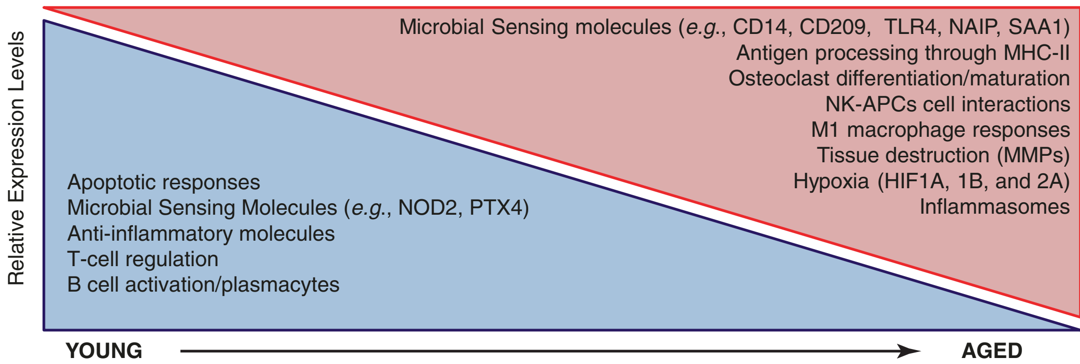

Fig. 8.3 Overview of outcomes of reports from a nonhuman primate model of naturally occurring and ligature-induced periodontitis related to the gingival transcriptome and aging [46–56]

Table 8.1 Cellular senescence genes examined

ABL1	CDKN2C	GSK3B	MKK3/6	RBL1	TFAP2
AKT1	CDKN2D	HSD17B12	MDM2	RBL2	TFDP1
ATM	CHEK2	HRAS	MORC3	RP3-398	TGFB1
ATMIN	CITED2	ID1	MYC	RRM2B	TGFB1I1
BMI1	CIZ1	IFNG	NAB1	SERPINB2	THBS1
CALR	CLASPN	IGF1	NFKB1	SIRT1	TP53
CCNA2	COL1A1	IGF1R	NLGN4X	SMAD3	TP53BP1
CCNB1	CREG1	IGFBP3	NONO	SMAD4	TPT1
CCND1	DKKL1	IGFBP7	NOX4	SMOC1	TSP
CCNE1	E2F	ING1	OCN	SMU1	TWIST1
CD44	E2F1	IRF3	PAI-1	SOD1	uPA
CDC25C	ETS1	IRF5	PCNA	SOD2	VAMP3
CDK2	ETS2	IRF7	PIK3CA	SPOCK2	VIM
CDK6	FBN	KIAA0907	PM20D2	SPOCK3	
CDKN1A	FN1	MAPKAP1	PRKCD	TBX2	
CDKN1B	GADD45A	MAP2K1	PTIRM1	TBX3	
CDKN2A	GFI1B	MAPK14 (p38)	PTBP1	TERF2	
CDKN2AIP	GRM7	MAP2K3	RB1	TERT	

vides a listing of the gene IDs ($n = 103$) included in this analysis. Figure 8.4 provides a heatmap description of the differences in cellular senescence gene expression profiles across ages in healthy tissues. Compared to healthy adult tissues, a set of genes were elevated or decreased in expression in even the healthy gingival tissues from aged animals. Generally minimal differences were observed in the expression of these genes in young or adolescent animals compared to the adult tissues. Fig. 8.4 also demonstrates a comparison of the cellular senescence genes in periodontitis tissues of adult and aged animals compared to healthy tissues from the same age groups. The most obvious differences were a widespread increase in the expression of cellular senescence genes in periodontitis tissues from both adult and aged animals. We summarized these findings using a Principal Components Analysis with the data demonstrating the rather distinctive expression of cellular senescence genes in the healthy aged gingival tissues (Fig. 8.5a), and even more pronounced separation of the aged periodontitis samples (Fig. 8.5b). The genes that were contributing to this variation in health included 14/17 primarily related to enhanced cellular senescence and with periodontitis; 13/14 gene transcripts were associated with enhanced cellular senescence.

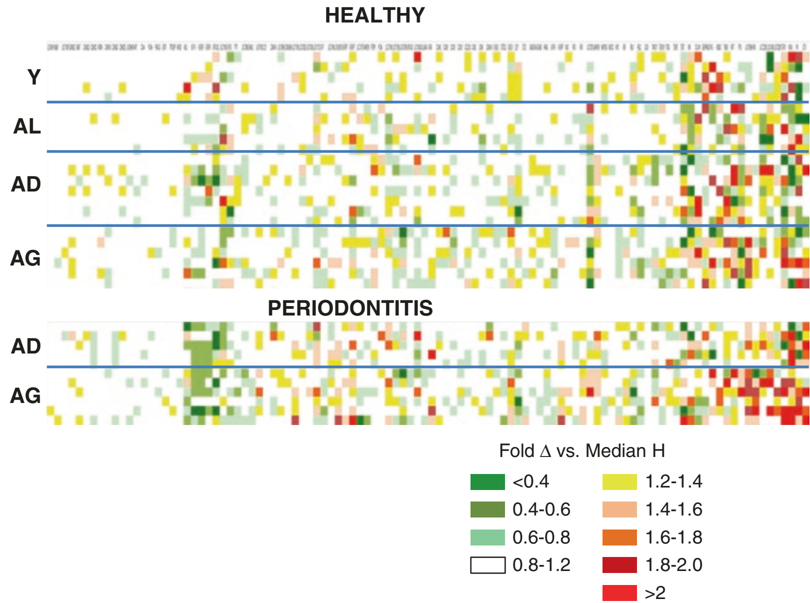

Fig. 8.4 Heatmap of fold-differences in gene expression profiles (compared to population median) for 103 cellular senescence genes in healthy gingival tissue from young (Y; <3 years), adolescent (ADO; 3–7 years), adult (AD; 12–16 years), and aged (AG; 18–23 years) rhesus monkeys. Also similar data presented for periodontitis tissues from adult and aged animals. Each box in a row represents the results from a single animal

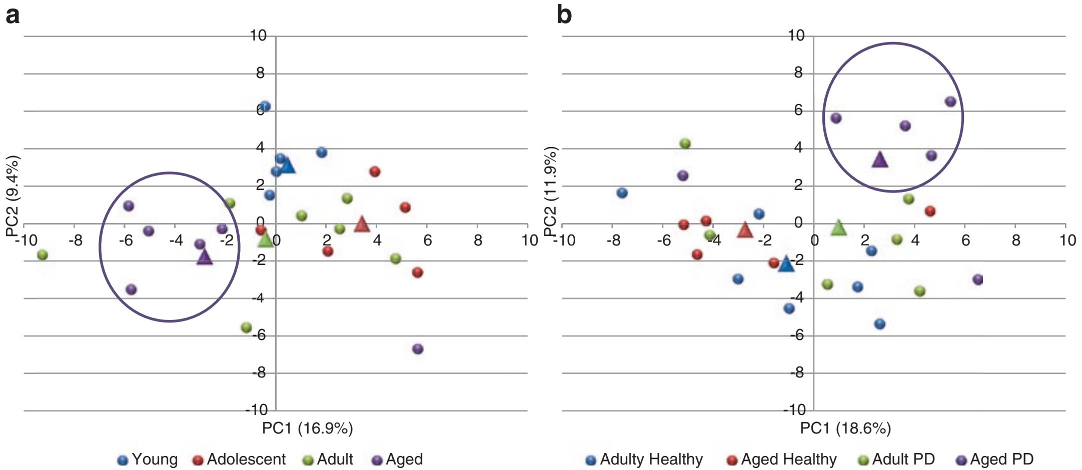

Fig. 8.5 Principal components analysis of cellular senescence gene expression patterns in the groups of nonhuman primates. Each point denotes the results from the gingival transcriptome from one animal. The matching colored triangles denote the group means

A KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of the altered gene expression patterns is depicted in Fig. 8.6 highlighting the genes that were up- or down-regulated in gingival tissue samples with periodontitis. As can be noted the majority of genes related to the cellular senescence were up-regulated across multiple functional processes. Only the DNA damage response gene interactions showed a predilection for being down-regulated.

Thus, a summary of these experimental findings support alterations in the gingival tissue transcriptome tending toward increased cellular senescence even in clinical healthy tissues from aged individuals. These patterns of senescence are enhanced in the tissues from periodontitis lesions. However, this cross-sectional study does not determine if elevated cellular senescence predisposes the tissues to disease initiation and progression, or if an aspect of the biology of

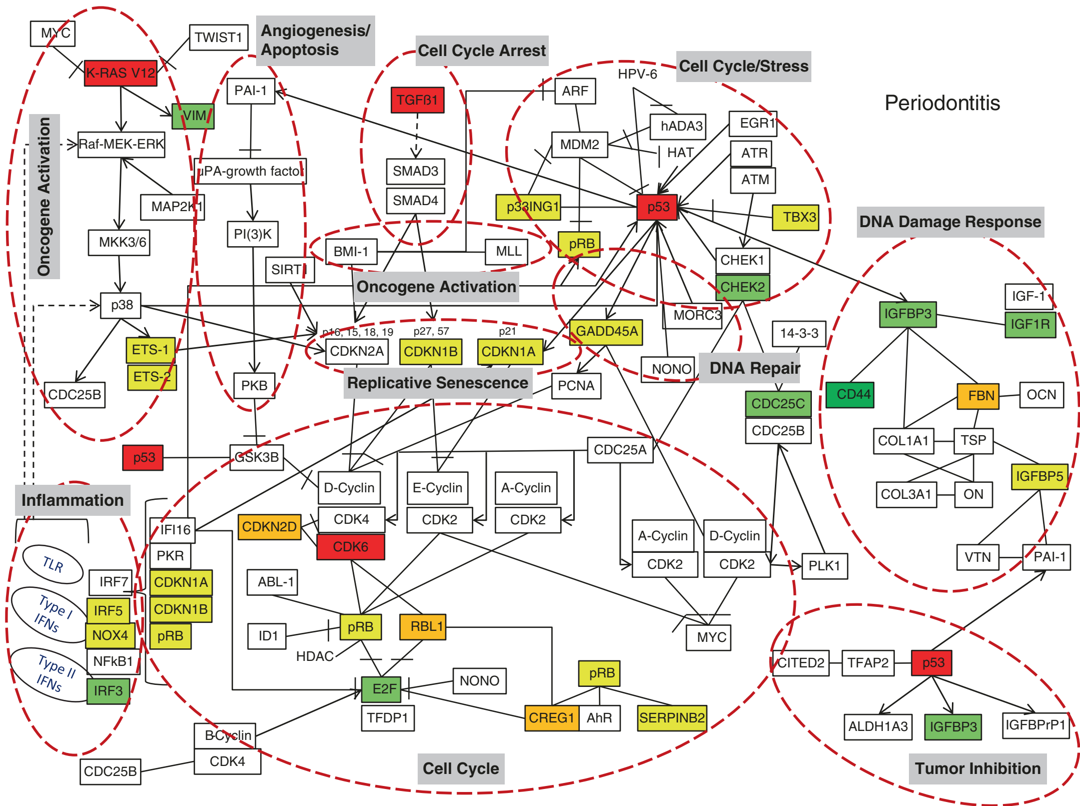

Fig. 8.6 Pathway schematic demonstrating the cellular senescence genes whose expression was increased (yellow→orange→red) or decreased (light green→dark green) in adult/aged periodontitis versus levels in clinically healthy tissues from the same age groups

periodntal disease triggers the cells and tissues toward senescence, leading to a greater extent and severity of disease in aging. Longitudinal studies will be required to more definitively document the temporal nature of the biologic and clinical changes.

8.3 Senolytics and Geroprotective Therapeutics in Periodontitis

The human population demonstrates extensive variation in aging effects that are a composite of genetics and environmental exposures at the individual subject level [58]. Cellular senescence is an irreversible process that arrests cell growth, leading to cells escaping the normal cell cycle and losing the ability to proliferate [3]. The normal replicative processes for cells can be short-circuited into a senescent state by assorted extrinsic and intrinsic stimuli, such as shortened telomeres, unrepaired DNA damage, oxidative stress and radiation, and molecularly via enhanced expression of oncogenes coupled with the loss of effective tumor suppressor gene functions [59] contributing to neoplastic and degenerative changes, although senescence is also a critical process in development, wound healing, and tissue repair [11]. Thus, this pleiotropic process is beneficial in early life but detrimental in late life and is reflected by a senescence-associated secretory phenotype (SASP) with senescent cells demonstrating heightened harmful pro-inflammatory responses [14, 60].

Within the context of immune senescence, these intrinsic and extrinsic factors are modulated and modified via the "immunobiography" of each individual related to the individuals "antigenic ecospace" triggering all immunological experiences across the lifespan and dependent upon the immune system plasticity and memory [39, 61]. The summation of this population variation has recently been portrayed within the treatises of "biological aging" [4, 58, 62–65]. Evolving strategies based on biologic aging are focusing efforts into development of "geroprotective" and "senolytic" therapeutics to enhance healthy biologic aging [66–71].

Geroprotective drugs focus on anti-aging targets that are consider effectors on the fundamental physiological causes of aging and age-related diseases, and thus these might be expected to extend the lifespan. Caloric-restriction (CR) and CR-mimetic drugs have been shown to have some geroprotective effects on aging [72, 73]. Examples of current molecules undergoing studies as geroprotective agents include melatonin, L-carnosine, metformin, and histone deacetylase inhibitors. A common theme of this approach is that calorie restriction works by reducing insulin and insulin-like growth factor-1, as well as increasing insulin sensitivity with gene expression changes consistent with longevity. However, CR has some challenges being accepted by large populations, thus the strategy of development of CR-mimetics to target this IGF-1 pathway.

Metformin (dimethylbiguanide) is currently a preferred hyperglycemic agent to manage type 2 diabetes mellitus (T2DM). Metformin activates AMP kinase (AMPK), which is a cellular energy sensor and inhibits the mammalian target of rapamycin (mTOR), thus reducing protein synthesis [70, 74, 75]. More recently this drug has been shown to significantly increase lifespan and delay the onset of age-associated decline and have therapeutic potential for the prevention and treatment of various aging-associated pathologies. In addition, related to periodontitis and the description of "inflammaging," metformin has clear beneficial effects in lowering chronic inflammation [74].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a pineal indole hormone that orchestrates numerous physiological functions. Melatonin's actions are partially mediated by the circadian multi-oscillator system, as well as exerting a strong antioxidant activity by scavenging OH, O2–, and NO. *Loss of mitochondrial function* contributes to aging throughout the body, which contributes to age-related diseases such as neurodegeneration, diabetes, and obesity [76–79]. Melatonin also increases the expression of some mitochondrial genes and by preserving mitochondrial function and promotes longevity-associated proteins (e.g., **SIRT1)**, thus exerting a specific action on a fundamental cause of aging processes. During aging, melatonin secretion declines and a progressive deterioration of the circadian system is observed. This melatonergic dysfunction in aging has been suggested to support that melatonin administration may positively impact senescence-associated pathologies. This concept was supported by data showing the ability to extend the lifespan of multiple species, from insects to mammals, although in mammals melatonin does not seem to strongly decelerate the basal aging processes.

Increasing life expectancy is accompanied by an increased risk for the development of age-associated diseases (coronary heart disease, cerebrovascular disease, cancer, arthritis, dementia, cataract, osteoporosis, type II diabetes, hypertension, Alzheimer's disease, Parkinson's disease, etc.), which negatively impact healthy aging. A general concept of natural antioxidants as geroprotectors has been proffered. L-carnosine is a dipeptide of the amino acids β-alanine and L-histidine that it is a potent natural hydrophilic antioxidant, which preserves human tissues from oxidative stress via its activity as a scavenger of hydroxyl and superoxide radicals and, even more efficiently, of the singlet oxygen molecule. Carnosine levels in the body decline with age. Facets of its function are hallmarks of aging in that it rejuvenates connective tissue cells, with positive effects on wound healing, and beyond its capacity as a potent antioxidant, it provides a broad-spectrum effect to minimize protein degradation [80–82].

Mechanisms of epigenetic regulation continue to increase as interesting targets contributing to a variety of processes related to aging (e.g., senescence, frailty, genomic instability, and carcinogenesis) [83–85]. The methylation and acetylation processes that occur at the genome level of regulation gene expression profiles have provided the impetus for drug targeting related to aging. Histone deacetylases (HDAC) are critical components of these processes, and HDAC inhibitors are considered promising as geroprotective pharmaceuticals. HDAC inhibitors include chemical classes, such as cyclic peptides, hydroxamic acids, short chain fatty acids (e.g., butyrate), and synthetic benzamides, that vary in particular HDAC class specificity [86]. HDAC inhibitors reverse the deacetylation of histone tails and activate the expression of particular genes. "Connecting-the-dots" suggests that since many biosynthetic and metabolic gene expression patterns are decreased with aging [87], improving their transcriptional expression through therapy with HDAC inhibitors could alter these age-associated functional declines. Moreover, targeted inhibition of HDACs could help regulate inflammatory and stress responses to improve aging and longevity [88]. Thus, these geroprotective therapeutics may reduce the risk of developing aging-related diseases, including periodontitis and enhance healthy aging.

It is well demonstrated that senescent cells accumulate in mammalian tissues during aging and help to trigger age-related pathologies [15]. While the senescent cells comprise a small number of the total body population, they have a propensity for the production of pro-inflammatory cytokines, chemokines, and extracellular matrix proteases, the portfolio of which drive the senescence-associated secretory phenotype SASP and contribute to aging [89, 90]. Recent studies in animal models have demonstrated that clearance of senescent cells extends lifespan by delaying aging-associated maladies [91–93]. Senolytic drugs are generally small molecule therapeutics designed to selectively induce the death of senescent cells to ameliorate age-related diseases. One of the most promising of these types of drugs is based on the compound rapamycin. Rapamycin acts by the inhibition of mTOR that is involved in sensing cellular nutrient levels regulating energy conservation and contribution to anti-aging effects. This crucial cellular protein is also a major target in caloric restriction effects on aging. While initially employed as an immunosuppressive agent, it was noted to extend lifespan in yeast and worms, and later in mice [94–96]. The field has expanded into the development of "rapalogs." Everolimus, an anti-cancer agent, was found in humans to help alleviate age-associated immune deterioration [96–98]. Since immune system aging is a major cause of disease and death, a recent study also demonstrated that everolimus treatment improved active immune responses to influenza vaccines in older subjects [99].

Transcript analysis of senescent cells showed an up-regulation of pro-survival genes and apoptosis resistance phenotype [100]. Dasatinib, an anti-leukemia drug, and quercetin, one of the most abundant dietary plant polyphenol flavonoids, could selectively kill senescent cells; and a combination of the drugs was synergistic in this effect [101]. Treatment with dasatinib and quercetin prevented cell damage, delay physical dysfunction, and extend the lifespan of mice, which would promote healthy longevity [102, 103].

An additional category of senolytic drugs have been identified that are classified as Heat shock protein (Hsp) 90 inhibitors [104, 105]. Hsp90 is an ATP-dependent molecular chaperone exploited by malignant cells to support activated oncoproteins and stabilizes proteins required for cell survival and thus is a target considered in cancer therapeutics. These inhibitors include natural products like geldanamycin (1,4-benzoquinone ansamycin) a macrocyclic polyketide from *Streptomyces hygroscopicus* [106, 107]. Geldanamycin induces the degradation of proteins that are mutated in tumor cells through the function of HSP90 and has led to the development of analogues, such as 17-*N*-Allylamino-17-demethoxygeldanamycin (17AAG). Radicicol, e.g., monorden, is also a natural product from *Pochonia chlamydosporia* produced by polyketide synthases and binds to Hsp90 [106, 108]. Effective cancer treatment, and likely

improved management of the adverse effects of senescent cells, includes strategies that would target multiple signaling pathway networks, and Hsp90 is a key element responsible for features of protein folding in multiple signaling networks that can prevent apoptosis. Gamitrinib-triphenylphosphonium (G-TPP), an anti-cancer agent, is a synthesized small molecule that acts on mitochondrial Hsp90 to enable the initiation of apoptosis and effects cancer and senescent, but not normal cells [109], and thus may have a use in therapeutics of aging.

8.4 Chronological and Biological Aging

The immune system in aging demonstrates alterations in both innate and adaptive immune cell functions and effector biomolecules, which constitute the description of immunosenescence and inflammaging, albeit, the actual cause(s) of these abnormal processes for immunoactivation remain to be established [110, 111]. These alterations represent only one aspect of a broad array of features of age-related diseases, and the evolving concept of "Geroscience" that represents an interdisciplinary approach to address aging in the context chronic age–related diseases and geriatric syndromes [61, 112]. A component aspect underlying these interrelationships is the evolving area of biologic aging. An increasing number of investigations have begun to explore the variation in healthy/unhealthy aging across the population and have suggested the existence of arrays of variable that could explain and/or predict biological aging and model the rate of senescence [4, 58, 65, 113], including specific metabolic and genetic/epigenetic markers [76, 77, 114, 115], coupled with telomere shortening [62] and genes controlling the aging process [5], as examples of targets in this research discipline.

Existing approaches considered in preventing/managing periodontitis in aging have included dietary manipulation, pharmaceuticals for impacting both the microbiome and host responses, and behavioral strategies [34, 116]. However, with regard to the local and systemic chronic inflammation characteristic of periodontitis, there is clearly a litany of systemic diseases related to periodontitis that likely reflects bacterial translocation through damaged periodontal tissues accompanied by chronic elevation in systemic inflammatory responses as co-morbid contributors with other risk factors for general disease processes [117, 118]. The unremitting elevated inflammation observed in untreated periodontal lesions of aged individuals may reflect an ineffective/dysregulated host response, allowing the persistence of pathogenic biofilms [28]. However, chronic periodontitis exists over decades in individual subject, generally demonstrating a clinical onset by the third-fourth decade of life [26, 119]. As indicated in Fig. 8.7

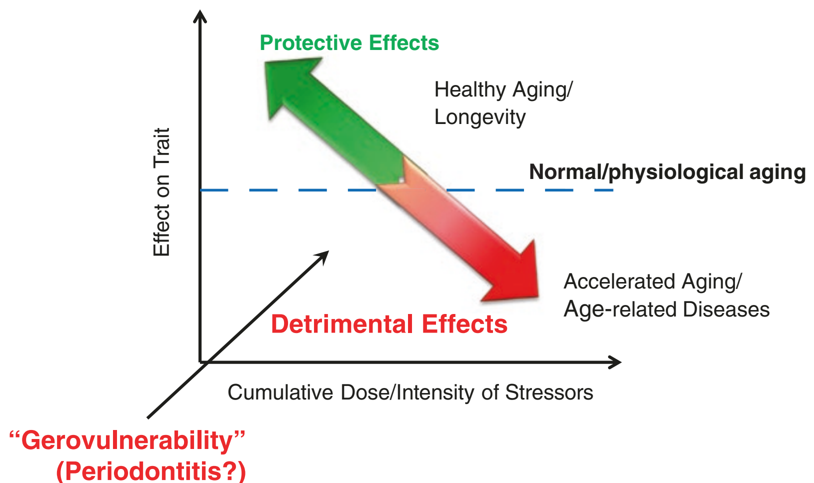

Fig. 8.7 Graphic considering the balance of protective and detrimental stressors that contribute to healthy versus accelerated aging. The theory of periodontitis existing as a detrimental factor enhancing gerovulnerability is presented. Adapted from Franceschi et al. [61]

there is a balance of protective and detrimental stressors within the intrinsic (e.g., genetics, metabolic) and extrinsic (e.g., exposome, antigenic ecospace) bionetwork of each individual that proscribes healthy or accelerated aging. Our tenet is the potential that periodontitis, rather than simply being an age-related disease, actually represents a detrimental effector and contributes to individual '*Gerovulnerability*'. This theory emphasizes the observed individual differences within the population related to the onset and progression of aging processes and age-related diseases. It also accentuates advancing evidence regarding fundamental differences in chronological versus biological aging at the individual level. In support of this data from Belsky et al. [65] identified gum health as a component of biologic aging. We have also recently noted a significant decrease in telomere base pairs in patients with periodontitis [39] across the lifespan. Thus, this chronic infection and persistent local and systemic inflammation could be a major component in the portfolio of risk factors for the expression of features of unhealthy aging. Consequently a restricted perspective that aging processes only effectuate an oral environment conducive to enhancing periodontitis [65] may limit the importance of broader strategies to improve healthy aging and should influence a future holistic approach to prevention, management, and interventional clinical decisions in periodontal disease and aging [34].

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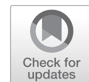

9 Peri-implant Disease

Mitchell W. Ponsford and Thomas G. H. Diekwisch

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9.1 Osseointegration: An Almost Perfect Relationship Between Metals and Living Tissues

According to the Centers for Disease Control and Prevention, the number of US adults with complete tooth loss has decreased from 49% in 1960 to 13% in 2012 [1, 2]. In addition, elderly adults are motivated to maintain their dentition since tooth loss has an impact on their oral health-related quality of life [3]. Some benefits to having a full complement of teeth include improved esthetics, function, nutrient intake, and self-esteem. The number of people that are keeping their teeth is on the rise, and when patients are missing individual teeth, implants have become a therapy of choice.

The replacement of missing teeth is one of the most challenging treatment modalities in dentistry. The traditional approach is to maintain the patient's existing dentition for as long as possible prior to

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resorting to tooth replacement strategies. Some of the conventional tooth replacement options include complete dentures, removable partial dentures, and fixed partial dentures. All of these options require rigorous maintenance and repair regimen and fall short of ideal replacement strategies in terms of function and esthetics. One of the functional benefits of implants as opposed to dentures is the continuous mechanical load exerted on the surrounding alveolar bone, resulting in bone maintenance and prevention of bone loss.

In general, loss of functional use and mechanical stress results in gradual resorption of bone, both in terms of height and width [4]. This has been referred to as disuse atrophy, which suggests that the body eliminates bone that is not actively stressed. According to Wolff's law, bone adapts its mass and structure to the mechanical demands placed on it [4]. One of the functional benefits of dental implants is the continuation of the mechanical stress exerted on alveolar bone, resulting in the prevention of bone resorption. While humans have attempted to replace natural teeth with implants for more than 1500 years, implants only became a reliable treatment option during the 1970s [5, 6].

Early dental implant technology consisted of blade and transosteal implants, and it was thought that both of these implant types relied on mechanical retention [7]. A wide array of metals and implant designs were used unsuccessfully. One implant design that is frequently referenced is the subperiosteal blade implant developed by Dahl in the 1940s [8]. This implant was inserted between the bone and the soft tissue and therefore relied on soft tissue anchorage. These implants were fraught with complications and were typically removed soon after placement due to infection, inflammation, and foreign body response [9].

Early endosseous implant studies revealed the remarkable ability of bone to tolerate metal implants and even tightly surround the inserted metal shaft. This phenomenon was first described by Bothe in 1940 and by Leventhal in 1951; however, it was not until 1952 that Per Ingvar Brånemark coined the term "osseointegration" [6, 10, 11]. Brånemark was studying blood flow in rabbits and discovered that titanium chambers placed in the rabbit tibia and fibula could not be removed from the bone after implantation. These studies prompted Brånemark to develop a dental implant fixture using pure titanium screws. Further studies demonstrated that these titanium implants demonstrated predictable long-term results [12].

Years after the original Brånemark implants were produced, Schroeder and Straumann in Switzerland worked with various alloys used in orthopedic surgery to develop their own dental implant [13]. In 1980, Schroeder initiated the International Team for Implantology (ITI), which helped stimulate advances in implant research and development. Several implant designs were developed and tested, including the Core-Vent, Stryker root form, and IMZ implants [7]. After years of testing, mainly through trial and error, some implants left the market and others withstood the test of time. The most popular dental implant designs used today are threaded, root-form implants with various surface treatments to facilitate osseointegration (Fig. 9.1).

The original Brånemark implants had a smooth, machined surface, while most modern-day implants have a roughened surface. The original Brånemark implants called for a 6-month healing time before loading while the modern-day roughened surface implants can be loaded in as little as 6 weeks [14]. The roughened implant surface results in an increase in surface area, allowing for increased bone apposition and better stress distribution along the implant body [15]. It has been shown that a roughened surface promotes bone formation by increasing the proliferation of cytokines, growth factors, and osteoblasts [16]. Some common surface treatments to create this roughened surface include sandblasting, acid etching, anodizing, electrochemical treatment, vacuum treatment, thermal treatment, and laser treatment [17]. Comparing smooth and rough surface implants side by side, it has been demonstrated that soft tissue adheres more readily to a smooth surface while bone tends to favor a roughened surface [18]. This concept has led to the design of a smooth collar at the top of the implant to facilitate soft tissue adherence.

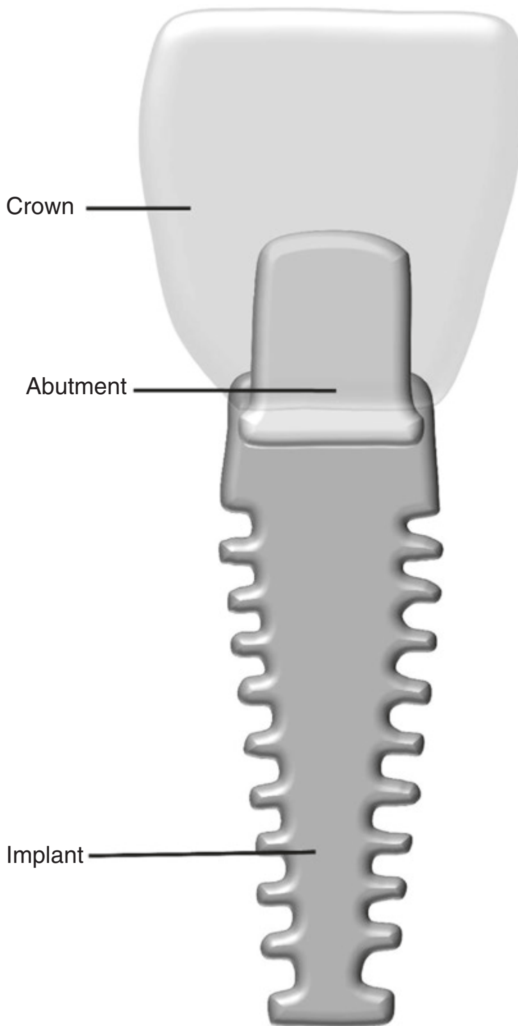

Fig. 9.1 Basic components of a typical dental implant. Dental implants usually consist of an implant fixture that anchors the implant in the bone (Implant), an abutment, that connects the implant fixture with the crown of the tooth (Abutment), and a prosthetic crown, that esthetically and functionally mimics a tooth crown of a healthy tooth (Crown)

The interface between bone and the oral cavity consists of a soft tissue barrier that includes sulcus, epithelial attachment, and connective tissue attachment [19]. This soft tissue barrier provides a protective seal between the bone and the outside world. In health, this soft tissue barrier prevents bacteria and debris from causing bone loss around the tooth. The integrity of this soft tissue seal is compromised in implants. According to Berglundh, the epithelial attachment is similar, while the connective tissue does not attach to the implant surface [20]. Other studies emphasized that the epithelium adheres to the implant via hemidesmosomes, but the connective tissue encircles the implant without attaching to the implant [21, 22]. These studies suggest that a tooth has a stronger biological seal than an implant, resulting in increased susceptibility of implants to invasion by bacteria and other debris.

Titanium became the material of choice for implants in both the dental and medical fields due to its perceived biocompatibility and its ability to form a seamless boundary between bone and implant, the process that Brånemark called osseointegration. Implants were considered biocompatible because of their ability to perform with an appropriate host response in a specific application [23]. Titanium is considered the most biocompatible metal due to its resistance to corrosion from bodily fluids, inertness, and relatively high fatigue limit. From an immunological perspective, implant biocompatibility is based on its containment in a tough, thin, avascular capsule that is quiescent [24].

Originally, the concept of osseointegration implied that bone is in intimate contact with titanium. However, in a typical dental implant, bone is in close proximity to the implant but does not adhere to it [24]. There is a thin biological layer between the bone and the implant, approximately 20–50 nm thick, is referred to as the “zone of tolerance.” [25, 26] This zone is composed of a titanium oxide layer, ground substance, and a cloud of zwitterionic forces that create enough friction to prevent implant movement. A zwitterion is a molecule that contains both a positive and a negative charge and therefore serves as a buffer between two dissimilar molecules. The titanium oxide layer is one of the key components responsible for titanium biocompatibility. The oxide layer insulates the titanium and serves as a buffer between the titanium and bone. Without a titanium oxide layer, titanium would become highly reactive and susceptible to corrosion [24].

Most modern-day implants utilize a design known as platform switching in order to maintain alveolar bone height over time. This design

comprises an implant design in which the abutment of the restored implant is of narrower diameter than the implant diameter. For example, if the implant is 6 mm in diameter, the portion of the crown that is attached to the implant is 4 mm in diameter. This concept was accidentally discovered when 3i Implant Innovations used abutments that were narrower than their implants. Lazzara and Porter reported that less bone loss was seen with platform switching [27]. The platform switch allows for the bone to form an implant–bone interface to the very top of the implant without a separate restorative component impinging on the bone to implant connection. This also will allow the oral mucosa to generate a soft tissue seal around the abutment and the crown as opposed to the implant body itself. Studies have demonstrated that platform switching, in contrast to platform matching, results in reduced bone loss after implant restoration [28, 29]. According to these and other studies, platform switched implants reveal minimal bone loss in the first year of service, and bone will even grow back to the coronal portion of the implant over time. Based on a study of platform switched Nobel implants that had been followed for 20 years, Chrcanovic reported that 11% of those implants displayed a gain in bone height and 36% experienced bone loss less than 1 mm [28]. In another study using platform switched implants, Froum detected an average of 0.8 mm of bone loss after 1 year, which decreased to only 0.3 mm of bone loss at 8 years [30].

In general, dental implants are regarded as a safe and highly effective treatment option for replacing missing teeth [24]. Compared to traditional modes of tooth replacement, dental implants have several benefits, including maintenance of bone height, stable anchorage for fixed restorations, and preservation of adjacent teeth when compared to bridge or denture-based applications. Moreover, implants allow for superior esthetics and function when compared to alternative tooth replacement options. In an effort to standardize the evaluation of implant health, Albrektsson et al. formulated five essential criteria for implant success in 1986 [31]. The criteria include: (1) immobility of the implant; (2) a lack of peri-implant radiolucency on a radiograph; (3) less than 0.2 mm vertical bone loss after the first year of service; (4) the absence of pain, infection, neuropathy, paresthesia, or violation of the mandibular canal; and (5) a minimum success rate of 85% at 5 years and 8% at 10 years. The authors also considered 1.5 mm of crestal bone loss within the first year a success and attributed this loss to the formation of soft tissue attachment.

Unfortunately, in recent years, the remarkable success rate of dental implants has turned them into a cure for all when only slightly compromised teeth have been replaced by implants without a clear prognosis for the implant to surpass the natural tooth in terms of longevity. A recent article in the *Journal of Dental Research* "Are Dental Implants a Panacea?" [32] asks whether the recent implant epidemic has led to the removal of teeth that could have been salvaged by conservative means. The article suggests that the longevity of even severely compromised teeth may far surpass that of the average dental implant if properly maintained [33, 34]. The readiness to apply dental implants without immediate clinical need raises eyebrows when clinical indicators of peri-implantitis have been reported in up to 45% of implant patients (Fig. 9.2) [35]. These recent studies by Giannobile, Derks, and others [36] have raised concerns about the unreflected use of implants as a means to an end for all dental health concerns regardless of the remarkable clinical success rates in many cases.

In addition to the increased readiness to place implants and potential side effects related to peri-implantitis, another worrisome trend has marred the once-untainted success story of dental implants: the changing training and skill levels of practitioners involved in placing implants. The high commercial profit margin associated with implant procedures has attracted a wide range of clinicians of various skill sets and training levels to participate in the profitable business. The original Brånemark implants were typically placed in a sterile operating room setting by oral and maxillofacial surgeons, while today, most implants are placed

in a private practice setting by a variety of dental professionals. Continuing education courses and dental school curricula include training on the placement and restoration of dental implants (Fig. 9.3). As a result, implants are being placed and restored by individuals with varying educational backgrounds. According to Adell, inexperienced surgeons had a 5-year implant survival rate of 75% while experienced surgeons had a 5-year survival rate of 98% [37]. Lambert found that inexperienced surgeons had implants fail twice as often as experienced surgeons [38]. Da Silva conducted a practice-based research network study where implant parameters were measured over time in multiple general dentists' offices [39]. That study found that after 4 years, 7% of the implants were classified as failures and 18.7% were considered to have excessive bone loss. The authors concluded that implants placed by general dentists have a higher failure rate when compared to those placed by specialists.

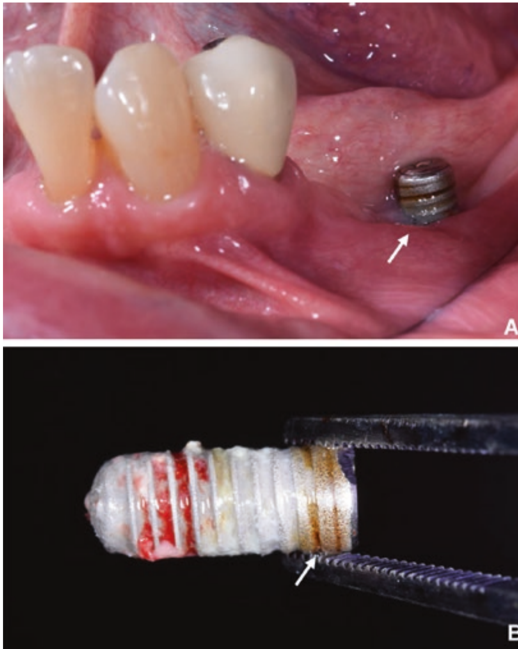

Fig. 9.2 Clinical signs of peri-implantitis. (**a**) Redness of the mucosal tissue immediately surrounding the implant surface (arrow). (**b**) Clinically visible signs of corrosion on the implant surface

According to the American Academy of Periodontics, implants displaying evidence of peri-implant disease suffer from either peri-implant mucositis or peri-implantitis [40]. Peri-implant mucositis entails the inflammation of the soft tissue around an implant without the loss of bone [41]. Peri-implantitis involves inflammation of the soft tissue and progressive bone loss around

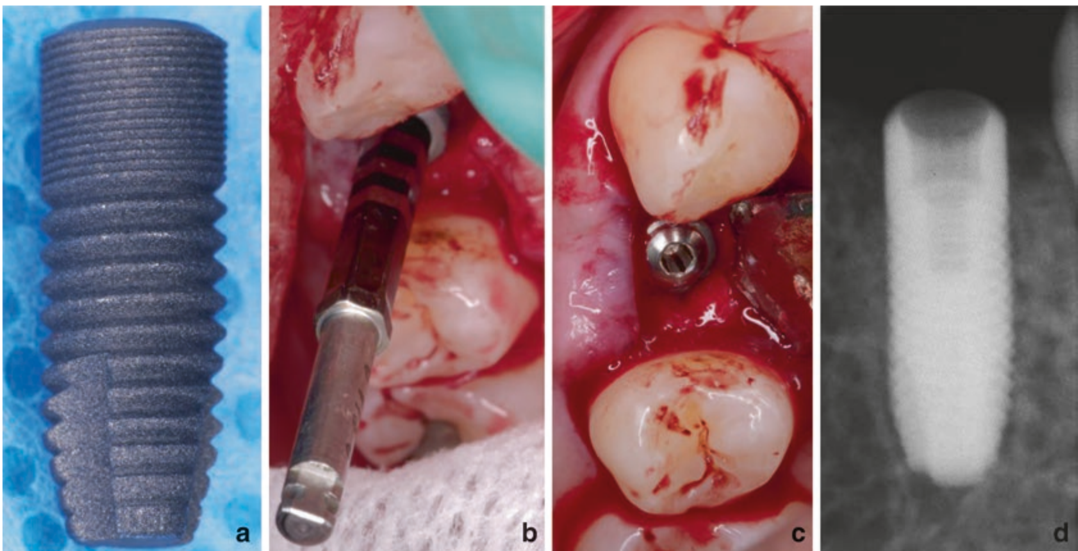

Fig. 9.3 Stages of implant placement. (**a**) Implant fixture, (**b**) insertion of the implant into the bone, (**c**) position of the implant between two adjacent teeth, and (**d**) radiograph of the successfully placed implant

the implant. According to a systematic review by Atieh et al. [42], peri-implant mucositis affects 63% of implant patients while peri-implantitis affects 19% of patients. To aid the clinician in determining the prognosis of a diseased implant, Froum et al. [43] have classified peri-implantitis into three different categories: early, moderate, and advanced. Early peri-implantitits is defined as an implant with a periodontal probing depth of greater than 4 mm, with bleeding upon probing and bone loss of less than 25% of the implant length. Moderate peri-implantitis entails probing depths from 6 to 8 mm with bleeding upon probing and 25–50% bone loss. Advanced peri-implantitis is an implant with a periodontal probing depth of greater than 8 mm, with bleeding upon probing and bone loss of greater than 50% of the implant length.

Peri-implantitis may eventually result in implant failure, which usually requires surgical removal of the implant in order to prevent further pain, infection, and bone loss. Becker et al. [44] described implant failure as the presence of implant mobility and radiolucency around the implant. In addition to these criteria, several other clinical observations such as pain, infection, tissue inflammation, and degree of bone loss help the clinician determine whether the implant is salvageable or needs to be removed.

Several studies have evaluated factors that could contribute to implant failure, yet in many cases the cause remains unknown. The timing of implant failure and an understanding of the healing process are useful tools that aid the clinician in determining the potential causes of failure. Chrcanovic et al. [45] define primary, or early, implant failure as the failure of an implant to osseointegrate after it has been placed in bone (i.e., failed to form a close union between the implant and surrounding bone during healing). Some studies speculate that primary implant failure may be due to overheating of the bone and/or poor surgical technique, even though a cause and effect relationship remains to be established [46, 47].

Chrcanovic et al. [45] call secondary implant failure a process that occurs later than primary implant failure and that is due to progressive bone resorption around the implant (i.e., advanced peri-implantitis). Studies demonstrate that bone loss around an implant could be associated with one or more of the following: poor clinical handling, poor implant design, complex patient medical history, poor oral hygiene, overloading of the implant due to the crown being too high, excess cement, or a response to foreign particles embedded in the tissue [45, 48–51]. Some of the clinical parameters for secondary implant failure include deep probing depths (using a periodontal probe), bleeding upon probing, purulence, pain upon palpation or percussion of the area, and radiographic bone loss.

9.2 Risk Factors Contributing to Implant Disease

Several patient-related risk factors are known to contribute to peri-implant disease, including smoking, diabetes mellitus, and pre-existing periodontal disease (Fig. 9.4). Smoking and its relationship to periodontal destruction has been discussed extensively in the literature [52, 53]. A longitudinal study by Miller et al. conducted statistical analyses of several variables that may contribute to tooth loss and found that smoking was the most important risk factor for tooth loss [54].

Several mechanisms by which smoking affects wound healing are discussed by Rivera-Hidalgo [55]. Nicotine decreases the proliferation, attachment, and chemotaxis of periodontal fibroblasts. Fibroblasts are a key cell that function in the healing and turnover of periodontal tissues. Smokers also suffer from decrease in oxygen delivery to the periodontal tissues, which leads to an increase in anaerobic bacteria. In smokers, polymorphonuclear leukocytes (PMN) cells have decreased motility and function, resulting in decreased periodontal immunity. Smokers typically experience severe xerostomia, which facilitates an increase in bacterial adhesion to the soft tissue and inadequate salivary flushing mechanisms. Smoking also reduces blood perfusion in the small capillary network of the periodontal soft tissues. As a result, periodontal connective tissues do not receive enough nutrients and are not able to rid themselves of waste products. Budunelli et al.

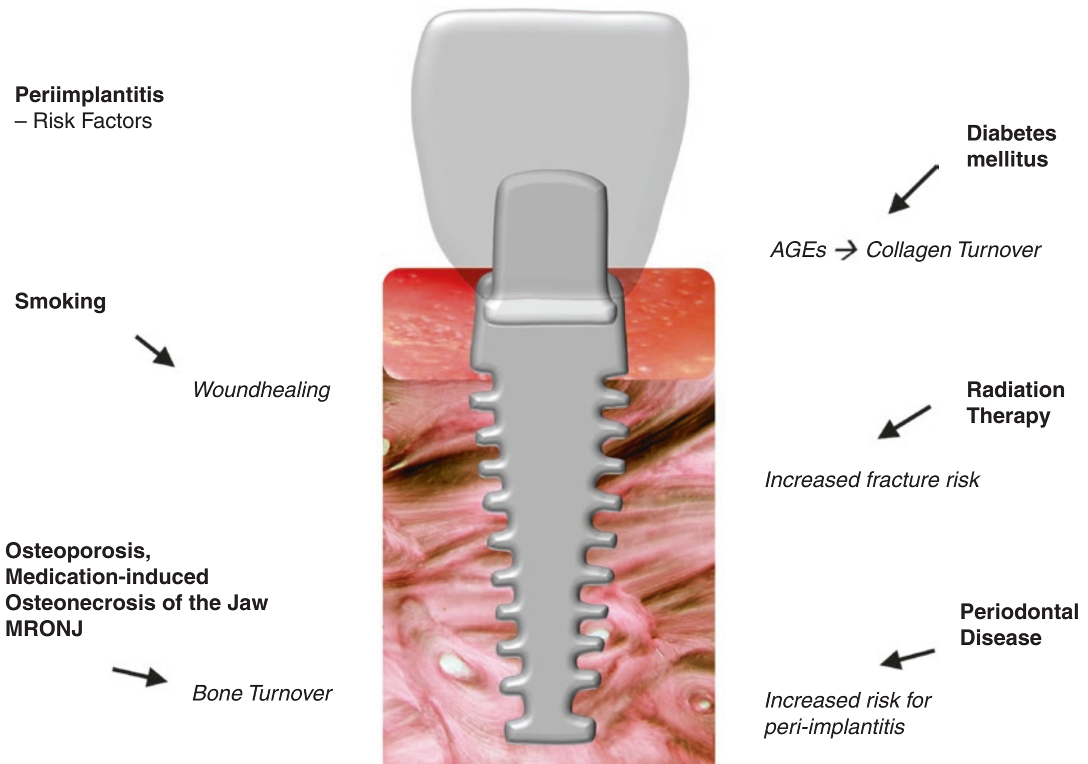

Fig. 9.4 Risk factors contributing to peri-implant disease. Factors contributing to peri-implant disease include diabetes, smoking, radiation therapy, periodontal disease, and osteoporosis and osteonecrosis

found that smokers have an altered RANKL to osteoprotegrin ratio [56]. RANKL is an acronym for receptor activator of nuclear factor kappa-B ligand, which binds to RANK in order to trigger bone resorption. Osteoprotegrin is a protein that can bind RANKL in order to minimize its effects. As a result, the catabolic bone resorption signaling surpasses the anabolic aspect of new bone formation in smokers, resulting in an imbalance of the bone regenerative periodontal homeostasis toward the katabolic aspect. Finally, a smoking-related increase in advanced glycation end-products (AGEs) results in a decrease in oxygen delivery to periodontal tissues and a decrease in collagen turnover [57].

Smoking appears to have a similar impact on dental implant health as well. Karbach et al. demonstrated that smoking was the most important risk factor for the formation of peri-implant mucositis [58]. It has also been reported that bone loss around implants in smokers is twice as high as in nonsmokers [59]. Chung et al. studied a variety of implant designs in smokers and nonsmokers placed over a 21-year period [60]. They found that smokers had almost 3 times more annual bone loss than nonsmokers. Another study that examined long-term results of implants found that the rate of implant failure was higher in smokers than in nonsmokers [61]. The authors concluded that the higher failure rate in smokers was due to a reduced healing capacity among patients who smoke.

Curiously, implant surface modifications may improve implant longevity more so in smokers than in nonsmokers. One study compared machined implants and oxidized implants in smokers and nonsmokers [62]. This study demonstrated that bone loss around machined implants was twice as high in smokers as in nonsmokers while there were similar bone levels and failure rates between smokers and nonsmokers when oxidized implants were used. In another

study, Balshe and coworkers found that rough surface implants in smokers had no significant failure rate, while there was a significant failure rate associated with smooth surface implants [63]. While some studies show reassuring results with rough surface implants, smoking is still considered a risk factor for peri-implant disease.

The effect of diabetes mellitus on periodontal health has been well established [64, 65]. There is evidence of a bidirectional relationship between diabetes and periodontal health in which the stability of one disease influences the other. Löe was the first to suggest that periodontal disease is the sixth complication of diabetes [66]. Some of the common complications found in diabetics include cardiovascular disease, neuropathy, nephropathy, retinopathy, and vascular changes. When a patient has prolonged elevated blood glucose, there is an increase in advanced glycation end-products (AGEs), which results in diminished oxygen delivery to tissue, poor collagen turnover, and reduced healing capacity. Prolonged diabetes is also associated with decreased PMN leukocyte motility and function, decreased fibroblast function, and increased RANKL/osteoprotegerin ratio [65]. A patient with well-controlled diabetes will typically have fewer of these sequelae and will hence heal better than an uncontrolled diabetic.

Elevated blood glucose as it occurs in diabetic patients and its level of control affect both periodontal therapy and implant therapy. In animal models, diabetic pigs have less bone-to-implant-contact and rats injected with AGEs exhibit a slower rate of osseointegration [67, 68]. Another study on diabetic rats reported decreased bone density around the implants [69]. Studies in humans have identified a correlation between uncontrolled diabetes and bleeding upon periodontal probing around implants, but they did not report an increase in bone loss or implant failure among diabetics [70–72]. Other studies in humans have suggested that periodontal wound healing occurs at near physiological levels in a well-controlled diabetic (Hemoglobin A1C ≤ 7) [73].

Based on their effect on bone density, osteoporosis and bisphosphonate treatment of osteoporosis and cancer have been tested for their relationship with implant failure. Osteoporosis is known for causing a decrease in bone density and is typically found in postmenopausal females [74]. In general, multiple cohort and meta-analysis studies have identified a slight correlation between osteoporosis and implant failure, but the correlation is weak and not statistically significant [75, 76]. Many osteoporosis and cancer patients are prescribed bisphosphonates, which decrease bone loss by inhibiting osteoclasts. Osteoclasts are bone cells that degrade bone into its mineral components. Without the help of osteoclasts, the jawbone is lacking in healing capacity and is therefore susceptible to a condition known as bisphosphonate-related osteonecrosis of the jaw (BRONJ). Several other medications, such as RANK ligand inhibitors and antiangiogenics, induce a similar phenomenon and so the term has been changed to medication-related osteonecrosis of the jaw (MRONJ) [77]. Some bisphosphonates, such as intravenous (IV) and nitrogen-containing oral bisphosphonates, are associated with a higher incidence of MRONJ [77]. Shabestari et al. conducted a case series on 21 patients taking oral bisphosphonates and reported that bisphosphonates had no effect on implant health [78]. A retrospective study on 362 patients treated with dental implants found no correlation between bisphosphonates and implant failure, but there was a correlation with implant thread exposure over time [79]. Together, these studies indicate that bisphosphonates do not have a substantial effect on implant failure.

Radiation therapy is often administered for the treatment of head and neck cancer [74]. This treatment can result in severe dry mouth and altered function of the bone and soft tissue. Similar to MRONJ, a history of radiation therapy can result in a condition known as osteoradionecrosis of the jaw. A systematic review based on 10,150 implants determined that implants placed in irradiated bone had a 174% higher chance of failure [80]. Thus, caution is advised when implant placement in irradiated bone is considered.

Periodontal disease not only affects the attachment and retention of natural teeth but is also implicated in the loss of implants due to peri-implantitis. Periodontal disease has a wide array of causes and risk factors but is most commonly

associated with bacterial plaque and the host immune response [81]. Periodontitis and peri-implantitis are both typically associated with a certain bacterial profile, namely, gram-negative anaerobic bacteria [82]. In addition, certain patients may be more susceptible to the deterioration of the periodontium due to individual variables such as medical history, social history, bacterial flora, and genetic profile [81].

A cross-sectional study including 109 volunteers resulted in a significant correlation between implant failure and periodontitis [83]. Swierkot et al. conducted a prospective long-term study on patients with a history of generalized aggressive periodontitis, formerly known as juvenile periodontitis [84]. Despite the fact that the aggressive periodontitis was controlled prior to implant placement, these patients were more susceptible to peri-implantitis, peri-implant mucositis, and implant failure when compared to healthy control patients [84]. Another longitudinal cohort study on adults reported a significant correlation between severe chronic periodontitis and late implant failure [85]. Costa et al. noted an increased likelihood to develop peri-implantitis when patients with peri-implant mucositis did not attend regular maintenance appointments [86]. Together, these studies established a significant correlation between periodontitis and peri-implantitis.

Based on these findings, the dental professional must remain abreast of current research with regard to risk factors for developing implant disease and implant failure. Smoking, diabetes mellitus, antiresorptive therapy, antiangiogenic therapy, radiation therapy, and periodontal disease are some of the more common risk factors discussed in the literature. Of these risk factors, several studies suggest that smoking and periodontal disease are the most prevalent risk factors for developing implant disease [82, 83, 85, 87].

9.3 Etiology: Bacterial Plaque

One of the most controversial and highly studied questions in dentistry is "what causes implant disease?" [41, 42] Many authors consider a multifactorial etiology for peri-implant disease (Fig. 9.5). Assuming that all risk factors

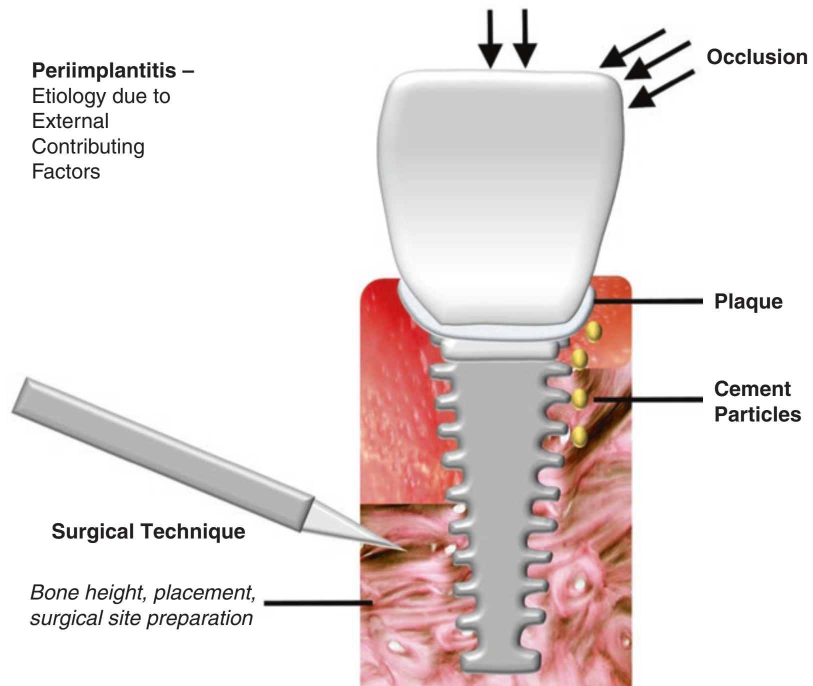

Fig. 9.5 External factors causing peri-implant disease. External factors contributing to the etiology of peri-implant disease include surgical technique, occlusion, plaque, and cement particles

are controlled and the patient is healthy, patients may still develop implant disease or implant failure due to yet-to-be-defined etiologies.

Bacterial plaque is among the most commonly discussed primary etiologies for gingivitis, periodontitis, and peri-implant disease. A well-organized biofilm on an implant surface appears to initiate and propagate peri-implant disease and peri-implant mucositis in a similar fashion as biofilms on the tooth surface cause gingivitis and periodontitis [41]. The early stages involve soft tissue inflammation and a shift from gram-positive aerobic bacteria to gram-negative anaerobic bacteria. If this early lesion is left unclean and uncontrolled, the plaque matures and the inflammation progresses and ultimately results in bone and tooth loss.

In 1965, Löe demonstrated in humans that the accumulation of bacterial plaque on teeth leads to gingivitis and that gingivitis resolves once oral hygiene is reinstituted [88]. Pontoriero et al. conducted a similar study on implants, using teeth in the same patients as a comparison [40]. After 3 weeks of plaque accumulation, the teeth and implants both displayed similar changes in bleeding, swelling, probing depth, and bacterial profile. There was no statistically significant difference between the teeth and implants after plaque accumulation. The teeth developed gingivitis as expected, and the implants developed peri-implant mucositis. Unfortunately, the authors did not take measurements after the patients resumed oral hygiene and therefore did not demonstrate whether peri-implant mucositis is a reversible process. Salvi et al. conducted a similar study and included clinical measurements 3 weeks after the reinstitution of oral hygiene [89]. Gingivitis and peri-implant mucositis were found to be reversible at the biomarker level, but the clinical parameters had not yet reached the pre-experimental levels. These parameters did, however, show trends toward resolution in both teeth and implants.

The term "peri-implantitis" was first used by Mombelli in 1987 when he discovered that implants with bone loss harbored gram-negative anaerobic rods, black-pigmented bacteroides, fusobacterium species, and spirochetes [90]. When evaluating the microbiota of healthy implants in the same patients, Mombelli reported predominantly coccoid cells. He referred to peri-implantitis as a site-specific infection, which has many features in common with periodontitis.

Peri-implantitis is thought to be initiated in a manner similar to periodontitis, namely, by a mounting bacterial insult and a host response [41, 42]. Some studies document a similar bacterial profile for both peri-implantitis and periodontitis, while others reveal a unique profile for peri-implantitis [91]. An independent study group of 30 clinical experts met in Italy to systematically review the literature on peri-implantitis [91]. They concluded that peri-implantitis is not comparable to periodontitis since several anatomical differences exist between the periodontium and the peri-implant environment. Among potential microbes associated with peri-implantitis, the review lists gram-negative anaerobes, opportunistic microbes, Epstein-Barr virus, anaerobic gram-positive rods, and *Staphylococcus aureus*. Some papers have identified *S. aureus* as the microbe that initiates peri-implantitis, but this notion was refuted by the aforementioned review in Italy [92, 93].

Other similarities between periodontitis and peri-implantitis include similar inflammatory cascades [41]. Both inflammatory processes exhibit an upregulation of proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-12, and tumor necrosis factor (TNF)-α [41]. However, peri-implantitis typically progresses more rapidly than periodontitis, most likely due to a less robust protective barrier around implants when compared to teeth. More specifically, teeth are protected through a connective tissue attachment and complex defense mechanism, while implants lack a connective tissue attachment and the interface between the implant and the bone is occupied by an avascular mucosa layer. A recent comparison noted a self-limiting process in teeth that separates the inflammatory lesion from bone through a protective connective tissue capsule, while such a separating barrier does not exist around implants [94].

Most modern implants feature a rough implant surface due to surface modifications including

sandblasting and surface etching. This roughened surface was introduced to enhance implant anchorage, adhesion, and stability. However, when it comes to plaque adhesion, the roughened surface provides a niche for bacterial plaque to firmly attach to the implant and create a mature bacterial colony [95]. Ultrasonic and hand instruments were designed to remove the majority of the plaque from a natural tooth, but they usually do not remove all of the hard deposits known as calculus [96]. Removal of bacterial plaque and calculus from implant surfaces is substantially more challenging due to the topography of the implant surface. Once the bacterial plaque has reached the implant itself, plaque removal becomes a challenge for the clinician, and there is a lack of universally accepted approaches for plaque removal from implant surfaces. Some implant companies supply “tissue-level” implants with a polished collar at the very coronal portion of the implant. This polished titanium is much easier to clean and allows for soft tissue adhesion. The drawbacks of this approach are poor esthetics and difficulties for crown design as it emerges from the implant.

9.4 Etiology: Occlusion

Occlusion is another potential factor that might contribute to implant disease and implant failure. While the effects of occlusion on teeth have been extensively studied, there is still a paucity of evidence regarding the effects of occlusion on implants [97]. A healthy tooth is suspended within its bony housing by the periodontal ligament (PDL). The PDL serves as a shock absorber, which distributes forces along the root [98]. The PDL also contains mechanoreceptors, which provide sensory feedback for the level of bit force and possibly monitor fine tuning. Implants, on the other hand, lack a PDL and are simply positioned in close proximity to the bone. As a result, implants lack the shock absorber effect of the PDL and do not provide occlusal feedback for micro-adjustments when the patient is chewing [99]. As another consequence of implant design, occlusal forces are concentrated at the crestal bone around implants [98]. Once loaded, teeth move between 25 and 100 micrometers (μm) in vertical direction and 56 and 150 μm in horizontal direction, while implants move only 3 and 5 μm vertically and 10 and 50 μm horizontally. The clinician is therefore faced with the challenge of creating a fine-tuned occlusal scheme that prevents excessive forces when the implants are in function.

The absence of a periodontal ligament as a resilient anchorage between implants and bone causes occlusal forces to directly affect adjacent bone. As a result, mechanical forces exerted on the implant supporting bone may either be physiological, relatively too high, or relatively too low. The level of forces transduced on implant carrying bone is of importance as bone is a tissue extremely susceptible to mechanical loading. To this date, Wolff’s law about the responsiveness of bone to mechanical stresses holds true [4]. Elaborating on Wolff’s law, Frost reported bone deposition or bone resorption depending on the direction and magnitude of the forces applied to bone [100]. Specifically, Frost determined that a very low amount of strain may result in disuse atrophy, a mild amount of strain maintains a “steady state,” and an increased level of strain results in bone resorption and even bone fracture.

The resulting tissue damage from excessive occlusal forces on natural teeth and their supporting structures is called occlusal trauma. Occlusal trauma may result in bony changes, occlusal wear, widened PDL, and tooth mobility [101]. The effect of excessive occlusal forces on implants is called occlusal overload. Occlusal overload occurs when either normal function or parafunctional habits result in structural or biological damage, including damage to the prosthesis, implant, or surrounding bone [102]. It has been suggested that peri-implantitis and occlusal overload are the two most common causes of late implant failure [97]. Several authors have correlated occlusal overload with crestal bone loss [103]. Kozlovsky et al. demonstrated in a dog model that occlusal overload with uninflamed mucosa resulted in a slightly reduced marginal bone level [104]. However, bone loss beyond the implant neck only occurred in the presence of both occlusal overload and peri-implant inflam-

mation. Other consequences of occlusal overload include prosthetic screw loosening, screw fracture, prosthesis failure, and implant fracture [105, 106]. Implant fracture can lead to peri-implant bone loss, resulting in complete implant failure [107].

A dentition that no longer relies on natural teeth but rather on implants requires an appropriate occlusal design to maximize implant longevity and to prevent costly implant repair and replacement procedures. From a biomechanical perspective and according to Wolff and Frost, occlusal designs to support implant integration will minimize the amount of cantilever forces [4, 100]. In other words, vertical bite forces are preferred over torqueing forces as these push heavily on one side of the implant. Cantilever forces are minimized by using an implant prosthesis that is slightly narrower than a normal tooth. Occlusal designs that do not extend too far in any direction beyond the diameter of the implant itself are preferable [97]. Non-axial shearing forces resulting from the cusp inclination of the crown should be minimized.

There is no general agreement about the implant length necessary to support a crown that matches adjacent teeth in length and width [108]. A number of authors have reported equal success rates when using short versus long implants, while others have found inferior results with short implants [109, 110]. Authors who favor a short implant base argue that the apical length is less important since the majority of the forces exerted on the implant occur at the cervical bone–implant interface. A consensus to this debate remains to be seen, but most clinicians and implant companies prefer implants that are at least 8 mm in length [111].

In addition to narrow crown designs to avoid torque forces, optimal implant design from an occlusal perspective also includes very light or no occlusal contact with the opposing tooth when the dentition is in maximum intercuspation (i.e., biting down) [107, 112]. Such design compensates for the lack of PDL around the implant. When a dentition transitions from a physiological bite to a heavy bite, the PDL will allow the teeth to compress, but the implant will remain stationary. In addition, when the patient is moving their jaw in a lateral or excursive direction, there should be no contact on the implant crown. Parafunctional habits must also be considered during implant therapy. Patients who brux (grind their teeth) or clench their teeth have a higher risk of implant failure [113]. These patients may benefit from wearing an occlusal night guard in order to prevent excessive forces from parafunctional habits. An optimized occlusal design will have a profound impact on wear patterns and on the longevity of teeth and implants alike.

9.5 Etiology: Surgical Technique

Another potential factor contributing to peri-implant disease is the clinical technique used during implant therapy. The great demand for dental implant treatment and the high profit margins have led some practitioners to place implants in ways that do not follow the biological, surgical, and mechanical principles that were adhered to during the early years of implant treatment.

One of the key requirements for successful implant placement is the presence of a stable bony ridge to support the implant. Implants will be at a high risk for failure if the implant is not placed into bone of sufficient quality and quantity [111, 114]. Primary stability is also a requirement for osseointegration. A number of bone classification systems have been developed to aid the clinician in implant planning. Leckholm and Zarb distinguished between type I bone as compact cortical bone, type II as dense trabecular and cortical bone, type III as dense trabecular bone with thin cortical bone, and type IV as low-density trabecular bone surrounded by thin cortical bone [115]. Seibert created a classification system for the shape of the defect in edentulous sites [116]. A class I defect entails a loss of defect width, class II is a loss of defect height, and class III is a loss of both width and height. The maxilla typically has less dense bone than the mandible, and the posterior jaws are typically less dense than the anterior regions. As a result, the mandible typically has higher implant success rates and the posterior maxilla has higher failure rates [117].

The condition of the soft tissue is another critical variable for implant therapy. The soft tissue crevice around implants does not compare well to the highly differentiated attachment apparatus of healthy teeth. As a result, the mucosal periphery surrounding implants lacks the resistance against bacterial infection, resulting in inflammation of the healthy periodontium. In addition, some studies emphasize the need for a keratinized mucosa surrounding implants. The lack of keratinized tissue (gingiva) surrounding teeth has been demonstrated to result in inflammation, recession, and even tooth loss [118]. It is not clear to what extent the presence of a keratinized mucosa is a requirement for implant health. Wennström demonstrated that health can be maintained around both implants and teeth that do not have keratinized mucosa [119]. These results were obtained in patients with adequate homecare and periodic professional cleanings. Others have reported a greater degree of plaque accumulation and mucosal inflammation even though the lack of keratinized mucosa did not affect implant survival [120]. Block et al. demonstrated that a lack of keratinized mucosa was associated with crestal bone loss of 2 mm or more and that the presence of keratinized mucosa was directly correlated with soft and hard tissue health [121]. Therefore, the lack of keratinized mucosa due to anatomical or surgical conditions may affect implant health.

Surgical trauma during implant placement should be minimized in order to maximize the likelihood of proper healing. Bone is a living tissue, sensitive to heat, and overheating of bone during the preparation of the site for an implant can lead to necrosis [122]. The clinician must use the proper drilling sequence, and cooling aids to minimize trauma to the bone. Occasionally, the surgeon will inadvertently create a fenestration in the bone, resulting in a direct contact between implant and soft tissue during healing [111]. Such a condition may negatively impact the osseointegration of the entire implant.

Reports of bacteria associated with failed implants underscore the need for rigorous aseptic surgical conditions during implant therapy. An aseptic surgical field will help minimize bacterial contamination and will result in lower implant failure rates as well [111]. The widespread trend for implants to be placed by practitioners lacking proper surgical training may thus be one contributing factor to the rise in peri-implant disease. It is recommended that any practitioner placing implants uses sterile instruments, proper draping, and careful handling of the implant after removal from its package.

The flapless strategy for implant placement has become a popular surgical technique due to its simplistic approach and potential for better healing and esthetics. This technique typically entails creating a small hole in the soft tissue and then preparing the implant bed through this hole. Other benefits of this approach include less post-operative pain and less trauma to bone and soft tissue [111]. Froum et al. conducted a study comparing flapless and flap protocols for implant placement [30]. Contrary to popular belief, there was no difference in bone levels, probing depths, bleeding on probing, or papilla height 8 years after implant placement. The authors concluded that both protocols were equally successful. However, with advances in radiology and three-dimensional implant planning, it is feasible to use the flapless protocol as long as proper surgical technique is exercised.

Two approaches toward implant placement are commonly distinguished: the one-stage and the two-stage protocol. The one-stage protocol entails placing an implant and a transmucosal healing abutment at the same time. This allows the implant to osseointegrate and the surrounding tissue around the abutment to heal. With the two-stage protocol, the implant is buried underneath soft tissue and later uncovered for the attachment of a healing abutment. The benefits to the one-stage protocol are reduced time, money, and surgical trauma [111]. The healing abutment also allows for the early formation of a mucosal barrier while the implant is healing. The drawbacks to the one-stage protocol are the potential for bacterial contamination of the implant during healing and the potential for trauma to the implant by the patient. With the two-stage protocol, the implant is allowed to completely integrate prior to its exposure to the bacterial flora and mechanical forces of the oral cavity. Several studies have

reported a decreased risk of implant failure using the two-stage protocol; however, since the two-stage protocol involves additional time, money, and surgical trauma, it is up to the patient to choose one option over the other [123, 124].

A third surgical approach toward implant placement involves placing the implant into a fresh extraction socket and is commonly referred to as an immediate implant [111]. Benefits of this approach include a reduced number of surgeries and a faster result when compared to conventional therapy. The drawbacks to this procedure include increased risk of infection, low bone to implant contact, more bone resorption, and a higher risk of implant failure [125]. It is also likely that implant placement on the same day will further traumatize the alveolar ridge and the surrounding soft tissue after already suffering initial trauma due to the tooth extraction procedure. This enhanced trauma on the surrounding bone and soft tissues are likely the cause for the increased bone resorption and failure rates associated with immediate implant placement versus delayed implant placement [126]. A benefit to immediate implants that is worth noting is the ability to create a temporary crown or custom healing abutment on the implant. This will help to preserve the soft tissue dimensions that were present around the tooth prior to extraction. Nonetheless, implants placed using the immediate approach are more prone to implant failure than implants placed using delayed strategies.

Surgical technique is especially important to remove inflammatory tissues from the implant site prior to implant placement. One example are the periapical lesions that often occur at the apex of extracted teeth. Proper surgical protocol requires thorough debridement and cleaning of the lesion prior to implant placement. However, many dentists have successfully placed immediate implants in sockets containing periapical lesions, and randomized controlled trials have shown similar failure rates when implants were placed immediately in sockets with periapical lesions compared to those placed in healthy sockets [127, 128]. However, a concern about primary stability and osseointegration in such inflamed sites remains. Interestingly, a periapical lesion on a tooth adjacent to the implant poses a high risk for infection around the apex of the implant [129]. Thus, proper surgical site preparation is an important strategy to prevent future implant infection and failure.

9.6 Etiology: Cement

The prosthetic components that attach to an implant comprise an abutment, which screws directly onto the implant, and a crown or bridge prosthesis. Two types of implant surgeries are generally distinguished: the one-stage and the two-stage implant procedure. For the two-stage implant, the prosthesis is cemented onto the abutment in the clinic, while for the one-stage implant, the prostheses including the abutment are fabricated as one piece in the lab. The one-piece prosthesis is also referred to as screw-retained implant since it can be screwed directly into the implant without the need for dental cement. Both cement and screw-retained prostheses are used routinely in the dental office, but some dentists prefer the cement-retained approach since it is typically more affordable. The screw-retained prosthesis is distinguished by a hole in the final crown for access to the screw. The location of the screw access hole relies heavily on proper implant placement so that the hole does not affect the cosmetics or function of the restoration.

The drawbacks to a cement-retained prosthesis include difficulties for crown removal after cementation and a potential for extrusion of excess cement into the surrounding tissue. This excess cement is very difficult to remove and may be inadvertently left embedded in the soft tissue. In 1999, Pauletto et al. reported four cases in which excess cement was associated with inflammatory lesions around the implants [130]. Deep probing depths, bone loss, and purulence were noted during surgical removal of the excess cement, and the lesions resolved after cement removal. Another case report demonstrated implant failure that occurred 1 month after crown cementation [131]. During surgical removal of the failed implant, significant bone loss was detected adjacent to an area with excess

cement and inflamed granulation tissue. Wilson conducted a case-control study in which he compared 42 test implants with peri-implantitis to 20 healthy control implants [48]. He used a dental endoscope to explore the condition of the peri-implant mucosa. Excess cement was found in none of the controls and in 34 of the test sites. 30 days after removal of excess cement, 25 of 33 test sites had no clinical signs of inflammation. The author concluded that excess cement was associated with peri-implant disease.

Burbano et al. studied 19 human biopsies that were taken from implants with peri-implantitis and cement-retained crowns [51]. These biopsies were analyzed using scanning electron microscopy and elemental analysis in order to determine the presence of dental cement embedded in the soft tissue. All 19 of the specimens displayed evidence of cement in the soft tissue, and findings were correlated with five different commercially available cements. Penarrocha-Oltra et al. studied the presence of different bacteria present around screw-retained and cement-retained implants [132]. After sampling 55 cement-retained implants and 46 screw-retained implants, the authors detected a significantly higher bacterial load in the cement-retained group.

An in vitro study by Rodriguez et al. studied the effects of different dental cements on human gingival fibroblasts (soft tissue–forming cells) and on pre-osteoblasts (bone–forming cells) [133]. In this study, various dental cements displayed only minute effects on pre-osteoblasts, while effects on fibroblasts were significant. There was a statistically significant decrease in the number of human gingival fibroblasts when exposed to all cements with a singular exception. The cement with a lesser effect on fibroblasts contained zinc oxide noneugenol, with the trade name “Temp-Bond.” Studies reviewed so far indicate that dental cement affects soft tissue health, bacterial load, and bone height in the implant periphery. The effect of cement on soft tissue inflammation would suggest a correlation between cement and implant failure; however, three different controlled clinical studies reported no correlation between cement-retained crowns and implant failure [134–136]. Thus, excess cement may have an effect on implant health, but not necessarily on implant failure.

9.7 Etiology: Titanium Allergy

Since its inception, titanium has been regarded as an extremely inert and biocompatible material. However, more recently, titanium has been associated with allergies, foreign body reactions, and particle release (Fig. 9.6). Reports related to allergic reactions to titanium have been on the rise [137]. The most common allergic reactions to titanium including types I, III, and IV. Type I hypersensitivity reactions are reactions in which the patient has been previously exposed to the allergen (i.e., titanium) and will mount a specific immune response to the allergen via IgE antibodies upon secondary exposure. This classic allergic reaction typically occurs within a short period of time. Type III hypersensitivity reactions are characterized by an excess of antigen-antibody complexes, which the body is unable to clear them from an affected area. This type of reaction develops within days or weeks. Type IV hypersensitivity reactions are cell-mediated and not antibody-mediated. Cell-mediated immune reactions occur when T helper cells recognize an allergen and secrete cytokines that cause a chain of events to occur. As a result of this immune reaction, the environment is infiltrated with aggressive and destructive cells such as macrophages, T lymphocytes, and mast cells, which cause damage to the surrounding area. Type IV reactions are delayed and take several days to develop.

Several authors have reported allergic reactions against orthopedic titanium implants associated with implant failure [137]. Examples include allergic symptoms in patients after the placement of titanium plates for fixation of bone fractures [138]. These patients were characterized by discoloration and titanium fragments surrounding these titanium plates as well as T lymphocytes and macrophages indicative of a type IV reaction in the proximity of the fracture prosthesis. In another study in patients with failing prosthetic hips, tissue samples once more contained T cells and macrophages indicative of

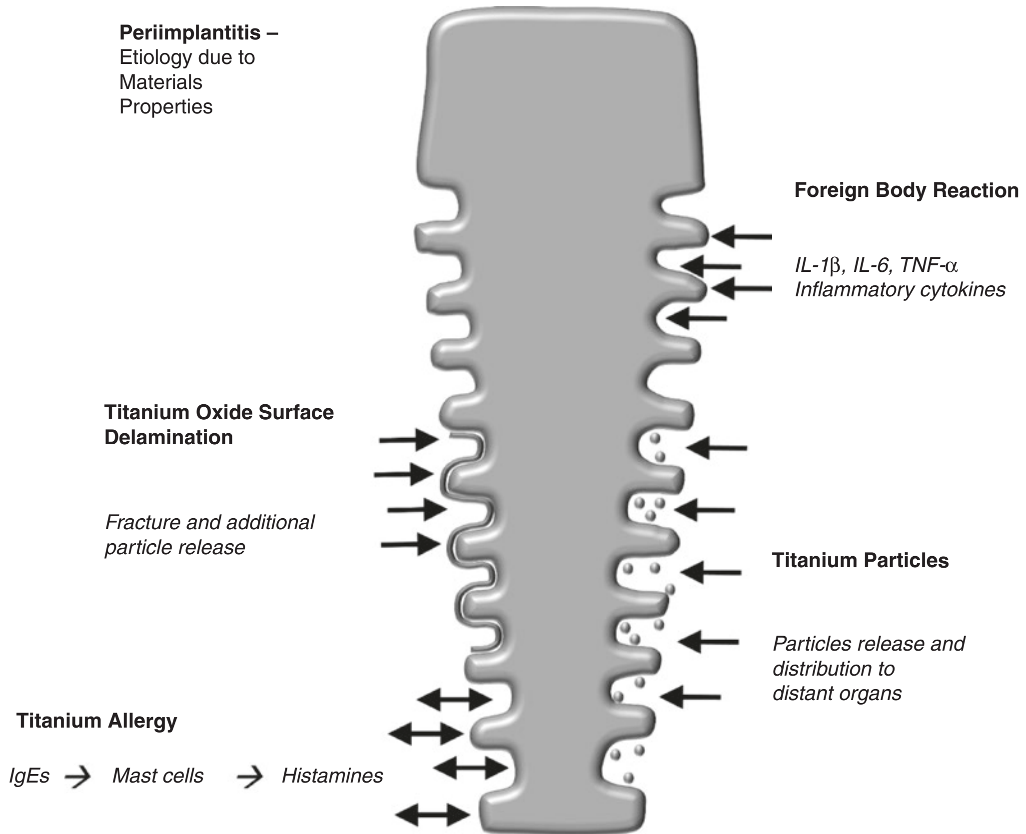

Fig. 9.6 Factors causing peri-implant disease related to the materials properties of the titanium surface. Several factors directly related to the metal implant and its major component, titanium, that have been attributed to play a significant role in the etiology of peri-implant disease, including implant surface delamination, titanium particles, foreign body reaction, and titanium allergy

a type IV allergic reaction [139]. Interestingly, all five of these patients revealed a negative result to a skin patch test using titanium. However, a titanium ointment test yielded positive results in two of these patients [139].

A cohort study evaluated 1500 implant patients in Spain for potential titanium allergies [140]. Thirty-five of these patients were suspected of having a titanium allergy based on a history of multiple allergies and a clinical appearance of an allergic reaction. Sixteen of these patients displayed allergic symptoms after implant placement or unexplained implant failure. Nine of these patients displayed positive reactions to a titanium allergy tests. Based on these findings, the authors calculated an estimated titanium allergy prevalence of 0.6% [140].

Implant surface modifications may further affect the allergic effects of titanium implants on surrounding soft tissues and bone. For example, a titanium nitride–coated implant abutments has been associated with an allergic reaction, and the allergic reaction subsided after the removal of the titanium nitride abutment [141]. There have also been reports of exfoliative cheilitis (exfoliation of the lips) after implant placement [142]. Implant placement has also resulted in facial eczema, while implant removal resolved the eczema, confirming the positive relationship between implant materials and allergic reactions [143]. These allergic reactions are somewhat surprising in light of the widespread use of titanium oxide in dermatological products, toothpaste, icing, salad dressing, chewing gum, candy, milk, tattoo ink, and paints [144].

9.8 Etiology: Foreign Body Reaction

All titanium implants trigger foreign bodies in humans, regardless how well they might integrate. The "zone of tolerance" between the bone and the implant provides an equilibrium between the implant and the human body [145]. In some cases this equilibrium is shifted from normal osseointegration to a foreign body reaction. Supportive of the concept of the implant as a foreign body, a study comparing the levels of periodontal pathogens and pro-inflammatory cytokines around healthy teeth and healthy implants demonstrated approximately twice as many pro-inflammatory cytokines around healthy implants as around healthy teeth [146]. The most prominent cytokines around implants included IL-1ß, IL-6, IL-8, and TNF-α. Cytokine levels in the periphery of healthy teeth and implants were higher when bacteria were detected.

The presence of bacterial plaque around failing dental implants makes it difficult to determine with certainty whether inflammatory reactions in the implant periphery are due to foreign body reactions or microbial-triggered inflammation caused by dental plaque. In contrast, orthopedic implants are not exposed to a microbe-rich environment and thus are fairly free of bacterial contamination. Loss of osseointegration in orthopedic implants is thus due to some form of "foreign body reaction." [147] Albrektsson et al. claim that initial marginal bone loss around implants is a reaction to treatment and not a disease process [148]. They state that the initial foreign body response can be sustained and aggravated, leading to significant bone loss and implant failure. In these cases, once severe bone loss has occurred, a secondary bacterial infection may follow. The authors suggest that marginal bone loss around an implant should not be regarded as a periodontitis-like disease, but instead as a "disbalance" caused by a foreign body response.

9.9 Etiology: Titanium Particles

The foreign body reaction against a titanium implant may either be directed against the entire implant or against the small titanium particles on the implant surface. Titanium ions have been located in the tissues surrounding both dental and orthopedic implants, and these in turn have been associated with tissue discoloration and foreign body reactions to these particles [137, 149]. Once an implant has been placed, the presence of titanium particles may not be limited to the immediate implant periphery but, by ions, may also migrate to distant organs through the blood vessels in the nearby soft tissue and bone. One study reported a slight increase in titanium within the lungs and regional lymph nodes after implant placement in sheep mandibles [150]. Two of these implants failed, resulting in a much higher level of titanium in the lungs and lymph nodes (7–9.4 times the levels in controls). In the orthopedic literature, numerous articles have discussed the possibility of metal debris traveling to distant organs, often referred to as "metallosis." [147, 151] A study on human cadavers with joint replacements determined metallic wear particles in the lymph nodes near the aorta in 68% of the patients [152]. An additional 38% had metallic particles in their liver and/or spleen. These particles were detected in aggregates surrounded by macrophages, a cellular response to rid the body of debris. These particles were more prevalent in patients with failed implants, similar to the findings in the sheep mandible study mentioned above.

Titanium particles can be released from the implant surface in numerous ways. Titanium can simply dissipate from the implant surface during and after placement, it can flake off of the implant due to mechanical forces, and it can exfoliate due to oxidative corrosion of the implant surface. Titanium particles released from implants vary in size from small ions to large titanium pieces [152].

It is not clear whether titanium exfoliates from the implant during surgical placement. Most modern-day implants have a surface that is treated and roughened, a process which has the potential to facilitate the exfoliation of small pieces of titanium. Senna et al. inserted three different implant designs (Nobel, Straumann, and Astra) into bovine ribs to evaluate the presence of loose titanium particles [153]. In this study, all three implant designs revealed a decrease in both surface area and

surface roughness after insertion into bone. Loose titanium and aluminum particles were observed, mainly at the crestal portion of the bone. A separate study on the titanium plasma sprayed (TPS) implant surface reported titanium granules in the soft tissue and bone after implant insertion [154]. Suarez et al. studied five different implant surfaces with the outcome that the grit blasted surface resulted in the greatest degree of titanium exfoliation during placement into bovine ribs [155]. Sridhar et al. simulated surgical placement of Straumann dental implants into foam blocks of varying densities designed to match different bone densities seen in the mouth [50]. The authors of this study reported that implant insertion did not result in exfoliation of titanium particles into the surrounding osteotomy site.

Localization of titanium particles in tissues surrounding implants poses the question whether particles were exfoliated during or after implant placement. Some studies have detected titanium particles in the surrounding soft tissue after the implant has been in function. Olmedo et al. conducted exfoliative cytology of the peri-implant mucosa and detected metal particles embedded in the soft tissue of both healthy and diseased implants [156]. The diseased implants displayed a higher concentration of metal within the soft tissue. Another study screened the plaque around healthy and diseased implants for titanium particles [157]. All of the implants screened displayed titanium particles within the plaque, but the diseased implants exhibited significantly more titanium per unit area of plaque. However, it is not clear whether these titanium particles were exfoliated during implant placement, as a result of metal fatigue, or simply dissolution of the titanium surface over time.

A phenomenon known as fretting corrosion occurs at the interface of two closely fitting surfaces when they are subjected to repeated micro-motion or vibration [151]. In the dental field, fretting corrosion may occur between the implant and the abutment that is attached to it [158]. Modern implant designs have attempted to minimize this micro-motion [159]. A very small gap between the implant and abutment, known as the microgap, allows for metal fatigue over time.

Fretting corrosion results in surface irregularities on both the implant and the abutment and leads to metal exfoliation into the surrounding tissue. When metal-on-metal wear occurs, there is a chance that the titanium oxide layer on the implant will be mechanically destroyed [151]. The implant will then be at risk for true oxidative corrosion, and only a newly formed titanium oxide layer on the implant surface would counteract oxidative corrosion. Tawse-Smith et al. collected exfoliative cytology samples from the tissue of implants restored with zirconia abutments and crowns [160]. Elemental analysis revealed that in these samples, high numbers of titanium particles were present at the implant abutment interface and in the soft tissue adjacent to the crown. Other studies demonstrated that the implant is at risk for a galvanic reaction between dissimilar metals when nonprecious metals are used for the abutment, resulting in corrosion and a loss of the titanium oxide layer [161].

The original Brånemark implants were made of commercially pure titanium, while modern implants are alloyed with other metals, including iron, aluminum, and vanadium. Iron is added for corrosion resistance, aluminum is added for increased strength, and vanadium acts as an aluminum scavenger to prevent corrosion [162]. Steineman has demonstrated that titanium alloys (TiAlV) are not as well integrated as pure titanium and have an enhanced corrosion rate [145]. According to Khan, titanium alloys have a better combination of corrosion and wear resistance, while pure titanium shows better corrosion resistance but inferior wear characteristics [163]. Modern titanium alloys are touted to be highly resistant to corrosion, but the extent to which stress and wear accelerate the corrosion rate of titanium remains understudied [24].

Continuous loading, micro-motion, and acidic environments may result in a permanent loss of the titanium oxide (TiO_2) layer on the implant surface and eventual corrosion of the implant [158]. Oxidative corrosion involves losing metal due to a chemical reaction that takes place with an electrolyte or acid as the metal repassivates or reforms an oxide layer [151]. Tribocorrosion refers to the combination of both fretting corrosion and oxida-

tive corrosion. With metals in general, this phenomenon occurs either along the entire surface or only in select locations. Typically, the majority of the titanium implant is stable and only a select area that lost its TiO_2 layer will experience corrosion. This phenomenon is referred to as pitting corrosion since it forms small pits in the areas that experience corrosion. Olmedo et al. installed both sterile titanium implants and implants with pitting corrosion into rat tibiae [164]. The implants with pitting corrosion displayed decreased bone–implant contact, and corrosion products were detected within the bone.

The microbe-rich oral cavity constitutes a challenging environment for implant placement, completely different from the sterile environment that prevails during the placement of orthopedic implants. Dental implants are constantly exposed to a variety of insults on a daily basis. Dental implants are susceptible to corrosion once exposed to an acidic environment and in the presence of micro-motion. There are two known situations in the oral cavity in which a dental implant is exposed to an acidic environment: acidic byproducts of oral bacteria and decontamination solutions used by the dentist or patient [165, 166].

Lactic acid is a waste product of the oral bacterial metabolism. The release of lactic acid may result in dental caries, gingivitis, periodontitis, or, in this case, peri-implantitis. Sridhar et al. immersed sterile dental implants into either a bacterial medium or a control medium in vitro [166]. In this study, the bacteria created a sustained acidic environment, leading to discoloration, deformation, corrosion, pitting, and rusting of the implant surface. In a follow-up study by the same authors, physiological mechanical forces on the implant in combination with a bacterial medium resulted in accelerated corrosion and dissolution of metal ions [159]. These results were corroborated by a University of Washington study that detected elevated levels of titanium within the plaque around implants with peri-implantitis when compared to the plaque around healthy implants [157]. In an in vitro study, implants were exposed to healthy human saliva for incremental lengths of time, resulting in significant dissolution of metallic particles already after 1 week [167]. Interestingly, trace amounts of vanadium were detected as well, questioning the stability of the TiAlV alloy used in modern implants.

Acidic medicaments used to decontaminate the implant surface provide another potential mechanism for implant corrosion. Wheelis et al. conducted an in vitro study to evaluate the corrosive effects of several detoxification solutions on Ti and TiAlV dental implants [165]. The solutions included citric acid, hydrogen peroxide, chlorhexidine gluconate, tetracycline, doxycycline, sodium fluoride, peroxyacetic acid, and CO_2 laser treatment. The treatments consisted of either immersing the implant in the solution or rubbing the implant with a cotton swab soaked in solution. Implants that were immersed in a solution with a pH less than three displayed corrosion and pitting of the implant surface. The authors also noted a color change in the acidic solutions, suggesting that titanium exfoliated from the implant. When rubbing was used, any solution with a pH less than 5.5 caused significant discoloration and pitting. The cotton swabs after solution administration contained remnants of titanium. Commercially pure Ti displayed less corrosion compared to the TiAlV alloy when subjected to the immersion protocol. These results suggest that the safest treatment modalities for implant surface decontamination include sodium fluoride and 3% hydrogen peroxide application as well as CO_2 laser treatment. Chlorhexidine may be applied to the implant surface but may lead to corrosion if it is burnished with a cotton swab.

Another source for titanium particles may be due to implant surface delamination. Delamination refers to the exfoliation or cleavage of a portion of the implant surface, resulting in the formation of a large titanium layer in the vicinity of the implant surface and exposure of the underlying implant body to corrosive environments. Rodrigues et al. reported corrosion in conjunction with surface delamination in both orthopedic and dental implants [158, 168]. Delamination of dental implants may be caused by micro-motion in an acidic environment, resulting in the exposure of the inner titanium body and accelerated dissolution [158]. After implant surface delamination, the underlying titanium body is unable to form a tita-

nium oxide layer if it is not exposed to oxygen. This results in a highly reactive surface that will interact with nearby acids and electrolytes in order to stabilize. Sridhar et al. determined that cyclic occlusal forces may result in surface delamination as well, providing additional evidence for the occurrence of micro-motion and fretting corrosion as causative factors for implant disintegration [159].

Based on the present data, there are several mechanisms contributing toward titanium dental implant corrosion (Fig. 9.7). At this point, it is not clear whether a corroded implant surface

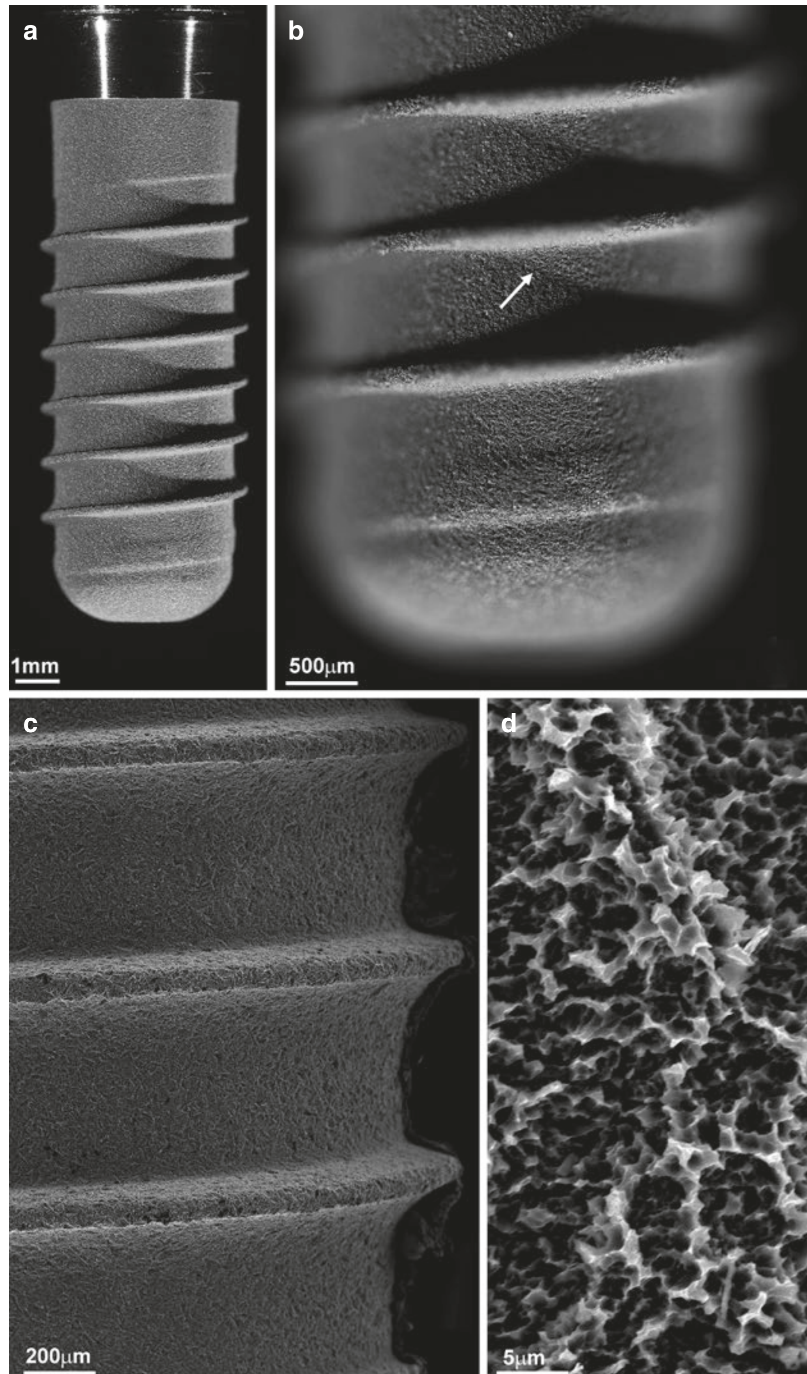

Fig. 9.7 Microscopic structure of a titanium implant surface (straumann standard plus implant). (**a**, **b**) are light micrographs and (**c**, **d**) are scanning electron micrographs. The arrow points to the roughened implant surface structure

is compatible with a healthy implant. However, there is emerging evidence suggesting that foreign particles embedded in the tissue provoke an inflammatory response. A study of orthopedic implants has demonstrated that metal debris trigger inflammation in vivo [169]. Wilson et al. obtained soft tissue biopsies around dental implants with peri-implantitis and evaluated them with light microscopy and SEM [49]. In this study, titanium and/or dental cement were detected in 34 of 36 biopsies, and particles were surrounded by plasma cells, giant cells, and other inflammatory cells. Another study demonstrated that titanium debris trigger a DNA damage response in oral epithelial cells [155]. Together, these studies suggest that foreign debris around titanium implants are not well tolerated and provide a baseline explanation for dental implant failure.

9.10 Summary: Osseointegration—Wishful Thinking or Oxymoron?

In the early years of implantology, osseointegration was the perfect term for the seemingly ideal junction between a living tissue, bone, and a block of metal, titanium. However, decades later, research demonstrated that the very interface between bone and metal became the cause for biological reactions against titanium particles and inflammation of the surrounding tissues, ultimately leading to bone loss and implant failure. While until today approximately 80% of all implants are considered clinically successful, even after 10 years, dentists are now seeking clinical solutions to treat peri-implant disease, how to prevent per-implantitis in the first place, and asking the question how safe titanium implants are for the health of their patients overall. While titanium implants remain a highly successful and lucrative treatment option, it is no longer clear whether the concept of osseointegration truly reflects the highly reactive interface between bone and titanium over the long time period of their exposure to the oral cavity.

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Part II

Periodontal Therapies Targeting Oral Microbiome

10 The Function of the Oral Microbiome in Health and Disease

Jorge Frias-Lopez and Ana E. Duran-Pinedo

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The term "microbiome" refers to the whole community of microorganisms as well as the environmental conditions and their functions and interactions that live in different habitats of the human body. Although in most places the coinage of the term is assigned to Joshua Lederberg [1], as early as 1988 Whipps JM et al. gave the first formal definition of the term: "*A convenient ecological framework in which to examine biocontrol systems is that of the microbiome. This may be defined as a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity.*" [2].

The oral cavity contains one of the most diverse microbiomes in the human body, in part because it provides significantly different niches that select for a different set of microorganisms. Of all bacterial species identified by the Human Microbiome Project, 26% were oral associated. Only the Gastrointestinal (GI) tract had a slightly higher number (Fig. 10.1) [3]. The microbial diversity of the oral cavity it is not just restricted to bacteria, a wide range of other microorganisms inhabit the human oral cavity, including fungi [4], viruses [4–6], archaea [7], and protozoa [8]. The oral microbiome forms a complex ecological community that influences oral and systemic health. The most common oral diseases, dental caries, and periodontal disease are microbiome-driven diseases. Recent studies have suggested that patients with longstanding periodontal disease are at higher risk of suffering systemic conditions such as diabetes, cardiovascular, respiratory diseases, and cancer as well as adverse reproductive outcome [9, 10]. The current control of dental plaque–related diseases is non-specific and is centered on the removal of plaque by mechanical means. However, due to our increasing understanding of the role that the oral microbiome plays in health,

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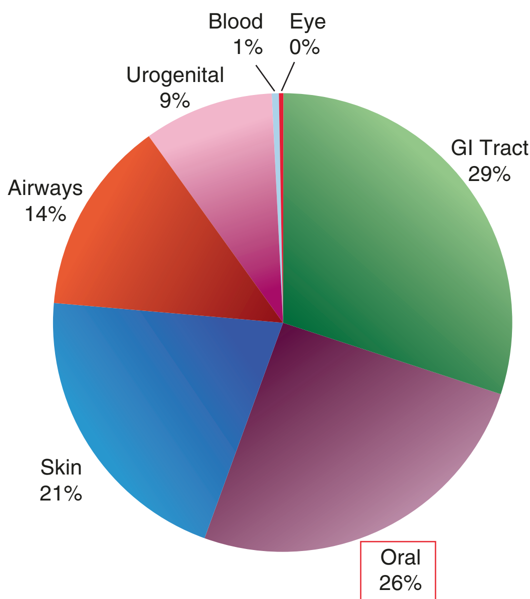


Fig. 10.1 The percentage of bacterial species distribution by body site. This figure shows the distribution by body site of bacteria that have been sequenced under the Human Microbiome Project (HMP). In red, the percentage of species in the oral cavity. (Reproduced from The NIH HMP Working Group et al. 2009. The NIH Human Microbiome Project. Genome Res. 19(12): 2317–2323)

the goal should be to develop methods based on the control of the microbiome aiming to maintain a healthy oral community instead of removing the whole microbial biofilm. This section of the book focuses on the modulation of the microbiome as a way of maintaining periodontal health.

To the newcomer, it might look that the study of the human microbiome did not exist before the establishment of the Human Microbiome Project by NIH. However, in the case of the oral microbiome, nothing could be further from the truth. The reason may be that the term "microbiome" was not in use when those studies were performed. In fact, among the first observations performed by Antonie van Leeuwenhoek using the simple microscope is the description of organisms of the oral microbiome [11].

Clinical needs drove the early studies on microbiology, and the early stage of development in oral microbiology was not an exception. The first comprehensive effort to describe the oral microbiome was made by W. D. Miller (1853–1907). He studied at the University of Ann Arbor, Michigan, and graduated as Doctor of Dental Surgery from the Philadelphia Dental College. In Berlin, he continued his studies, especially in microbiology. Between 1881 and 1907 Miller published more than 150 scientific articles, but his most famous work was the book *The Micro-Organisms of the Human Mouth: The Local and General Diseases Caused by Them*, which was first published in German in 1889 [12]. Miller recognized the critical elements for our modern concept of the etiology of dental caries: an acidic environment and the presence of specific microbes. However, it was JK Clarke in 1924 who the first to isolate a bacterial species from dental caries. The microorganism was named *Streptococcus mutans* and was shown to be capable of fermenting several sugars and producing a pH of 4.2 in glucose broth [13]. The other significant infectious human oral disease is periodontitis, the sixth most prevalent disabling health condition in the world, affecting 743 million people worldwide [14]. One of the early achievements in oral microbiology was the association between the accumulation of dental plaque and oral diseases. Dental plaque was one of the first samples described in detail by Antony van Leeuwenhoek, showing a highly diverse community with organisms presenting different morphologies and movement [11]. However, it was not until 1898 that G.V. Black and J.L. Williams linked the activity of the oral biofilm, which they described as the "gelatinous microbic plaque," with dental caries [15, 16].

In this chapter, we will briefly describe the role that the oral biofilm plays in health and disease, with particular emphasis on the new knowledge we have acquired in the last few years thanks to the application of new technologies such as next-generation sequencing (NGS) techniques to the study of the oral microbiome. The final goal would be to manipulate the oral microbiome to the host's advantage. However, we are not near that goal in part because understanding the biological properties that confer stability to the microbiome is a highly challenging task due to the enormous microbial diversity and interactions between microbes and host. If we want to manipulate the oral microbiome to our advan-

tage, we should understand the kind of interactions that occur in the oral cavity and their role in the stability of the oral microbiome.

10.1 The Oral Microbiome in Health

The oral ecosystem is a very complex system with several significantly different niches that lead to very different communities on various surfaces of the oral cavity [17–19]. Through antagonistic as well as mutualistic inter-species interactions, an ecological balance is reached that allows maintaining these complex microbial communities. Disruptions in the equilibrium of this ecosystem may lead to the changes in species composition and function that could lead to the development of oral disease [20, 21]. A transition from a commensal to a pathogenic oral microbiome causes an imbalance of oral homeostasis, a phenomenon called "dysbiosis" [22].

Until recently, most of the efforts in studying the human microbiome have been focused on the pathological aspects of specific organisms. However, there has been an enormous shift in the way we perceive the role of the microbiome, and we have now started to move from a pathogen-centric view of the microbiome to the idea that preserving a "healthy" microbiome could prevent the development of infectious diseases. We now know that the commensal microbiota plays an essential role in maintaining oral and systemic health and that a healthy oral microbiome exerts its functions on a series of different strategies.

The mere presence of the healthy oral biofilm in the oral cavity inhibits colonization by pathogens. The host-associated microbial communities interfere with the colonization and establishment of microbes of foreign origins through a phenomenon known as *bacterial interference* or *colonization resistance* [23]. Several mechanisms have been proposed to explain the colonization resistance, including stimulating the host immune response against invaders, competition for substrates and host-binding sites, and generating a microenvironment that is inhibitory to potential competitors [23]. One of the features that characterize the classical pathogens is their ability to overcome "colonization resistance" by using their arsenal of virulence factors. The example of the importance of "colonization resistance" as a mechanism to maintain health can be seen in the disruption of the oral microbiome by antibiotic treatments. Immunosuppressed patients or patients with diabetes all of which have an altered oral microbiome that allow for colonization by opportunistic pathogens such as *Candida* [24], *Staphylococcus aureus* [25, 26], and enterococci [26, 27].

One significant and less studied aspect of maintaining a healthy oral microbiome is the effect that it may have on other body sites away from the oral cavity. Thus the oral microbiome is an essential player in maintaining low blood pressure by the reduction of nitrate to nitrite, which is taken up into the bloodstream via gastric absorption and converted into nitric oxide. Nitric oxide is essential for vascular health and helps to keep blood vessels in good health and thus has an antihypertensive effect [28–30]. The essential role of oral bacteria has been confirmed by the observations that the increase in plasma nitrite following nitrate ingestion is markedly reduced by the use of an antimicrobial mouth rinse [29, 31]. Thus overzealous oral hygiene could have negative implications for what appears to be an essential natural mechanism for maintaining cardiovascular health. Among the members of the oral microbiome members of the genera *Veillonella*, *Neisseria*, *Prevotella*, *Actinomyces*, and *Haemophilus* are the most prevalent taxa isolated and thus may make a significant contribution to nitrate reduction in the oral cavity [32, 33].

10.1.1 Microbial Interactions

In a very comprehensive review, Marsh and Zaura define the types of interactions that occur in the oral cavity based on an extensive search on the PubMed database [34]. They identify two classes of significant interactions in health: (1) *synergistic interactions*, which include enzyme complementation or sharing, food webs, co-adhesion, cell–cell signaling, gene transfer, and modification of the environment; and (2) *antagonistic*

interactions, which include bacteriocin production, hydrogen peroxide production, organic acid production and generation of inhibitory pH conditions, bacteriophage release, competition for essential nutrients, and predation [34]. One of the critical features of the oral biofilm is that it presents a well-defined architecture derived from specific physical interactions between its members [35, 36]. One of the mechanisms by which these specific interactions occur is through co-adhesion, the adherence of planktonic cells to already attached organisms on a surface, facilitating the formation of multispecies biofilms [37] and thus the establishment of food webs and cell–cell signaling.

In the oral cavity, the primary source of nutrients for the microbiome are host proteins and glycoproteins, and these are obtained mainly from saliva in supragingival plaque [38, 39] and the gingival crevicular fluid (GCF) in subgingival biofilms [40]. Given the diversity of the oral microbiome, it is not surprising that bacteria cooperate in the degradation of those proteins, helping to maintain the complexity of the oral biofilm. In general, isolated oral bacteria do not grow well in proteins, but the coordinated action of proteolytic microorganisms leads to the formation of simpler products that could be used by other organisms. Thus the presence of *Prevotella intermedia* supports the growth of *Eubacterium lentum*, *Fusobacterium nucleatum, Parvimonas micra*, and *Streptococcus intermedius* in serum, organisms that do not grow well by themselves in the same medium [41]. Moreover, oral bacteria express a wide range of glycosidases, including sialidases, *N*-acetyl-β-D-glucosaminidase, β-D-galactosidase, and α-L-fucosidase that could result in the complete degradation of host glycoproteins [42–45].

10.1.2 Microbial Communication

Communication is one of the critical elements in the successful organization of the oral biofilm. Therefore cell–cell signaling interactions are an essential aspect of maintaining a stable, healthy microbiome. We now know that many bacteria communicate in a process generally referred to as "quorum sensing" (QS) and also by cyclic dinucleotides as signal molecules [46]. QS refers to microbial signaling systen that is mediated by molecules secreted by the bacteria themselves, and when they reach a specific threshold, either the producer or other organisms trigger a response [47]. The number of bacterial taxa harboring QS systems has grown to include hundreds of species across most known bacterial phyla [48]. QS is involved in controlling biofilms formation and gene expression in oral bacteria [49, 50]. Gram-positive bacteria use peptides as QS signal molecules, which generally have a narrow spectrum of activity. Among those peptides, the competence stimulating peptide (CSP) is an essential signal in a variety of *Streptococcus* species (*S. mutans*, *S. gordoni*i, and *S. intermedius*), whose function is to control the levels of proteins involved in biofilm formation, competence development, bacteriocin synthesis, fraticide, and autolysis [51–55]. Autoinducer-2 (AI-2) is synthesized by the luxS gene in several genera of oral Gram-positive and Gram-negative bacteria and is considered a "universal language" for inter-species communication. As an example, *F. nucleatum* AI-2 significantly enhanced the biofilm growth of *S. gordonii* and attachment of *F. nucleatum* to pre-formed *S. gordonii* biofilms. By contrast, *F. nucleatum* AI-2 reduced biofilm growth of *S. oralis* and attachment of *F. nucleatum* to pre-formed *S. oralis* biofilms [56]. Interestingly, AI-2 also plays a role in inter-kingdom communication in oral biofilms. Thus the ability of a *Porphyromonas gingivalis* ΔluxS mutant to induce an inflammatory response is severely impaired in fibroblasts [57]. More recently, cyclic dimeric guanosine 3′,5′-monophosphate (c-di-GMP), a bacterial secondary messenger, has become a molecule of high interest, since elevated concentrations of c-di-GMP regulate many processes affecting cell wall homeostasis, fatty acid synthesis, and the initiation and maturation of bacterial biofilms [46, 58, 59].

10.1.3 Antagonistic Interactions

The second class of interactions that shape the commensal or pathogenic nature of the oral biofilm is antagonistic interactions, where one of the interacting members of the microbiome benefits at the expense of the other organisms. Microorganisms synthesize and release antagonistic compounds that can give them a competitive advantage during colonization and when competing with other microbes. Among those compounds, bacteriocins are ribosomally synthesized proteinaceous antibiotics that kill or inhibit species closely related to the producer bacterium and are produced by a large number of non-pathogenic streptococci, which makes some of them ideal candidates for development as the model probiotic for the oral cavity [60, 61].

Another compound used by the members of the oral microbiome in their antagonistic interactions is the production of hydrogen peroxide. Oral streptococci can produce growth-inhibiting amounts of hydrogen peroxide as a byproduct of aerobic metabolism [62, 63]. Although the antagonistic effect of streptococcal hydrogen peroxide production is well known, its long-term ecological effects on shaping the oral microbiome are more complicated. At the initial stages of hydrogen peroxide production competitors are killed, thus promoting the selection of compatible species into the developing biofilm. As a consequence of cell lysis, there is a release of DNA into the environment. This extracellular DNA contributes to the stability of the oral biofilm [64–66].

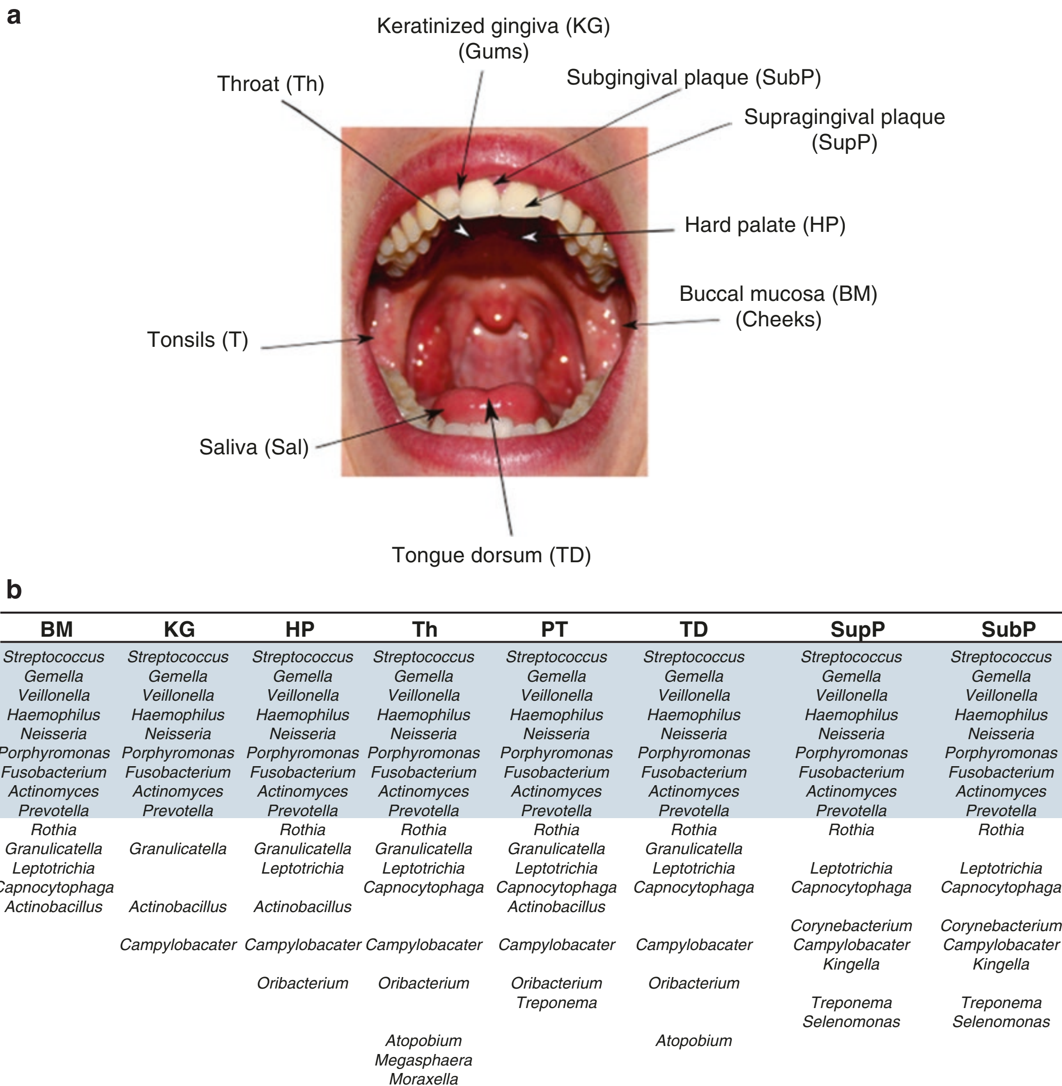
Furthermore, the extracellular matrix protects against the activity of antimicrobial compounds [22]. Hydrogen peroxide causes DNA damage, which in turn could lead to beneficial mutations in competent oral streptococci uptake of extracellular DNA. Extracellular DNA could, therefore, support adaptation processes to changing environmental conditions and promote the evolution of oral biofilm development [63]. Additionally, there may be varying concentrations of hydrogen peroxide in different regions of the biofilm, and the balance between a healthy microbiome and dysbiosis may be the result of multiple antagonistic microbial interactions.

10.2 Composition and Function of the Commensal Oral Microbiome

A wide range of microorganisms colonize the human oral cavity. Besides, bacteria and fungi, Archaea, viruses, and protozoa are part of a healthy microbiome [4]. Given the vast number of different organisms, we will focus our interest on the "bacteriome" (subsequently referred to as "microbiome") because it is the best described of the communities in the human microbiome in general. Current knowledge on the role of fungi, viruses, and protozoa as part of a healthy oral microbiome is beyond our goals for this chapter.

We can distinguish between two types of communities that can be found in the oral microbiome: the *indigenous community*, which is characterized by bacterial species that are found in virtually every human adult of all populations, irrespective of environmental conditions; and the *transient exogenous bacteria,* which are members of the community that are detected in the oral cavity but whose natural habitat is other [27, 67]. To distinguish between transient species and endogenous species, we cannot directly rely on human sampling studies. Rather, it has to come from comparing the human studies with environmental studies to determine the frequency with which clones of a particular genus are recovered as host associated or the environment associated [67–70].

Fortunately, there is vast wealth of knowledge related to the composition of the indigenous flora in the oral cavity compared to our understanding regarding other body sites, in significant part due to the pioneer work of people such as F. Dewhirst, B. Paster, S. Socransky, and A. Hafajjee [67–73]. The HMP assessed microbiome composition of nine intraoral sites (buccal mucosa, BM; hard palate, HP; keratinized gingiva, KG; palatine tonsils, PT; saliva, Sal, subgingival plaque, SubP; supragingival plaque, SupP; throat, Th; and tongue dorsum, TD) showing that the oral habitat is the most stable of all sites in the human body [74]. Specific genera are present in all oral sites, as shown in Fig. 10.2. Members of the genera *Streptococcus, Gemella, Veillonella, Haemophilus, Neisseria, Porphyromonas, Fusobacterium, Actinomyces,* and



BM	KG	HP	Th	PT	TD	SupP	SubP
Streptococcus	*Streptococcus*	*Streptococcus*	*Streptococcus*	*Streptococcus*	*Streptococcus*	*Streptococcus*	*Streptococcus*
Gemella	*Gemella*	*Gemella*	*Gemella*	*Gemella*	*Gemella*	*Gemella*	*Gemella*
Veillonella	*Veillonella*	*Veillonella*	*Veillonella*	*Veillonella*	*Veillonella*	*Veillonella*	*Veillonella*
Haemophilus	*Haemophilus*	*Haemophilus*	*Haemophilus*	*Haemophilus*	*Haemophilus*	*Haemophilus*	*Haemophilus*
Neisseria	*Neisseria*	*Neisseria*	*Neisseria*	*Neisseria*	*Neisseria*	*Neisseria*	*Neisseria*
Porphyromonas	*Porphyromonas*	*Porphyromonas*	*Porphyromonas*	*Porphyromonas*	*Porphyromonas*	*Porphyromonas*	*Porphyromonas*
Fusobacterium	*Fusobacterium*	*Fusobacterium*	*Fusobacterium*	*Fusobacterium*	*Fusobacterium*	*Fusobacterium*	*Fusobacterium*
Actinomyces	*Actinomyces*	*Actinomyces*	*Actinomyces*	*Actinomyces*	*Actinomyces*	*Actinomyces*	*Actinomyces*
Prevotella	*Prevotella*	*Prevotella*	*Prevotella*	*Prevotella*	*Prevotella*	*Prevotella*	*Prevotella*
Rothia		*Rothia*	*Rothia*	*Rothia*	*Rothia*	*Rothia*	*Rothia*
Granulicatella	*Granulicatella*	*Granulicatella*	*Granulicatella*	*Granulicatella*	*Granulicatella*		
Leptotrichia		*Leptotrichia*	*Leptotrichia*	*Leptotrichia*	*Leptotrichia*	*Leptotrichia*	*Leptotrichia*
Capnocytophaga			*Capnocytophaga*	*Capnocytophaga*	*Capnocytophaga*	*Capnocytophaga*	*Capnocytophaga*
Actinobacillus	*Actinobacillus*	*Actinobacillus*		*Actinobacillus*			
						Corynebacterium	*Corynebacterium*
	Campylobacter	*Campylobacter*	*Campylobacter*	*Campylobacter*	*Campylobacter*	*Campylobacter*	*Campylobacter*
						Kingella	*Kingella*
		Oribacterium	*Oribacterium*	*Oribacterium*	*Oribacterium*		
				Treponema		*Treponema*	*Treponema*
						Selenomonas	*Selenomonas*
			Atopobium		*Atopobium*		
			Megasphaera				
			Moraxella				

Fig. 10.2 Genera detected in the sampled digestive tract microbiome sites based on similarities in microbial composition. (**a**) Physical oral sites sampled in this work. (**b**) Taxonomic composition of the microbiota in eight digestive tract body habitats investigated based on the average relative abundance of 16S rRNA pyrosequencing reads assigned to a genus. Highlighted are genera common to all the sites. Buccal mucosa (BM), keratinized gingiva (KG) and hard palate (HP), throat (Th), palatine tonsils (PT), tongue dorsum (TD), supragingival (SupP), and subgingival plaques (SubP). (Modified from Segata et al. 2012. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat, and stool samples. Genome Biol. 13, R:42)

Prevotella can be found in all oral sites of a healthy person [68, 74–77]. Despite those similarities, the oral microbial composition is well differentiated by niche location. Certain species are only found at significant levels in a particular location of the oral cavity, as indicated in Fig. 10.2b. For instance, *Corynebacterium* and *Kingella* represent a substantial fraction of the microbiome in the subgingival and supragingival biofilm while members of the genus *Granulicatella* are absent in those sites and present in the rest of sites studied [77]. However, what defines the different niches in the oral cavity is the relative abundance of the various members of the community. As shown in Fig. 10.3a, the majority of genera are shared by the different sites, but their relative abundance is very differ-

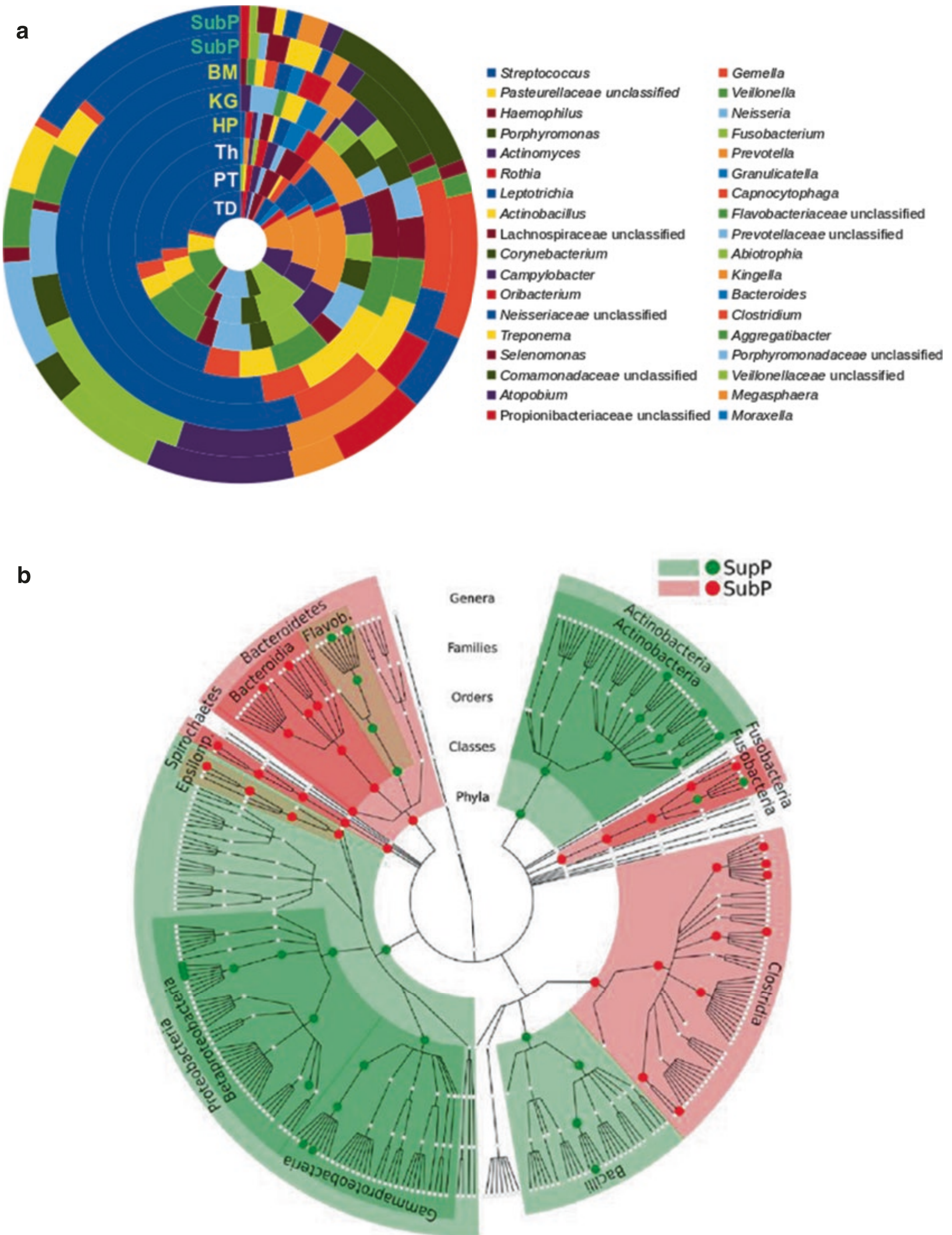


Fig. 10.3 Niche specialization in the oral cavity even among adjacent body sites. (**a**) Distribution of the genera detected in the sampled digestive tract microbiome sites based on similarities in microbial composition as described in Segata et al. [78]. (**b**) Circular cladogram based on the RDP Taxonomy [79] reporting taxa significantly more abundant in supragingival (red) and subgingival plaque (green) and demonstrating the extensive specialization even at these highly related sites. (Reproduced from Segata et al. 2012. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat, and stool samples. Genome Biol. 13, R:42)

ent. Thus, members of the genus *Capnocytophaga* are relatively abundant in the subgingival and supragingival plaque while, although present in other sites, representing just a small fraction of the total biofilm. Niche specialization is present even among adjacent body habitats. Figure 10.3b shows taxa significantly more abundant in supragingival and subgingival plaque and indicates the high specialization even at these highly related sites. At the class level, Actinobacteria, Bacilli, Gamma-proteobacteria, Beta-proteobacteria, and Flavobacteria are characteristic of the supragingival plaque, whereas Fusobacteria, Clostridia, Epsilon-proteobacteria, Spirochaetes, Bacteroidia, and unclassified Bacteroidetes are biomarkers for the subgingival plaque [77].

Recently with the use of next-generation sequencing approaches the functional potential of the oral microbiome can be assessed. Information on the genes present in the community by shotgun metagenomic analysis allows for comparing the potential activities carried out by the oral microbiome in different sites and environments [77, 80]. Due to the complexity of the oral microbiome, instead of assessing the potential of specific species, it is more logical to determine the functional activities of the community as a whole. Given the specificity of the niche of the oral community composition, it is not surprising that in health it seems to be a partitioning of functions associated with different oral sites [77, 80]. Figure 10.4 summarizes our cur-

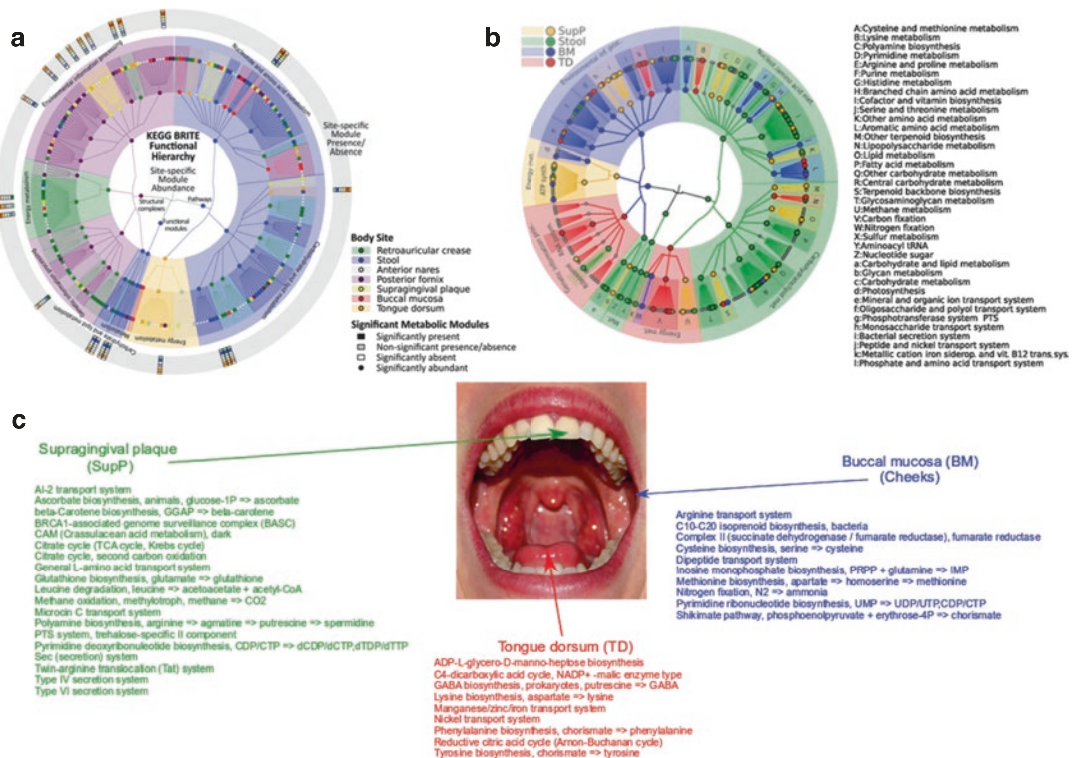


Fig. 10.4 Bacterial metabolic activities associated with oral sites. (**a**) Metabolic modules are differentially present or abundant in at least one body habitat of the human microbiome. Metabolic modules and pathways from the KEGG BRITE hierarchy [81] found to be differentially abundant (inner cladogram) or differentially covered (outer ring, presence/absence) in the human microbiome. Differentially abundant modules are colored by their most abundant body habitat. (Reproduced from Abubucker et al. 2012 Metabolic Reconstruction for Metagenomic Data and Its Application to the Human Microbiome. PloS Comput Biol.;8(6):e1002358). (**b**) Functional characterization of the digestive microbiota based on metabolic pathway abundances in the buccal mucosa, supragingival plaque, tongue dorsum, and stool from metagenomic shotgun sequencing. (Reproduced from Segata et al. 2012. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat, and stool samples. Genome Biol. 13, R:42). (**c**) Oral site specificity of microbiome activities [77]

rent knowledge on the topic. The supragingival plaque is enriched in metabolic functions associated with environmental information processing (e.g., secretion systems, signal transduction, and signaling molecules and interaction) and energy metabolism (e.g., oxidative phosphorylation, methane metabolism, and nitrogen and sulfur metabolism). Buccal mucosa activities are also enriched in environmental information processing but of different nature (e.g., peptide and amino acid transport).

Interestingly, one of the increased activities in the tongue dorsum was γ-aminobutyric acid (GABA) biosynthesis. GABA is the primary inhibitory neurotransmitter known to counterbalance the action of the excitatory neurotransmitter glutamate. What could be the role of the GABA produced in the oral cavity in the health of the host is still not known.

In one study that focused on the metatranscriptome during biofilm formation and after meal ingestion [82], results showed that changes in bacterial activity during plaque development and after meal ingestion were person-specific. In some cases, over 80% of active bacteria corresponded to only three genera (*Actinomyces*, *Corynebacterium,* and *Rothia*) whereas other individuals did not show any dominant genera in their active microbial community. As could be expected, the predominant genera of active members of the community were *Streptococcus* (12–19%) and *Actinomyces* (3–12%). Actinomyces showed higher frequencies in early plaque samples, in agreement with its known role as an early colonizer. Other frequent active genera were the Actinobacteria, *Rothia*, *Angustibacter,* and *Kineococcus*; the Proteobacteria *Neisseria*, *Kingella,* and *Alysiella*; the Firmicutes *Gemella*, *Paenibacillus,* and *Veillonella* and finally *Capnocytophaga* and *Fusobacterium*. *Kineococcus*, *Alysiella*, and *Paenibacillus* are genera commonly found in environmental samples. Nonetheless, members of these genera have been identified in oral samples, although generally at low numbers [83–88].

In early stages of supragingival plaque development, genes involved in the metabolism of carbohydrates, energy, amino acids, cofactor/vitamins, and xenobiotic degradation were predominantly up-regulated. In late stages, the up-regulation is associated with genes involved in quorum sensing response, in particular genes, identified as belonging to Type II secretion systems. Since type II secretion systems promote the secretion of folded periplasmic proteins that typically play a role in survival, this finding indicates that the community is adapting as it develops. Among the significant results of this work is that the metatranscriptomic profiles during biofilm formation and after meal ingestion were person-specific. Some individuals showed virtually no changes in the active bacterial population after food ingestion, suggesting that their microbiota is not affected by food ingestion, potentially reducing the risk of acidic pH and promoting dental health. They also showed that the expression of genes linked to translation machinery is higher in early biofilm stages, whereas more specialized genes are expressed in the mature biofilm. Among them, genes involved in competence, quorum sensing, mutacin production, and DNA uptake were over-expressed in late biofilm, indicating a more sophisticated level of interactions in mature biofilm than at earlier stages of biofilm formation.

We are just at the dawn of functional studies of the oral microbiome. In the next few years, an increased effort should be placed on understanding how the distinct functional activities in the different oral sites are linked to a healthy oral microbiome.

10.2.1 From a Commensal to a Dysbiotic Microbiome

The transition from a commensal microbiome to a dysbiotic one is an essential step in the role that the microbiome plays in oral diseases. Dysbiosis in the oral cavity occurs when environmental conditions are altered for enough period of time, thus affecting the ecology of the ecosystem, leading to an overgrowth of certain indigenous microorganisms, which become the dominant species at the affected site at the expense of health-associated taxa [34, 89]. The primary oral

diseases of humans caused by bacteria, that is, caries and periodontal diseases, are associated with dysbiotic microbiomes, and dysbiotic communities have also been associated with systemic diseases such as cancer [90].

In the present section, we will discuss the microbial activities and environmental changes that lead to dysbiosis in a cariogenic microbiome and perio-pathogenic microbiomes.

10.2.2 Dental Caries

Dental caries is one of the most common infectious diseases, and it is associated with an increased frequency of dietary sugar intake. Saccharolytic bacteria in the oral microbiome metabolize those sugars, and a low pH is generated within the biofilm. To explain the evolution of the disease and the role that the oral microbiome plays in caries, Marsh proposed the "ecological plaque hypothesis" [91, 92]. This hypothesis posits that selection of cariogenic organism is linked to changes in environmental conditions that lead to shifts in community composition and function. If the pH remains below a specific pH value (5.5) for extended periods, a change in the bacterial populations to more cariogenic organisms that are acidogenic and acid-tolerant (aciduric) can occur [92]. During this progression, there are a loss of diversity and a reduction in levels and activity of beneficial bacteria [93–95], although the diversity may increase when the lesion penetrates dentine, which reflects the importance of the differences of activities in different oral niches [96, 97].

Historically acidogenic species of the genus *Streptococcus,* mainly *S. mutans,* have been considered the etiological agent of dental caries. Indeed, numerous metagenomic studies have shown that certain acidogenic and aciduric species such as *S. mutans* and *Lactobacillus* spp. are highly correlated with active caries [93–95, 97, 98]. However, recent studies show evidence that many other species are likely to be relevant. They include members of the genera *Actinomyces, Fusobacterium, Porphyromonas, Rothia, Granulicatella, Gemella, Selenomonas, Bifidobacteria, Scardovia*, and *Haemophilus* [95, 99–101]. Moreover, the bacterial communities present at different stages of caries development change. Microbial composition at the initial phase of caries is significantly different from that found at subsequent stages [19, 102]. The relative proportion of *S. mutans* increases from 0.12% in healthy dental plaque to 0.72% in caries affecting enamel. Nonetheless, *Streptococcus mitis* and *Streptococcus sanguinis* were the dominant streptococci in enamel lesions.

We now know that the number of active species of bacteria in dental caries lesions is high, supporting the idea that a complex consortium multiple microorganisms cooperate to initiate and expand the disease [96, 99] rather than a single species being responsible for the condition. Thus, in a pioneer metatranscriptomic study focused on the active bacterial communities in caries lesions, Simón-Soro et al. [97] showed that active caries lesions contained between 70 and 400 metabolically active species of bacteria. Agreeing with those results, in a more recent study, Kressirer et al. found that gene expressed in caries mapped 108 named species [99].

Active communities in different caries environments are very distinct. Thus non-cavitated ("white spot" lesions), open dentin, or enamel-dentin caries presented different active communities. While members of the genera *Streptococcus* and *Veillonella* were highly active in all three types of caries, *Lactobacillus* spp. were only highly active in the enamel-dentin caries sites, where the carious lesion might extend into dentin without a clinically visible crack at the enamel surface [97]. Kressirer et al. found that *Lactobacillus* spp. were highly active in coronal caries, while in dentin caries, the most representative members of the active community were *Neisseriales, Prevotellaceae*, and *Actinobacteria*. Moreover, although *S. mutans* was elevated in coronal and dentin caries compared with caries-free, another important cariogenic organism, *S. wiggsiae* was associated with dentin caries, suggesting that *S. wiggsiae* was primarily active in dentin lesions [99]. By contrast, Peterson et al., when looking at community-wide expression profiles of dental plaque samples from dental car-

ies, found that a limited number of species were responsible for the transcripts in the community. Streptococci represent the majority of the active members in caries. *S. sanguinis* was the most active member of the community with 16% of the transcripts, followed by *S. mitis* (10%), *V. parvula* (9%), *Capnocytophaga* sp. (9%), *S. oralis* (8%), *Streptococcus* spp. (7%), *G. haemolysans* (5%), *S. gordonii* (4%), and *Neisseria* sp. (3%) [103].

More interesting is the fact that despite interpersonal variation in the composition of active communities, when looking at functional activities, distinct patterns of expression emerge. A large number of activities are associated with oxidative stress with high expression of proteins that metabolize superoxides, and peroxides are present in enamel/coronal caries [102, 104]. Functional profiles of caries-associated bacterial communities indicate that genes involved in this kind of activities are over-represented only at the initial stage (enamel caries). In later stages expression of genes coding for osmotic stress tolerance proteins as well as collagenases and other proteases [102] and arginine deaminase and urease that counterbalance acidic pH [99] enable dentin degradation in dentin cavities.

An analysis of already-existing metatranscriptomic libraries to identify functional differences between health and caries showed that deregulated metabolic sub-networks of KEGG Orthology (KOs) groups were indeed significantly different [105]. *S. mutans* and *Lactobacillus casei,* which frequently are associated with dental caries, were among the species that showed the most significant up-regulation of the KOs in the dental caries sub-network in disease. Nonetheless, in addition to the well-known cariogenic species, a more extensive range of species showed a similar pathogenic expression profile in the maximum-scoring sub-network. The disease-associated parts of the caries sub-network included nine KOs from the pathways of the phosphotransferase system and fructose and mannose metabolism. Among them are sugar phosphotransferases involved in the beta-glucoside metabolism that are critical elements in the process of colonization [106, 107]. Another set of disease-associated pathways was a pathway that converts sorbitol to fructose 6-phosphate. Unlike many other oral species, *S. mutans* can metabolize sorbitol as a carbon source [108, 109]. In contrast, only one KO was down-regulated in disease in the same pathway. This KO converts fructose to fructose 6-phosphate, suggesting that while in health fructose may be the primary carbon source, in dental caries sorbitol might be used as an additional source of carbon [105].

Underlying how much is still unknown on the mechanisms of dental caries, a recent study that applied metatranscriptomics and metabolomics approaches found a much higher diversity in alkali-generating pathways within complex oral biofilms than previously known. The classical mechanism against acidification of the ecosystem was thought to be alkali production through ammonia production from arginine and urea [110, 111]. However, Edlund et al. found that glutamate dehydrogenase, threonine and serine deaminase, and up-regulation in membrane proteins involved in ammonia gas conduction were acting as a way to control pH besides the urease activity and arginine deaminase system [112]. Additionally, this study revealed that *Veillonella* species are well adapted toward acid stress by up-regulating various metabolic pathways associated with the control of pH.

10.2.3 Periodontal Diseases

Periodontal diseases (gingivitis and periodontitis) are polymicrobial diseases caused by the coordinated action of a complex microbial community that lead to inflammation of tissues supporting the teeth. Gingivitis, the mildest form of periodontal disease, is perhaps the most common bacterial disease of humans with a prevalence in adults of over 90% [113]. Gingivitis is characterized by a buildup of the oral biofilm in the subgingival crevice and inflammation of the gums [114–116]. Its symptoms can be eliminated through professional dental cleaning. However, if untreated, it can progress to chronic periodontitis, a severe form of the periodontal disease characterized by chronic inflammation, destruction of gum tissue, and ultimately loss of both tooth

attachment and alveolar bone [117]. Periodontitis is the sixth most prevalent disabling health condition in the world, affecting 743 million people worldwide [14].

In the case of periodontitis, the inflammatory response of the host is a significant driver of the environmental changes that would select for a dysbiotic community. There is host–microbiome cross-talk that leads to inflammation and bone loss [118]. Changes in local environmental conditions will alter the competitiveness and outcome of multiple interactions among the organisms in the subgingival microbiota, leading to substantial changes in the microbial composition of the biofilm.

Periodontal diseases are an example of what van Steenbergen described as "pathogenic synergism" [119] in which the outcome of the dysbiotic process is the result of the combined activity of an interacting network of organism in which each member is only partly responsible for the virulence of the whole. Different species would undertake a distinct role or function for the consortium to persist and cause disease. Consistent with the new concept of low abundance species ("keystone pathogens") having a disproportionate effect of the virulence of the whole community [120–122].

Models of periodontal disease progression posit that tissue destruction progresses through periods of acute exacerbation (activity) followed by periods of remission [123–125]. Goodson et al. found that in 22 untreated patients whose attachment level was measured every month for 1 year, 5.7% of the sites became significantly deeper while 11.5% of the sites became significantly shallower during that period. Among sites with increased pocket depth, approximately half showed spontaneous recovery to their original depth, and half of these sites exhibited cyclic deepening followed by spontaneous recovery to their original depth [126]. Others have also described patterns of "exacerbation" and "remission" [127, 128]. It has been postulated that changes in the composition of subgingival biofilms could explain these periods of disease activity. In fact, a few studies have found differences in the levels of subgingival species when comparing progressing and non-progressing sites using cultural [129, 130] and molecular approaches [131–133]. These studies also demonstrated a considerable overlap in the composition of the microbial communities associated with progressing and non-progressing lesions, suggesting that the difference in the periodontal status of the sites could not be explained solely by the reported differences in the subgingival microbial composition.

10.2.3.1 Gingivitis

Studies on the function of the oral microbiome in *gingivitis,* the mildest form of periodontal disease, are limited. Just recently metatranscriptome has begun to be used to analyze community-wide gene expression in the human microbiome [134–138]. It is known that there are changes that are characteristic of this condition and distinct from both health and periodontitis [139, 140]. At least eight taxa become particularly dominant, including TM7, *Leptotrichia, Selenomonas, Streptococcus, Veillonella, Prevotella, Lautropia*, and *Haemophilus* [140, 141].

Nowicki et al. studied the metatranscriptome changes associated with the transition from health to periodontitis and showed that changes in the overall activity of oral microbiota during the early stages of periodontal disease progression promote enhanced destruction of host tissue and survival within the oral cavity [137]. Interestingly, the list of most highly abundant genera was similar to results based on community composition mentioned above, with *Leptotrichi*a, *Prevotella*, *Streptococcus*, *Fusobacterium*, and *Actinomyces*. They also showed that virulence-related gene expression is elevated during the transition from oral health to gingivitis and that metabolic pathways more strongly associated with health include genes involved in ascorbate and aldarate metabolism, porphyrin and chlorophyll metabolism, carbon fixation in prokaryotes, the pentose phosphate pathway, antibiotic biosynthesis, and pyruvate metabolism. Metabolic pathways more strongly associated with gingivitis include genes involved in pyrimidine metabolism, vitamin B_6 metabolism, glycolysis and gluconeogenesis, and propanoate and butanoate metabolism [137]. Genes with virulence-related activities with sig-

nificant changes in expression in gingivitis relative to health comprise peptidases, nucleases, and hydrolases as well as genes involved in chemotaxis and cell surface modifications.

Periodontal pathogens, representative of the most abundant genera in the study (*Leptotrichia buccalis*, *Prevotella nigrescens, S. constellatus, F. nucleatum,* and *Actinomyces israelii*), up-regulate the expression of specific and general virulence-related genes during gingivitis relative to health. Included within the particular virulence genes category are collagenases, gingipains, and hemagglutinins, and in the generalized virulence-related gene are nonspecific peptidases or proteases and stress response proteins). *L. buccalis* virulence-related gene products up-regulated during gingivitis include several genes involved in antibiotic resistance and nonspecific proteases. *P. nigrescens* and *F. nucleatum,* two members of the orange complex according to Socransky and Haffajee [142], significantly over-expressed a wide variety of virulence-related genes in plaque samples from teeth at a time point showing clinical indications of disease, including those involved in antibiotic resistance, proteolysis, breakdown of collagen, and iron uptake. Virulence factors endothelin-converting enzyme 1 and a gingipain were found to be highly up-regulated by *P. nigrescens*. These first findings regarding the functional changes seen between health and gingivitis are primarily corroborated by similar results in previous work analyzing samples from healthy and diseased (periodontitis) teeth and seem to indicate a transition state regarding the gene expression of virulence-associated genes in the community.

10.2.3.2 Periodontitis

Periodontitis is the most severe form of periodontal disease that, if left untreated, causes destruction of gum tissue and loss of both tooth attachment and alveolar bone by a chronic inflammation process. As shown in the next section of this chapter, there is a good body of research that links periodontitis to several systemic diseases, including cardiovascular disease, pre-term delivery, and low birth weight, diabetes mellitus, respiratory infections, and osteoporosis.

The oral microbiome is highly diverse, and for that reason, most studies on the microbiome of periodontitis have focused on describing microbial communities or on the immunological response of the host to the bacterial challenge. Others studies are starting to integrate biofilm composition and host response [143, 144], and just a few have begun to identify certain commonalities in the way the function of the microbial community shifts in dysbiosis. To this date, the model for periodontitis progression suggests that changes in periodontal microbiota lead to dysbiosis by deregulating the host-immune response, leading to chronic inflammation. We know very little about the process that initiates dysbiosis leading to periodontal disease progression. Recently, some studies have identified the functional signatures that characterize the periodontal disease sites, highlighting the hypothesis that the oral microbiome as a meta-organism can induce disease progression.

The current advances in high-throughput sequencing technology have tremendously enriched our knowledge not only on the composition of the oral microbial community but on the gene expression. It is now known that the periodontal microbiota is more diverse than previously thought, with over 700 microorganisms identified [67, 69, 70]. The vast diversity of periodontal microbiota suggests that the pathogenic mechanisms supporting periodontitis are the results of a coordinated synergy of more than a small group of organisms.

Based on sequencing-based findings, a polymicrobial synergy and dysbiosis (PSD) model has been proposed to explain the periodontal pathogenesis [120]. This model suggests that periodontal diseases are initiated by a dysbiotic microbial community, rather than by a specific group of periodontal pathogens. The whole community expresses distinct genes that synergize to lead the microbiome to disease. The dysbiosis of the microbial community can disrupt the host-microbe homeostasis and facilitate its transition to a chronic inflammatory state. Thus, the whole microbial community modulates the disease progression, and the study of changes in metabolic activities is critical for our understanding of the

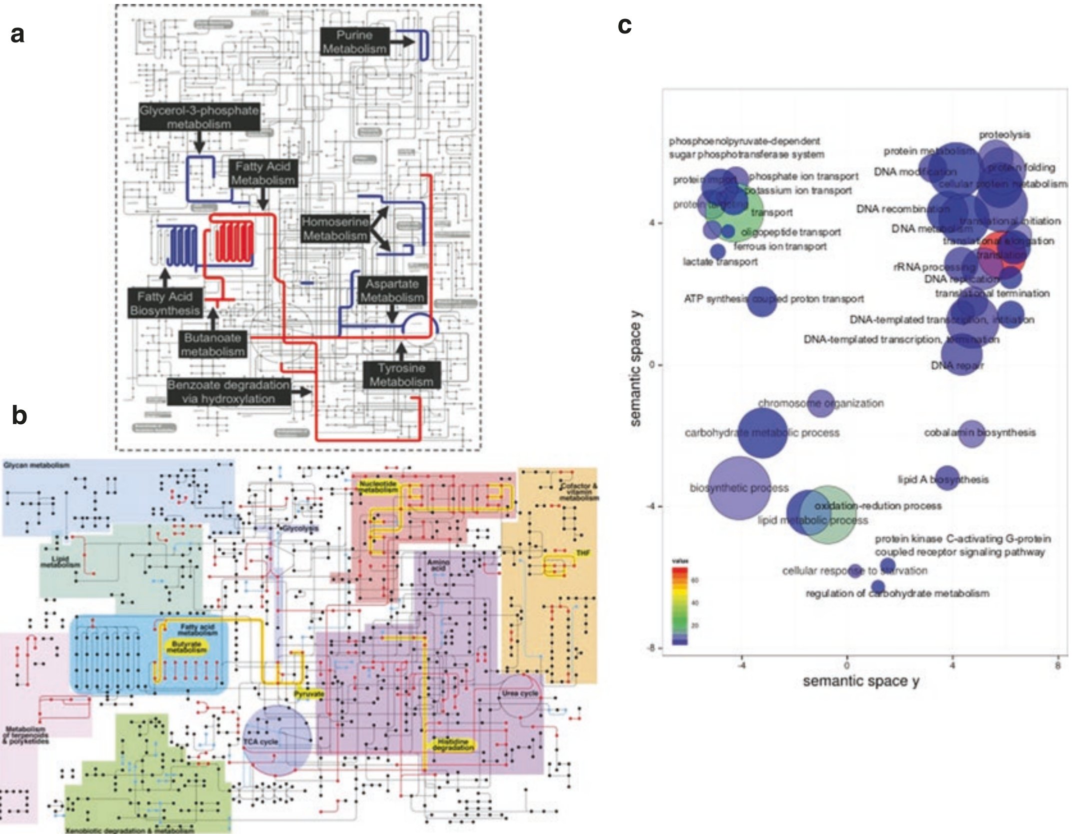


Fig. 10.5 Functional signatures of periodontitis. (**a**) Enrichment in metabolic pathways present in healthy and periodontitis samples identified by metagenomic analysis. Dark blue—significantly enriched in healthy samples; Dark red—significantly enriched in diseased samples. (Reproduced from Liu et al. 2012. Deep Sequencing of the Oral Microbiome Reveals Signatures of Periodontal Disease. PloS One 7(6):e37919). (**b**) Differential metabolic gene expression in the diseased periodontal microbiome. Black lines indicate enzyme-encoding genes that were expressed and unchanged in health and disease; red lines indicate genes up-regulated during disease, and blue lines indicate genes up-regulated during health. Colored regions identify different sections of the metabolic pathway map. (Reproduced from Jorth et al. 2014. Metatranscriptomics of the human oral microbiome during health and disease. MBio 5(2):e01012–14). (**c**) Metabolic activities associated with the progression of pocket depth identified by metatranscriptomic analysis. (Reproduced from Yost et al. 2015. Functional signatures of oral dysbiosis during periodontitis progression revealed by microbial metatranscriptome analysis. Genome Med. 7(1):27)

mechanisms by which the oral microbiome modulates the disease.

Among the different metabolic activities of the oral microbiome, lipid metabolism in the microbiome is highly influenced by periodontitis. In several independent studies, changes in lipid metabolism of the members of the microbiome have been identified as a signature in disease [135, 136, 138, 145] (Fig. 10.5). Fatty acid metabolism has been proposed as a metabolic function important in the virulence of several human pathogens [146, 147]. A metagenomic analysis comparing disease and periodontitis found that the diseased microbiome is enriched in metabolic functions belonging to fatty acid metabolism (Fig. 10.5a) and with anaerobic metabolism (e.g., ferrodoxin oxidation and acetyl-CoA degradation) [145]. Also enriched in disease were many virulence factors such as the presence of conjugative transposons, type IV secretion systems, and the biosynthesis of toxic compounds such as acetone, butanol, and ethanol, as well as the Lipid-A of lipopolysaccharide (LPS) biosynthesis. Metatranscriptomic studies

of periodontal microbiota have confirmed the importance of the activities mentioned above. In those studies, the results represent the profiles of synthesis of mRNA, and thus, it is a representation of actual changes in gene expression. In three different studies of periodontitis metatranscriptome the metabolism of lipids and lipid A biosynthesis was altered (Fig. 10.5b, c) [135, 136, 138].

Metatranscriptomic profiles of subgingival plaque from active and inactive sites in patients with chronic periodontitis identified functional signatures that could explain the initial stages of dysbiosis [138]. The microbiome of the progressing sites is already dysbiotic at the initiation of the study [138]. There was an over-representation in the baseline of progressing sites of terms related to cell motility, transport (iron, potassium, chloride, citrate, and amino acids transport), lipid A and peptidoglycan biosynthesis, and protein kinase C-activating G-protein-coupled receptor signaling pathway, as well as the synthesis of aromatic compounds. On the other hand, in the baseline samples from non-progressing sites, there was an over-representation of GO terms related to tricarboxylic acid cycle, metal ion transport, phosphoenolpyruvate-dependent sugar phosphotransferase system, and protein secretion. Interestingly, histidine biosynthesis was over-represented at the initial stages of the progressing sites while Jorth et al. found that histidine catabolism was up-regulated in the disease sites of their study [136].

A key altered metabolic activities in periodontitis, and its progression seems to be potassium ion transport [135, 138]. Its importance was confirmed by demonstrating that potassium levels increased the virulence of the oral community as a whole. At the same time, it has altered the immune response of gingival epithelium, increasing the production of TNF-α and reducing the expression of IL-6 and the antimicrobial peptide human β-defensin 3 [148].

The results of metatranscriptomic analyses seem to indicate that the genes expressed by the oral microbiome and their functions are more relevant to periodontitis pathogenesis than the microbiota composition, perhaps due to the high genomic redundancy in the oral microbiome.

10.2.4 The Oral Microbiome in Systemic Diseases

Over the years, many studies have linked periodontal disease with the predisposition of individuals to suffer systemic diseases such as cardiovascular disease, oral cancer, gastrointestinal diseases, adverse pregnancy outcomes, diabetes, and more recently neurodegenerative diseases such as Alzheimer's disease [149–152]. However, it remains to be established whether specific periodontal pathogens stimulate the development of the systemic disease or if the systemic disease causes the abundance of periodontal pathogens to change. If the pathogens cause non-oral disease, then they would represent obvious targets for therapeutic intervention. It is proposed that the presence of periodontal pathogens could be used as diagnostic markers to predict susceptibility to non-oral disease. The oral microbiome has the potential to develop a wide variety of non-oral conditions. For instance, about 30 abundant species in the oral cavity, mainly Gram-negative anaerobe bacteria, are known to produce endotoxins, which could directly contribute to systemic diseases.

Oral pathogens can migrate from the oral cavity to distant sites of the body *P. gingivalis* is capable of invading various cell types, including epithelial, endothelial, and smooth muscle cells. Daily bacteremias through tooth brushing and chewing, especially in those with periodontitis, can contribute to the entry of oral bacteria into the bloodstream and thus to the direct contact with distant tissues. It should be noted that other bacteria in the oral microbiota such as F. nucleatum and Filifactor alocis can invade endothelial cells and the cariogenic bacterium S. mutans can invade vascular cells on human coronary artery endothelial cells (HCAEC) [153–155].

In most cases, the breach between oral health and systemic diseases is mediated by an increase in inflammation. The association between oral inflammation and systemic inflammation is crucial to understanding the detrimental effects of oral inflammation on several organ systems. Oral microbiota can cause oral inflammation and also contribute to systemic inflammation through the release of toxins or microbial subproducts into the bloodstream.

10.2.4.1 Cardiovascular Disease

Cardiovascular disease remains the leading cause of death in the U.S accounting for almost one in every four deaths [156]. One-third of Americans have some form of the disease, including coronary disease with myocardial ischemia, a cerebrovascular illness with strokes, and peripheral arterial disease with gangrene [157]. However, half of those patients do not have the classic disease risk factors such as obesity, hypercholesterolemia, hypertension, history of smoking, or genetic background [158, 159], leaving the cause of atherosclerotic plaque progression and disease unknown. Several studies have reported a positive correlation between periodontal disease and atherosclerotic vascular disease [160, 161]. On the other hand, a recent statement by the American Heart Association stated that current data are insufficient to support a causal relationship between periodontal disease and atheroesclerotic vascular disease [157]. Even though different studies have suggested that there may be an association between periodontitis and cardiovascular disease, the mechanisms by which oral infections impact cardiovascular diseases have remained unclear.

In a large meta-analysis of five cohort studies combined that included 86,092 patients, the results showed that individuals with periodontal disease had 1.14 times higher risk of developing coronary heart disease than the controls and showed an even higher risk of developing coronary heart disease (2.22 times). This study showed that both the prevalence and incidence of cardiovascular disease are significantly increased in patients with periodontitis [162]. Furthermore, an association between edentulousness and serum antibodies against *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* with coronary heart disease was observed in a study that included 1163 patients [163]. An additional study confirmed the presence of oral bacterial DNA species in 42 atheromatous plaques retrieved by endarterectomy. The bacterial species most commonly found in this study was *P.gingivalis*, followed by *A. actinomycetemcomitans*, *T. forsythia*, *Eikenella corrodens*, *F. nucleatum*, and *Campylobacter rectus*. Moreover, live *P. gingivalis* has been found in various tissues [164].

Furthermore, studies in an animal model of atherosclerosis using hyperlipidemic mice infected with *P. gingivalis* and *Treponema denticola* demonstrated that infection with these bacteria is associated with alveolar bone loss and aortic atherosclerosis [165, 166]. After oral infection in an animal model, *P. gingivalis* and *T. denticola* induced a systemic immune response, and bacterial genomic DNA was found in the oral epithelium and aorta and within systemic organs. Additionally, *P. gingivalis* evades innate immune detection via Toll-like receptor (TLR)-4, facilitating chronic inflammation in the vasculature [167]. It was also demonstrated that *P. gingivalis*, through its secreted outer membrane vesicles, can induce platelet aggregation in human samples, which could contribute to thrombus formation in vivo [168]. Interestingly, other oral pathogens such as *A. actinomycetemcomitans*, *T. forsythia, C. rectus, F. nucleatum, P. intermedia*, and *T. denticola* failed to aggregate platelets when tested for aggregation activity, suggesting that only *P. gingivalis* expresses the virulence factors involved in platelet aggregation [169]. As shown above, several oral pathogens are associated with a higher risk of cardiovascular disease in humans, and studies in mice support the possibility that infection with the oral pathogens may lead to the disease.

10.2.5 The Oral Microbiome and Cancer

In the early 1990s, *Helicobacter pylori* was recognized as a causative agent of human gastric cancer [170], becoming the first major bacterial pathogen to be associated with human cancer [171, 172]. *H. pylori* leads to chronic inflammation due to the failure of the host to eradicate the infection and thus to oxidative stress, deriving from immune cells and from within gastric epithelial cells, which is a leading contributor to DNA damage, apoptosis, and neoplastic transformation [173]. In addition to *H. pylori*, various bacterial infections have also been found to correlate with an increased risk of developing cancer, e.g., gallbladder carcinoma with *Salmonella typhi* infection [174] and colon cancer with

Streptococcus bovis infection [175]. More importantly, it is now well established that members of the oral microbiome contribute to a variety of oro-digestive cancers [176–179].

10.2.5.1 Oral Cancer

Oral cancer is the term that usually includes cancer of the lip, tongue, salivary glands, and other sites in the mouth (gum, the floor of the mouth, and other unspecified parts of the mouth). Over 90% of all oral cancers correspond to oral squamous cell carcinoma (OSCC), and 10% are due to adenocarcinoma. Despite therapeutic advances, the 5-year survival ratio is approximately 50%, making OSCC one of the most aggressive malignancies [180]. OSCC is considered the eighth most common cancer worldwide and is among the three most common cancers in South-Central Asia [181]. The site of the occurrence of this disease depends on region-specific epidemiological risk factors. In South Asian countries, the cheek (buccal mucosa) and gingiva are the leading sites of involvement, whereas, in Western societies, the tongue is most commonly affected [182].

Along the years, research has suggested a link between periodontal disease and cancer. A meta-analysis study, including 3183 subjects, showed that patients with periodontal disease have an increased susceptibility to oral cancer [183]. More recent studies found a positive correlation between periodontal disease and pancreatic, head and neck, and lung cancers [172]. Another study examined one million randomly selected insurance cases in Taiwan and found that patients in the periodontitis cohort exhibited a higher risk of developing cancer than those in the gingivitis cohort [184]. The major etiological factors contributing to risk to develop OSCC are alcohol and smoking. Eradication of these factors and early diagnosis are the most desirable prevention. However, bacteria might play a crucial role in the etiopathogenesis of esophageal cancer. Petters and co-workers studied, for a decade, the oral microbiota of over 122,000 patients, and found asociation between *T. forsythia* and a higher risk of esophageal adenocarcinoma, while *P. gingivalis* was linked to a higher risk of esophageal squamous cell carcinoma. Interestingly, Streptococcus and Neisseria were linked to a lower risk of esophageal cancer [185].

Yang et al. evaluated the profile of oral microbiome during oral cancer's progression from the early stage to the late stage [186]. The diversity of the oral microbiomes was significantly higher in stage 4 patients than that in healthy controls. *Fusobacteria* abundance *showed a*n increase with the progression. Fusobacteria abundance showed an increase with the cancer progression. In OSCC stage 1 reached to 4.35% and in stage 4 incresed to 7.92% of the entire community, while in healthy controls represented 2.98% of the total community. At the genus level, the abundance of *Fusobacterium* increased, while the number of *Streptococcus, Haemophilus, Porphyromonas*, and *Actinomyces* decreased with cancer progression. *At the species level F. periodonticum, P. micra, S.constellatus, H. influenza*, and *Filifactor alocis* increased in abundance from stage 1 to stage 4. Based on those results, the use of bacterial marker panel of three bacteria was suggested: *F. periodonticum,* which increases the numbers and decreases the abundance of *S. mitis*, and *P.pasteri*. Inaba et al., also found *P. gingivalis* at significantly elevated levels in OSCC [177] and esophagus squamous cell carcinoma (ESCC) patients, but not in healthy mucosa [171].

Although the exact mechanisms involved in tumorigenesis by periodontal bacteria have not been completely elucidated, local inflammatory effects triggered by the bacterial infection have been associated with the cellular transformation [187].

Two important periodontal bacteria, *P. gingivalis* and *F. nucleatum*, possess all the attributes consistent with a role in cancer development and progression [151]. Chronic *P. gingivalis* infection is associated with oro-digestive cancer [188], an increase in oral cancer invasion [177], epithelial to mesenchymal transition [189] and production of oral cancer stem cells [190].

Several mechanisms of carcinogenesis promoted by *P. gingivalis* have been proposed. First, it has been associated directly with the activation of specific oncogenic pathways. Among them is the promotion of survival in gingival epithelial cells through both the activation of the PI3K/Akt pathway and the inhibition of cytochrome c release [191], as well as with the reduction of the

expression of proapoptotic proteins [192]. *P. gingivalis* is capable of blocking apoptosis through the JAK/STAT pathway in gingival epithelial cells and thus can modulate the cell death pathway [193]. The LPS of *P. gingivalis* contributes to the inhibition of apoptosis and induces proliferation in gingival epithelial cells. This effect is associated with an increased expression of TLR4 [194]. *P. gingivalis* was also shown to cause gingival epithelial cells' migration in a manner dependent on the overexpression of the activator of the epithelial-mesenchymal transition (EMT) Zeb1 [189]. Moreover, *P. gingivalis* increases proliferation and promotes invasion and migration [195] and inhibits the activity of glycogen synthase kinase 3 (GSK3b), a critical EMT regulator, in primary oral epithelial cells [196].

The potential role for periodontal pathogens in the induction of oral cancer was confirmed in an oral-specific chemical carcinogenesis animal model. The study showed that the periodontal pathogens *P. gingivalis* and *F. nucleatum* stimulate tumorigenesis via direct interaction with oral epithelial cells and that the effect is mediated by the host innate immune system [197]). In the case of OSCC, it was demonstrated that *P. gingivalis*, but not *F. nucleatum,* promotes invasion and metastasis of oral squamous cells by inducing matrix metalloproteinase 9 (pro-MMP9) expression [177]. Another study showed that prolonged and repetitive exposure to *P. gingivalis* increases the aggressiveness of oral cancer cells via epithelial-mesenchymal transition-like changes in the cells [190].

Several mechanisms have been proposed by which the oral bacterium *F. nucleatum* induces tumorigenesis in the colon [176, 178]. *F. nucleatum* generates a pro-inflammatory microenvironment that is conducive for colorectal neoplasia progression [198] and induces oncogenic responses through a FadA adhesin [178]. Furthermore, the expression of pro-inflammatory cytokines in periodontal disease has been linked to microbial-triggered carcinogenesis [199]. However, the mechanisms by which oral bacteria may contribute to cancer development are still unknown.

Regarding the study of changes in functions of the whole microbiome during OSCC progression, our knowledge is more limited. Using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) as a proxy to predict the oral microbiome functions [200], in two different studies, Perera et al. [201] and Al-Hebshi et al. [202] found that despite the differences in community composition, certain the results from functional prediction analysis were consistent between both studies. Genes involved in bacterial motility, flagellar assembly, and bacterial chemotaxis synthesis were enriched in the tumors, and particularly, LPS biosynthesis pathways were enriched in both cohorts. Moreover, in another independent study genes related to protein and amino-acid metabolism, such as valine, leucine and isoleucine, phenylalanine, tyrosine, and tryptophan biosynthesis, were inversely associated with OSCC progression [186]; the same results were observed by Perera et al. Where genes are responsible for phenylalanine, tyrosine, and tryptophan, biosynthesis were significantly associated with the controls [201].

A more direct method to characterize community-wide gene expression profiles is the use of metatranscriptomics, which is based on the set of transcripts being synthesized by the microbial community under different conditions. This approach has been extremely informative in providing new insights into microbial functions and active communities in caries [97, 103], periodontitis [135, 136, 138], and gingivitis [137] and during biofilm formation and after meal ingestion [82]. In a pilot study of community-wide gene expression analysis of microbiome in OSCC Yost et al. [203] found that regardless of the community composition, specific metabolic signatures were consistently found in disease (Fig. 10.6a) and that Fusobacteria, again, was the most active group of bacteria in the microbiome of OSCC (Fig. 10.6b) and *F. nucleatum* was the organism that showed a higher up-regulation of putative virulence factors on the OSCC samples (Fig. 10.6c). Among the functional activities that characterized the metatranscriptome of the oral microbiome in OSCC, iron ion transport, tryptophanase activity, peptidase activities, and superoxide dismutase were over-represented in tumor and tumor-adjacent samples when com-

Fig. 10.6 The metatranscriptome of OSCC. (**a**) Gene ontology enrichment analysis for the metatranscriptome profiles of the oral microbiome associated with cancer status; Over-represented biologic processes in the OSCC tumor sites. (**b**) Cladograms report the taxa showing different abundance values for the transcripts comparing healthy control matching tumor sites vs. OSCC tumor sites. (**c**) Comparison of healthy control vs. tumor sites of the phylogenetic origin of up-regulated putative virulence factors in OSCC. The relative abundance of hits from a specific bacterium about the total differentially expressed virulence factors in each of the comparisons. (Reproduced from Yost et al. 2018. Increased virulence of the oral microbiome in oral squamous cell carcinoma revealed by metatranscriptome analyses. Int J Oral Sci. 10(4):32)

pared to the healthy controls. The expression of putative virulence factors in the oral communities associated with OSCC showed that activities related to capsule biosynthesis, flagellum synthesis and assembly, chemotaxis, iron transport, hemolysins, and adhesins were up-regulated at tumor sites [203].

10.2.6 The Oral Microbiome and Non-oral Cancers

It has been shown that patients suffering from periodontal disease have a higher risk of developing some type of cancer [204]. Periodontitis is characterized by a localized chronic inflammation produced in response to a dysbiotic process of the oral microbiome in the subgingival region. Although the inflammatory processes occur locally in the oral cavity, the inflammatory mediators synthesized and released during periodontitis, can migrate from the oral cavity to other body sites, thus contributing to the initiation of extraoral diseases like cancer. Interestingly, some types of cancer have associated carcinogenesis inflammatory molecules migrating to distal sites in the human body [205] while showing a direct association between the disease and carcinogenic effect mediated by periodontitis-associated bacterial species either directly in oral cells or by migrating from the oral cavity [206]. The spread of oral bacteria to distal body sites, either after

routine activities or dental procedures, was early reported by Cobe [207]. Mainly, oral anaerobes are released to circulation after some daily activities, such as tooth brushing, flossing, and chewing [208], and also immediately after oral therapeutic procedures such as scaling and root planning [209]. Therefore, dental or oral surgery has been considered predisposing factors for bacteremia of oral origin in both adults and children [210]. In periodontal disease, the migration of bacteria from the oral cavity to other organs in the human body is likely to occur through the blood circulation [211, 212], providing a portal of entry for oral bacteria into the vessels and thereby allowing them to spread to distant sites [168]. These bacteremias of oral origin are usually polymicrobial, with higher numbers of Gram-negative bac*teria* [213]. In addition to *P. gingivalis*, other periodontitis-associated taxa have been associated with orodigestive cancers. *A. actinomycetemcomitans* correlates with a higher risk of pancreatic cancer [214], and *T. denticola* has been detected in both tongue squamous cell carcinoma [215] and esophageal cancer tissues [216]. As it is the case in OSCC, the question of how species of oral bacteria contribute to the process of carcinogenesis has not been fully understood. It is still unknown how oral bacteria affect the local microbiome in distal sites and therefore alter host cell responses. Although the exact mechanisms involved in cancer progression by the activity of the oral microbiome have not been completely elucidated, local inflammatory effects triggered by the bacterial infection have been associated with the cellular transformation [187].

Colorectal carcinoma (CRC) is the fourth leading cause of cancer deaths worldwide. A polymicrobial signature of Gram-negative anaerobic bacteria was associated with colorectal carcinoma in 130 tissues analyzed [217]. CRC has been associated with a high abundance of *F. nucleatum* and *Clostridium difficile* in the intestinal microbiota of colorectal carcinoma patients [218]. A polymicrobial signature of Gram-negative anaerobic bacteria was associated with colorectal carcinoma in 130 tissues analyzed, and Gram-negative anaerobic oral pathogens such as *Fusobacterium*, *Leptotrichia*, and *Campylobacter* species were identified in individuals with tumors [217]. Extensive pieces of evidence associating bacteremia caused by *F. nucleatum* with underlying malignancy have been reported [219]. This organism is considered as a risk factor for colorectal cancer (CRC) [220–222], as the bacterium is over-represented in colorectal tumor tissues versus healthy tissues in CRC patients [217, 223, 224]. Because *F. nucleatum* is commonly found in CRC in association with other oral species (e.g., *P. micra*, *Peptostreptococcus stomatis*, *Gemella morbillorum*, *Porphyromonas* spp., *Leptotrichia* spp., and *Campylobacter* spp.), it is reasonable to conclude that the source of these microbes is most likely the oral cavity [217, 225–228].

It has been proposed that *F. nucleatum* triggers cancer on CRC through three virulence factors: the adhesin FadA, the LPS, and the autotransporter protein Fap2 [221]. FadA induces inflammation and activation of pro-carcinogenic pathways directly in colorectal cells, activating E-cadherin-β-catenin signaling [178]. The LPS of *F. nucleatum* is a trigger for the production of inflammatory cytokines both in the gingiva and in the colonic tissue [223, 229]. *F. nucleatum*-enriched colorectal adenoma subjects show an increased expression of proinflammatory cytokines such as IL-6, IL-12, IL-17, and TNF-α when compared to non-adenoma controls [230]. Finally, Fap2 decreases the cytotoxicity of immune cells, favoring cancer progression [231]. Moreover, in vivo studies have demonstrated that F. nucleatum increases tumor multiplicity and the recruitment of tumor-infiltrating immune cells. F. nucleatum generates a proinflammatory microenvironment associated with an NF-κB-mediated response (COX-2, IL-1β, IL-6, IL-8, IL-10, and TNF-α) [198], providing the link between inflammation and cancer [232]. Besides, *F. nucleatum* increases the proliferation and invasion ability of colonic epithelial cells by activating NF-κB signaling and increasing the production of pro-inflammatory cytokines (IL-6, IL-1β) and MMP-13 [233].

There are even fewer studies on the association of other periodontitis-associated bacteria with cancer. Among them, *T. denticola* is a highly

invasive anaerobic bacterium that presents a chymotrypsin-like proteinase (CTLP) as a major virulence factor. CTLP has been detected within orodigestive tumor tissues, including OSCC, tongue, tonsil, and esophagus [234]. CTLP converts pro-MMP-8 and pro-MMP-9 to their active forms, which are associated with metastasis in the tongue, esophageal, gastric, pancreatic, and CRC cancers [235–237].

10.2.7 The Oral Microbiome and Adverse Pregnancy Outcomes

It is well established that maternal infections are associated with adverse pregnancy outcomes [238]. Infection is considered one of the major causes of preterm labor and low birth weight deliveries, responsible for somewhere between 30 and 50% of all cases [239–241]. Although earlier literature does not include periodontitis as a risk factor for preterm birth, the importance of this condition has been increasingly recognized for its association with systemic diseases. In a 2002 study 18.2% of all registered cases of preterm birth were linked to periodontal disease [242]. Thus, research into the association between periodontal disease and adverse birth outcomes has gained relevance at a clinical level and within the field of public health [243–245]. Moreover, more than half of all permanent sequelae that infants suffer at neurological, cardiovascular, respiratory, and congenital levels have also been attributed to preterm birth. Babies with a low birth weight (LBW) are 40 times more likely to die than healthy birth weight babies, and the risk is even higher when associated with preterm birth complication, such as respiratory distress syndrome, chronic lung disease, cardiovascular disorders, a compromised immune system among others [246–248].

Bacterial infection of the extraplacental membrane may lead to chorioamnionitis, a condition strongly associated with premature membrane rupture and preterm delivery [249, 250]. The biological mechanisms involve bacterially induced activation of cell-mediated immunity, which leads to the production of cytokines (such as IL-1, IL-6, and TNF-α) and synthesis of prostaglandins (especially prostaglandin E2[PGE2]) [249, 251].

During normal pregnancy, the intra-amniotic levels of these mediators increase until reaching a threshold at which point labor is induced [252]. The abnormal production of these mediators during infection triggers preterm labor and low birth weight [253–256]. However, in many cases of confirmed chorioamnionitis, no active infection of the genitourinary tract could be found, indicating that local infection is not the sole cause of this condition [249, 250]. These findings led to speculation that a distant infection from the placental complex or the genitourinary tract still presents a risk for premature labor and low birth weight babies. The theory that remote sites of infection might contribute to early labor and low birth weight outcomes was started by several studies using a hamster model [257]. Pregnancy outcomes were evaluated in that animal model after either the establishment of experimental periodontitis, subcutaneous tissue infection with *P. gingivalis* [258] or intravenous injection of LPS from *P. gingivalis* [257]. In those studies, the fetal weight was lower in the experimental animals, and the severity of the effects was directly associated with the levels of PGE2 and TNF-α.

With the advent of evidence-based medicine, in 1996 Offenbacher et al. [252] performed the first case-control study that reported a 7.5-fold higher risk of preterm birth in mothers with periodontal disease, reviving the interest in this disease and its association with pregnancy and birth outcomes. In a subsequent case-control study, Offenbacher and others measured the levels of PGE2 and IL-1 and the levels of 4 periodontal pathogens (*T. forsythia, P. gingivalis, A. actinomycetemcomitans*, and *Treponema denticola*) in the gingival crevicular fluid (GCF) of 48 mothers of preterm labor and low birth weight infants. GCF levels of PGE2 were significantly higher in mothers of preterm labor and low birth weight infants than in mothers of infants with normal birth weight (controls). The four periodontal pathogens, characteristically associated with mature plaque and progressing periodontitis, were detected at significantly higher levels in

the case of the study group [240]. However, the association of periodontal disease and adverse outcomes are conflicting [252, 259, 260]. This inconsistency can be partly attributed to the heterogeneity of the studies in terms of their design, statistical analyses, sample sizes, and the definitions of periodontitis used, generating uncertainty and imprecision around the conclusions drawn in most of the work published to date.

Due to hormonal changes in pregnant women, they are more susceptible to gingivitis and periodontitis than non-pregnant women. Indeed, approximately 40% of pregnant women demonstrate clinical evidence of periodontal disease [261].

Two different mechanisms have been proposed to explain how oral health is associated with adverse pregnancy outcomes. The first hypothesizes that the systemic dissemination of endotoxins or inflammatory mediators derived from periodontal disease could affect the development of the fetus or spontaneous abortion [262]. Bacterial pathogens, antigens, endotoxins, and pro-inflammatory cytokines produced during periodontal disease can cross the placental barrier, resulting in disturbances in the maternal fetal unit that could contribute to adverse pregnancy outcomes [263, 264].

The second proposes that oral pathogens themselves can translocate from an unhealthy oral cavity and cross the placenta, reaching the intra-amniotic fluid and fetal circulation [261, 265]. Indeed, oral bacterial species involved in the pathogenesis of periodontal disease have been reported in amniotic fluid of women with preterm labor or preterm premature rupture of membranes [266–270]. *F, nucleatum* is also one of the most frequent species involved in the microbial invasion of the amniotic cavity [266, 270], and it is the most common oral pathogen found in a variety of placental and fetal tissues, amniotic fluid, and cord blood in cases of preterm birth and neonatal sepsis [238, 271, 272]. A case report of term stillbirth suggested that *F. nucleatum* could translocate from the mother's mouth to the uterus when her immune response was weakened during a respiratory infection [273]. Furthermore, *F. nucleatum* is often detected along with other oral subspecies in intrauterine infections, which are likely from the same infectious origin, implying co-translocation from the oral cavity. It has been postulated that *F. nucleatum* translocates from the maternal oral cavity to the intrauterine cavity via a hematogenous transmission [274–276]. This hypothesis was tested on animal models on which *F. nucleatum* was hematogenous injected. After inoculation, specific colonization and proliferation of the bacteria in the fetoplacental unit without causing systemic infection were reported. The bacteria colonize initially in the decidua by crossing the endothelium. After that, they spread to the amniotic fluid, fetus, and fetal membrane, mimicking chorioamnionitis and eventually leading to preterm and term fetal death [277]. The pattern and duration of infection, as well as the pathology of the mouse placenta, correspond to the stillbirth case reported on a term stillbirth caused by *F. nucleatum* [273]. In mice, a placental infection caused by *F.nucleatum* was characterized by localized bacterial colonization, inflammation, and necrosis. *F. nucleatum* was shown to activate both TLR2 and TLR4 in vitro. In vivo, the fetal death rate was significantly reduced in TLR4-deficient mice (C57BL/6 TLR4$^{-/-}$ and C3H/HeJ (TLR4$^{d/d}$), but not in TLR2-deficient mice (C57BL/6 TLR2$^{-/-}$), following *F. nucleatum* infection. The reduced fetal death in TLR4-deficient mice was accompanied by decreased placental necroinflammatory responses in both C57BL/6 TLR4$^{-/-}$ and C3H/HeJ. The decrease of bacterial colonization in the placenta was observed in C3H/HeJ, but not in C57BL/6 TLR4$^{-/-}$. These results suggest that inflammation, rather than the bacteria per se, was the likely cause of mice fetal loss. TLR2 did not appear to be critically involved, as no difference in bacterial colonization, inflammation, or necrosis was observed between C57BL/6 and C57BL/6 TLR2$^{-/-}$ mice. A synthetic TLR4 antagonist, TLR4A, significantly reduced fusobacterial-induced fetal death and decidual necrosis without affecting the bacterial colonization in the placentas. TLR4A had no bactericidal activity, nor did it affect the birth outcome in sham-infected mice. TLR4A could have promised as an anti-inflammatory agent for the treatment or prevention of bacterial-induced preterm birth [277, 278].

FadA adhesin from *F. nucleatum* has been proposed as a virulence factor involved in the cell attachment and invasion of the placenta [279]. FadA is a small-helical peptide consisting of 129 amino acid residues in its non-secreted form (pre-FadA) and 111 residues in the secreted form (mFadA). It is a unique adhesin in that both pre-FadA and mFadA are required for oligomerization and function [280]. FadA was shown to be involved in *F. nucleatum* 12,230 colonization of the mouse placenta [178, 279, 281]; however, its inactivation did not abolish placental colonization, suggesting the involvement of an additional adhesin(s).

Using a system for transposon mutagenesis, Coppenhagen-Glazer S. et al. created a galactose-sensitive adhesin mutant in *F. nucleatum*. They found that the positive mutants harbored the transposon in the gene coding for the Fap2 outer membrane autotransporter. The *fap2* mutants failed to show lactose-inhibitable coaggregation with *P. gingivalis* and were defective in cell binding. A *fap2* mutant also showed a 2-log reduction in murine placental colonization compared to the wild type. This work suggests that Fap2 is a galactose-sensitive hemagglutinin and an adhesin that is likely to play a role in the virulence of fusobacteria during pregnancy [282].

Recent studies have reported that the human placenta harbors a low abundant microbiome closely mimicking the human oral microbiome provides further support for hematogenous transmission [283]. Although the advancement of microbial detection technologies has allowed us to characterize the oral microbiota, many species are still underestimated because a large number of species are unculturable and hence cannot be detected by the conventional culturing methods employed by the hospital microbiology laboratories. For instance, an uncultivated oral species, *Bergeyella*, which had never been identified in intrauterine infection, has been repeatedly detected in amniotic fluids associated with preterm birth using 16S rRNA gene-based culture-independent technology [269, 284, 285]. It is tempting to think that a specific portion of the unexplained stillbirths may be caused by previously unrecognized oral bacteria, translocated to the uterus independent of the ascending vaginal route. Further systematic analysis is needed to determine the prevalence of oral bacteria in stillbirth [269, 284]. *S. mutans* and *Aspergillus*, two microorganisms associated with oral infections (caries and fungal infection), have been reported to be present in intra-amniotic samples [286].

Other oral pathogens, such as *P. gingivalis* and its endotoxins, were also found in the placenta of preterm delivery patients [287, 288]. Chorionic and intra-amniotic infection with *P. gingivalis* has been involved in several cases of preterm birth [289]. Studies in animal models demonstrate the ability of *P. gingivalis* to negatively impact pregnancy: LPS from *P. gingivalis* induced placental and fetal growth restriction and resorption in rats [290] and antibodies raised against *P. gingivalis* caused fetal loss when passively administered into mice [291].

The maternal-fetal interface represents an immunologically unique site that must promote immune tolerance to the fetus while at the same time maintaining a robust host defense against possible infections. Even though little is known about the role of innate immune receptors during pregnancy, it was known that the placenta expresses toll-like receptors (TLRs) during normal pregnancy [292]. Periodontal disease or the presence of periodontal pathogens such as *T. denticola* and *P. gingivalis* has been shown to increase the expression of TLRs, suggesting increased innate immune responses. Chaparro et al. demonstrated the presence of *P. gingivalis, A. actinomycetemcomitans, F. nucleatum, T. denticola*, and *T. forsythia* using the polymerase chain reaction (PCR) technique on placental biopsy of pregnant women with periodontal disease obtained after aseptic placental collection at the time of delivery. They also showed a significant increase in the expression of TLR-2 in the placentas of the case-patients but not in the control group [293]. Although more studies will be required to establish conclusively that there is a cause-and-effect relationship between periodontal disease and adverse pregnancy outcomes, the results so far suggest that preventive measures against periodontal disease in pregnant women will be protective to the babies.

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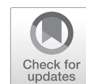

Bacterial Peptides Targeting Periodontal Pathogens in Communities

11

Hua Xie and Richard J. Lamont

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11.1 Introduction

The major, or more accurately keystone, pathogen in periodontitis is *Porphyromonas gingivalis*, an indigenous organism found in the oral cavity of most adults. *P. gingivalis* is well adapted to the subgingival ecosystem, where it can be located on the root surface, in the gingival crevicular fluid, as well as in and on gingival epithelial cells [1, 2]. A variety of properties underpin the success of *P. gingivalis* in the complex subgingival microenvironments. *P. gingivalis* expresses fimbrial and other surface protein adhesins that mediate attachment to other biofilm constituents and to host surfaces such as epithelial cells [2–7]. An asaccharolytic organism, *P. gingivalis*, secretes proteases, including the arginine- and lysine-specific gingipains, to provide peptides and amino acids for growth. Hemin released from heme-containing proteins such as hemoglobin is acquired by a number of hemin transport systems as the primary source of iron [1, 8, 9]. While normally in a homeostatic relationship with the host, if uncontrolled the factors that contribute to colonization and survival can also contribute to dysbiosis and tissue destruction. Moreover, the usual ecological context of *P. gingivalis* is a

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constituent of a polymicrobial biofilm, and other community inhabitants can exacerbate or restrain pathogenic potential [1, 5, 10]. Similarly, *P. gingivalis* as a keystone pathogen can modulate overall community pathogenicity, or nososymbiocity [10, 11], and it is the community that is the fundamental etiological unit initiating periodontal diseases. The concept of nososymbiocity gives rise to novel approaches for the control of the bacterial component in periodontal diseases. Community engineering to reduce nososymbiocity may be a more viable strategy than attempting to eliminate *P. gingivalis*, which is difficult to achieve as the organism is embedded in biofilms and within epithelial cells. Favoring the growth of organisms that are antagonistic to *P. gingivalis* and reducing the levels of synergistic or accessory pathogens would impede the transition to a homeostatic community to a pathogenic entity. More specifically, targeting the molecules that effectuate the interaction of *P. gingivalis* with other community participants and the increase in nososymbiocity have shown promise in both in vitro and in vivo studies.

11.2 *P. gingivalis* Community Development

The accumulation of periodontal bacteria into heterotypic communities is driven by co-adhesive and physiological compatibility. Thus, organisms that can provide nutrients for mutual growth tend to attach to each other and associate together, and this underlies structural partitioning within communities [2, 5]. As a strict anaerobe, *P. gingivalis* also benefits from the metabolic activity of the antecedent community which reduces oxygen levels. *P. gingivalis* binds avidly to the early biofilm colonizers such as actinomyces and streptococci, and the importance of attachment of *P. gingivalis* to a streptococcal substratum is evident from human studies. Oral introduction of *P. gingivalis* in human volunteers results in the organism initially localizing almost exclusively on streptococcal-rich supragingival plaque [12], and *P. gingivalis* is frequently detected in the supragingival plaque of healthy humans [13–23]. Oral streptococci also comprise a significant portion of the microbial population of subgingival plaque [24–34], and *P. gingivalis* is often isolated together with streptococci, including *S. gordonii,* from subgingival plaque samples [35]. Moreover, imaging studies of human dental plaque have revealed highly organized microbial consortia, within which *Porphyromonas* associates with streptococci and a number of other taxa [36, 37].

The molecular basis of attachment to *Streptococcus gordonii* has been defined in detail [38–40]. Co-adhesion is mediated by two sets of adhesin-receptor pairs: the FimA and Mfa1 component fimbriae of *P. gingivalis* which interact with streptococcal GAPDH and SspA/B surface proteins, respectively (Fig. 11.1). The accumulation of *P. gingivalis* also requires sensing of the streptococcal metabolite para-amino benzoic acid (pABA) [40] which is utilized in the tetrahydrofolate pathway. Both chemical sensing through pABA and physical attachment interface with a tightly regulated signal transduction cascade within *P. gingivalis* based on protein tyrosine (de)phosphorylation [41, 42] (Fig. 11.2) which controls the expression of fimbrial adhesins and the extent of community development [40]. Communities of *P. gingivalis*-*S. gordonii* are synergistically pathogenic in the murine model of alveolar bone loss [43], and hence the association of *P. gingivalis* with *S. gordonii* provides a therapeutic target for restricting the pathogenic potential of *P. gingivalis*.

11.3 Inhibition of Fimbrial Assembly

The fimbriae of *P. gingivalis* mediate most of the adhesive properties of the organism and can bind to other bacteria, host cells, and extracellular matrix proteins [5, 44, 45]. The Mfa1 fimbriae of *P. gingivalis*, which engage with the streptococcal SspA/B surface protein, are assembled by a unique mechanism that entails proteolytic processing of lipidated precursor subunits. Polymerization of processed subunits occurs on the cell surface by a donor strand exchange (DSE) mechanism which can involve either the N- or

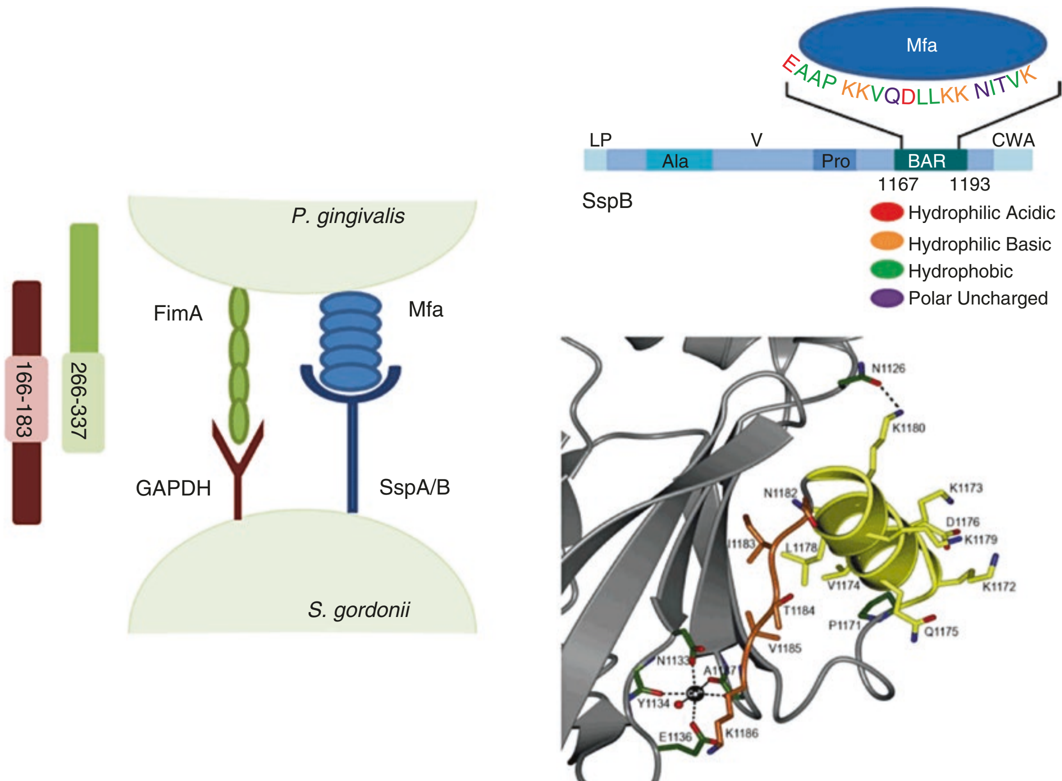

Fig. 11.1 Structures required for the maintenance and stabilization of *P. gingivalis*–*S. gordonii* co-adhesion. Center panel shows the FimA–GAPDH and Mfa1–SspA/B adhesin–receptor pairs. The left panel shows the interacting domains of the FimA and GAPDH proteins with the amino acid (aa) residues indicated. The upper right panel shows the domain structure of the SspB protein and the aa residues involved in recognition of Mfa1. *LP* leader peptide, *Ala* alanine-rich repeats, *V* variable region, *Pro* proline-rich repeats, *BAR* Mfa1-interacting domain, *CWA* cell wall anchor. BAR spans aa residues 1167–1193, and the EAAP, KKVQDLLKK, and NITVK sequences are involved in Mfa1 recognition. The lower right panel shows the structure of the SspB C-terminal region with the protruding BAR handle. Reproduced with permission from [44] https://doi.org/10.1111/j.1365-2958.2011.07707.x

C-terminal regions [46, 47]. Given their novel extracellular assembly mechanism and their critical role in *P. gingivalis* colonization, the Mfa1 fimbriae are potential targets for disruption or prevention of *P. gingivalis* community development. Alaei et al. [48] established that a 9-residue peptide derived from the Mfa1 C-terminal region significantly inhibited fimbrial polymerization through competing with the endogenous subunit pro region and donor strand for binding in the cognate hydrophobic groove. Moreover, the Mfa1 C-terminal peptide caused a significant reduction in *P. gingivalis* adhesion to *S. gordonii* in a dual species community model. Since the assembly of the Mfa1 fimbriae occurs on the extracellular surface, inhibitory molecules do not need to cross the bacterial membranes. Hence molecules based on the Mfa1 C-terminal region have promise for delivery in the oral cavity.

11.4 Mimicking the Streptococcal Receptor for *P. gingivalis*

The SspA/B adherence-mediating domain (designated BAR, SspB Adherence Region) is also well characterized, and spans amino acid (aa) residues 1167–1193. BAR is fully conserved between SspA and SspB [49] and contains three distinct functional domains that encompass aa residues 1182–1186 (NITVK), 1174–1178 (VQDLL), and 1168–1171 (EAAP). Within

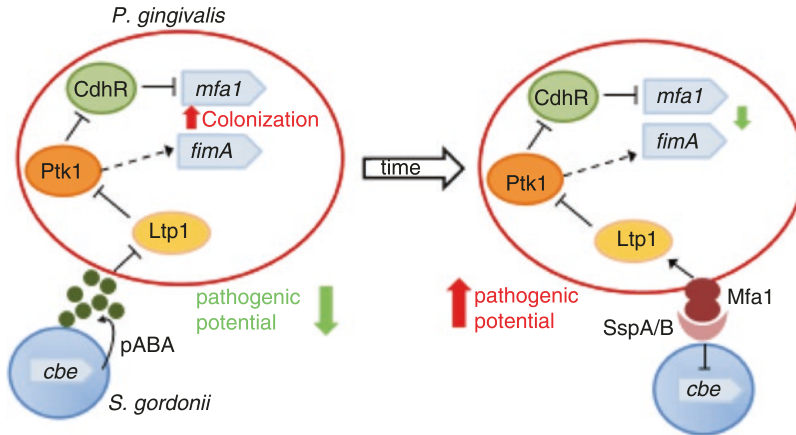

Fig. 11.2 Communication in *P. gingivalis-S. gordonii* communities. Interactions between *P. gingivalis* and *S. gordonii* resulting from metabolite (pABA) perception (left) and direct contact (right). pABA secreted by *S. gordonii* inactivates the *P. gingivalis* tyrosine phosphatase Ltp1. Dephosphorylation and inactivation of the tyrosine kinase Ptk1 are thus reduced. Ptk1 phosphorylates and inactivates the transcription factor CdhR, which is a repressor of the *mfa1* gene. Ptk1 activity also converges on the expression of the *fimA* gene. The expression of both fimbrial adhesins is increased, and in this mode *P. gingivalis* is primed for attachment to *S. gordonii*. However, nososymbiocity is reduced, and pABA-treated *P. gingivalis* cells are less pathogenic in animal models. The engagement of Mfa1 with the streptococcal SspA/B surface protein increases Ltp1 and reverses information flow through the Ltp1–Ptk1 axis. In addition, Mfa1–SspA/B binding suppresses the expression of chorismate binding enzyme (Cbe), which is responsible for pABA production. Prolonged physical interaction between *P. gingivalis* and *S. gordonii* leads to increased nososymbiocity, and dual infection of animal models causes more alveolar bone loss than *P. gingivalis* infection alone. Reproduced with permission from [2] https://doi.org/10.1038/s41579-018-0089-x

the NITVK domain, the N1182 and V1185 residues are essential for recognition by Mfa1 [50]; however, it is the physical properties of the amino acids occupying the 1182 and 1185 positions which dictate activity. Substitution of basic amino acids for N1182 and substitution of hydrophobic residues for V1185 enhance binding to Mfa1, indicating that both electrostatic and hydrophobic interactions contribute to the BAR-Mfa1 interaction [51]. The VQDLL motif of BAR constitutes an α-helix that resembles the eukaryotic nuclear receptor box domain which is involved in protein–protein interactions through a hydrophobic or amphipathic α-helical motif [52]. The VQDLL domain is flanked on either side by two lysine residues which may stabilize the initial binding through a charge clamp mechanism. EXXP, which is a Ca^{2+} binding motif, is represented in BAR by EAAP [43], and crystallography data show that Pro1171 constrains the amphipathic VQDLL α-helix such that hydrophobic residues V1174, L1177, and L1178 face a hydrophobic contact surface [53]. Further, the crystal structure shows that the NITVK and VQDLL motifs protrude from the polypeptide in a configuration described as an attachment "handle," and this structure is stabilized by Ca^{2+} [53]. A synthetic peptide based on the BAR region inhibits *P. gingivalis-S. gordonii* co-adhesion and community development and diminishes the pathogenicity of *P. gingivalis* in a mouse model of periodontitis. In order to develop clinical applicability, mechanisms that allow the delivery of sustained high concentrations of BAR have been explored [54]. The incorporation of BAR into poly (lactic-co-glycolic acid) (PLGA) and methoxy-polyethylene glycol PLGA (mPEG-PLGA) nanoparticles (NPs) resulted in sustained release of active BAR concentrations [55]. The BAR-encapsulated NPs were capable of inhibiting *P. gingivalis-S. gordonii* community formation and disrupting pre-existing communities [55]. BAR-encapsulated NPs may thus provide a potent platform to disrupt the association between *P. gingivalis* and *S. gordonii*. However, the use of peptides as topically applied therapeu-

tic agents in the oral cavity is problematic due to the cost of peptide synthesis and susceptibility to proteases which are produced in abundance by oral bacteria. Small-molecule mimetics of BAR are therefore an attractive alternative. Patil et al. [56] synthesized stable small-molecule mimetics of the BAR region by joining mimics of VXXLL and NITVK through the “click” reaction. Molecules with a 2,4,5-trisubstituted oxazole framework blocked *P. gingivalis* adherence to *S. gordonii* in vitro when “click” coupled with substituted arylalkynes. Moreover, community development was also inhibited in the presence of *Fusobacterium nucleatum*, which can bind to both *P. gingivalis* and *S. gordonii*, indicating the potential for efficacy in the more complex multispecies plaque biofilm environment in vivo [57]. Significantly, several of the BAR-derived compounds reduced alveolar bone loss in mice infected with *P. gingivalis*-*S. gordonii* communities. The most-active compounds were compact molecules with short intraatomic distances between the fluorine and methoxy groups.

11.5 Antagonistic Interactions Among Oral Bacteria

In addition to polymicrobial synergy, antagonistic interactions among oral organisms also occur. Interbacterial antagonistic interactions may arise through competition for nutrients or space, or can be mediated by specific toxic compounds such as bacterocins and antibiotics, or by type VI secretion systems. In the oral cavity, pH is also important and *Streptococcus mutans* which is strongly acidogenic can inhibit the growth of *Treponema denticola* and *P. gingivalis* [58]. Hydrogen peroxide produced by oral streptococci is also toxic to other organisms such as *Aggregatibacter actinomycetemcomitans* [59, 60]. Another example of rivalry is illustrated by *P. gingivalis* and *Streptococcus mitis*. *P. gingivalis* is able to induce DNA fragmentation and death in *S. mitis* [61], consistent with the overarching keystone pathogen concept that *P. gingivalis* can orchestrate the composition and function of oral microbial communities.

11.6 Inhibition of Virulence Expression in *P. gingivalis* by *S. cristatus*

Thee expression of the virulence genes is regulated in *P. gingivalis* in response to precarious environments. Noteworthy results from oral bacterial cell-cell communication studies indicate that the expression of FimA, the major subunit protein of the long fimbriae of *P. gingivalis,* is suppressed in the presence of *S. cristatus*. Consequently, *P. gingivalis* binds poorly to the surface of *S. cristatus* and the two organisms do not accumulate into dual species communities [62]. These data suggest that *S. cristatus* may have an ability to re-direct the development of pathogenic dental biofilms by preventing *P. gingivalis* attachment and colonization. This concept is supported by the findings that, unlike the case for *S. gordonii* [43], oral infection of experimental animals with *P. gingivalis* and *S. cristatus* together results in less alveolar bone loss compared to *P. gingivalis* alone [63]. Additionally, the examination of patients with periodontitis demonstrated an inverse relationship between the number of *S. cristatus* and *P. gingivalis* cells in dental plaque [64].

The investigation of the molecular basis of the antagonistic interactions between *P. gingivalis* and *S. cristatus* identified a surface protein of *S. cristatus*, arginine deiminase (ArcA), as the signaling molecule to which *P. gingivalis* responds by repressing the expression of the *fimA* gene [65]. ArcA catalyzes the hydrolysis of L-arginine to L-citrulline and ammonia, and the latter is believed to be important for oral biofilm pH homeostasis and also caries prevention [66]. The reduction in arginine levels can also impede the ability of *P. gingivalis* to form communities with oral streptococci [67].

Many species of streptococci are arginine deiminase-positive [68]; however, the expression of the *arcA* gene varies significantly. Under standard growth conditions, *S. cristatus* transcript levels are almost 15-fold higher than those in *S. gordonii* [69]. Relatively lower ArcA expression in *S. gordonii* compared to *S. cristatus* may be one factor that contributes to the different relationships

of each of these organisms with *P. gingivalis*. The inhibition of FimA production in *P. gingivalis* may require a relatively high expression of *arcA*, and the limited expression of *arcA* in *S. gordonii* consequently is not able to induce a response in *P. gingivalis*. The mechanistic basis of differential expression of *arcA* in various streptococcal species is not well defined, but sequence variation in the promoter regions of *arcA* likely plays a key role in dictating the level of gene expression [69]. ArcA from *S. intermedius* also downregulates *fimA* transcription as well as the expression of the *mfa1* gene encoding the Mfa1 minor fimbrial adhesive subunit protein [67, 70]. Despite the overall similarity in outcomes, the mechanisms of action of ArcA in *P. gingivalis* antagonism may differ between *S. intermedius* and *S. cristatus*. In the case of *S. intermedius* the enzymatic function of ArcA is necessary for antagonistic activity. For *S. cristatus,* the converse may be the case as the arginine deiminase inhibitors, aminoguanidine and L-lysine, while being able to block the arginine deiminase activity of ArcA, had no effect on the inhibition of *fimA* expression [65]. Moreover, transwell experiments show that the *P. gingivalis*–*S. cristatus* antagonistic interaction requires direct cell–cell contact [71].

A systematic analysis of the active domains of ArcA from *S. cristatus* identified an 11-mer peptide with the native amino acid sequence (NIFKKNVGFKK) which could bind tightly to the surface of *P. gingivalis* [71]. Strikingly, in addition to the inhibition of *fimA* transcription, this peptide also inhibited the expression of several virulence genes in *P. gingivalis*, including *mfa1* and genes encoding the gingipain proteases (*rgpA/B* and *kgp*), with half-inhibitory concentrations (IC50) of around 10 μM. Consistent with the suppression of transcripts, the production of fimbrial proteins and gingipains was also significantly reduced when *P. gingivalis* was treated with this peptide, which thereby was designated Streptococcal-derived Anti-*P. gingivalis* Peptide (SAPP).

The ability of SAPP to prevent community development has been demonstrated through in vitro and ex vivo studies. Treatment of *P. gingivalis* with SAPP significantly reduced the formation of monospecies biofilms of *P. gingivalis* strains including both fimbriated and afimbriated lineages. This result is significant as strains of *P. gingivalis* devoid of fimbriae have been be isolated from periodontal pockets. SAPP can also inhibit the development of heterotypic *P. gingivalis*-*S. gordonii* communities and attachment of *P. gingivalis* to saliva-coated surfaces [71]. Another important finding is that SAPP not only inhibits *P. gingivalis* attachment to saliva-coated or to streptococcal surfaces but also disrupts established *P. gingivalis*-*S. gordonii* biofilms. Although the mechanism is unclear, it is speculated that SAPP inhibits re-entry of the detached *P. gingivalis* cells into the biofilm. Dispersed *P. gingivalis* cells will then have a greater chance of being eliminated from the oral cavity due to swallowing or expectoration. SAPP also works effectively to impede multi-species biofilm formation. In an ex vivo study, dental plaque retrieved from periodontitis patients was grown in the presence or absence of SAPP [72]. Bacteria bound to culture well surfaces were collected, and the number of sessile bacteria was determined using qPCR. The amount of *P. gingivalis* detected in complex multi-species biofilms cultured with SAPP was significantly lower than that found in wells without SAPP. This inhibitory activity is not strain-specific, as the effects of SAPP on the inhibition of *P. gingivalis* attachment were found with four distinct fimA genotypes (I, II, III, and IV). More remarkable was the finding that along with *P. gingivalis*, other chronic periodontitis-associated bacteria, *Tannerella forsythia* and *Treponema denticola*, were also significantly reduced in abundance in biofilms in the presence of SAPP. However, there was no significant difference in the numbers of streptococci in biofilms cultured in the presence or absence of SAPP. These data suggest that SAPP may affect colonization of other periodontal pathogens in highly orchestrated oral microbial biofilms and that *P. gingivalis*, as a keystone bacterium, plays an important role in modulating the composition of multispecies biofilms, as has been demonstrated in animal studies [11].

Another important aspect to the pathoecology of *P. gingivalis* in the gingival compartment

is the ability of the organism to enter into gingival epithelial cells and reside intracellularly for extended periods [73]. The invasion of *P. gingivalis* is initiated following the stimulation of epithelial cell integrin receptors by the FimA adhesin [74]. The invasion efficiency of *P. gingivalis* strains grown with SAPP at 2 × 50% of inhibitory concentration of *fimA* expression was reduced by approximately five-fold compared with *P. gingivalis* without SAPP treatment. Intracellular *P. gingivalis* impinge on a number of eukaryotic signal transduction pathways some of which converge on cytokine and chemokine expression, a phenomenon known as immune paralysis [2, 75, 76]. Further, *P. gingivalis* gingipains can degrade several cytokines/chemokines. Another feature of SAPP is its ability to reverse impaired secretion of cytokines and chemokines induced by *P. gingivalis*. The accumulation of IL-6, IL-8, and MCP-1 (Monocyte chemoattractant protein 1), in the extracellular milieu of gingival epithelial cells, was restored when *P. gingivalis* was pretreated with SAPP (Fig. 11.3). IL-8 and MCP-1 are both well-known chemoattractant cytokines. MCP-1 plays important roles in regulating the migration and infiltration of monocytes/macrophages [77], while IL-8 is involved in the recruitment of neutrophils to the site of infection [78]. By preventing *P. gingivalis* antagonism of these compounds, SAPP may be able to reduce the impairment of host immunity by *P. gingivalis*, which in turn may facilitate the maintenance of periodontal tissue homeostasis.

SAPP is an 11-mer peptide with molecular weight of 1322.62 Da and theoretical isoelectric point (pI) of 10.48. The secondary structure of SAPP as determined by circular dichroism (CD) spectroscopy shows it exists as a random coil in water. SAPP also appears to have no significant helical propensity, as helix structures were not formed in the presence of tri-fluoroethanol, a helix-inducing agent [72]. SAPP is soluble in

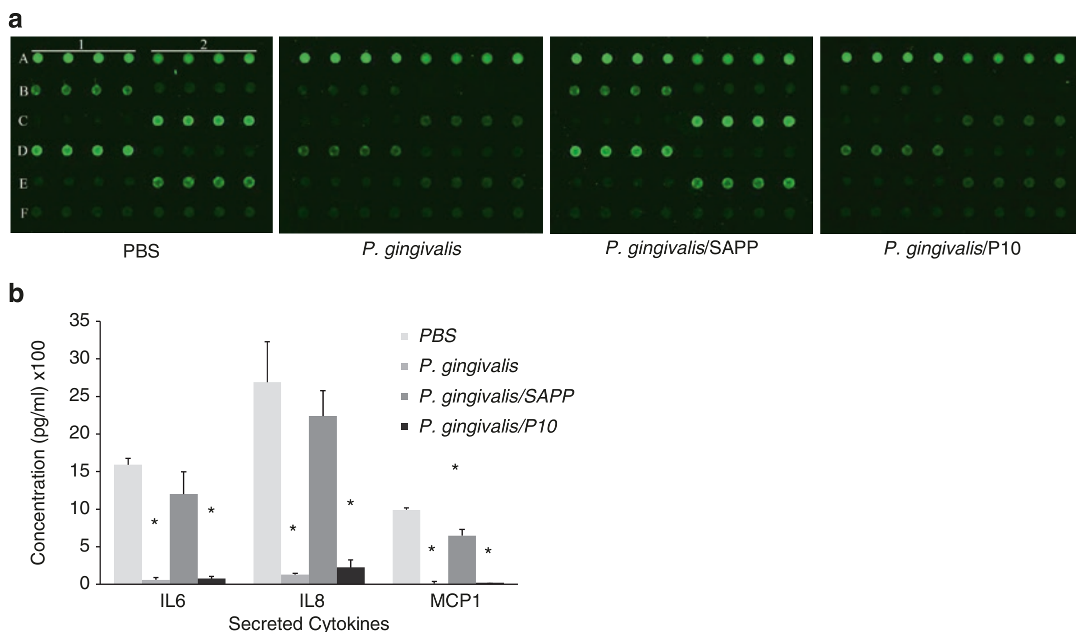

Fig. 11.3 The role of SAPP in reversing *P. gingivalis* induced immune paralysis. (**a**) Gingival epithelial cells were reacted with PBS, *P. gingivalis* 33277, *P. gingivalis* treated with SAPP (30 μM), or *P. gingivalis* treated with a SAPP analog (P10). Levels of 10 selected cytokines secreted into the media were determined by a protein array. Each antibody is printed in quadruplicate horizontally: A1 and A2 are biotin-labelled IgG (positive control); B1 is IL-1α; B2 is IL-1β; C1 is IL-4; C2 is IL-6; D1 is IL-8; D2 is IL10; E1 is IL-13; E2 is MCP-1; F1 is INF-γ; and F2 is TNF-α. (**b**) Quantitation of array data for three selected cytokines. Asterisks indicate significant differences in the levels of cytokines detected with or without *P. gingivalis* treatment (*t* test, $p < 0.05$)

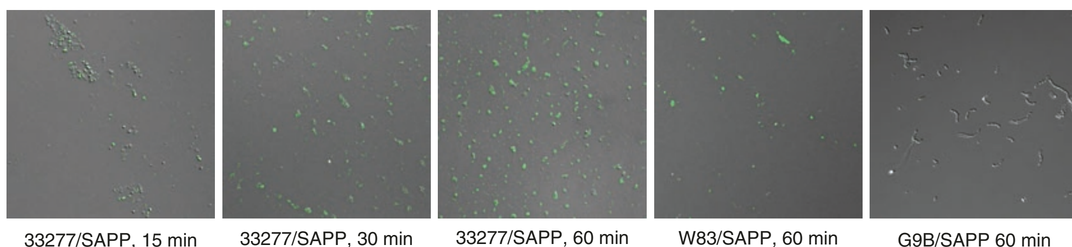

Fig. 11.4 Specific interaction of SAPP and *P. gingivalis* strains. Biofilms of *P. gingivalis* 33277, W83, and *S. gordonii* G9B were formed on saliva-coated glass and exposed to FITC-labeled SAPP (5 μM) for 15, 30, or 60 min. Bacterial-associated SAPP (green) was visualized using confocal microscopy

water and PBS, and is relatively stable for at least 24 h at 25 °C. However, gradual degradation of SAPP occurs in diluted whole saliva within a 24 h period. Toxicology studies detected no plasma membrane impairment in gingival epithelial cells or in human periodontal ligament fibroblasts (HPLFs) after exposure to SAPP at concentrations as high as 800 μM (approximately 80 times greater than the IC50 for *fimA* and *rgpA* expression) for as long as 48 h. Moreover, SAPP treatment also did not induce apoptosis or necroptosis of these cell types. These properties of SAPP make it a promising agent for therapeutic development against chronic periodontitis.

The mechanism by which SAPP initiates signal transduction in *P. gingivalis* is currently not clear, although direct interaction between SAPP and *P. gingivalis* cells appears to be required. Fluorescently labeled SAPP can be detected on *P. gingivalis* surfaces after a 15 min exposure, and a time-dependent increase in SAPP binding was observed during a 1 h exposure (Fig. 11.4) [72]. Two functionally inactive SAPP derivatives that have one or two residues substituted with alanine failed to bind efficiently to *P. gingivalis* cells. SAPP binds specifically to *P. gingivalis*, and as shown in Fig. 11.4, SAPP interacts with both fimbriated and afimbriated stains (33277 and W83) but not with *S. gordonii*. Two surface proteins of *P. gingivalis*, a major immunodominant antigen (RagB, PGN_0294) and a protein of the MotA/TolQ/ExbB proton channel family (PGN_0806), were identified as putative receptors of SAPP. The deletion of the genes encoding these proteins significantly reduced the *P. gingivalis* response to SAPP. Previously, the *Pseudomonas aeruginosa* TonB–ExbB–ExbD protein complex was reported to be involved in signal transduction [79]. Moreover, RagA, which is thought to associate with RagB on the *P. gingivalis* surface, is a TonB-dependent receptor [80–82]. Hence TonB-dependent transportation may be necessary for SAPP action.

11.7 Conclusion

Treatment of chronic periodontitis conventionally involves surgical and nonsurgical mechanical therapies that physically remove the plaque biofilm. In cases of severe and refractory manifestations of the disease, the treatments are sometimes supplemented with systemic or local administration of antibiotics [83–85]. Concerns regarding the use of antibiotics include the disruption of symbiotic or mutualistic relationships between the host and commensal microbiota [86], and the emergence of oral bacteria resistance to antibiotics [87]. In either event, currently available treatments for periodontitis are often only temporarily effective and recurrence of the disease is common [88, 89], possibly due to incomplete pathogen elimination [90]. Therefore, therapeutic agents that specifically interfere with the colonization of periodontal pathogens are an attractive and advantageous alternative over conventional antibiotics. Compounds that target fimbrial assembly or the Mfa1-Ssp interaction show substantial promise, particularly with regard to the development of active small-molecule mimetics. The advantage of SAPP is that it inhibits surface attachment, host invasion, and biofilm formation

of *P. gingivalis* in vitro by repressing the expression of a plurality of virulence factors including the fimbrial adhesins and gingipain proteases. Importantly, SAPP has a broad, though species-specific, activity against a spectrum of *P. gingivalis* strains but not oral commensal bacteria. Selectively targeting *P. gingivalis* has the added advantage of preventing the pathogenic influence of the keystone pathogen on the entire community, and thus potentially maintaining a healthy oral microbiota.

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Periodontal Pathogen Sialometabolic Activity in Periodontitis

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Periodontitis (PD) is an inflammatory disease characterized by progressive destruction of the tooth-supporting structures, often leading to tooth loss. It affects over 700 million people worldwide and is one of the major oral conditions globally after caries that is estimated to cost $442 billion per year [1–3]. PD is induced by a subgingival polymicrobial community in which a bacterial triad known as the red complex comprising of *Porphyromonas gingivalis* (Pg), *Treponema denticola* (Td), and *Tannerella forsythia* (Tf) is strongly represented. The environmental niche that these bacteria inhabit, as with most human mucosal infections and colonizable surfaces such as the airways, gut, and female reproductive tract, is rich in glycoproteins decorated with *N*- or *O*-linked sugar-glycan chains, of which most are capped at the terminal end with the 9-carbon sugar, sialic acid (Neu5Ac) [4]. A wide variety of pathogens from different genera inhabiting a range of niches within the body, including the oral cavity, are known to utilize Neu5Ac as a source of carbon and nitrogen or to cloak their surface to avoid immune attack (please see review [5]). Likewise, the red-complex bacteria have been shown to produce sialidase that cleaves the terminal sialic acid from glycoprotein-linked glycans on the surface of epithelial cells, immune cells, and oral secretions such as gingival crevicular fluid (GCF). In this review, we summarize accumulating evidence demonstrating that sialic acid harvesting by oral pathogens is not only key to their survival in the oral cavity but might also be responsible for immune dysfunction and disrup-

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tion of tissue integrity observed in periodontitis. Further, we propose that sialidase inhibition with sialidase-targeting pharmacological drugs, such as those currently employed for influenza and also from a range of other sources, may be an attractive adjunct therapy in controlling periodontitis. While sialidase production is a prominent feature of the red-complex bacteria, it is not limited to these species in the oral cavity. For example, several oral commensal and opportunistic organisms such as *Streptococci* spp. (mitis, oralis, intermedius), *Capnocytophaga* spp., *Actinomyces naeslundii*, *Actinomyces oris*, *Prevotella oralis*, *Bifidobacterium dentium*, and *Propionibacterium acnes* produce related sialidases [6, 7]. It is likely that both the nature of physical niche in the mouth and the physiology of individual species dictate the importance of sialidase activity in a given setting. Among the species that might be considered as early or primary colonizers of tooth surfaces in the mouth such as *Streptococci*, *Actinomyces*, *Bifidobacterium*, and *Propionibacterium* are saccharolytic organisms where their primary carbon source is often glucose or other dietary sugars, meaning sialidase activity might chiefly be present to access underlying sugars as part of a more versatile catabolic profile. However, as these organisms are not in direct contact with mucosal surfaces, or immune cells, the impact of their sialidases on inflammation is expected to be limited at best.

Moreover, some bacteria in the oral microbiota that do not produce their own sialidase likely benefit from sialidase activity of other bacteria. For example, some *Fusobacterium* spp. can catabolize exogenous sialic acid for energy or reprocess sialic acid to decorate their surface with the sugar [5]. Still others can also benefit from community sialidase activity, which might allow them to utilize sialic acid or bind to underlying sugars (cryptic epitopes) on glycans, e.g., *S. gordonii*, *S. mutans*, *S. sanguinis*, and *S. salivarius* [7–10]. The contribution of sialidase activity in periodontal inflammation has recently come to light from clinical observations of raised levels of sialidase activity in GCF of periodontitis patients [11]. The negative impact of sialidase activity on periodontal disease is heightened since in the context of the subgingival environment of the periodontal pocket, sialidase activity can disrupt the integrity (structure-function) of host glycoproteins, disrupt pattern-recognition receptor (TLRs) signaling in infiltrating immune cells as well as epithelial layers, and promote the survival, persistence, and pathogenesis of periodontal bacteria.

12.1 Sialic Acid Foraging by Periodontal Pathogens

Current evidence indicates that the key periodontal pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* strictly rely on host-derived sialic acid for their survival in the oral cavity and virulence. This is unlike many other human pathogens such as *Campylobacter jejuni*, *Neisseria meningitidis*, and some *Fusobacterium* spp. that have dedicated biosynthetic pathways to synthesize their own sialic acid [5]. The work from our groups showed that *T. forsythia* mutants lacking sialidase and the sialic acid transporter NanT are unable to acquire environmental sialic acid and are severely attenuated in biological activities such as survivability in biofilms on sialoglycoprotein substrates [12] and interactions with and survival on epithelial cells [13, 14]. In addition, other groups have shown that sialidase deletion in a capsulated strain of *P. gingivalis* results in reduced capsule thickness phenotype [15] and gingipain protease expression [16]. These studies showed that compared to the wild-type capsulated strain, a sialidase-deficient mutant in vitro formed less biofilms and was less resistant to killing by the host complement [15]. Moreover, while the wild-type strain was able to spread to multiple organs and cause mouse mortality following subcutaneous infection, the sialidase-deficient mutant was found to be highly attenuated showing only localized spreading and ineffective in causing mortality in mice. In the case of *T. denticola*, sialidase deficiency caused an increased surface deposition of complement attack complex and reduced virulence in a mouse model [17]. In addition, though it is yet to be established in the periodontal setting, our groups have preliminary evidence of sialidase-dependent modulation of

epithelial and monocyte responses to LPS stimulation, and as such sialic acid scavenging might exacerbate inflammation by affecting TLR signaling. In this scenario, periodontal pathogen-secreted sialidases might contribute to subgingival inflammation through increasing TLR4 responsiveness to LPS as it has been shown that desialylation of TLR4 promotes its dimerization and activation at other human mucosal layers [18]. Moreover, sialidase-mediated desialylation is a mechanism through which one of the immunosuppressive circuits is compromised in immune cells dependent on Siglecs (sialic acid-binding immunoglobulin-type lectins) [19]. In a normal homeostatic state, Siglec-G/10 (G, mouse/10, human) binds a sialylated receptor CD24 and induces an inhibitory circuit that attenuates TLR signaling. Indeed, the data from our lab show that *T. forsythia* sialidase treatment in vitro can increase TLR4 activation in macrophages (unpublished). Thus, sialic acid utilization by pathogens can take roles in immune evasion and dysregulation, community biofilm development, and pathogen survival. Plausibly, the sialidase activity might also assist periodontal pathogens in mitigating the toxic effects of ROS (reactive oxygen species) in the inflamed periodontal pockets as released sialic acid residues can act as scavengers of peroxide residues [20, 21]. Taken together, these findings demonstrate that sialidase is an important and common virulence determinant that may contribute to the pathogenicity of periodontal pathogens.

As mentioned above, both *P. gingivalis* and *T. denticola* possess sialidases that have been shown to be key to their survival and virulence [15–17]. Both *P. gingivalis* and *T. denticola* sialidases display a similar domain organization with a C-terminal catalytic domain with homology to glycosyl hydrolase 33 (GH33) family carbohydrate-active enzymes in the CAZy database [22] that is preceded by an N-terminal domain, which in the case of *T. denticola* is a putative peptidoglycan binding domain [17] while in the case of *P. gingivalis* shows no homology to any of the known protein domains in the sequence or structural databases (Fig. 12.1). Despite clear data existing that *T. denticola* seems to require sialidase activity or monomeric sialic acid for growth in serum, its genome sequence lacks any homologs of sialic acid catabolic genes [17], suggesting the existence of novel pathways.

In contrast, biochemical and functional knowledge of the *T. forsythia* NanH sialidase is well advanced, mostly from work in our teams. As with *P. gingivalis* and *T. denticola*, we have revealed that *T. forsythia* sialidase is key to the bacterium's microbiology and virulence [5, 12–14]. However, unlike *P. gingivalis* and *T. denticola*, it expresses its sialidase, NanH, encoded by the *nanH* gene, as part of a dedicated sialic acid scavenging, transport, and utilization operon (Fig. 12.1). Of note, this operon is present in all of the *T. forsythia* strains sequenced to date, including the genomes of three clinical isolates

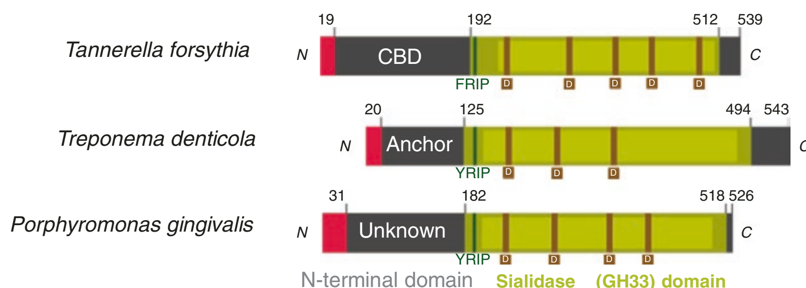

Fig. 12.1 Schematic representation of periodontal pathogen CAzymes. A. sialidases: The GH33 sialidase domains are shown in yellow, with the Asp (D) boxes indicated alongside the catalytic FRIP domains. Signal sequences are indicated in red. N-terminal domains are indicated in gray, with the *T. forsythia* confirmed carbohydrate-binding domain (CBD); *T. denticola* putative cell wall anchor (Pfam PF09479) indicated; alongside the *P. gingivalis* N-terminus, which has no homology in databases

recently sequenced by our group [23] and in metagenomic reconstruction from ancient calculus [24]. Notably the *nan* operon is missing from the related *Tannerella* BU063 strains isolated to date. NanH is a 62 kDa secreted enzyme again comprising two domains with the C-terminal domain comprising a catalytic domain that is a member of the GH33 family mentioned above. It contains five Asp-box motifs, a conserved catalytic Arginine triad, and FRIP-motif (Fig. 12.1). Once again *T. forsythia* NanH possesses a 170–180-amino-acid-long, N-terminal domain with no sequence or structural homology in the PDB database outside of sialidase enzymes found within members of the *Bacteroidetes*. The NanH N-terminal domain has now been characterized by our groups as a novel carbohydrate-binding module (CBM) with broad specificity for host glycans but which prefers sialylated glycans and those with alpha-2,3 glycosidic linkages [25]. As mentioned above, NanH is associated with a novel sialic acid utilization system which contains a novel transporter system (NanOUT) [26–28] for the uptake of monomeric sialic acid and a 9-0-sialate-acetylesterase (NanS) [26]. This NanS enzyme is novel in its class as it contains two SGNH-like Sialate-esterase domains and acts to enhance release of Neu5Ac (sialic acid) from host glycoproteins containing diacetylated sialic acids (Neu5,9Ac) that block the action of sialidases (ref). Furthermore, in the *nan* operon, NanH is followed by a β-hexosaminidase [29], which may act to release subsequent sugars within host glycans [26], and a predicted sialic acid mutarotase that improves utilization of the alpha anomeric form of sialic acid [30].

In addition, our studies have shown that the release, transport, and utilization of sialic acid are critical to the interaction and survival of *T. forsythia* with epithelial cells as well as bacterium's growth in glycoprotein-based biofilms [12–14, 29]. These data shed light on the ability of the bacterium to cleave and utilize host Neu5Ac as a survival strategy whereby removal of terminal sialic acid residues in host glycoproteins in salivary secretions and on epithelial cells allows the bacterium to colonize and utilize the liberated sialic acid as a source of carbon and nitrogen and possibly as a precursor for the peptidoglycan (PGN) synthesis (Fig. 12.2). This is pertinent as PGN synthesis pathways are notably lacking in *T. forsythia*, which cannot de novo synthesize the PGN amino sugar *N*-acetylmuramic acid (MurNAc) from simple nonamino sugars. While

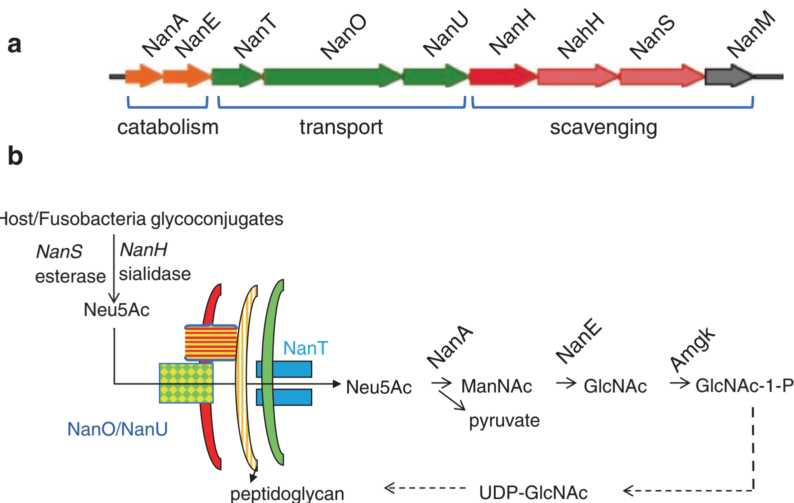

Fig. 12.2 Sialic acid scavenging as a means of peptidoglycan biosynthesis and survival by *T. forsythia*. (**a**) Genetic organization of sialic acid utilization operon. (**b**) Putative metabolic pathway of sialic acid utilization to peptidoglycan biosynthesis. Dashed line arrows indicate canonical enzymes involved in the conversion of various sugar intermediates to peptidoglycan have not been found in *T. forsythia*

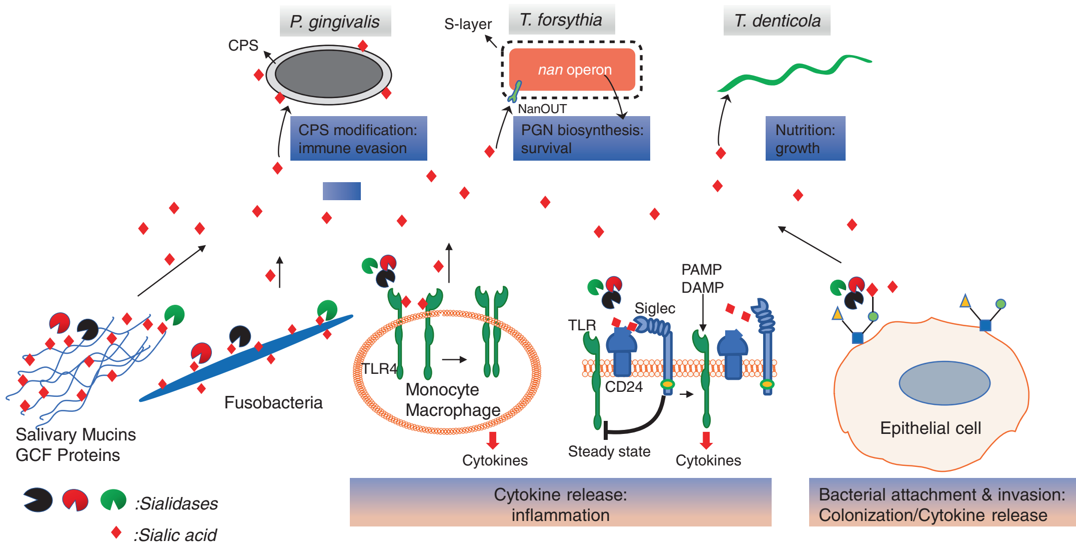

Fig. 12.3 Model of sialic acid-dependent lifestyle of periodontal pathogens. Key: *CPS* capsular polysaccharide, *PGN* peptidoglycan, *S-layer* surface layer, *NanOUT* sialic acid transporter system

it is able to uptake muropeptides released by other bacteria in the oral cavity, e.g., *Fusobacterium nucleatum* [31], we postulate that the role of *T. forsythia* sialidase in liberating free monomeric sialic acid might be critical for the bacterium's survival in the oral cavity as sialic acid could serve as an alternative source for MurNAc synthesis as we have suggested previously [28]. This is significant as the human host is also unable to synthesize and thus provide MurNAc to the bacterium. We postulate that by harvesting sialic acid as an alternative nutritional source, *T. forsythia* is able to gain a competitive edge over the subgingival cohabiting microbiota. In support of a notion that sialic acid foraging by *T. forsythia* might be critical in this respect, a community-wide transcriptome analysis of the subgingival microbiome has indicated that the *T. forsythia nanH* transcript levels likely increase in subjects with periodontitis as compared to healthy controls [32].

One by-product of this use of sialic acid would be a potential increase in availability of peptidoglycan fragments comprising NOD (nucleotide-binding oligomerization domain)-like receptor ligands such as muramyl dipeptide (MDP; NOD2 ligand) and γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP; NOD1 ligand) [33] in the subgingival environment which may heighten NOD-mediated inflammation. Thus, sialic acid scavenging may potentially exacerbate inflammation by affecting TLR signaling as well as putatively increasing NOD-mediated inflammation.

Taken together, sialic acid cleavage by periodontal pathogens contributes to immune dysregulation and evasion, community biofilm development, and pathogen survival (Fig. 12.3). Importantly, support for the notion that sialic acid acquisition by the subgingival microbiome might be important in disease progression comes from our studies demonstrating a heightened expression of microbial sialidase activity in diseased sites compared to healthy sites and the heightened sialidase activity as a predictor of poor standard treatment outcomes [11].

12.2 Global Sialidase Activity and Impact on Health and Disease

It is important to take into account the contribution of sialidase activity of commensal oral organisms which may be critical for microbial ecology and health or disease in ways that have yet to be fully understood. In this regard, the sial-

idase activity of commensal bacteria may also be required for their growth/colonization and thus may have roles in maintaining healthy microbiota. The damaging effects of sialidase from commensal bacteria colonizing the supragingival niche might be mitigated both due to sialic acid-rich salivary mucins acting as a coating layer on hard surfaces. The delicate balance between the host and the sialidase activity in health is thus likely maintained by appropriate host barrier functions of salivary proteins including mucins and innate immune responses. This is in contrast to the subgingival niche and inflamed periodontal pockets where sialidases from periodontal pathobionts can directly encounter infiltrating immune cells (monocytes/macrophages) and influence inflammation. In addition, as mentioned above, sialidase activity might promote the survival of pathobionts in the harsh subgingival environment; in the case of *T. forsythia*, sialidase activity promotes its growth, and for *P. gingivalis* and *T. denticola*, sialidase activity is important in protecting these bacteria against the complement attack while it may also allow them easier access to underlying protein substrates that are key for their virulence and nutrition [15, 17]. Thus, the role of sialidase might be context dependent. In a periodontitis setting, inflammation and dysbiosis due to pathobionts *P. gingivalis*, *T. forsythia*, and *T. denticola* and proliferation of commensals might further increase global sialidase activity and thus synergistically exacerbate periodontitis. This notion is supported by observations that sialidase activity is elevated in plaque biofilms [34] and is high in diseased GCF in patients [11].

12.3 Translational Potential of Sialidase Inhibition

The dependence of key periodontal pathogens on sialic acid for survival, virulence, and immune modulation provides treatment opportunities for periodontitis by targeting sialidase activity with sialidase inhibitors. The potential translatability of blocking periodontal pathogen sialidase activity, heightened by the availability of several current FDA-approved (Tamiflu, Relenza, Peramivir) or novel sialidase inhibitors, to alleviate periodontitis and associated dysbiosis development and inflammation is very promising. Sialidase inhibitors could be used as an adjunct therapy in situations where periodontal pathogens present resistance to antibiotics. This may also minimize the use of antibiotics and development of antibiotic resistance. Our published and preliminary studies have demonstrated that inhibiting sialidase function with pharmacological inhibitors (FDA-approved zanamivir and oseltamivir) can block the ability of *T. forsythia* to form biofilms and survive on sialoglycoconjugates and epithelial cell monolayers, both in mono-species and mixed-species infections with *P. gingivalis* and *Fusobacterium* spp. [12, 14]. Importantly, sialidase inhibition can block the availability of sialic acid for peptidoglycan biosynthesis in *T. forsythia*, and therefore may reduce any selective advantage *T. forsythia* might have over other bacteria in biofilms in vivo. In the case of *P. gingivalis*, lack of sialidase activity in the bacterium results in increasing sensitivity to hydrogen peroxide and reduced gingipain protease activity, suggesting that sialidase activity might be involved in regulating the virulence potential of this keystone pathogen [16]. In a capsulated strain of *P. gingivalis*, sialidase activity seems to influence capsule formation and confer the bacterium the ability to resist complement attack [15]. Moreover, based on in vivo studies in a mouse model, the role of sialidase activity has been highlighted. It has been shown that while wild-type *P. gingivalis* has the ability to disseminate to multiple organs following infection, sialidase deficiency abrogates this ability causing only localized spreading around the site of infection [15]. In *T. denticola*, sialidase activity seems to be responsible for the acquisition of sialic acid as a nutrient as well as for sialic acid modification of surface glycoproteins as a means of blocking deposition of membrane attack complex and killing [17]. An obvious next step is therefore to test the in vivo potential of these synthetic as well as other naturally occurring plant-derived inhibitors (berberine and palmatine) [35] against sialidase-producing pathogens. In this regard, it is tempting to first test the efficacy of sialidase inhibitors/

inhibitor cocktails found effective in vitro in conferring protection to pathogen-induced periodontal bone loss in a mouse model, including in polymicrobial situations. The inhibitors might include both those that block the NanH sialidase's enzymatic activity (Tamiflu) and inhibitors that block NanH's lectin function (S-Lewis oligosaccharides or glycan mimics). The use of sialidase inhibitors for the treatment of periodontitis is not without risks due to potential off-target effects of these inhibitors on endogenous host sialidases (neuraminidases) playing important roles in the physiology of the host [36–38]. However, in principle one can design specific inhibitors that target the bacterial sialidases while not compromising functionality of the host sialidases [39]. We envisage a situation where topical administration of glycosidase inhibitory compounds could be deployed as gels applied as part of nonsurgical root debridement or within mouthwashes.

12.4 Summary

In this review we provided accumulating evidence demonstrating that sialic acid foraging by oral pathogens is not only key to their survival in the oral cavity but is also responsible for immune dysfunction and dysbiosis observed in periodontitis. We envisage that sialidase neutralization with pharmacological drugs, such as those currently employed for influenza, and also plant derived sialidase inhibitors in development may be an attractive adjunct therapy in controlling periodontitis.

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Ecological Approaches to Periodontal Therapy

13

Patricia I. Diaz and Anilei Hoare

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13.1 Introduction

Dysbiosis consists of an imbalance in the species that form a microbiome community with depletion of beneficial species and outgrowth of pathogenic ones. Dysbiosis is a feature of periodontitis [1, 2]. Current periodontal therapies are not efficient at restoring a health-like community, and therefore there is a need to better delineate the factors that control the stability of subgingival communities associated with health and those events that promote dysbiosis. In particular, ecological factors, which refer to the relationships of microorganisms with one another and their interactions with the environment, are considered drivers of dysbiosis and could represent modifiable targets to develop ecology-based therapeutics. Ecological therapies to control dysbiosis are already in effect for other mucosal-associated diseases, with the best examples seen in the lower gastrointestinal tract [3, 4].

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This chapter will discuss the importance of understanding events leading to subgingival dysbiosis and the stage of development of different ecology-based preventive or therapeutic strategies for periodontitis.

13.2 Subgingival Microbiome Dysbiosis Is Associated with Progression of Periodontitis

The inflammation and tissue destruction associated with periodontal disease occur as a response to a perturbed (dysbiotic) subgingival microbiome. Although it is clear that genetic and environmental determinants shape the host inflammatory response to bacteria present in the gingival sulcus [5, 6], a dysbiotic microbiome community able to trigger pathology seems to be required for the development of periodontitis [7–9]. A recent human clinical longitudinal study characterized the subgingival microbiome in active sites that showed disease progression (attachment loss) in comparison with stable sites [9]. This study showed differences in the composition and functional activities of the subgingival microbiome in active sites prior to attachment loss. Sites that went on to progress were enriched for Gram-negative anaerobic and proteolytic species from the genera *Prevotella*, *Porphyromonas*, *Tannerella*, and *Treponema*, while stable sites had higher proportions of aerobic and facultative species from the genera *Neisseria* and *Streptococcus*, among others. Moreover, a characterization of the genes expressed by the whole community showed higher metabolic activity and expression of virulence factors in the sites that went on to progress. Longitudinal clinical studies in patients presenting localized rapidly progressing forms of periodontitis have also shown that the presence of a specific virulent strain of *Aggregatibacter actinomycetemcomitans* is associated with future risk of attachment loss [10]. A more recent study showed that the presence of single bacterial species was not as useful to predict future risk of bone loss as the presence of a consortium formed by *A. actinomycetemcomitans*, *Streptococcus parasanguinis*, and *Filifactor alocis* [8]. Altogether, findings from these longitudinal clinical studies suggest that a microbial community with pathogenic properties precedes disease progression. Importantly, progression could not be solely attributed to the presence of a single microbial species but seems associated with the properties of the subgingival community as a whole.

Experimental animal models have also been useful to discern the specificity of the microbial stimuli associated with periodontitis. In a mouse model of ligature-induced periodontitis, Dutzan et al. [7] showed that the sole increase in total bacterial burden close to the gingiva is insufficient to drive disease-associated Th17 immune responses and bone loss. Increased load in combination with disruptions in the community structure consisting of enrichment of Gram-negative species was required for a disease phenotype. In another model, oral inoculation of mice with the human periodontitis-associated microorganism *Porphyromonas gingivalis* results in bone loss, despite low levels of pathogen colonization [11]. In this model, the potent proteases of *P. gingivalis* contribute to dysregulation of host-protective mechanisms including the complement cascade and neutrophil phagocytosis, with these changes leading to an environment that allows quantitative and qualitative disruptions in the whole microbial community, which is ultimately responsible for induction of bone loss [11, 12].

The data discussed above suggests that communities with specific virulence attributes precede the occurrence of periodontitis. Since the microbial communities associated with disease are formed by indigenous species (native to the mouth), the question that emerges is what factors serve as triggers for dysbiosis at specific gingival sites. The section below reviews the ecological triggers hypothesized to initiate and maintain subgingival microbiome dysbiosis. These events are reviewed with a focus on the community behavior and its interaction with the environment.

13.3 Ecological Drivers of Subgingival Dysbiosis

An important characteristic of subgingival communities is their polymicrobial nature. The subgingival crevice harbors around 500 different

bacterial species with each site inhabited by a complex community [1, 13]. Microorganisms in these communities form metabolic webs in which products are exchanged with species complementing the metabolic needs of each other. It is therefore likely that specific interspecies metabolic exchanges are required for the occurrence of the microbiome shifts associated with periodontitis. Microorganisms hypothesized to be key "metabolic anchors" of subgingival communities include core species such as *Fusobacterium nucleatum*, which is an ubiquitous species found in health and an important component of communities in periodontitis [1]. *F. nucleatum* has been shown to coaggregate with a range of other species [14]. In biofilm and continuous culture planktonic models, *F. nucleatum* forms net-like structures in which other bacteria are retained, and therefore it is likely that *F. nucleatum* is an important structural component of plaque [15, 16]. In continuous culture chemostat models, *F. nucleatum* has been shown to be important for the survival of other anaerobes under aerated conditions [16, 17]. *F. nucleatum* has the ability to adapt to oxygenated conditions reducing the environment through the activity of an NADH oxidase, which allows growth of other anaerobes more sensitive to oxygen, such as *P. gingivalis* [16, 18]. *F. nucleatum* also supports the growth of *P. gingivalis* by supplying it with carbon dioxide [16].

Another ubiquitous subgingival plaque species, *Veillonella parvula*, has been shown to promote the biofilm growth of late colonizing pathogens such as *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* when part of polymicrobial communities containing streptococci [19]. In vitro work has shown that *V. parvula* is less sensitive to oxygen than *F. nucleatum*, and via its catalase activity, it can reduce the environment and facilitate *F. nucleatum*'s growth [20]. *Veillonella* is particularly important when hydrogen peroxide-producing streptococci are part of the community as it can detoxify this product allowing the growth of *F. nucleatum*. *Veillonella* can also support the in vitro growth of *P. gingivalis* partly by producing hemin, a preferred iron source of the latter [21].

In another example of metabolic cooperation that leads to the establishment of a pathogenic species, the early colonizer *Streptococcus gordonii* has been shown to facilitate the in vitro biofilm growth and in vivo fitness and survival of *Porphyromonas gingivalis*, by providing it with 4-aminobenzoate/para-amino benzoic acid (pABA) [22]. Moreover, in agreement with the cooccurrence of streptococci and *A. actinomycetemcomitans* in clinical samples [8], in vitro and animal studies have shown a synergy between *S. gordonii* and *A. actinomycetemcomitans* [23, 24]. *S. gordonii* promotes the virulence of *A. actinomycetemcomitans* by providing it with the metabolite L-lactate, a preferred carbon source for *A. actinomycetemcomitans* [23]. *A. actinomycetemcomitans* is capable of aerobic respiration and benefits from the hydrogen peroxide produced by *Streptococcus gordonii* [24]. Like *Veillonella*, *A. actinomycetemcomitans* is also catalase positive. Detoxification of hydrogen peroxide by catalases produces water and oxygen, which *A. actinomycetemcomitans* uses as an electron acceptor in its respiratory metabolism [24]. It seems therefore that the establishment of reduced microniches that can promote the growth of species with lower oxygen tolerance and the exchange of metabolic by-products, including respiratory electron acceptors and carbon sources, are important drivers of microbiome shifts. Figure 13.1 summarizes these webs of metabolic interactions. It should be noted, however, that the webs of metabolic exchanges among subgingival species are likely to be complex and redundant. There is a lack of studies evaluating the role of specific microbiome components during dysbiotic shifts in complex in vivo-like communities.

Higher flow of gingival crevicular fluid (GCF) due to inflammation is also thought to drive subgingival dysbiosis. Clinical longitudinal studies show levels of gingival inflammation are associated with subsequent attachment loss [25]. GCF is thought to promote dysbiosis due to its high content of complex glycoproteins which favor the growth of species that rely on protein catabolism. In vitro investigations have shown that the presence of serum proteins in the culture medium promotes the enrichment of proteinase-rich Gram-negative pathobionts [26–28]. A recent study demonstrates that species typical of periodontal pockets can be enriched from oral samples using a growth medium that simulates the nutritional aspects of the inflamed subgingival environment [29]. Some of the

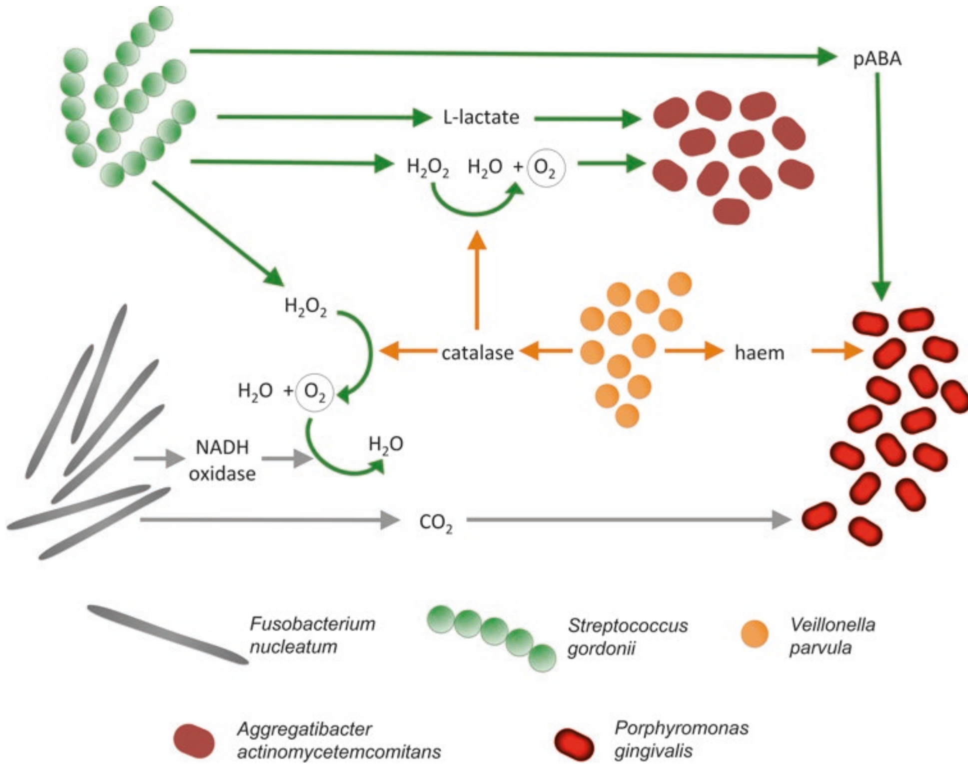

Fig. 13.1 Cartoon summarizing documented interspecies interactions among subgingival community members that result in enhanced growth of periodontitis-associated species. Mitis-group streptococci such as *Streptococcus gordonii* supply L-lactate to *Aggregatibacter actinomycetemcomitans*. *S. gordonii* also produces hydrogen peroxide (H_2O_2), which catalase-positive species, such as *A. actinomycetemcomitans* or *Veillonella parvula*, metabolize. The resultant oxygen enhances growth of *A. actinomycetemcomitans*. However, oxygen and H_2O_2 can limit the growth of anaerobes such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. *Veillonella*-derived catalase allows growth of *F. nucleatum* in the presence of streptococci by metabolizing H_2O_2. *F. nucleatum* does not have peroxidase activity but can contribute to further reduce the environment via its NADH oxidase activity detoxifying oxygen and enhancing the growth of *P. gingivalis*. *F. nucleatum* supplies CO_2 to *P. gingivalis*. *Veillonella* produces heme, which is the preferred iron source of *P. gingivalis*. *S. gordonii* provides 4-aminobenzoate/para-amino benzoic acid (pABA), which *P. gingivalis* uses for folate biosynthesis. Cartoon is based on the work of Diaz et al. [16, 18], Zhou et al. [20, 21], Stacy et al. [23, 24], and Kuboniwa et al. [22]

enriched periodontitis-associated species were not detectable in the initial inoculum but grew to form an important proportion of the total community biomass after a 3-week enrichment period in a protein-rich medium. Increased inflammation is also associated with bleeding, which in turn promotes the growth of several Gram-negative black pigmented anaerobes that use hemoglobin-derived heme as their preferred iron source [30, 31]. In vitro studies also suggest that the peroxidase activity present in inflammatory exudates and in blood can promote growth of hydrogen peroxide-sensitive pathobionts in a polymicrobial community containing streptococci, by neutralizing the antimicrobial effect of hydrogen peroxide produced by these species [32].

Potassium has also recently been identified as a signal that promotes the pathogenicity of the subgingival microbiome. In a study that analyzed the genes expressed by subgingival communities, it was shown that communities at healthy or periodontally stable sites express higher number of genes involved in transport of potassium compared to communities of diseased sites [9, 33]. This finding is in agreement with previous investigations that suggest potassium is scarce in health and becomes elevated as dysbiosis and tis-

sue destruction progress [34]. Potassium, which is released from the inside of host cells as tissue destruction occurs, has been shown to increase the virulence potential of an oral bacterial community triggering upregulation of genes related to iron transport and motility and increasing the community hemolytic activity [35]. Figure 13.2 shows a graphical representation of the nutri-

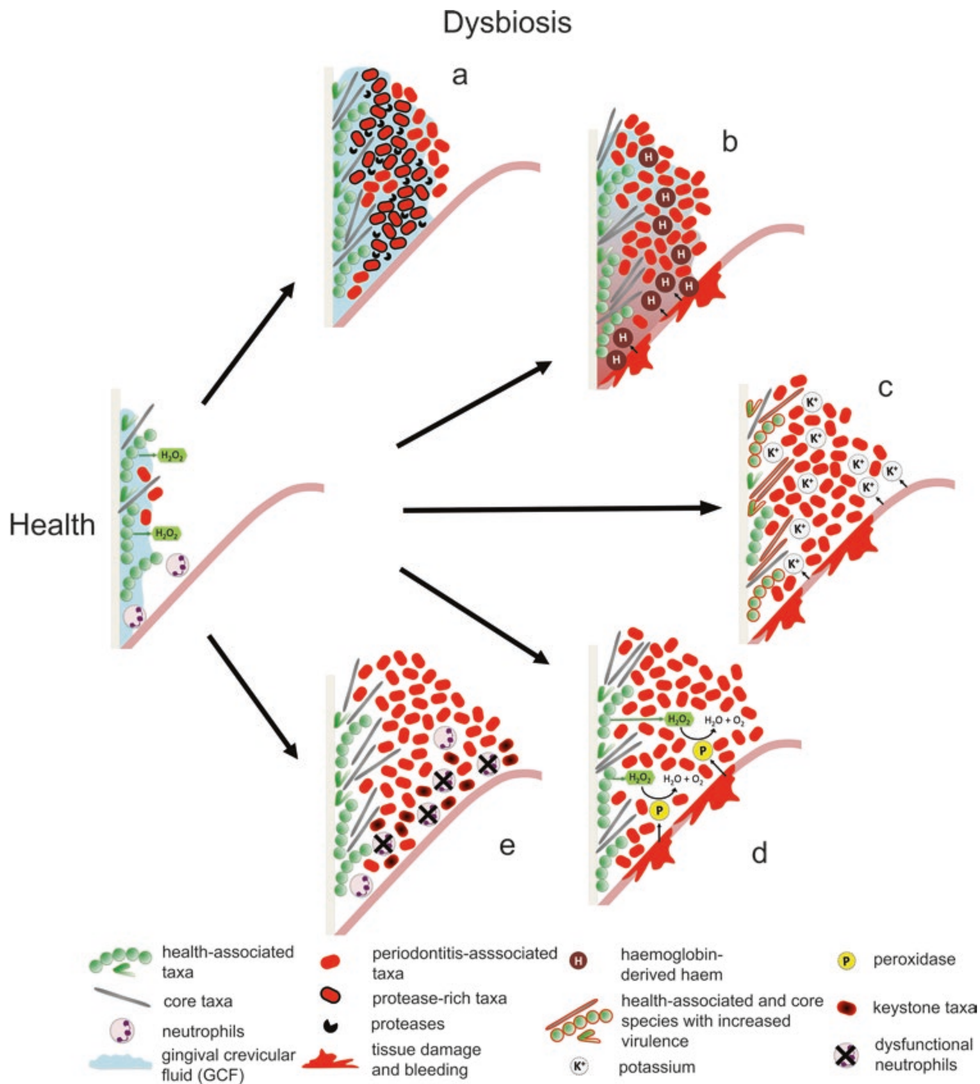

Fig. 13.2 Inflammation-associated changes in the subgingival environment suggested to act as drivers of microbiome dysbiosis. Health is characterized by low inflammation with minimal gingival crevicular fluid flow and the presence of potentially beneficial species such as hydrogen peroxide-producing streptococci. In panel (**a**), an increase in gingival crevicular fluid results in higher availability of complex glycoproteins, which select for taxa rich in proteases. Peptides released by these taxa aid in the growth of the whole community. In panel (**b**), inflammation and bleeding increase the availability of heme, which allows growth of black-pigmented periodontitis-associated anaerobes. In panel (**c**), tissue damage induces release of potassium which increases the virulence of the whole community. In panel (**d**), peroxidase activity present in inflammatory exudates and in blood neutralizes hydrogen peroxide produced by streptococci, thereby promoting the growth of hydrogen peroxide-sensitive pathobionts. In panel (**e**), keystone taxa dysregulate host-protective mechanisms such as neutrophil phagocytosis allowing the growth of the whole community and dysbiosis to occur. Cartoon based on several studies referenced in the text

tional and environmental cues that result from inflammation and their effect on subgingival microbiome shifts.

The species important for the formation of dysbiotic communities are not only those capable of establishing key partnerships that support the metabolic function of pathobionts. Other species may play a crucial role for the survival of the whole community and the emergence of dysbiosis by disabling an effective host response (Fig. 13.2e). One example is *P. gingivalis*, which in an oral inoculation mouse model, dysregulates host responses in such a manner that the growth of the whole community is enhanced and qualitatively altered [11]. *P. gingivalis* interferes with effective bacterial clearance by blocking phagocytosis in neutrophils, which are essential sentinels controlling the growth of subgingival communities. By disabling effective neutrophil surveillance, *P. gingivalis* promotes microbiome dysbiosis and inflammation and leads to bone loss [12].

13.4 Can Ecology Be Used to Prevent or Reverse Dysbiosis?

Considering microbiome shifts associated with periodontitis result from interspecies interactions and environmental influences on the community, it seems logical to investigate if manipulating the community ecology could be of benefit to prevent or reverse dysbiosis. Several therapeutic strategies are currently under investigation to prevent or resolve inflammation in the adjacent gingiva [36–38]. Strategies to manipulate the host response could also result in a reversal of the microbiome dysbiotic shifts. Indeed, a study in a rat model of ligature-induced periodontitis found that topical application in diseased sites of a promoter of inflammation resolution, Resolvin E1, not only had a positive effect reversing tissue destruction and inflammation but also partially resolved microbiome dysbiosis [37]. Below we will discuss ecological strategies aimed at preventing or reversing subgingival dysbiosis by manipulating the community composition rather than inflammation (promising strategies are summarized in Table 13.1). These approaches need further development and would be complimentary to anti-inflammatory therapies.

Table 13.1 Ecological approaches to periodontal therapy and stage of development

Therapy	Description	Stage of development
Probiotics	Administration of probiotic species as adjuncts to periodontal therapy	Randomized controlled trials show small but positive effects of *Lactobacillus* spp. probiotics as adjuncts to scaling and root planing on selected clinical and microbiological parameters [42–44]
Prebiotics	Administration of selected substrates to enhance growth of health-associated species and suppress pathogens	In vitro studies have identified candidate substrates with beneficial effects [46, 47]
Oral inoculation with beneficial oral species	Administration of indigenous health-associated species as adjuncts to periodontal therapy	In vivo study in a beagle dog model of periodontitis shows that administration of a mixture of streptococci after scaling and root planing has positive clinical and microbiological effects [53]
Targeted killing of pathogens	Species-specific antimicrobials targeted against periodontal pathogens	A prototype fusion antibiotic with specific binding to a *P. gingivalis*-expressed protein shows greater in vitro killing efficiency than the antibiotic alone [55]

13.5 Probiotics and Prebiotics

The World Health Organization and the Food and Agriculture Organization of the USA define probiotics as “live microorganisms which, when administered in adequate amounts, confer a

health benefit on the host." Probiotics have been extensively utilized to diminish symptoms of gastrointestinal diseases such as infectious childhood diarrhea, antibiotic-associated diarrhea, pouchitis, and ulcerative colitis, with specific strains of *Lactobacillus* and *Bifidobacterium* spp. recommended in these situations [39–41]. Probiotics are thought to exert their beneficial effects through enhancement of the mucosal barrier, modulation of the immune response, and displacing or antagonizing pathogenic species. *Lactobacillus* spp. probiotics have been tested as adjuncts to scaling and root planing in the treatment of periodontitis [42–44]. These studies report that the probiotic group showed greater improvements in selected clinical parameters and microbiological outcomes in comparison to the placebo group. The effects of these trials, however, are small and these protocols have not been widely adopted in clinical practice.

An alternative approach to community manipulation is prebiotics, which are defined as "nondigestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract" [45]. Prebiotics with beneficial gastrointestinal effects include dietary carbohydrates such as fructo-oligosaccharides, galacto-oligosaccharides, and lactulose [45]. This concept has been applied to oral microbial communities with a recent study performing a high-throughput screening of 742 nutritional compounds for their ability to stimulate the in vitro growth and biofilm formation of 16 oral species grown individually or in dual species competition assays [46]. The compounds beta-methyl-D-galactoside and *N*-acetyl-D-mannosamine were identified as the ones with the greatest potential. In a follow-up study, three substrates, *N*-acetyl-D-mannosamine, succinic acid, and the dipeptide Met-Pro, were seen to increase the proportions of beneficial species and lower the percentages of pathogens after their application to a 14-species biofilm [47]. Further testing of these compounds under in vivo conditions and in the presence of communities of higher diversity is needed to confirm their potential clinical applicability.

13.6 Is Whole-Microbiome Transplantation a Feasible Approach to Revert Subgingival Dysbiosis?

Whole-microbiome transplantation to restore mucosal health is a strategy successfully applied in the lower gastrointestinal tract. Administration of stool from a healthy donor is able to resolve symptoms of *Clostridium difficile*-associated diarrhea in a more efficient manner than vancomycin treatment, the standard of care [3, 4]. Fecal transplantation has been shown to restore gut bacterial diversity and induce shifts in the microbiome community structure toward that of the healthy donor [48]. Stool donor type (related or unrelated) and degree of engraftment have not been shown to be determinant for successful treatment of *C. difficile* infection by fecal transplants [49]. However, if subjects have concurrent inflammatory bowel disease (IBD), the success of fecal transplantation is compromised as shown by higher proportions of the original community and increased frequency of episodes of *C. difficile* infection on long-term follow-up in comparison with subjects unaffected by IBD [49]. This is an important finding that underlies the role of inflammation as a modifier of the mucosal microbiome.

Whole-microbiome transplantation has not been tested as a treatment strategy in periodontitis. Before this approach is considered, several questions need to be resolved. First, there is a possibility that transplantation of a microbiome community from a periodontally healthy donor would result in dysbiosis once grafted in the diseased recipient, which has a genetic background and environmental stimulants that promote inflammation. It is also not clear if all communities from healthy individuals have the potential to become dysbiotic or if certain individuals carry health-associated communities of greater resilience. Another issue to address is feasibility. An exploratory research study recently evaluated an antimicrobial approach to decrease oral bacterial load in preparation for future whole-microbiome transplantation [50]. Decreasing the load of bacteria in the recipient mouth would indeed be

desirable to promote better engraftment of the donor microbiome. Other issues to resolve regarding feasibility include donor selection, method of collection to ensure sufficient donor material, method of delivery, and biosafety concerns.

13.7 Targeted Microbiome Manipulations

The identification of specific strains within the indigenous human microbiome able to promote health and restore the composition of a healthy community either by modulating the host response or by antagonizing pathogens is another strategy recently investigated in experimental animal models. In a murine model, the administration of a cocktail of human intestinal clostridia is able to decrease signs of colitis and allergic diarrhea by induction of anti-inflammatory responses [51]. Discovery of oral species with a similar immunomodulatory potential could represent a potential avenue in the treatment of subgingival dysbiosis.

It is clear that currently available treatment modalities for periodontitis neither fully restore a health-like community nor promote the growth of beneficial species [52]. To address this issue, investigators have evaluated the effect of administration of health-associated species as adjuncts to scaling and root planing using a beagle dog model of periodontitis [53]. Although significant reductions in pocket depth, bleeding on probing, and clinical attachment levels were observed with scaling and root planing alone, there were greater improvements when a mixture of streptococci (*Streptococcus sanguinis*, *Streptococcus salivarius*, and *Streptococcus mitis*) were topically applied after scaling and root planing. The group that received the streptococci also showed more dramatic reductions in anaerobic and black-pigmented species including *Porphyromonas gulae* (a canine form of *P. gingivalis*), *P. intermedia*, and *Campylobacter rectus* and a lesser tendency for reemergence of these pathogens after 12 weeks [53]. This proof of concept study could serve as the basis for future work to investigate whether inoculation with beneficial species could oppose the reemergence of dysbiosis after treatment.

Targeted antimicrobial approaches to selectively kill pathogens or keystones within complex communities are also emerging. In the caries field, an antimicrobial peptide designed to selectively kill the cariogenic pathogen *Streptococcus mutans* within a human saliva-derived in vitro oral multispecies community has been developed [54]. Interestingly, *S. mutans* elimination provoked a shift in the overall community composition toward one compatible with health [54]. Targeted approaches against pathogens associated with periodontitis are also under development. For instance, recent work described that *P. gingivalis* relies on a hemophore-like protein (HusA) to bind extracellular heme, its preferred iron source, under conditions of low iron availability such as when *P. gingivalis* invades epithelial cells [55, 56]. HusA homologues are limited to the phylum *Bacteroidetes*, and highly homologous sequences are only present in the genus *Porphyromonas* [55]. Apart from binding hemin, HusA is able to bind to deuteroporphyrin IX (DPIX), with significantly higher affinity than to heme. The taxonomic specificity of HusA and its binding properties has been exploited to design a deuteroporphyrin-lysine-metronidazole antibiotic, which effectively binds HusA and kills *P. gingivalis* with greater effectiveness than metronidazole alone. The antibiotic was particularly effective under iron-limited conditions in which HusA is upregulated [55]. Further experiments to test the antibiotic in mixed biofilms are required to assess its specificity. This work, however, represents an example of how a better understanding of the molecular physiology of oral pathogens can lead to the design of targeted strategies to promote their eradication.

13.8 Conclusions

Our current understanding of the etiology of periodontitis points to dysbiosis of the subgingival microbiome as a triggering factor and also a consequence of the disease process. Factors that con-

tribute to the emergence and perpetuation of dysbiosis include interspecies interactions, changes in the environment due to inflammation, and dysregulation of the host response by keystone pathogens. Apart from several emerging strategies to control inflammation, which could also result in restoration of a health-like microbiome community, other approaches to prevent dysbiosis or restore a community compatible with health are under investigation. These include probiotics, prebiotics, use of indigenous beneficial species as adjuncts to periodontal treatment, and the design of antibiotics targeted to pathogens. With the exception of probiotics, these strategies have only been tested in vitro or in animal models. Further research is required to develop approaches to manipulate subgingival communities and restore a health-like community, which is an outcome current therapies do not achieve.

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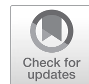
Part III

Tissue Engineering for Periodontal Regeneration



Protein- and Cell-Based Therapies for Periodontal Regeneration

14

Zhao Lin, Young-Dan Cho, Frederic Kauffmann, Lea Sarment, Jill Beitz, Darnell Kaigler, and William V. Giannobile

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An important therapeutic endpoint of periodontal therapy is the complete structural and functional reconstruction of the affected periodontal apparatus. Histologically, this includes the formation of new periodontal ligament (PDL), cementum, and alveolar bone [1]. Clinically, it is indicated by pocket reduction and radiographic bone fill of the defect with the presence of PDL space [1]. Conventional nonsurgical (scaling and root planing) and surgical (open flap debridement (OFD) and osseous surgery) periodontal therapies have established the effectiveness of arresting the disease progression and resuming the periodontal ecosystem to some extent [2–7]. However, such therapies often heal by tissue repair (so-called scaring), which is characterized by connective tissue attachment and the formation of long junctional epithelium on the root surface without the regeneration of the cementum-PDL-bone complex [8–12].

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14.1 The Biology of Periodontal Wound Healing

Similar to wound healing in other tissues in the body, periodontal tissue healing includes four major phases: hemostasis (formation of blood clot), inflammation, proliferation and new tissue formation, and remodeling [13–17]. Injuries to the blood vessels during periodontal procedures cause

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blood extravasation. Within minutes, a fibrin-rich clot attached to the root surface is developed by blood coagulation [18, 19]. The clot consists of red blood cells and platelets in a network of cross-linked fibrin fibers along with plasma fibronectin, vitronectin, and thrombospondin [14–17]. It has been shown that the linkage of fibrin fibers to the root surface and their stability are key factors for new connective tissue attachment as the fibrin network serves as a provisional matrix for cell migration [20]. Another important function of the blood clot is that it is a reservoir of growth factors and cytokines. Degranulated platelets release a series of growth factors including platelet-derived growth factor (PDGF), transforming growth factor-β1 (TGF-β 1), vascular endothelial growth factor (VEGF), interleukin-1β (IL-1β), etc., which provide the start signals for a cascade of cellular events associated with wound healing [21]. Within hours, a large number of inflammatory cells, predominantly neutrophils and monocytes, migrate to the wound edge and root surface [16, 18, 19]. Neutrophils clean the wound by removing foreign particles, necrotic tissue debris, and bacteria. Cytokines released by these early inflammatory cells further amplify the healing signals and more inflammatory cells are recruited. Within 3 days, the wound healing is dominated by the late phase of inflammation as macrophages infiltrate and granulation tissue starts to form [16]. Macrophages continue to phagocytize bacteria and debris from cells and extracellular matrix. It is worth noting that tissue degradation enzymes and toxic products are often released when inflammatory cells clean the wound site. Therefore, prolonged inflammation, which may be caused by persistent bacterial infection, can lead to healthy tissue destruction. As the healing progresses, a subpopulation of macrophages and other cells produce cytokines that are critical for inflammation resolution and tissue regeneration [22, 23]. Fibroblasts, mesenchymal stem cells, pericytes, etc. are recruited into the wound area. These cells proliferate and differentiate to different cell types in periodontium. Within a week, connective tissue attachments can be seen at the root surface. The newly formed bone is usually woven bone, which is less organized and mechanically weak [13–15]. Over time, the newly formed periodontal tissues will be remodeled to form stronger attachments with denser, well-organized collagen fibers and lamellar bone to meet the needs of occlusal loading [13–15]. This happens within weeks to months, and sometimes even years.

In 1976, Melcher presented the concept of “compartmentalization” and divided the periodontal structures into four compartments: the lamina propria of the gingiva, the periodontal ligament, the cementum, and the alveolar bone [24]. It is believed that to regenerate the cementum-PDL-bone complex, cells from those three compartments should occupy the periodontal defect, but not the cells from gingiva. Guided tissue regeneration (GTR) procedures have been developed based on this concept, and a barrier membrane was used to exclude gingival tissues and prevent the epithelial downgrowth [25–27]. A large body of literature supports the effectiveness of GTR in regenerating periodontia [1, 28]; however, increasing evidence suggests that periodontal regeneration can also occur without the exclusion of gingival epithelium and connective tissues. Some regeneration of bone- and the tooth-supporting structures after conventional therapies has been documented in isolated reports [29–31]. With the emergence of protein and peptide products, a large amount of bone fill is often seen in patients that received protein-/peptide-based periodontal therapies, which were often performed without a barrier membrane [32–38]. True periodontal regeneration after such therapies has also been reported by human histological studies [39–46]. Therefore, it is reasonable to think that during the healing process, cells from different origins (e.g., PDL, bone marrow, alveolar bone surface, cementum, gingiva) penetrate into the wound area. Many of these cells may have the potential to differentiate into different tissues. Appropriate signals from the microenvironment can drive the differentiation/transdifferentiation of local cells and induce the self-organization of these cells to form the cementum-PDL-bone complex with proper blood supply and innervation. Identification of the signaling molecules that control the tissue proliferation and differentiation may promote the regenerative potential of periodontium.

Collectively, periodontal regeneration is a complex process with a cascade of cellular and

molecular events. In the past decades, research has been focused on the understanding of this process, and different techniques have been developed to promote the tissue regeneration through targeting at different steps/phases of the healing. Some of the various regenerative techniques include GTR (epithelial exclusion), minimally invasive surgery (blood clot stabilization), protein/peptide therapy (enhancing cell migration, proliferation, and differentiation), cell therapy (increasing the number of stem cells in the microenvironment), and anabolic agents (improving the bone formation and remodeling), to name a few. In this chapter, we will highlight some of the major developments in the protein-/peptide-based and cell-based periodontal regenerative therapies.

14.2 Protein- and Peptide-Based Periodontal Regenerative Therapies

Over the last 20 years, significant advances have been made in using biological proteins and peptides to treat periodontal osseous defects [47, 48]. To date, three products are commercially available: enamel matrix derivative (EMD), recombinant human platelet-derived growth factor-BB (rhPDGF-BB), and P-15 peptide. Molecules in the R&D pipeline include recombinant human fibroblast growth factor-2 (rhFGF2), recombinant human growth and differentiation factor-5 (GDF-5), bone morphogenetic proteins (BMPs), teriparatide, and sclerostin neutralizing antibodies among others. A summary of these proteins and peptides is shown in Table 14.1.

Table 14.1 Summary of peptides/growth factors for periodontal regeneration

Growth factors/ peptides	Development stage	Biologic function
Emdogain	FDA approved	Peptide mixture from immature enamel layer of 6-month-old piglets. Enhances cell adhesion and stimulates cell proliferation, angiogenesis, osteogenesis, cementogenesis, and ECM synthesis
P-15/ABM	FDA approved	A polypeptide consisting of 15 amino acids that mimics the cell-binding domains of type I collagen. Enhances cell adhesion. It was also approved by the FDA for orthopedic application
rhPDGF-BB	FDA approved	Major factor released from platelets during blood clotting. Strong chemotactic and mitogenic effects on inflammatory cells and mesenchymal cells. Enhances angiogenesis
rhFGF-2	Phase II/III clinical trial	A heparin-binding growth factor. Stimulates fibroblasts proliferation and ECM synthesis, increases chemotaxis, proliferation, and differentiation of endothelial cells
rhGDF-5	Phase II clinical	A member of the bone morphogenetic protein family. Promotes cell proliferation, increases chemotaxis of osteoblast progenitor, and enhances osteoblast differentiation
rhBMP-2	Preclinical	A potent growth factor for bone formation and the first growth factor approved by the FDA for bone regeneration. Associated with root resorption and ankylosis in periodontal regenerative model in large animals
OP-1 (BMP-7)	Preclinical	A limited FDA approval under a humanitarian device exemption for treatment of recalcitrant tibial nonunions. Increases mitogenesis and differentiation of osteoblasts. Enhances periodontal regeneration in a dog model without root ankylosis
rhBMP-6	Preclinical	Enhanced formation of new bone, cementum, and functionally oriented PDLs was observed in murine
rhBMP-12	Preclinical	Induces expression of tendon and ligament specific genes, stimulated periodontal regeneration; however, it has limited effect on osteogenesis
rhBDNF	Preclinical	A member of the neurotrophin family. Stimulates osteogenesis and angiogenesis
Teriparatide	Clinical trial	N-terminus 34 amino acids of PTH. Anabolic agent; stimulates bone formation. Improved periodontal healing after systemic teriparatide administration compared to OFD
Sclerostin Ab	Preclinical	Neutralizing antibody for Sclerostin—a negative regulator in Wnt signaling. Anabolic agent; promotes bone formation, increases bone mass, and enhanced the regeneration of periodontal tissues in murine models

14.2.1 Enamel Matrix Derivative (EMD)

EMD is made of a mixed protein/peptide fraction derived from the immature enamel layer of 6-month-old piglets and delivered in a propylene glycol alginate (PGA) carrier [49]. Early reports showed that enamel-derived proteins are deposited at the dentinocemental junction (DCJ) and the surface of developing roots prior to cementum formation, suggesting that they may play an important role in the formation of periodontal tissues [50–53]. This led to a series of studies to investigate the potential of EMD in treating periodontal defects [54–58]. The majority of EMD (>90%) is different versions of amelogenin protein derived from different splice variants and post-secretory regulation. The function of EMD is known to influence the behavior of many cell types (i.e., cementoblasts, PDL cells, osteoblasts, MSCs, T cells) by mediating cell migration, attachment, spreading, proliferation, and differentiation [49, 59, 60]. Studies showed that EMD also inhibits osteoclast formation and stimulates angiogenesis. More than 30 randomized controlled trials (RCTs) and several meta-analyses and systematic reviews have been published reporting the clinical outcomes following application of EMD in the treatment of intrabony defects, including recent position papers published by the AAP Periodontal Regenerative Workshop [28, 47, 49]. Evidence supports the efficacy of EMD as a single therapeutic agent in treating periodontal infrabony defects. The use of EMD resulted in a significant gain in clinical attachment level (CAL), reduction in periodontal probing depth, and improvement in radiographic bone level when compared to OFD, EDTA, or placebo. Human histological studies also indicate a true regeneration of the periodontal apparatus. However, the impact of EMD in combination therapies is still not clear. Meta-analysis showed that there was no significant difference when comparing the outcome of EMD only to EMD + barrier membrane or EMD + bone graft. Furthermore, no superior effect was seen in EMD treatment compared to other treatment modalities, such as bone grafting and GTR. Recently, EMD has also been used to treat gingival recession [61]. In summary, clinical use of EMD alone or combined with bone graft materials is safe and can provide long-term clinical outcomes comparable to other regenerative treatments (GTR and bone grafting). Figure 14.1 shows a clinical case using EMD to treat an infrabony defect.

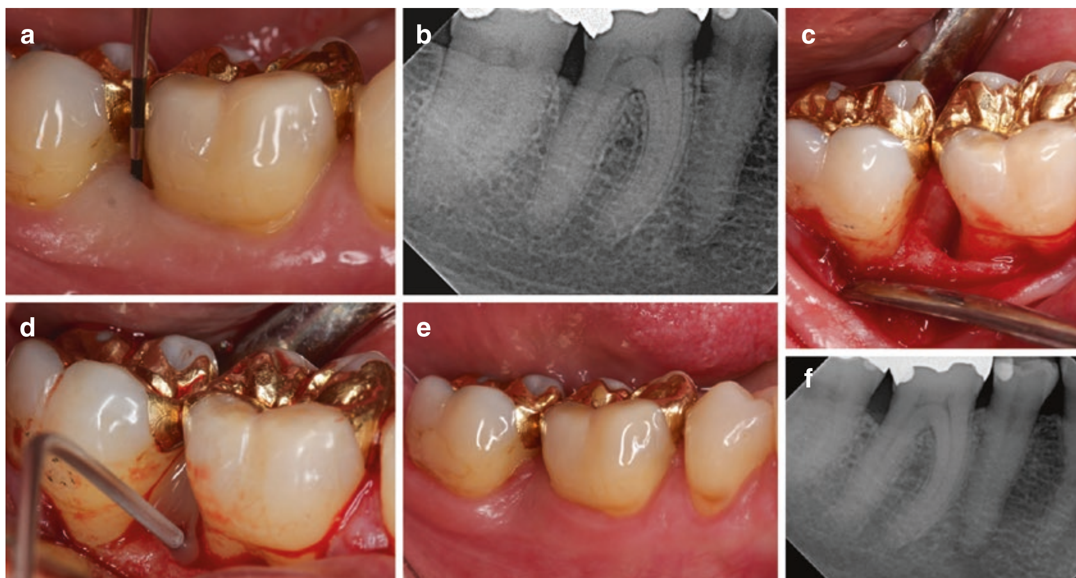

Fig. 14.1 A clinical case treated by enamel matrix derivative (EMD). (**a**) Baseline probing. (**b**) Baseline radiography. (**c**) Infrabony defect shown during the surgery. (**d**) The application of EMD. (**e**) Clinical view at 3 years later. (**f**) Radiographic view 3 years postoperation. (This case was contributed by Dr. Frederic Kauffmann)

14.2.2 Platelet-Derived Growth Factor-BB (PDGF-BB)

PDGF-BB is one of the major factors released from platelets during blood clotting [62]. It is a potent mitogen for cells of mesenchymal origin, which exerts broad wound-healing activities through enhancing cell migration, proliferation, and angiogenesis [63]. Early studies in large animals suggested that PDGF-BB stimulated periodontal hard and soft tissue regeneration [64–66]. This was later confirmed by five RCTs [34, 37, 67–69]. In the first multicenter phase III RCT (60 patients/group), application of rhPDGF-BB with β-tricalcium phosphate (β-TCP) carrier resulted in significantly greater CAL gain, linear bone gain, and percent defect fill at 3 months and 24 months compared to OFD + vehicle control [37]. Using a composite analysis, higher rates of clinical and radiographic success, defined as the percentage of cases with CAL ≥ 2.7 mm and linear bone growth ≥1.1 mm, continued to be seen over 36 months. Interestingly, the low-dose rhPDGF-BB (0.3 mg/mL) demonstrated a stronger effect than high dose (1 mg/mL) [70]. In a report of a subset of the study population who had good compliance, the periodontal tissues were stable with proper physiological function even after 5 years [71]. The second multicenter RCT (27 patients/group) confirmed the significant improvements in CAL gain, linear bone growth, and probing depth after rhPDGF-BB treatment [34]. Results from three more recent RCTs with relative smaller patient numbers were also in line with the aforementioned findings [67–69]. Meta-analysis of these five RCTs demonstrated statistically significant improvement in all the primary outcomes (bone fill and linear bone gain) and secondary measures (CAL gain and probing depth reduction) when comparing the 0.3 mg/mL rhPDGF-BB treatment to control [72]. Histologically, periodontal bony defects treated with rhPDGF-BB with bone allograft showed formation of new bone, cementum, and functionally oriented PDL fibers, which indicated a true periodontal regeneration [39, 41, 43]. In contrast, β-TCP treatment alone healed only by fibrous connective tissue repair and a long-junctional epithelial attachment. So far, there are no studies to compare the outcome of rhPDGF-BB to other regenerative treatments. Taken together, rhPDGF-BB demonstrates an adequate safety profile in treating periodontal defects and a comparable regenerative impact compared to other techniques currently being used. It is worth mentioning that, compared to EMD, there is a consistency of the concentration of recombinant protein applied to each patient, which may produce more consistent clinical results. Figure 14.2 shows a clinical case using rhPDGF-BB to treat an infrabony defect.

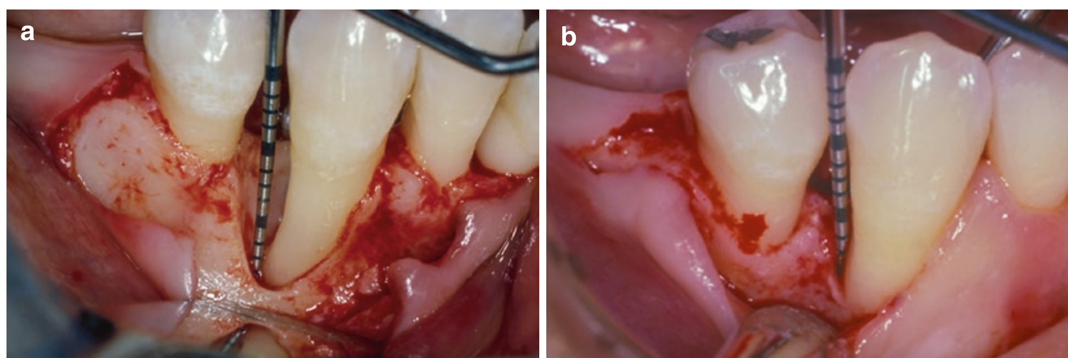

Fig. 14.2 A patient with a severe localized bony defect was treated by rhPDGF-BB. The baseline defect (**a**), the 1-year reentry (**b**), the baseline radiograph (**c**), the 3-year postoperative radiograph (**d**), and the 10-year postoperative radiograph (**e**) and clinical photograph (**f**), demonstrating periodontal repair and stability of the result. (Reprint with permission [70])

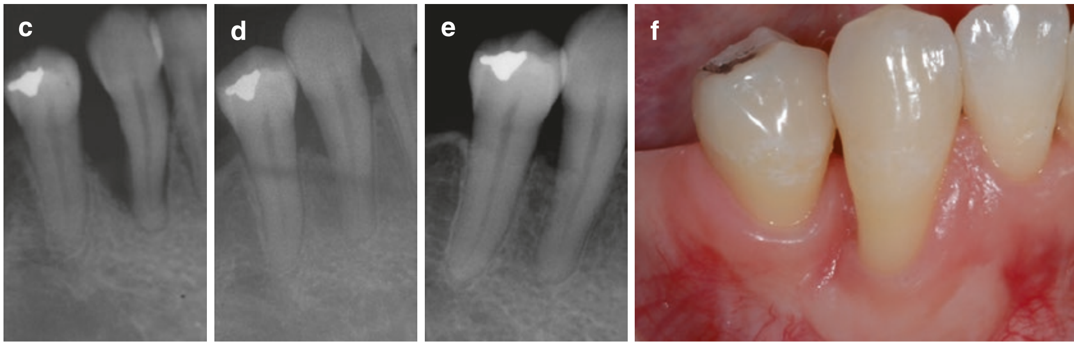

Fig. 14.2 (continued)

14.2.3 Fibroblast Growth Factor-2 (FGF-2)

FGF-2 is a heparin-binding growth factor that acts in a variety of developmental and healing processes [73]. It exerts proliferative and antiapoptotic effects on cells, including osteoblasts, and is a potent stimulator of angiogenesis [21, 73]. Local application of rhFGF-2 improved the bone union in tibial fracture in a randomized, placebo-controlled trial [74]. Regarding periodontal regeneration, five RCTs have been conducted (four in Japan and one in the USA) [32, 35, 36, 75]. Early trials in Japanese patients with a large patient number showed that high doses (0.3 and 0.4% in 3% hydroxypropylcellulose carrier) of rhFGF-2 resulted in a significantly greater percentage of bone fill (BF%) than OFD + vehicle alone at 36 weeks after administration [35, 36, 75]. In a recent multicenter RCT performed in the USA, a similar trend was observed although the difference was not statistically significant, which may be related to a smaller patient number ($n = 21–23$/group) in this study [32]. Meta-analysis based on the data from available RCTs found that a statistically significant difference was seen at the BF% in the 0.3% rhFGF-2 group compared to control group (~22.37% higher in rhFGF-2 group, $p < 0.00001$), as well as the linear bone gain. However, no significant difference was observed in CAL gain [72]. Interestingly, the study B of the phase III trials by the Japanese group was designed to compare rhFGF-2 and EMD [75]. The primary endpoint, which was linear alveolar bone gain at 36 weeks, was 1.927 mm (95% CI, 1.6615–2.1920; $n = 108$) in the rhFGF-2 group and 1.359 mm (95% CI, 1.0683–1.6495; $n = 109$) in the EMD group. The superior effect of rhFGF-2 was statistically significant. Therefore, rhFGF-2 can be another protein-based regenerative therapy for periodontal defects, which is safe and capable of producing comparable clinical outcomes. Figure 14.3 shows a clinical case using rhFGF-2 to treat a furcation defect.

14.2.4 P-15

P-15 is a polypeptide consisting of 15 amino acids that mimics the cell-binding domains of type I collagen, which facilitates cell attachment and migration to root surfaces through the interaction with integrins [76]. P-15 mixed with bovine-derived hydroxylapatite (anorganic bone matrix or ABM) is commercially available. It was approved by the US Food and Drug Administration (FDA) in 2015 as a bone graft in anterior cervical discectomy and fusion (ACDF) procedures with a brand name of “i-Factor.” In the area of periodontal regeneration, Yukna et al. reported that the combination of ABM and P-15 resulted in a significantly better mean defect fill of 2.9 ± 1.2 mm (72.9%) versus 2.2 ± 1.4 mm (50.67%) for ABM alone [38]. Others reported similar findings and the clinical results of ABM/P-15 were stable for at least 3 years [77–81]. In an earlier clinical trial, ABM/P-15 demonstrated a superior effect than demineralized freeze-dried

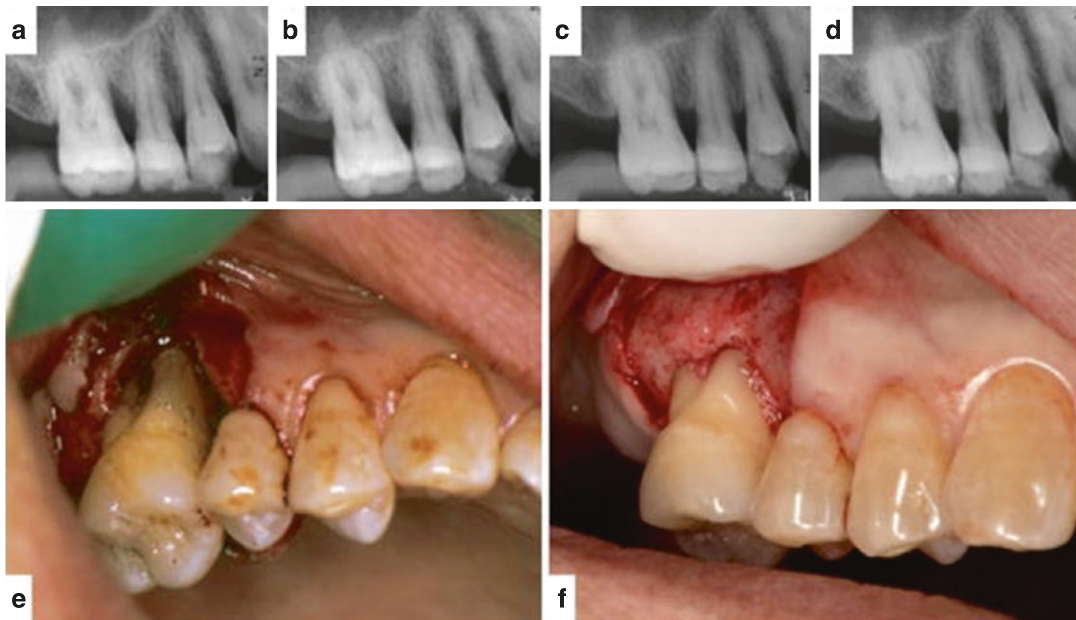

Fig. 14.3 A clinical case treated by rhFGF-2. A 52-year-old female nonsmoker. Radiographic and clinical evidence of new bone formation for tooth #3 suggesting periodontal regeneration after 9 months of local delivery of 0.3% rhFGF-2/cellulose into the furcation and vertical periodontal defect. The baseline radiograph shows vertical bone loss on the mesial side and the furcation area (case courtesy of SM). (**a**) Through (**d**) standardized periapical radiographs before surgery (**a**) and at the 3-month (**b**), 6-month (**c**), and 9-month (**d**) reevaluations. (**e**, **f**) The advanced furcation lesion observed at baseline (**e**) contrasted with the evidence of bone fill at clinical reentry (**f**). (Reprint with permission [48])

bone allograft (DFDBA) [82]. One human histologic evaluation showed evidence of new cementum, PDL, and bone after ABM/P-15 treatment, suggesting periodontal regeneration [46]. The use of xenograft as a carrier raises the question as to whether the results could be due to the osteoconductive/osteo-inductive effects or the long degradation time of the matrix itself. However, it is worth of note that the ABM used as a P-15 carrier is derived from thermally treated bovine bone at a much higher temperature (>1000 °C) than that which is used in some other xenografts (300 °C) [83]. The higher processing temperature used in ABM is believed to destroy the normal bone structure, limit any osteo-inductive effect, and accelerate the graft material degradation, which makes it a reasonable carrier in the studies. In summary, ABM/P-15 is a promising agent in treating periodontal infrabony defects; however, additional studies with larger patient numbers are needed to further assess its clinical value.

14.2.5 Platelet-Rich Plasma/Platelet-Rich Fibrin (PRP/PRF)

PRP, PRF, and other related products have been tested for periodontal regeneration. Platelets are one of the major cell types in blood clots and serve as a natural reservoir for growth factors, which is very appealing for regenerative medicine [84]. Depending on the processing technique, platelet concentrates can be divided into two major groups: platelet-rich plasma (PRP, with anticoagulants) and platelet-rich fibrin (PRF, without anticoagulants) [84, 85]. Techniques have also been developed to enrich more monocytes/buffy coat cells into the concentrate, including leukocyte- and platelet-rich plasma (L-PRP) and leukocyte- and platelet-rich fibrin (L-PRF) [84, 85]. Although PRP is considered an economical source for growth factors, inconclusive clinical results were found in a meta-analysis. Roselló-Camps et al. reported that PRP treatment

favored the probing pocket depth reduction; however, the difference did not reach statistical significance ($P = 0.09$) [86]. For attachment level (AL) changes, 12 articles were included. The weighted mean difference was 0.58 mm favoring the PRP, with a 95% CI = 0.24–0.91 mm ($P = 0.0008$). A significant change was also seen in bone level; however, this parameter was only reported by four studies (two measured BL in millimeters and two measured in percentage). In regard to PRF, a recent meta-analysis evaluated its effect on infrabony and furcation defects [87]. Significant probing depth reduction (1.1 vs. 0.5 mm, $p < 0.001$), CAL gain (1.2 vs. 0.6 mm, $p < 0.001$), and bone fill (1.7 vs. 0.7 mm, $p < 0.001$) were found when comparing L-PRF to OFD. A similar effect was also seen in furcation defects. Studies have also been done to compare PRF to other grafting materials or biologics; however, the conclusion is still not clear [88–91]. Taken together, at this moment, platelet-derived concentrates have not shown sufficient evidence to promote periodontal regeneration. It is worth mentioning, however, that standardization of the protocols to generate these platelet concentrates are needed to assure an optimal effect. The components of PRP/PRF are highly dependent on the donor. More studies are also needed to further determine more comprehensively the key components of the concentrates.

14.2.6 Bone Morphogenetic Proteins (BMPs) and Others

Other growth factors have also been studied as local delivery agents to promote periodontal regeneration, but most of these studies are still in the preclinical (animal) level. Here, we highlight some of the promising candidates that have been tested in large animals or early clinical trials. GDF-5, a member of the bone morphogenetic protein family, plays important roles in joint development and promotes the healing of ligament/tendon damage and pure bone defects [92–97]. It has chemotactic, mitogenic, and osteogenic effects to cells derived from PDL and bone tissues [98, 99]. Application of rhGDF-5 has been shown to stimulate the formation of new cementum-PDL-bone apparatus in dogs and nonhuman primates [100–102]. In an FDA phase IIa RCT, rhGDF-5 in a β-TCP carrier resulted in greater probing depth reduction and CAL gain; however, the differences didn't reach statistical significance, possibly due to the small patient number of the study ($n = 10$) [45]. Bone morphogenetic protein-2 (BMP-2) is profound growth factor for bone formation and is the first growth factor approved by the FDA for bone regeneration. Over the years, studies in murine, dog, and monkey models have also demonstrated its impact on the regeneration of periodontal tissue [103–105]. However, root resorption and ankylosis were frequently observed in teeth receiving BMP-2 treatment [106]. Bone morphogenetic protein-7 (BMP-7), also known as osteogenic protein-1 (OP-1), is a potent stimulator of bone formation. However, it seems that its function and mechanism may not be completely the same as BMP-2 [106]. Giannobile and others reported pronounced stimulation of osteogenesis, regeneration of cementum, and new attachment in dogs and nonhuman primates [106, 107]. Interestingly, no root ankylosis was found after BMP-7 treatment. Bone morphogenetic protein-6 (BMP-6) is another morphogen that is associated with bone formation. Enhanced formation of new bone, cementum, and functionally oriented PDLs was observed after the application of rhBMP-6 in a murine model [108]. Similar to BMP-7, no ankylosis was found and root resorption was limited. Bone morphogenetic protein-12 (BMP-12), also known as GDF-7, is an interesting member of the BMP family. Application of BMP-12 induces the differentiation of MSCs to tendon lineage, evident by the upregulation of tendon/ligament specific genes such as scleraxis (SCX) and tenascin C (TN-C), and promotes tendon regeneration [109–112]. Wikesjo et al. compared the impacts of rhBMP-12 and rhBMP-2 in periodontal regeneration in a dog model [113]. Functionally oriented PDL fibers bridging the gap between newly formed bone and cementum were found after receiving rhBMP-12, whereas this was a rare observation in the rhBMP-2 group. On the other hand, ankylosis was more frequently seen in the latter group. Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is important for the survival and

differentiation of central and peripheral neurons. In addition, it is also expressed in a variety of nonneural cells, including PDL cells and odontoblasts [114]. It has been shown that BDNF delivery was able to promote angiogenesis and stimulate formation of periodontal supporting structures (newly formed cementum, PDL, and alveolar bone) in large animal models [114, 115].

14.2.7 Anabolic Agents (Sclerostin Neutralizing Antibody and Teriparatide)

Sclerostin antibody and teriparatide have been representative approaches to increase bone formation ("anabolic" therapy) in the treatment of osteoporosis [116–124] and received attention as promising protein drug candidates in alveolar bone regeneration. Given that both are related to the Wnt/β-catenin pathway, which has a critical role in the osteogenesis and pathogenesis of bone-related diseases, a lot of research interest has moved toward activation or inhibition of this pathway to modulate bone formation [125].

14.2.7.1 Sclerostin Antibody

The use of neutralizing antibodies against sclerostin can aid in stimulating bone formation [126]. The SOST gene encodes the osteocyte-secreted sclerostin glycoprotein, which belongs to a family of genes that antagonize BMP or Wnt activity, both critical for bone formation [127, 128]. Wnt signaling is key for many pathways regulating cell growth, differentiation, function, and death [129]. Sclerostin has been shown to bind to LRP5/6 and inhibit Wnt signaling, impairing osteoblast differentiation and function [130, 131]. The sclerostin antibody binds and inhibits sclerostin, resulting in the dual effect of increased bone formation and decreased bone resorption [121]. Systemic application of Scl-Ab has been shown to significantly increase the osseointegration of implant in a rat model [132]. In addition, there were evident differences in the trabecular bone architecture in the femur of the tested animals compared to that of the control rats. Similarly, Scl-Ab was tested for bone regeneration in experimental periodontitis and to promote dental implant osseointegration [133]. Systemic injection of Scl-Ab dramatically increased the alveolar bone mass and serum bone formation markers at both 2 and 4 weeks in rats. More importantly, 6 weeks of Scl-Ab significantly improved the maxillary bone healing and restored the alveolar bone mass following experimental periodontitis, which provided early preclinical evidence to support the therapeutic potential of Scl-Ab in periodontal disease treatment [134].

14.2.7.2 Teriparatide

Teriparatide is a biosynthetic recombinant protein of human parathyroid hormone (PTH) consisting of N-terminus 34 amino acids, which acts as an anabolic agent in bone formation [135]. In 2002, the FDA approved an indication for teriparatide injection (*Forteo*, Eli Lilly and Company, Inc.) for the treatment of osteoporosis, and occasionally it has been used off-label to promote bone healing. PTH is a primary regulator of calcium maintenance in the bone and kidneys, and the PTH-induced anabolism is also involved in Wnt signaling pathway [136]. When given by intermittent injection, teriparatide binds directly to osteoblasts and exhibits anabolic effects, which include the increase of osteoblast number and activity and a decrease of osteoblast apoptosis [117]. However, continuous treatment could result in hypercalcemia and abnormal bone histology [137]. In the dental application, some preclinical studies showed an anabolic effect of teriparatide in alveolar bone defects [138, 139]. In a RCT, 40 patients with severe chronic periodontitis who underwent periodontal surgery were randomly assigned to two treatment groups: daily injections of teriparatide (20 μg) or placebo for 6 weeks. Improved clinical outcomes (probing depth reduction and CAL gain), greater resolution of alveolar bone defects, and accelerated osseous wound healing in the oral cavity were observed after systemic teriparatide administration as compared to placebo, suggesting that teriparatide is a promising drug candidate to improve periodontal tissue regeneration and oral reconstruction [140, 141]. Ongoing research is focused on developing a better drug delivery system to replace the current regimen, which requires daily subcutaneous injections in the thigh or abdomen.

14.2.8 Challenges and Future Directions of Protein-Based Therapies

Over the last few decades, there has been considerable progress in periodontal tissue regeneration. With a better understanding of the development and healing of periodontal tissues, biomimetic products, as well as an array of growth factors, have been or are being developed to enhance the periodontal regeneration. Increasing clinical data from RCTs and case series supports the application of such products, and more clinicians have adapted their clinical practice in treating periodontal defects to reflect the exciting advances in this field. It is worth noting that studies to compare different factors are still largely lacking. Clinicians usually choose these products based on their training and clinical experiences. A major concern for the growth factor/peptide-based therapy is the supraphysiological doses that are needed. For example, the concentration of PDGF-BB in the commercially available product is a few thousand times higher than that in our bodies. The clinical dose for BMP-2 to stimulate bone regeneration is a millionfold higher than the biological concentration [142]. Therefore, increasing side effects may be seen if a large dose of growth factor is used, which has been reported in the BMP-related products [142]. It would be important for clinicians to know the different mechanisms of these growth factors and their potential side effects, which may provide some guidance for them to choose different products in different clinical situations. Future development of local delivery systems that can control the spatial and temporal release of growth factors would make it easier to apply such biologics and, more importantly, enhance the regenerative outcomes and reduce the side effects.

14.3 Cell Therapy in Craniofacial and Periodontal Regeneration

Among the main factors of the tissue engineering triad (cells, scaffold, and signals), cells provide the viability needed to generate a stable, living tissue. Cell therapy, defined as a treatment with cellular materials, is considered an emerging therapy with high therapeutic potential [143]. Historically, primary cells from the patient (i.e., liver cells, bone cells from autogenous graft, etc.) were used to produce target tissues. However, invasive methods are usually needed to collect the cells and the sources of primary cells can be very limited, which limits the clinical application of such a strategy. To solve these problems, stem cells, including embryonic stem (ES) cells, postnatal stem cells, and adult stem cells, have received great attention because of their capacities to replicate themselves (they can be grown to large numbers) and differentiate into other cell types (making it possible to replace any damaged cell and tissue) [144]. In addition, increasing evidence supports that stem cells, especially mesenchymal stem cells (MSCs), are a reservoir for a variety of growth factors, cytokines, and microvesicles, which could have profound effects on wound healing. In fact, it is believed that such paracrine or trophic effect, rather than direct differentiation, is the primary mechanism of the regenerative outcomes associated with many stem cell therapies.

Except for hematopoietic stem cells, bone marrow mesenchymal stem cells (BM-MSCs) are the most studied stem cell population in cell therapy. Specifically, in stem cell-mediated bone regeneration, they have historically been a popular cell source to use because they are derived from the bone and are easy to harvest. However, the number of BM-MSCs in peripheral blood and bone marrow is relatively small, and it is difficult to expand them to a number large enough for clinical effectiveness. With the advancement of cell programming technologies that enable generation of pluripotent stem cells (iPSCs) from somatic cells, the idea of using mature somatic cells is coming back to the field [145]. As an example, iPSCs derived from dental tissues can be readily reprogrammed to periodontal cells because of their epigenetic status [146]. Additionally, a direct cell programming technology has been introduced to induce the trans-differentiation of somatic cells to a target cell type in order to overcome the limitations of iPSC-based therapy, namely, that it is labor-intensive and time-consuming, there is epi-

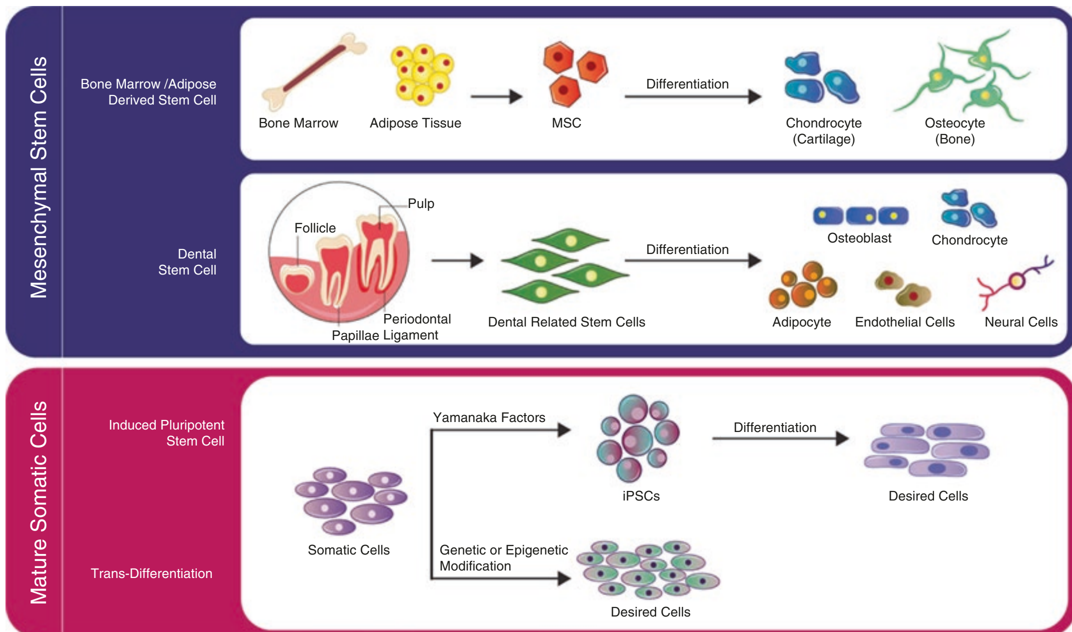

Fig. 14.4 Cell sources for cell therapy. Mesenchymal stem cells from the bone marrow, adipose, and dental tissues are being studied for their application in periodontal regeneration. Somatic cells can also been reprogrammed to iPSCs or directly to periodontal cells by trans-differentiation

genetic memory from the donor cells, and there is the potential of tumorigenesis associated with the stemness of the cells [147–149]. Here, we review the development of stem cell-based therapy in the area of periodontal and craniofacial regeneration (Fig. 14.4) and discuss the challenges and future directions toward the translation of cell therapy to clinical practice.

14.3.1 Types of Cells Used in Periodontal Tissue Regeneration

14.3.1.1 Bone Marrow-Derived Mesenchymal Stem Cells (BM-MSCs)

First identified by Friedenstein, BM-MSCs have a multi-potency to differentiate to various specialized cells [150]. They directly participate in tissue regeneration through cell differentiation but more importantly have an impact on wound healing through paracrine mechanisms [151]. As such, BM-MSCs have been widely explored as a potent cell source in tissue engineering [152].

14.3.1.2 Dental Stem Cells

Dental pulp stem cells (DPSCs). Dental pulp contains highly proliferative stem cells with a self-renewal and multi-potential property, which can differentiate into osteoblasts, odontoblasts, adipocytes, chondrocytes, or neural cells [153]. The fact that DPSCs have the ability to form a vasculature and differentiate into odontoblasts in response to tissue damage makes it a promising stem cell source in regenerative medicine, especially in dental pulp regeneration [154]. Preclinical and early clinical evaluations of cell therapy using DPSCs are ongoing and show early promise [155–157].

Periodontal ligament stem cells (PDLSCs). The periodontal ligament (PDL) is a specialized connective tissue which establishes the attachment between the tooth and alveolar bone [158]. A population of MSCs have been isolated from PDL tissues, named PDLSCs, which play a critical role in the development and healing of periodontium [159]. PDLSCs can differentiate into

multiple cell lineages including osteoblasts, chondrocytes, neurons, etc. and have the potential to form new cementum, PDL, blood vessels, and alveolar bone when implanted in vivo, indicating possible applications in bone- and tooth-associated tissue engineering [160–162].

Stem cells from human exfoliated deciduous tissue (SHED). Partially due to the young age of the donors, SHED are characterized as unique stem cells with a high proliferation rate and plasticity for multi-potent differentiation [163]. Many studies have demonstrated the multi-potency of SHED and their potential for dental tissue regeneration [164, 165].

Dental follicle stem cells (DFSCs). The dental follicle (DF) is a loose connective tissue sac that is derived from the unerupted tooth and plays an important role in tooth development [166, 167]. During tooth development, Hertwig's epithelial root sheath induces the differentiation of DFSCs into PDL fibroblast, osteoblasts, or cementoblasts [168, 169]. Because DFSCs can be easily harvested from extracted third molars, increased interest has been given to investigate the potential application of DFSCs in root/periodontal regeneration, and ex vivo model systems have been developed to further study these applications [170].

14.3.1.3 Induced Pluripotent Stem Cells (iPSCs)

iPSCs are pluripotent stem cells, which are generated from somatic cells by the ectopic expression of Yamanaka factors *Oct4*, *Sox2*, *Klf4*, and *Myc* [145]. iPSC-derived mesenchymal cells from dental tissue have been studied to compensate for a disadvantage of iPSCs, such as de-differentiation. Many studies have demonstrated the regenerative potential of iPSCs from dental-derived tissues such as dental pulp, PDL or gingival fibroblasts, apical papilla, and exfoliated deciduous teeth, to name a few [171].

14.3.1.4 Trans-differentiation

Trans-differentiation is a conversion from one mature somatic cell into another somatic cell via genetic or epigenetic modification without a pluripotent state [172]. It is known that trans-differentiation can be induced experimentally and also occur naturally in response to tissue damage [149]. The possibilities of trans-differentiation from adipocytes or fibroblasts to osteoblasts were proven in in vitro and in vivo studies [147, 173, 174]. In this regard, stimulating mature somatic cells to trans-differentiate to a desired cell type for a treatment purpose could be the next generation of cell therapy technology for tissue regeneration based on its variety of advantages over the use of iPSCs.

14.3.2 Clinical Studies with MSCs for Periodontal/Alveolar Bone Regeneration

In the past decade, stem cell therapy has been actively investigated in the field of craniofacial regeneration. According to a recent systematic review from May 2018, 47 controlled clinical studies have been published, including 22 RCTs [175]. Additionally, 30 uncontrolled studies and case series were reported. Most of these studies (74%) were designed to investigate the effect of stem cell implantation in sinus augmentation and/or ridge augmentation. Others tested the outcome of stem cell therapy in treating alveolar clefts and cranial defects. A small number of the studies used stem cells to reconstruct the defects associated with fractures or tumor treatment. Regarding the cell source, about half of the controlled studies harvested stem cells from iliac bone marrow aspiration. MSCs harvested from periosteum, maxillary tuberosity, and adipose tissues were popular too. Stem cells from dental pulp and PDL were also reported. In 19 out of 47 controlled studies, the isolated cells were not cultured before implantation, whereas others used different techniques to expand the cells in vitro before delivery to patients. Overall, despite the different cell sources, harvest techniques, culture conditions, expanding methods, cell seeding numbers, carriers, and clinical applications, stem cell therapy seems to be safe in all these studies without major adverse effects. Meta-analysis was able to be performed to determine the effect size of cell therapy. In sinus augmentation, higher-quality bone regeneration was observed after cell

implantation with carriers compared to carriers alone; however, this difference was rather small. Here, we highlight some studies using stem cells to treat alveolar defects and diseases.

Bone regeneration. The use of stem cells has a positive influence on wound-healing processes, accelerating tissue proliferation, differentiation, and maturation and reducing patient morbidity [176, 177]. Kaigler et al. isolated a group of MSCs and macrophage-like cells from the bone marrow (CD90+ and CD14+) and expanded them by a single-pass perfusion process [178]. These cells demonstrated a strong osteogenic and angiogenic potential in a pilot study. In a phase I/II feasibility RCT, the implantation of these ex vivo expanded BMSCs for sinus floor augmentation appeared safe [179]. Through clinical, radiographic, tomographic, and histologic analyses, stem cell therapy had a positive impact on alveolar bone healing, especially in the most severe defects (Fig. 14.5). Interestingly, it was determined that the regenerative outcomes correlated with the CD90+ cell population transplanted. Other studies with expanded cells or whole-tissue fractions also support the beneficial effect of stem cell transplantation in orofacial bone regeneration [180–183]. In severe atrophic alveolar ridges, it has been shown that stem cell delivery can be useful to rebuild the bone for implant-supported prostheses [180, 184, 185].

Periodontal regeneration. Although a number of clinical studies have evaluated the impact of stem cell delivery on bone formation, there are only a few clinical trials that have been done on periodontal regeneration. Yamada et al. completed a study to use autologous BMSCs from iliac crest to treat infrabony defects and furcations [164, 186]. The cells were expanded before periodontal surgeries and mixed with PRP gel. The average reduction in PD, gain in CAL, and radiographic bone gain were 5.12 ± 2.45, 4.29 ± 1.32, and 3.12 ± 1.23 mm, respectively. The treatment was safe in all the patients. Autologous PDL cells from extracted third molars were used by some groups and promising results were reported [187, 188]. It is interesting to mention that PDL cells have been incorporated in different scaffolds to regenerate the periodontal complex in various small and large animal models. By mixing the PDL cells, apical papilla stem cells, and a pre-made, root-shaped biphasic hydroxyapatite scaffold, Sonoyama et al. were able to generate a bioengineered tooth root (“bioroot”) [189]. In combination with the imaging-based, computer-aided design technique, we fabricated customized scaffold for periodontal defects. The implantation of stem cells in this scaffold induced the new formation of periodontal complex, evident by the insertion of defined, oriented fibrous fibers [190, 191].

Soft tissue regeneration/recession coverage. Soft tissue healing is clinically important in dentistry not only for cosmetic reasons but also to provide additional tissue volume to prevent further recession of gingiva and papillae. Harvesting autologous tissues for recession coverage is usually unpleasant for patients. Therefore, artificial soft tissue materials have been developed to replace autologous grafts. A bilayer tissue-engineered cell sheet (allogeneic cultured keratinocytes and foreskin fibroblasts) has been approved by the FDA to treat gingival recession. The cell layers produce an array of regenerative molecules, including cytokines and growth factors, to stimulate wound healing [192–195]. Stem cells can also be printed and grown into soft tissue-like material which can be used as soft tissue grafts [196]. Interestingly, injection of MSCs with a hyaluronic acid scaffold has shown to decrease the interproximal “black triangle” [197].

Dental pulp regeneration. Although root canal treatment has significantly extended the longevity of infected teeth, it is not a perfect solution due to the increased possibility of fracture and other complications. The potential to regenerate the pulp tissue holds many promises in the clinical setting, especially in children when the root is not yet mature [198]. Root revitalization procedures have been used with the concept that stem cells from the apical region “bleed” into the root canal and form a new pulp and blood vessels. Furthermore, promising results have been shown when stem cells are added into the root canal as part of the revitalization process [157, 199, 200].

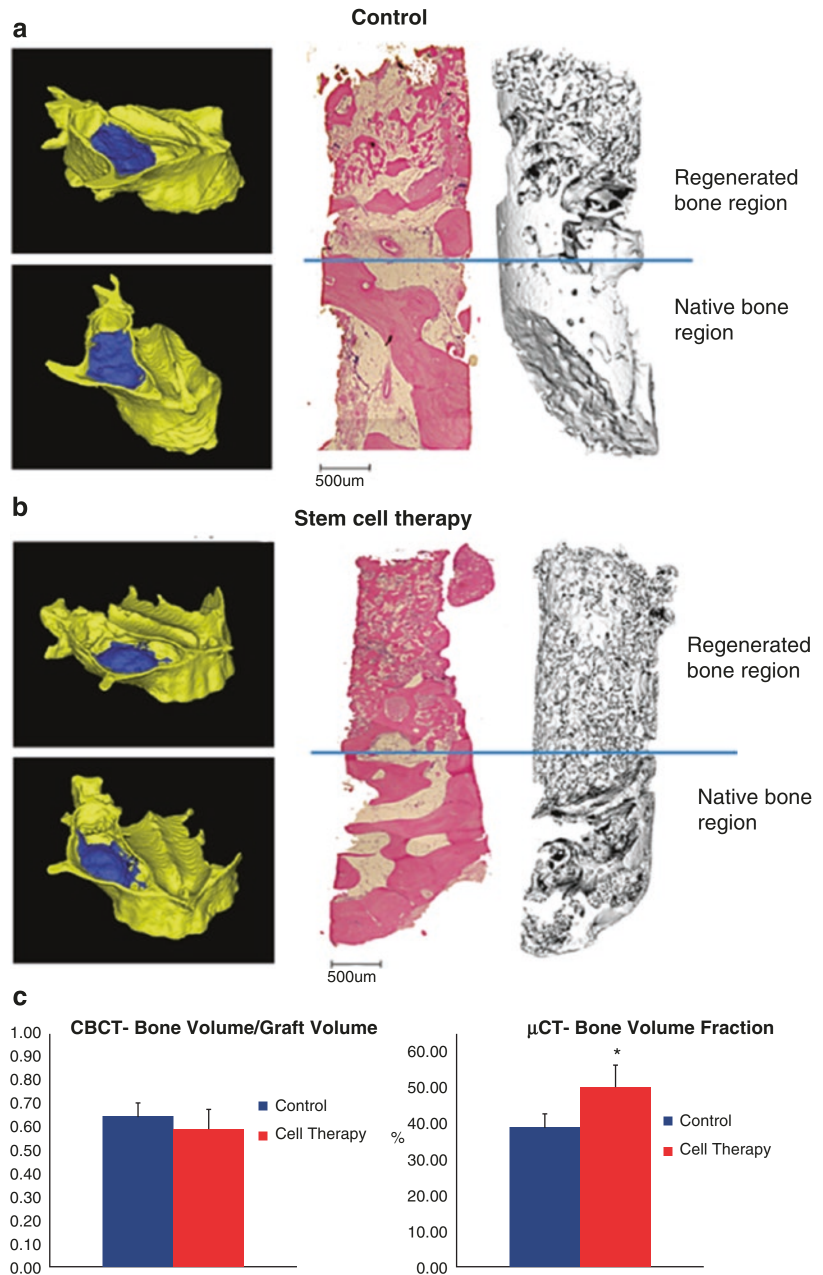

Fig. 14.5 Bone engineering of maxillary sinus bone deficiencies using enriched CD90+ stem cell therapy: a randomized clinical trial. Better bone quality with stem cell therapy in treating severe defects (>50% bone height deficiency). Representative images of 3D reconstructions of occlusal and lateral open views into the maxillary sinus cavity of the skull show the bone volume that was grafted (blue) in the control (**a**) and stem cell therapy (**b**) groups in severe bone defects. Histological and corresponding µCT images of bone biopsies harvested from the grafted regions of the two groups show a greater degree of mineralized bone tissue in the stem cell therapy group. (**c**) CBCT analysis of the bone volume/graft volume ratio was no different between the control and stem cell therapy groups in treating severe defects; µCT analyses of the bone biopsies revealed that compared with the control, BVF was significantly higher in the stem cell therapy group in treating severe defects. (Reprint with permission [179])

14.3.3 Future Direction for Cell Therapy in Periodontal Regenerative Medicine

Cell therapy continues to be an important research focus in periodontal and craniofacial regeneration, and increasing evidence from preclinical and clinical studies has supported their efficacy. Before cell therapy is clinically accepted for widespread use in practice, many challenges must be addressed, namely, identifying the best way to deliver the cells on carrier scaffolds depending on the target tissue of interest. Emerging technologies in personalized medicine, namely, computer-

aided scaffold design technology and 3D printing, now make it possible to fabricate customized scaffolds for each individual. Different materials (hydroxyapatite, calcium phosphate, polymers, collagen, etc.) and fabrication techniques have been evaluated to develop the optimal carrier to support cell attachment, viability, and differentiation of the stem cells [201, 202]. Another issue that the field needs to consider is the cost-benefit ratio of cell therapy, which is an important practical consideration for treating non-life-threatening (disabling) diseases, like periodontitis and bone loss. Current cell therapy requires a centralized cell culture facility, proper shipping conditions, and storage technology, which will be very expensive for the typical dental clinic setting. The next generation of cell therapy, which is based on the stem cell secretome, like conditioned media and exosomes, may hold great promise in their ability to mitigate the risks associated with cell transfer while still maintaining some key therapeutic benefits of cell therapy.

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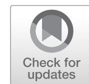

15 Lasers in Periodontal and Peri-implant Therapy: Challenges and Opportunities

M. A. Reynolds, M. E. Aichelmann-Reidy, and P. S. Rosen

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15.1 Introduction

Multiple nonsurgical and surgical approaches have been applied in the management and control of inflammatory periodontal and peri-implant diseases. The primary goal of treatment is to achieve periodontal and peri-implant health and to reduce risk of future disease recurrence and/or progression. A common clinical objective in the management of these inflammatory conditions is to reduce the burden of pathogenic bacteria and, presumably, risk for progressive inflammation and disease recurrence. Periodontal bacterial pathogens, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans*, exhibit strong associations with periodontitis [1]. Evidence suggest that the microflora of the oral cavity prior to implant placement determines the composition of the microflora in the peri-implant area [2]. Consistent with the foregoing premise is the observation that the primary bacterial species implicated in the pathogenesis of peri-implantitis are recognized as periodontal pathogens [3]. In a recent systematic review, Perez-Chaparro et al. [3] concluded that there is moderate evidence to support an association of

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P. gingivalis, *T. denticola*, and *T. forsythia* in the etiology of peri-implantitis. Additionally, there is some evidence to implicate *P. intermedia* and *Campylobacter rectus* in the etiology of peri-implantitis. Conventional active therapy has targeted the disruption and removal of dental plaque (biofilm) and calculus, root/implant surface decontamination and/or modification (e.g., root planing), with often a concomitant goal of reducing pocket depth. The management of contributing etiologic factors, such as cigarette smoking, inclusion of a conscientious regimen of daily oral hygiene, and regular professional supportive periodontal maintenance remain essential [4]. Surgical treatment approaches are commonly applied to manage moderate and advanced periodontitis and peri-implantitis, often with the objective of achieving either periodontal or peri-implant bone regeneration, respectively [5]. Longitudinal studies document the stability of clinical outcomes achieved with regenerative periodontal therapy; however, available data on long-term (>5 years) outcomes are insufficient to meaningfully compare clinical improvements and tooth or implant survival following various treatment approaches. Nevertheless, several factors consistently appear to increase risk of tooth or implant loss, including uncontrolled diabetes and pretreatment severity of bone loss [6, 7]. Longitudinal studies suggest that the particular periodontal therapy is less important than thorough debridement of the diseased area, frequent professional care, and excellent oral hygiene practices by the patient [8]. Moreover, compliance with regular professional supportive care is associated with improved tooth retention [6, 9, 10] and implant survival [7, 11].

In general, the treatment of periodontal and peri-implant diseases targets the detoxification of root/implant surfaces alone or in combination with tissue regeneration and/or the elimination of periodontal/peri-implant pocketing as well as establishment of effective patient plaque-control regimens and regular professional supportive care [12]. Conventional surgical approaches, such as open flap debridement, provide critical access to evaluate and detoxify root and implant surfaces as well as establish improved periodontal form and architecture; however, these surgical techniques alone offer only limited potential in restoring or reconstituting bone or component periodontal tissues. Moreover, traditional therapeutic approaches are hampered by clinician-, patient-, and site-related factors. These factors include, among others, a clinician's surgical skills, patient habits such as smoking, defect configuration, and clinical access for effective debridement and disinfection.

The introduction of LASER (light amplification by stimulated emission of radiation) technology has ushered in new therapeutic strategies and approaches to the treatment of inflammatory periodontal and peri-implant diseases [13]. Laser radiation (beam) is characterized by high directionality (collimation), coherence (photons are emitted in-phase), monochromaticity (narrow spectral width), and intensity (brilliancy). When laser light strikes a tissue surface, it can be reflected and refracted, scattered, absorbed, or transmitted. The fractional intensity that goes into these different processes depends on the optical properties of the tissue as well as the laser parameters, such as wavelength, energy, and pulse duration. The wavelength of light is the primary parameter determining the extent of energy absorption by a target tissue (Fig. 15.1). Each wavelength of laser energy exhibits unique absorption characteristics by cellular chromophores—including keratin, melanin, collagen, lipids, and certain proteins, among others—hemoglobin, oxyhemoglobin, and water. Laser energy is similarly absorbed by bacterial chromophores. The laser wavelength defines the mechanism of interaction, depth of penetration (Fig. 15.2), and absorption of photon energy which can include photothermal (i.e., heating), photodynamic (mediated by exogenous chromosphere molecules or photosensitizers), biostimulation, and photoablation (ablative decomposition) responses [15].

In a recent review of lasers and the treatment of periodontitis, Cobb succinctly summarized the key suppositions underlying the general rationale for laser periodontal therapy as well as evidence to support them. [16] The presumed clinical benefits of using lasers, or specific laser wavelengths, as a monotherapy or adjunct to traditional therapeutic

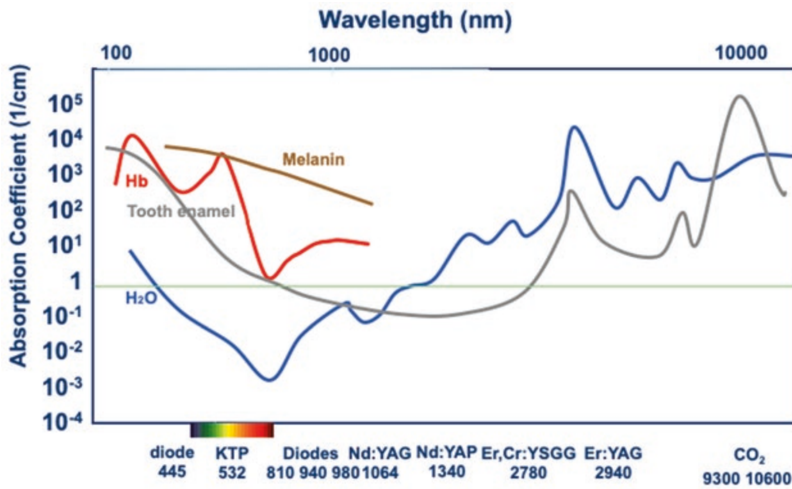

Fig. 15.1 Approximate net absorption curves of various tissue components, including water (H_2O), tooth enamel, melanin, and hemoglobin (Hb). *CO_2* carbon dioxide, *Er,Cr:YSGG* erbium, chromium-doped yttrium-scandium-gallium-garnet, *Er:YAG* erbium-doped yttrium-aluminum-garnet, *KTP* potassium titanyl phosphate, *Nd:YAG* neodymium-doped yttrium-aluminum-garnet, *Nd:YAP* neodymium-doped yttrium-aluminum-perovskite. (Image courtesy of Dr. Donald J. Coluzzi, and adapted from D. J. Coluzzi, Fundamentals of lasers in dentistry: basic science, tissue interaction, and instrumentation. J Laser Dent 16 (Spec. Issue): 4–10, 2008; with permission© 2008 Academy of Laser Dentistry [14])

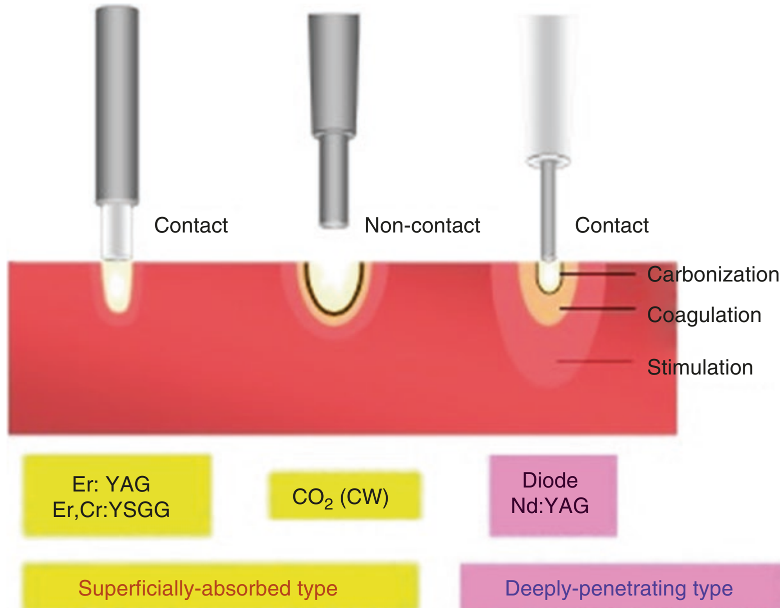

Fig. 15.2 Classification of lasers according to depth of light penetration in tissue—superficially absorbed type (shallow penetration and scatter) versus deeply penetrating type (deep penetration and scatter). *CO_2* carbon dioxide, *CW* continuous wave, *Er,Cr:YSGG* erbium, chromium-doped yttrium-scandium-gallium-garnet, *Er:YAG* erbium-doped yttrium-aluminum-garnet, *Nd:YAG* neodymium-doped yttrium-aluminum-garnet. (Image reproduced from A. Aoki et al., Periodontal and peri-implant wound healing following laser therapy. Periodontol 2000 68: 217–269, 2015; with permission© 2015 John Wiley & Sons A/S [14])

strategies, include significant potential for root surface debridement and detoxification, reduced subgingival bacterial burden, targeted eradication of pigmented anaerobic gram-negative bacteria, effectual subgingival curettage, suppression of inflammation, biostimulation, and periodontal regeneration [16]. Although peri-implant tissues differ from periodontal tissues in terms of composition and organization, these same suppositions apply and can be extrapolated to the application of lasers to peri-implant therapy.

This chapter provides an overview of laser science and clinical evidence on the therapeutic efficacy of lasers as a monotherapy or adjunct to traditional nonsurgical and surgical approaches in the treatment of periodontal and peri-implant diseases.

15.2 Impact of Local Environmental Cues

The ability of cells to sense and respond to their local environment is essential for normal cellular function and survival and provides an important therapeutic pathway to modulate wound healing. Bone grafts are intended to promote and/or accelerate natural regenerative processes at the site of the defect by providing architectural support and stability, serving as an osteoconductive scaffold for anchorage-dependent cells with osteogenic potential. Grafts containing growth factors or osteoblasts/osteoprogenitor cells also exhibit the ability to directly induce bone formation or promote osteogenesis, respectively. Evidence also points to the capacity for the graft, serving as a substratum, to function as an insoluble signal regulating the expression of the soluble osteogenic molecular signals of the TGF-β superfamily and initiating bone formation by induction [17–19].

The responsiveness of mesenchymal stem cells, osteoprogenitor cells, and osteoblasts to certain environmental cues, such as pulsed electromagnetic fields and nanovibrational fields, forms the basis for clinical practice of biophysical stimulation to increase and enhance reparative anabolic activity of the bone [20]. The molecular basis of bone mechanotransduction is complex and reflects a diverse interplay of ion channels, integrins, cell membrane, cytoskeleton, and other systems [21]. Recent studies suggest that laser energy can also modify the molecular dynamics of the membrane [22, 23]. These membrane alterations may be attributable in part to induction of free radical generation and to change in enzymatic and antioxidative activities of cellular components [23]. Laser energy has the potential to modulate a wide array of cellular and molecular pathways.

Hosseinpour et al. [24], for example, comprehensively reviewed in vitro and in vivo studies evaluating the effects of laser radiation on cellular and molecular activities, including osteogenic markers, angiogenic markers, growth factors, and inflammatory mediators, with the potential to impact bone regeneration. Photobiomodulation was found to significantly enhance expression of osteocalcin, collagen, RUNX-2, vascular endothelial growth factor (VEG-F), bone morphogenetic proteins (BMPs), and COX-2. Given the heterogeneity of the studies, Hosseinpour et al. [24] concluded that the effects of laser irradiation depend on multiple laser parameters; however, the most important parameter appears to be energy density. Furthermore, the authors concluded that there is insufficient evidence to guide the clinical therapeutic application of photobiomodulation. Emelyanov and Kiryanova [25], in contrast, concluded that cell type, rather than wavelength, was most important in choosing laser parameters. These authors also concluded that the highest increases in proliferation or differentiation were obtained using high power density, low energy density, and short exposure time. Bayat et al. [26] also concluded that low-level laser therapy causes a stimulatory effect on osteoblasts and osteocytes and enhances osteoblast proliferation and differentiation of different bone cell lines used in in vitro studies. Escudero et al. [27] similarly concluded that low-level laser therapy has positive photobiostimulatory effects on bone regeneration, accelerating its process regardless of parameters and the use of biomaterials. The results of these studies are consistent with other evidence that laser radiation has the ability to stimulate gingival fibroblast proliferation,

collagen synthesis, and wound healing [28–30]. Cell stimulatory effects have been demonstrated in response to irradiation by low-level lasers, including Ga-Al-As (805 or 650 nm), diode (810 nm, 870, or 940 nm), pulsed Nd:YAG, and Er:YAG lasers. [31–33] [34]

Preclinical investigations provide evidence for the potential of laser energy to influence bone metabolism and wound healing. Kim et al. [35], for example, examined bone repair following the application of a pulsed Nd:YAG laser, using a noncritical-sized calvarial defect model in rats and rabbits. The defects were left empty or filled with a collagen membrane prior to wound closure (Fig. 15.3). Starting the day after surgery, one defect in each animal was irradiated with a Nd:YAG laser once every 2 days for 2 weeks at a constant total fluence rate (344 J/cm^2), output power (0.75 W), pulse repetition rate (15 pps), and wavelength (1064 nm) holding the laser source 1–2 cm from the calvarial skin surface. Microcomputed tomography was performed after 4 weeks of defect healing. Laser irradiation resulted in significantly greater new bone area and percentage bone normalized to total defect area compared to nonirradiated control defects with and without scaffold in both animal models (Fig. 15.4).

Laser irradiation is absorbed by intracellular chromophores, presumably altering cellular activity, and is an important mechanism of action of low-level laser radiation. However, high-intensity pulsed laser irradiation can produce acoustic waves in the target tissue. Ninomiya et al. [36] examined the potential for high-intensity pulsed laser irradiation to accelerate bone formation using a femur model in rats. A Q-switched Nd:YAG laser was used to irradiate femurs either once a day, with the average fluence rate set at 100 mW/cm^2, or twice daily, with the average fluence rate set at 50 mW/cm^2. The mean bone volume and mineral apposition rate in the metaphysis was significantly higher following laser irradiation than the nonirradiated control group; however, the increase was highest for the lower fluence and higher-frequency condition, suggesting an important role for pulse frequency in bone formation. Ninomiya et al. [36] concluded that the formation of the bone induced by high-intensity pulsed laser irradiation might be due to laser-induced pressure waves. In a subsequent study, Ninomiya et al. [29] demonstrated that nanosecond pulsed laser irradiation of the rat femur, using a Q-switched Nd:YAG laser, resulted in an increased bone volume and min-

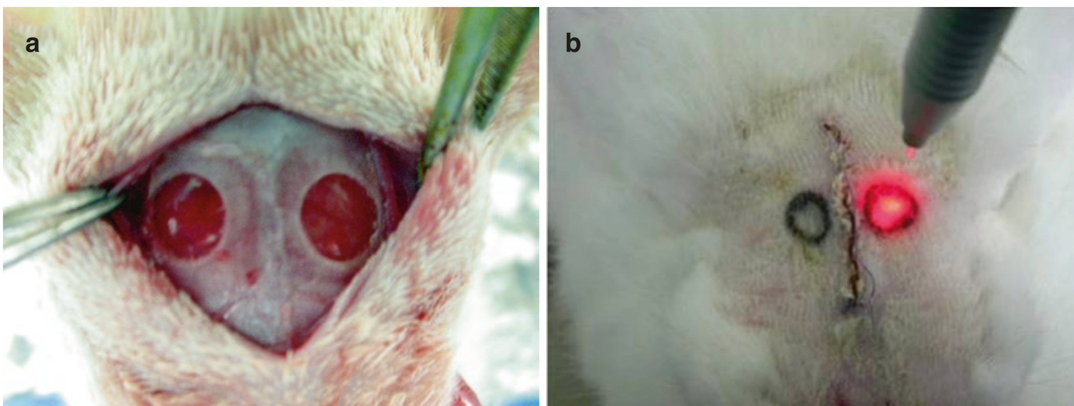

Fig. 15.3 Study of bone repair following application of a pulsed Nd:YAG laser, using a noncritical-sized calvarial defect model in rats and rabbits (**a**). In rats, a 5-mm-diameter bilateral calvarial bone defects were created and left empty or implanted with a collagen sponge. Starting the day after surgery, one defect in each animal was irradiated with a Nd:YAG laser once every 2 days for 2 weeks at a constant total fluence rate (344 J/cm^2), output power (0.75 W), and pulse repetition rate (15 pps), holding the laser source and adapted 1–2 cm from the calvarial skin surface (**b**). (Images courtesy of Dr. Soon Jung Hwang, and adapted from K. Kim et al., High-intensity Nd:YAG laser accelerates bone regeneration in calvarial defect models. J Tissue Eng Regen Med 9: 943–951, 2013; with permission© 2013 John Wiley & Sons [35])

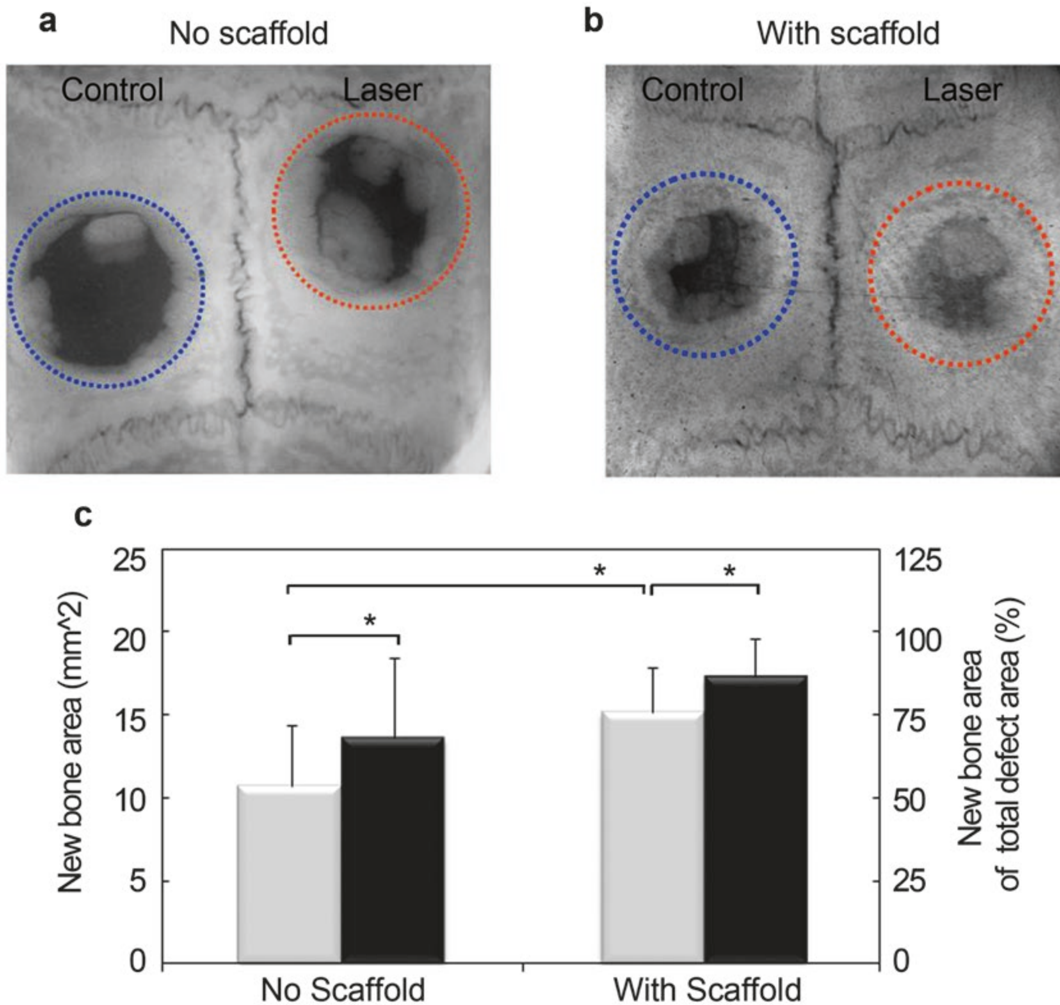

Fig. 15.4 Histomorphometry based on microcomputed tomography-based analysis of calvarial defect after 4 weeks of healing (**a**–**b**). Dotted circles in blue (nonirradiated control group) and red (irradiated laser group) indicate the position and dimensions of the 5 mm original defect size. (**c**) Histomorphometric analysis revealed significantly ($p < 0.05$) greater new bone area (mm^2; left axis) and percentage (%) normalized to total defect area (right axis) for laser-irradiated defects (dark bar) compared to nonirradiated control defects (light bar) with and without scaffold. Similar results were shown using an 8 mm calvarial defect model in the rabbit. (Images courtesy of Dr. Soon Jung Hwang, and adapted from K. Kim et al., High-intensity Nd:YAG laser accelerates bone regeneration in calvarial defect models. J Tissue Eng Regen Med 9: 943–951, 2013; with permission© 2013 John Wiley & Sons [35])

eral density based on morphometric analysis. Importantly, the histologic analysis revealed a decrease in number of osteoclasts, suggesting that the laser-induced increase in bone volume was partially attributable to a decrease in osteoclastic activity. Other preclinical studies, however, using different laser wavelengths, protocols, and experimental models, have provided varying results with respect to bone healing [37, 38].

15.3 Wound Healing: Windows of Opportunity

Lasers have the potential to affect each of the four highly integrated and overlapping phases of the wound-healing process: hemostasis, inflammation, proliferation, and tissue remodeling or resolution [39, 40]. High-intensity lasers, for example, have been shown to induce photothermal and hemodynamic responses that

lead to swelling and rupture of erythrocytes, ultrastructural perturbation of the endothelial cell membrane and denudation of the endothelial monolayer triggering primary and secondary hemostasis, and coagulation secondary to protein denaturation, conformational rearrangement, and cross-linking and aggregation (reviewed by [41]).

Cell culture and experimental animal models of inflammation provide evidence that low-level laser irradiation reduces inflammatory mediators and markers of inflammation. Lee et al. [42], for example, examined the anti-inflammatory effect of laser irradiation on human periodontal ligament cells cultured with or without lipopolysaccharide (LPS) from *P. gingivalis* or *Escherichia coli*, followed by irradiation with a gallium-aluminum-arsenide (GaAlAs) laser (660 nm) at an energy density of 8 J/cm^2. Laser irradiation was shown to inhibit the LPS-induced pro-inflammatory cytokine gene expression, including tumor necrosis factor-α (TNF-α), interleukin 1-ß (IL-ß), interleukin 6 (IL-6), and interleukin 8 (IL-8), decrease nuclear factor-κB (NF-κB) transcriptional activity, and elevate intracellular levels of cyclic adenosine monophosphate (cAMP) relative to the unexposed control cells. The results suggest that low-level laser irradiation might inhibit LPS-induced inflammation through the cAMP/NF-κB pathway. Wu et al. [43] investigated the anti-inflammatory effect of low-power laser irradiation using a GaAlAs laser (660 nm) on LPS-treated human adipose-derived stem cells. LPS exposure significantly induced the production of pro-inflammatory cytokines (cyclooxygenase-2, IL-1ß, IL-6, and IL-8). Laser irradiation markedly inhibited LPS-induced, pro-inflammatory cytokine expression at an optimal dose of 8 J/cm^2. Giannelli et al. [44] reported that low-intensity Nd:YAG laser irradiation significantly reduced *P. gingivalis* LPS-induced nitric oxide production and cell activation by macrophages and strongly attenuated intercellular adhesion molecule-1 and vascular cell adhesion molecule expression, as well as interleukin-8 production, by endothelial cells, thereby blunting the LPS-induced inflammatory response. These culture studies highlight the ability to modulate the inflammatory response through laser irradiation, presumably through common or overlapping cellular pathways.

Bortone et al. [45] examined the effect of low-level laser irradiation on kinin receptor messenger ribonucleic acid (mRNA) expression in the carrageenan-induced rat paw model of edema. The results demonstrated that laser irradiation (660 or 684 nm wavelength) significantly decreased Kinin B1 receptor mRNA expression and modestly decreased Kinin B2 receptor mRNA expression. Using an LPS-induced peritonitis model in mice, Correa et al. [46] examined the effect of an infrared low-level laser (GaAs; 904 nm, 4 mW) on the migration of inflammatory cells. Laser irradiation was found to diminish inflammatory cell migration in a dose-dependent manner, with the strongest effect on migration with the 3-J/cm^2 exposure, with reductions of 77% in neutrophil counts and 49% in leukocyte counts. Pires et al. [47] reported that low-level laser irradiation decreased IL-6 and cyclooxygenase-2 (COX-2) expression in both acute and chronic phases in collagenase-induced tendinitis in rats.

Boschi et al. [48] reported that low-level laser irradiation (660 nm) induced an anti-inflammatory effect characterized by inhibition of either total or differential leukocyte influx, exudation, total protein, nitric oxide (NO), IL-6, monocyte chemoattractant protein-1 (MCP-1), interleukin 10 (IL-10), and TNF-α, in a dose-dependent manner in carrageenan-induced pleurisy in a rodent model. Collectively, the results of these studies and others [49–51] document the potential for low-level laser (e.g., Nd:YAG, diode) to suppress mediators of inflammation and that this effect likely involves modulation of the NF-κB transcriptional pathway [52].

Of particular importance to regenerative wound healing is evidence that low-level laser (e.g., Nd:YAG, diode, CO_2) irradiation has the capacity to promote the proliferation and differentiation of mesenchymal stem cells as well as the proliferation of gingival fibroblasts, osteoblasts, and other cell types [25, 26, 30, 53–60]. Studies also document the potential for laser irradiation to augment the immune response, including lymphocyte stimulation, mast cell function, and dendritic cell mobilization [61, 62].

Recent systematic reviews generally conclude that laser irradiation promotes wound healing in

experimental animal models [63–65]. Gál et al. [66], for example, concluded that low-level laser irradiation, when applied to wounded animals, was associated with superior results for tensile strength (8 studies) and wound contraction analysis (11 studies) based on controlled studies. Posten et al. [67], however, noted that improvements in surgical wound healing in rodent models have not been duplicated in larger animals, such as pigs, which have skin more closely resembling that of humans. Nevertheless, other reviewers have concluded the results of cell studies and animal experiments show strong evidence to substantiate conducting large clinical trials to evaluate the efficacy of low-level laser in promoting wound healing. [68]

The unequivocal interpretation of available evidence is hampered by multiple factors, including heterogeneity of experimental models, protocols, and irradiation parameters, such as wavelength, irradiance, and pulse structure as well as the energy, energy density, irradiation time, and treatment interval [69]. The studies provide little insight into the mechanism of laser action, whether photothermal, photochemical, or photomechanical. Similar concerns have arisen with studies evaluating the efficacy of irradiation with different laser types, including carbon dioxide (CO_2), Nd:YAG, and diode, on wound healing following tooth extraction in animal models and humans. Systematic reviews generally conclude that there is limited evidence that certain lasers and protocols appear to improve bone and soft tissue wound healing [70]; however, there are insufficient well-designed and randomized controlled clinical trials with comparable study design to conclude that laser therapy enhances wound healing following tooth extraction [71]. The availability of such data is necessary for the development of evidence-based recommendations and clinical guidelines.

15.4 Therapy: Setting the Stage

A major challenge in treating periodontal and peri-implant diseases, such periodontitis and peri-implantitis, is the effective removal of bacterial toxins and disruption of tooth/implant-associated biofilms.

15.4.1 Decontamination and Detoxification

15.4.1.1 Root Surface

The effective decontamination and disinfection of root and dental implant surfaces by mechanical instrumentation, whether using hand instruments or powered devices, is often clinically challenging to achieve. The rationale for selecting laser therapy generally includes the expectation that lasers, whether used as a monotherapy or adjunctive to scaling and root planing (SRP), are effective in detoxifying root surfaces, in producing a significant reduction in subgingival bacterial load, and in reducing inflammation [16]. Another rationale for the selection of lasers is the premise that lasers can access deep periodontal pockets, furcation defects, and complex root topography, including grooves and concavities, better than scalers [72].

Lasers provide the ability to deliver large amounts of energy into relatively small, targeted regions of soft or hard tissue. Achieving a desired tissue or material modification is dependent on the proper selection of laser wavelength and parameters. The unique interaction of laser light with a tissue or material can lead to permanent changes in the tissue or material properties.

Early in vitro studies provided important information on the behavior of different laser parameters, such as wavelength and pulse duration, directed at root surfaces for the purpose of disrupting and removing calculus. The studies sought to characterize the efficiency and effectiveness of calculus removal as well as potential alterations in root surface topography and structure. The diode laser is one such laser that alters the root surface and has been shown to result in severe surface modifications, such as crater formation [73]. Additionally, the application of CO_2 and Nd:YAG laser resulted in morphologic changes in the root surfaces concordant with energy density, with or without air/water surface cooling [74]. Descriptions of laser-induced surface changes include, among others, cavitation, globules of melted and resolidified mineral, surface crazing, and production of a superficial char layer. The significance and impact of root surface

modifications on periodontal regeneration are unknown; however, there is evidence that such morphologic surface changes may hinder cell attachment [75–77].

More specifically, the Nd:YAG laser wavelength is minimally absorbed in water and exhibits minimal absorption by the tooth, bone, calculus, or enamel. As a result, it is not effective for the removal of calculus, and the energy density necessary to ablate the calculus has significant collateral thermal effects [78]. Similarly, the thermal effects of the CO_2 laser will lead to carbonization and root damage [78]. Schwarz et al. [73] reported diode lasers are overall ineffective at calculus removal and cause undesirable root surface alterations, such as grooves and cratering.

In contrast, the Er:YAG laser affects calculus removal without gross morphologic alterations in the cementum surface. Eberhard et al. [79] compared the non-surgical effectiveness of Er:YAG laser and conventional SRP in achieving calculus-free subgingival root surfaces on single-rooted teeth with untreated periodontitis. When residual calculus was measured by digitized planimetry following extraction and both treatments were performed for the same time duration, SRP produced a significantly greater area free of residual calcified deposits than with Er:YAG laser irradiation (93.9 ± 3.7% versus 68.4 ± 14.4%, respectively). When laser irradiation was performed for twice the time utilized for hand instrumentation, the mean area of root surface devoid of calculus increased but remained significantly less than with SRP (83.3 ± 5.7% versus 96.3 ± 3.5%, respectively). The effectiveness of both treatments in subgingival calculus removal was not related to the initial probing depth. Notable, too, was that laser-treated tooth surfaces exhibited no dentin exposure and minimal reduction of cementum, whereas hand instrumentation was associated with denudation of dentin.

In a similar study, Schwarz et al. [80] evaluated the effectiveness of an Er:YAG laser for subgingival calculus removal from root surfaces of single-rooted teeth treatment planned for extraction due to severe periodontitis. In this study, subgingival laser irradiation was performed using a fluorescent calculus detection system. Histologic evaluation revealed that Er:YAG laser application provided subgingival calculus removal comparable to that provided by SRP. Again, no detectable surface alterations were noted. Additionally, Crespi et al. [81] found smooth root surfaces and an absence of debris following Er:YAG laser application. In sum, Er:YAG laser can be effective in calculus removal without root surface alteration or major thermal side effects to adjacent tissue [82–84]. Furthermore, the resultant smooth root surface morphology was attained even at higher energy settings [80, 81, 85].

Similar results for calculus removal have been reported for the Er,Cr:YSGG laser [86]. Etemadi et al. [87], however, reported that the Er:YAG laser appears to have an advantage in terms of time and efficiency of calculus removal compared to the Er,Cr:YSGG laser. Stereomicroscopic examination revealed no carbonization or residual calculus in either treatment group; however, root surfaces exhibited craters, with significantly higher number of craters in the Er,Cr:YSGG laser group than the Er:YAG laser group. Ting et al. [88] found Er,Cr:YSGG laser irradiation produced root surface alterations without thermal alterations, such as carbonization and melting [88]. As summarized in a recent review by Lavu et al. [89], erbium lasers are suitable for calculus removal with minimal root surface alteration or thermal damage, providing a favorable surface for cell attachment.

15.4.1.2 Dental Implant Surface

Laser application to dental implant surfaces has significant potential to induce thermal alterations on the implant surface and impact to surrounding tissue. The CO_2 laser, diode laser, Er:YAG laser, and Nd:YAG laser have all been used clinically in the treatment of peri-implant diseases. With respect to surface implant decontamination, the Nd:YAG laser is not recommended for implant decontamination, since it alters and ablates the titanium surface at any applied energy level [90]. However, it has been used successfully for treatment of the surrounding peri-implant tissues. The CO_2, diode, and Er:YAG lasers all have been effective at decontamination in vitro [91]. The diode laser does not

damage the titanium surface and is also capable of decontamination of rough surface implants [92]. CO_2 lasers can be used without implant surface damage if appropriate power output is selected. Implant decontamination has been reported when applying a CO_2 laser at an energy density of 286 and 245 J/cm^2 in vitro [93].

The Er:YAG laser can effectively remove calculus and plaque from a variety of contaminated titanium surfaces of different characteristics [94, 95]. Schwarz et al. [94] reported that irradiation with an Er:YAG laser resulted in greater plaque biofilm removal on sandblasted and acid-etched titanium surfaces prepared in the oral cavity than with ultrasonic instrumentation or plastic curettes with chlorhexidine rinsing. In another clinical trial, Er,Cr:YSGG laser application to contaminated sandblasted and acid-etched surfaces resulted in effective biofilm removal [96]. Both Er:YAG and Er,Cr:YSGG lasers cause no visible changes to the implant surfaces, and the addition of a water spray minimizes the potential temperature changes at the implant material surface [97]. Overall, CO_2, diode, and erbium lasers appear effective for decontamination of implant surfaces. What remains to be defined is the biocompatibility of titanium surfaces and their potential to support re-integration after the laser decontamination.

15.4.2 Microbial Disinfection

In clinical practice, antimicrobial chemotherapeutic agents are widely used in an effort to reduce or change the quality of microbial pathogens in biofilms through local or systemic delivery. Laser radiation exhibits the ability to disrupt biofilms and exert broad-spectrum antimicrobial activity, although the effect and action appear dependent on laser beam parameters, dose, and bacterial species [98–102]. The antimicrobial properties of lasers and laser-activated photosensitizers have received considerable attention in the management of periodontal and peri-implant diseases.

15.4.2.1 Photodynamic Therapy

One means of disinfection is the use of low-level light energy and chemical photosensitizers. The photodynamic process is based on converting light energy to chemical, which requires an additional agent to transform the light energy, called a photosensitizer. This process has been termed antimicrobial photodisinfection or photodynamic therapy. The stimulation of a photosensitizer by an appropriate light wavelength produces highly reactive oxygen species, such as reactive singlet oxygen ($^1O_2$) [103, 104]. The singlet oxygen ($^1O_2$) and free radicals generated are highly reactive with extremely short lifespans (measured in μ seconds) due to their unstable electronic configuration.

A variety of exogenous compounds have been used that can be photoactivated in the ultraviolet and visible regions of the electromagnetic spectrum [105]. Multiple photosensitizing compounds, including phenothiazine chloride, toluidine blue, methylene blue, and tolonium chloride, have been used in photodynamic therapy for periodontal and peri-implant disease [106]. Photosensitizing molecules when delivered in a pocket may interact with different cellular constituents based on their affinities for these components, while the binding sites determine the localization of the photodynamic damage effect in situ due to the generation of reactive oxygen species [105].

Bactericidal efficacy of photodynamic therapy was explored by Akram et al. [106] in a systematic review of clinical trials that assessed bactericidal efficacy of photodynamic therapy when combined as an adjunct to SRP in patients with periodontitis. Seventeen prospective, randomized controlled clinical trials examining antimicrobial photodynamic therapy (aPDT) for the treatment of periodontitis met the inclusion criteria. Additionally, study inclusion required pre- and posttreatment microbial counts for any of the following periodontal bacteria: *P. gingivalis*, *T. forsythia*, *T. denticola*, and *A. actinomycetemcomitans*. Of the 17 studies, 13 clinical trials showed similar reduction in the selected periodontal pathogens for SRP and SRP plus aPDT. All studies utilized diode lasers but with differences in protocols that included irradiation wavelength (470 and 810 nm), exposure duration (60–300 sec), photosensitizing agent, and follow-up period, limiting the interpretation of outcomes regarding the effectiveness of aPDT as an adjunct

to SRP to reduce periodontal pathogens. In sum, only 24% of the clinical studies reported bacterial count reductions of key periodontal pathogens beyond that accomplished by SRP alone. Of note, in one study, *T. denticola* was actually significantly increased at 24 weeks following SRP plus aPDT [107]. Gandhi et al. [108] recently reported a 9-month clinical trial demonstrating significant reductions in *A. actinomycetemcomitans* and *P. gingivalis* counts using low-level laser therapy and aPDT as an adjunct to SRP in the treatment of periodontitis.

To establish whether aPDT can substitute for the incorporation of a systemic antibiotic during SRP, another comparative review of aPDT by Akram et al. [109] identified five clinical trials comparing aPDT to systemic antibiotics as an adjunct to SRP. These studies provided outcome data following irradiation of periodontal pockets with diode laser wavelengths with follow-up from 12 to 48 weeks. When compared to adjunctive antibiotics, aPDT did not produce any additional benefit in clinical outcomes. Therefore, it remains equivocal whether aPDT can substitute for systemic administration of antibiotics in the nonsurgical therapy of periodontitis, particularly in patients with more aggressive rates of disease progression. Overall, consistency is lacking with respect to the adjunctive impact of aPDT on microbial counts.

15.4.2.2 Dental Implant Surface Disinfection

Decontamination of dental implants was the focus of a recent review by Alasqah et al. [110]. This review of in vitro studies examined the effects of aPDT on bacterial colonization and dental implant surface topography, including titanium implants, zirconia implants, and titanium discs. All included studies used diode laser energy, ranging in wavelength from 625 to 810 nm. Photosensitizers applied included methylene blue, toluidine blue, indocyanine green, and phenothiazine chloride, and a variety of bacterial species were evaluated, including *P. intermedia*, *A. actinomycetemcomitans*, *P. gingivalis*, *Streptococcus gordonii*, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Campylobacter rectus*, *Filifactor alocis*, *Eikenella corrodens*, *Parvimonas micra*, *T. forsythia*, *T. denticola*, and *Staphylococcus aureus*. All studies showed a significant but incomplete reduction in the bacterial load. Implicated in other studies is the suggestion that the implant surface type may influence the effectiveness of photodynamic therapy, presumably due to differences in biofilm or biofilm access [111]. Regardless, most studies addressing different implant surfaces generally demonstrate some reduction of bacterial load following photodynamic therapy [112–116]. It has been suggested that, dependent on the implant surface, the combination of titanium brush application with aPDT might be more efficient for the reduction of bacteria [111]; however, this remains to be tested in vivo since the assessment with the combined application was in vitro and with only one seeded pathogen, *S. aureus*, over a short-incubation period. It is important to note that aPDT did not eliminate the bacteria; therefore, an important consequence is the persistence of residual LPS, which was not evaluated in these studies.

Huang et al. [117] evaluated osteoblast-like MG63 cell attachment, proliferation, differentiation, and mineralization on contaminated SLA (sandblasting, large grit, and acid-etching) titanium alloy surfaces after photodynamic therapy using different concentrations of methylene blue and the application of a 660 nm diode laser. The titanium alloy surfaces were first contaminated with *A. actinomycetemcomitans* or *Streptococcus mutans*. aPDT resulted in significant reductions in bacterial colonies. Importantly, the disinfected disc surfaces were found to support osteoblast-like MG63 cell attachment, proliferation, differentiation, and mineralization. The highest methylene blue concentrations (350 and 400 μg/mL) resulted in the lowest lipopolysaccharide (LPS) remaining quantity on the *A. actinomycetemcomitans*-contaminated surfaces. Notably, osteoblasts cultured on disinfected surfaces with the application of the higher methylene blue concentration photodynamic therapy achieved comparable osteoblast culture to that of the control without contamination. Thus, aPDT offers the potential to sufficiently reduce bacterial and LPS contamination to allow for bone growth or reintegration of the bone along the previously diseased dental implant. Clinical studies,

however, are necessary to extrapolate these and other experimental results with titanium and titanium alloy surfaces to patient care.

15.4.3 Implant Particulate and Debris

Orthopedic implant materials can undergo corrosion, degradation, and wear, releasing particles and debris into the surrounding tissues that can elicit inflammatory and immune responses [118]. Titanium dioxide (TiO_2) nanoparticles, for example, exhibit the potential to induce oxidative stress, cellular apoptosis, and inflammation [119]. Titanium particles and degradation products have been detected in oral tissues associated with dental implants (reviewed in [120]). Of growing concern is the potential for titanium particles to elicit an inflammatory response in oral tissues [120–122]. Although there is no direct evidence of a causal relationship between particulate titanium debris and inflammation in oral tissues, a growing number of reports have documented the presence of particulate debris in the soft tissues surrounding dental implants with peri-implantitis. One recent report describes two cases of peri-implantitis that initially responded poorly to regenerative therapy; however, when the sites were subsequently irradiated with either an Nd:YAG or CO_2 laser, demonstrable improvements emerged in clinical parameters and radiographic bone fill [123]. The authors suggested that the successful treatment of peri-implantitis may need to also incorporate decontamination of the soft tissues in addition to the implant surface. Particulate debris may also provide insight into the etiology of certain cases of refractory peri-implant mucositis. Future investigations appear warranted.

15.5 Periodontal Therapy

15.5.1 Nonsurgical

In a recent systematic review, Chambrone et al. [124] examined the use of infrared lasers (i.e., Diode, Er:YAG, and Nd:YAG) alone or as an adjunct to SRP for the nonsurgical treatment of chronic periodontitis. The clinical application of SRP plus infrared lasers as part of debridement procedures was found to promote significant improvements in bleeding on probing, clinical attachment level, and probing depth. Nonetheless, it was concluded that infrared laser (Er:YAG and Nd:YAG) alone did not show additional gains to those accomplished by SRP alone. Moreover, no overall differences were identified when comparing clinical outcomes between infrared laser alone and SRP alone (Diode, Er:YAG, or Nd:YAG); however, the results of studies evaluating SRP plus infrared laser (Diode, Er:YAG, or Nd:YAG) suggest modest additional clinical benefits in clinical attachment level gains (<1 mm) and probing depth reduction (<1 mm) to those achieved by SRP alone. Consistent with the clinical outcomes, Chambrone et al. [124] found the application of SRP alone, laser alone, and SRP plus laser was essentially comparable in reducing total colony-forming units and levels of different bacterial pathogens (e.g., *A. actinomycetemcomitans*, *T. forsythia*, *C. rectus*, *E. corrodens*, *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *T. denticola*) within 4–12 weeks after treatment. Consistent with studies following mechanical instrumentation, levels of the bacterial pathogens generally returned to levels comparable to baseline 6 months after treatment [125–127]; however, sustained superior reductions in selected periodontal pathogens after Er:YAG laser or SRP plus laser treatment have been reported at 12 months [128].

Zhao et al. [129] conducted a systematic review to evaluate the Er:YAG laser versus SRP as alternative or adjunctive treatment for chronic periodontitis. The meta-analysis, which included data from eight studies, showed that Er:YAG laser resulted in comparable short-term (3 months) improvements in clinical attachment level gain and probing depth reduction to those obtained with SRP. At 12-month follow-up, the comparison of the two treatment modalities (three studies) demonstrated no statistically significant difference in clinical attachment level gain or probing depth reduction.

Recent systematic reviews generally conclude that compared to SRP alone, the ER:YAG laser results in similar or improved short-term clinical outcomes; however, no significant treatment differences remained with respect to outcomes at the 6- and 12-month follow-up periods [72, 130]. Er:YAG laser and Er,Cr:YSGG laser result in modestly more surface roughness when compared with ultrasonic and hand instrumentation [86]. Interpretation of the results, however, was limited by heterogeneity and risk of bias [72]. The level of evidence for the nonsurgical application of lasers for the treatment of periodontitis was critically appraised in a more recent systematic review and meta-analysis [131]. The authors concluded that SRP plus aPDT will attain a 0.53 mm mean gain in clinical attachment level with moderate certainty. However, when compared to SRP alone, the adjunctive use of a diode laser (non-aPDT), Nd:YAG laser, or erbium laser had a low level of evidence or certainty of effect on clinical attachment level gain (0.21–0.41 mm benefit) in the reported critical appraisal [131, 132].

15.5.2 Subgingival Curettage (Pocket De-epithelialization)

Gingival curettage is a surgical procedure designed to remove the epithelial lining of the periodontal pocket, with the goal of denuding the subjacent gingival connective tissue. The original objective of the gingival curettage procedure was to remove the pocket lining and junctional epithelium, including any granulation tissue, thereby setting the stage for new connective tissue attachment to the tooth. Subgingival curettage has been performed using manual curettes, chemicals, and excisional gingival flap surgery [133, 134]. Clinical studies, however, have consistently failed to show any additional benefit of subgingival curettage, when compared to SRP alone, with respect to probing depth reduction, attachment level gain, or inflammation reduction [135, 136]. One of the limitations of mechanical curettage is the potential for epithelial remnants, such as at the gingival margin or near the epithelial attachment, or due to epithelial rete extensions. Centty et al. [137] histologically compared periodontal flaps elevated using an inverse-beveled incision, extending from the free gingival margin to the alveolar crest, with the goal of surgically excising the pocket epithelium. The experimental periodontal sites were next irradiated using a carbon dioxide laser to remove any remaining pocket lining and gingival (oral) epithelium. Following the procedure, soft tissue biopsies were obtained and submitted for histologic examination. The results revealed the remnant pocket epithelium on all the specimens. The histologic results of available studies, therefore, suggest that gingival curettage, regardless of procedural method, does not completely remove pocket lining epithelium. Moreover, the results of clinical studies suggest minimal benefit of subgingival curettage performed with lasers either as a monotherapy or adjunctive to traditional periodontal therapy. [124]

Lin et al. [138] compared gingival curettage performed with an 810 nm diode laser to curettage performed with hand instruments. Significant and comparable improvements in clinical measures were observed following curettage in both groups after 4 weeks. Notably, the investigators reported that laser curettage required less treatment time and was associated with less treatment discomfort than curettage with hand instruments.

Using a primate model, Rossman et al. [139] examined whether de-epithelialization with the CO_2 laser would increase the amount of connective tissue attachment to root surface. Elastics were used to create periodontal defects on the maxillary premolars and incisors of cynomolgus monkeys. Bilateral open flap debridement was performed. On the experimental side, CO_2 laser was used to remove the oral epithelium prior to flap replacement. Histologic examination revealed a delay in sulcular epithelialization (day 14 versus day 28, respectively) and a trend to less epithelium and more connective tissue attachment after 7 days on the experimental side than on the control side. The investigators concluded that the CO_2 laser may be a useful tool to retard epithelium and thereby enhance new connective tissue attachment.

Israel et al. [140] conducted a pilot study to evaluate whether pocket de-epithelialization with a CO_2 laser at the time of flap surgery and at 10-day intervals over the first 30 days of healing can enhance the formation of a connective tissue attachment. Six mandibular incisors in two patients were splinted prior to open flap debridement, when a notch was placed on the roots at the height of the crest of the alveolar bone, prior to flap closure. The experimental side received de-epithelialization of the outer (oral) gingiva with the carbon dioxide laser and the inner gingival flap. The de-epithelialization was repeated on the test side at 10, 20, and 30 days postsurgically. Block sections were taken at 90 days and processed for histologic analysis. The results showed that for both patients, junctional epithelium (JE) was formed on both test and control teeth. In all control teeth, the JE extended the entire length of the root to the base of the reference notch. In one patient, on the experimental side, the notch was filled with connective tissue and limited new cementum [140].

In sum, laser curettage can be performed efficiently with the application of lasers, such as diode or CO_2, but the supposition of complete de-epithelialization of the pocket lining remains elusive, and there is a lack of added benefit for surgical curettage beyond that attained with SRP.

15.5.3 Surgical Periodontal Therapy

15.5.3.1 Surgical Flap Access with Laser Treatment

A limited number of studies combine surgical flap access with laser treatment for the treatment of periodontitis. The majority of laser therapy clinical trials have focused on the inclusion of laser treatment in the nonsurgical management of the disease, where laser therapy is an adjunct to nonsurgical instrumentation of the teeth. Testimony to this clinical research focus is characterized in the comprehensive tabulation of studies in a recent review of lasers and the treatment of periodontitis [16]. Surgical flaps combined with adjunctive laser treatment have been reported for Er,Cr: YSGG, CO_2, diode, Er:YAG, and Nd:YAG lasers. Er:YAG and diode lasers, when used with mechanical debridement, have been compared to mechanical debridement following access flap surgery of 5 mm or deeper pockets. [141–144]. The studies found comparable or superior improvements in clinical outcome measures after either 3 or 6 months [142–144] or 6, 12, 24, and 36 months [141]. Gaspirc and Skaleric [141] reported significantly greater improvements in clinical attachment level and probing depth after flap access plus Er:YAG compared to conventional flap surgery.

Er:YAG studies, some of which also included guided tissue regeneration with enamel matrix protein, found minor differences favoring the laser treatment group when comparing probing depth reduction and clinical attachment level gain. However, the level of evidence is limited for the inclusion of laser treatment with regenerative surgical therapy and enamel matrix derivative, since no significant differences between the surgical therapies, with or without the laser treatment, were noted in a meta-analysis. [145] There is only one clinical trial with Er,Cr: YSGG laser, and the authors concluded that laser treatment resulted in significant reductions in probing depth and bleeding score but had similar gains in clinical attachment level [16]. Notable was one controlled clinical study with 15-year follow-up after coronally advanced flap surgery and CO_2 laser root treatment. The control group had modified Widman flap treatment. Significant reductions in probing depth and gains in clinical attachment level at initial sites with probing depth of 5 mm or greater were maintained over the 15-year period [146] providing evidence for maintenance of the attained treatment results. Overall, there is modest benefit reported for the inclusion of laser treatment with periodontal flap surgery for the treatment of periodontitis. In a meta-analysis [145] of laser application for surgical therapy, the weighted mean difference for probing depth was 0.56 mm and that of clinical attachment level was 1.34 mm favoring the laser treatment with flap surgery. However, this report found no statistical differences when comparing surgical outcomes with or without laser treatment. Thus, the benefit of the application of lasers to surgical periodontal therapy is surmised

but not clinically recognized in the limited number of available studies, reflecting, in part, the heterogeneity in study design and methodology.

Several suppositions underlie the rationale for incorporating lasers into periodontal surgery [16], including some evidence from preclinical studies. In addition to root surface decontamination and detoxification, as considered earlier, certain lasers and laser protocols are thought to contribute to more effective subgingival curettage, targeted and/or overall reduction in subgingival bacteria, reductions in inflammation, and rapid wound healing.

15.5.3.2 Regenerative Surgical Therapy with Laser Treatment

Regenerative therapies are designed to support *regeneration* of the attachment apparatus, namely, the formation of new bone, cementum, and periodontal ligament (Figs. 15.5, 15.6, 15.7, 15.8, 15.9, 15.10, 15.11, and 15.12). The biological goal of periodontal regeneration, therefore, is restoration of the lost periodontium. Conventional surgical approaches, such as open flap debridement, heal primarily through *repair*, characterized principally by the formation of a long junctional epithelial attachment to the previously diseased root surface. Repair is healing of the periodontal attachment apparatus by tissue, such as junctional epithelium, which does not fully restore architecture and function. Long junctional epithelium can be produced rapidly during wound healing, due to high proliferative activity of epithelial cells [148]. Limited evidence of formation of other component tissues, such as the bone, has been reported following open flap debridement surgery [149].

Contemporary therapeutic approaches to periodontal regeneration include bone replacement grafts, guided tissue regeneration (GTR), and biologics [150, 151]. These regenerative therapies have been used in combination and in conjunction with agents to modify and promote wound healing [149]. Evidence-based systematic reviews and meta-analyses support the efficacy of commercially marketed mammalian-derived bone grafts, guided tissue regeneration, and biologics for periodontal regeneration, as reflected in clinical improvements evidenced by probing depth reduction, clinical attachment gain, and reentry/radiographic defect fill. Histologic evidence of periodontal regeneration—new bone, cementum, and periodontal ligament on a previously diseased root surface—is available for each regenerative therapy. However, the most thoroughly and extensively characterized histologic outcome evidence in humans is for alloge-

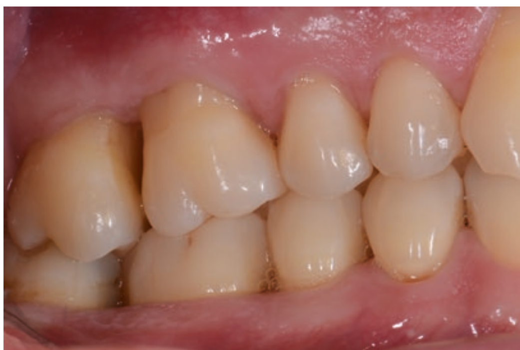

Fig. 15.5 Pretreatment clinical view of 44-year-old Caucasian man with a medical history of multiple drug allergies, including antibiotics (penicillin) and sulfa medications (treatment was provided by PSR). Periodontal examination revealed generalized bleeding upon probing and periodontal pocketing, with probing depths of 5–7 mm involving the maxillary molars

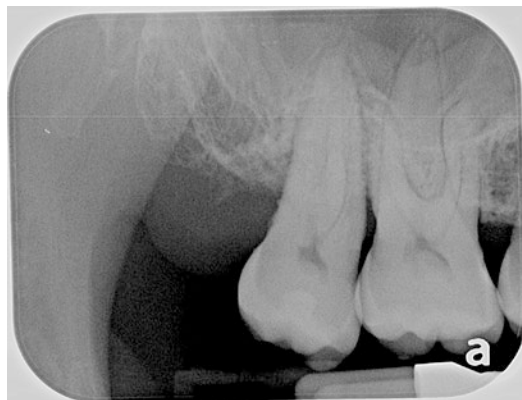

Fig. 15.6 The preoperative radiograph is consistent with stage III periodontitis, with evidence of calculus deposits on the roots. An angular osseous deformity is suggested on the distal of tooth # 2; note the close root proximity of the molars

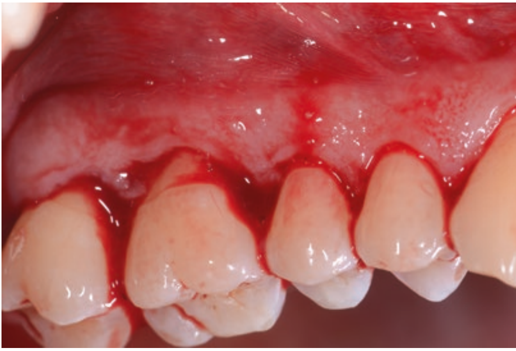

Fig. 15.7 Clinical presentation after second pass using the laser-assisted new attachment procedure with the Nd:YAG laser (3.8 W for each pass) prior to occlusal adjustment. A modification to postoperative protocol management was necessary because of patient concerns about his drug allergies; consequently, he was not placed on antibiotics, and a botanical rinse (PeriActive, Izun Oral Care) was prescribed for infection and plaque control. Supportive periodontal maintenance was performed on a 3-month interval

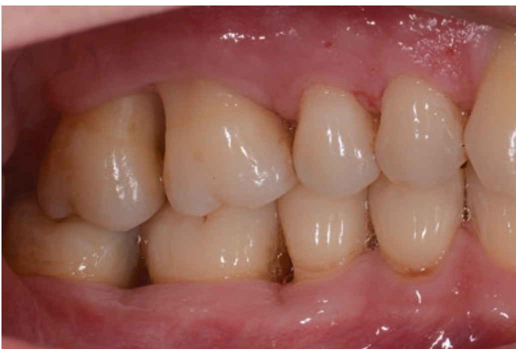

Fig. 15.8 Clinical presentation 1 year after treatment. Highly effective oral hygiene was consistently maintained during this period. Note the reduction in gingival volume and healthy tissue appearance. Probing depths range from 2 to 4 mm without bleeding

neic demineralized freeze-dried bone [152, 153]. Despite the histologic evidence, wound healing is characterized by a combination of periodontal regeneration and repair. Consequently, there currently exists a need for more robust and cost-effective regenerative strategies. Considerable interest in regenerative periodontics and medicine has focused on emerging technologies, including scaffolds and cell-based grafts [154, 155], biologics [156], and lasers [124].

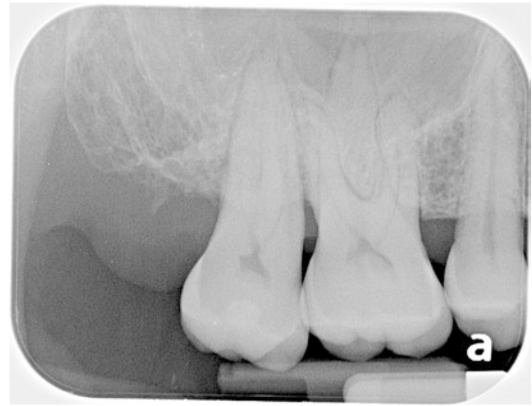

Fig. 15.9 Radiograph 1 year after treatment suggests osseous fill of the intrabony lesion on the distal of the second molar. The crestal lamina dura is well defined elsewhere

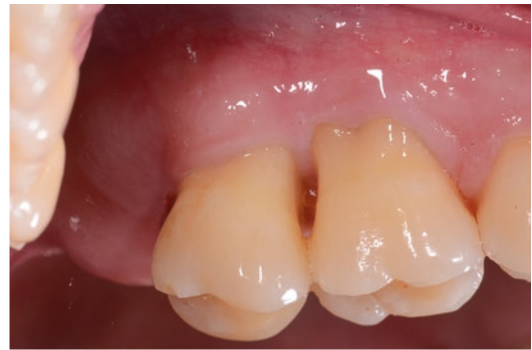

Fig. 15.10 Clinical improvements remain stable 5 years after treatment, excellent oral hygiene, and regular professional care

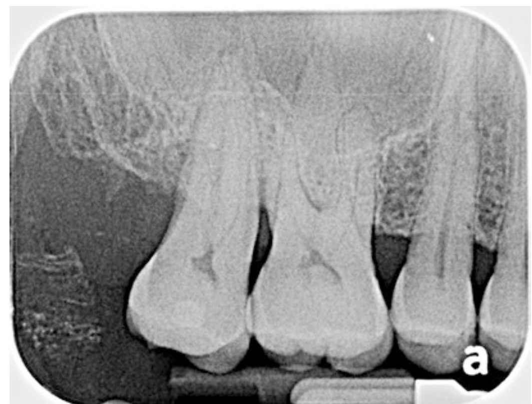

Fig. 15.11 Radiographic findings 5 years after treatment are consistent with the clinical findings of periodontal stability

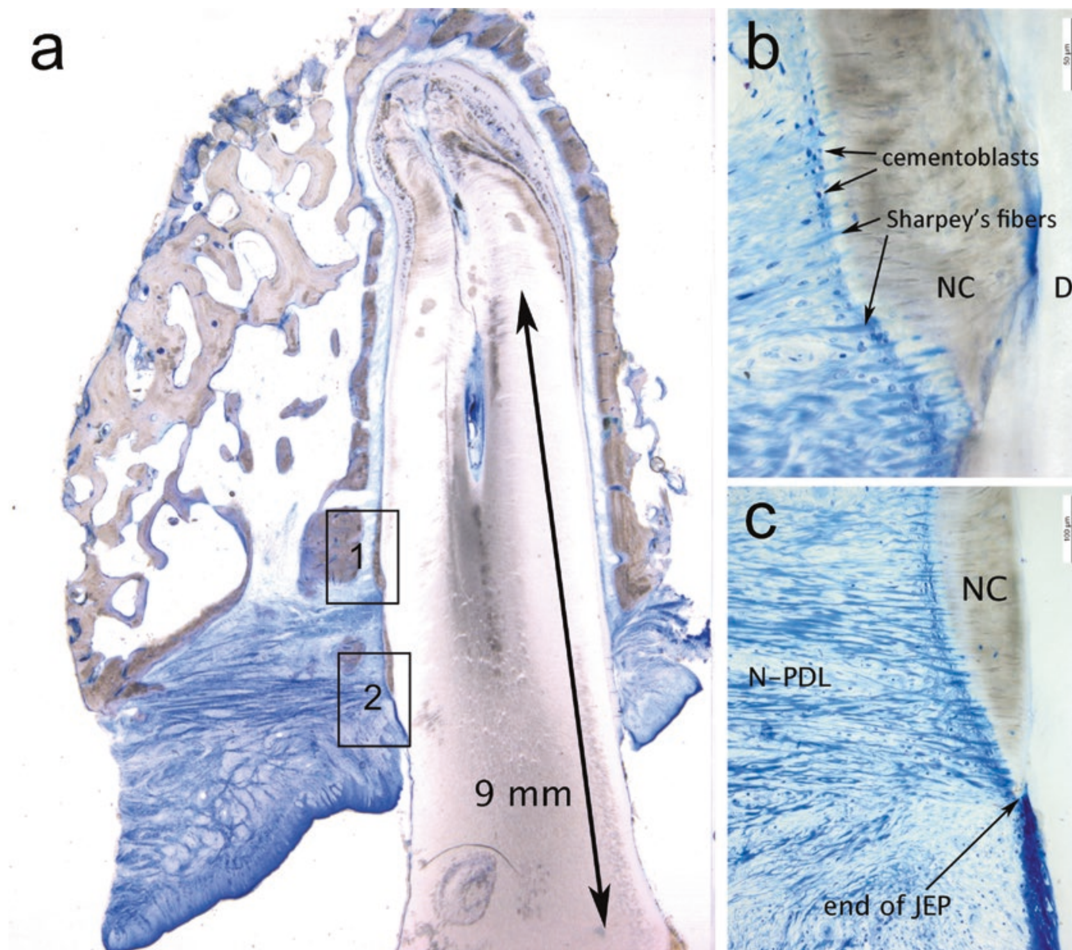

Fig. 15.12 (**a**) Panoramic histologic view showing bone fill and periodontal regeneration of an intrabony defect 9 months after treatment with laser-assisted new attachment procedure (LANAP). The arrow confirms the 9-mm notch (base of calculus) measurement from cementoenamel junction made at time of surgery. (**b**) Higher-magnification view of box 1 shows inserting Sharpey fibers into the new cementum (NC) and presence of cementoblasts. D (dentin). (**c**) Higher-magnification view of box 2 shows supracrestal collagen fibers inserting into the new cementum (NC) just apical to the junctional epithelium (JEP). The layer of new cementum extends to the coronal extent of the defect with adjacent new periodontal ligament (N-PDL). [Images courtesy of Dr. Marc L. Nevins. Reproduced and adapted from M. L. Nevins et al., Human clinical and histologic evaluation of laser-assisted new attachment procedure. Int J Periodontics and Restorative Dent 32: 497-507, 2012; with permission© 2012 By Quintessence Publishing Co, Inc. [147])

The exploration of laser irradiation for periodontal regeneration has been reported within clinical case series and, infrequently, within controlled clinical trials. Nevertheless, histologic evidence provides proof of principle that laser therapy, particularly the proprietary laser-assisted new attachment procedure (LANAP®), supports periodontal regeneration, including new bone, cementum, and periodontal ligament [140, 147, 157]. One such case series applied LANAP®, utilizing a Nd:YAG laser, for the surgical management of periodontitis and was pivotal in providing histologic evidence of periodontal regeneration following a laser surgical procedure on teeth that were determined to be hopeless in their prognoses [147] (Fig. 15.12). Clinical outcomes

reported for this series yielded a mean probing depth reduction of 5.4 ± 2.64 mm and mean clinical attachment level gain of 3.8 ± 2.38 mm after 9 months of follow-up. Another case series incorporated the Er:YAG laser into traditional regenerative periodontal surgery [158]. In this study, nine intrabony defects were surgically debrided via curettes and included the adjunctive use of the Er:YAG laser to complete degranulation; then the Er:YAG laser was applied to the root surfaces. After the application of enamel matrix derivative and bone graft, the laser was applied to establish a coagulated blood clot over the graft. At 12 months, the mean defect depth was reduced from a baseline of 6 mm to 1 mm, the mean probing depth was reduced from 6.2 mm to 2.0 mm, and the mean clinical attachment level improved from 7.5 mm to 3.4 mm [158] at 12 months. An assessment of the comparative efficacy of laser treatment was not possible in this study.

A meta-analysis of controlled clinical studies which included Nd:YAG or Er:YAG laser treatment groups and guided tissue regeneration with enamel matrix protein found only minor differences between the laser and non-laser treatment groups when comparing probing depth reduction and clinical attachment level gain favoring the laser group, weighted mean differences of 0.01 mm and 0.10 mm, respectively. No significant differences between the surgical therapies, with or without the laser treatment, were noted in this meta-analysis, and the authors concluded the level of evidence was limited for the inclusion of laser treatment in regenerative surgical therapy. [145]

15.6 Implant Therapy

15.6.1 Peri-implant Mucositis

Nonsurgical mechanical therapy, good oral hygiene, and regular professional care are only modestly beneficial in treating peri-implant mucositis [159]. A growing concern is that peri-implant mucositis may not be completely reversible with treatment [160]. Only three laser studies were identified in a recent review of laser treatment of peri-implant mucositis [161], and the authors conclude there was no evidence of added benefit of laser therapy for mucositis. Future clinical trials, therefore, are necessary to evaluate the potential benefit of this approach.

15.6.2 Peri-implantitis

15.6.2.1 Nonsurgical Treatment Outcomes for Peri-implantitis

Application of lasers for the treatment and decontamination of dental implants has been considered to improve nonsurgical outcomes and overcome the shortcomings of traditional mechanical debridement. Inclusion of local delivery of antimicrobials has not overcome the limitations of the surface topography of the infected dental implant surface. Two recent systematic reviews explored nonsurgical treatment of peri-implantitis with lasers. Chambrone et al. [162] focused on antimicrobial photodynamic therapy (aPDT) as a treatment modality. Overall, aPDT provides similar results to conventional nonsurgical therapy. One randomized controlled study, however, reported that the adjunctive use of aPDT resulted in about a 1 mm greater reduction in mean probing depth than debridement alone at implant sites with initial probing depth ≥ 4 mm [163]. While clinical improvement occurs following aPDT, when compared to scaling alone, it was concluded that no additional benefit was manifest. The authors noted that additional conclusions were not possible due to the restricted base of evidence for some treatment approaches and conditions [162].

Significant improvement in attachment level can occur when aPDT is combined with mechanical scaling, but probing depths, bleeding, and plaque levels did not reach significance in a network meta-analysis [164]. A network meta-analysis was conducted to compare across interventions, namely, a comparison of photodynamic therapy with mechanical debridement to local drug delivery with mechanical debridement, since no direct comparisons are available in existing clinical trials. The analysis allowed for indirect comparisons of pooled existing studies where no direct comparison was available by

study treatment groups using common outcome measures: probing depth, clinical attachment level, bleeding, and plaque scores. The quality of the evidence was still considered low in this reported network analysis, concordant with the conclusions of Chambrone et al. [124] in their report on the best-evidence consensus [164].

Nonsurgical adjunctive use of laser irradiation for the treatment of peri-implantitis with diode or erbium lasers results in a significant reduction in bleeding on probing [161] when compared to non-laser treatment. However, given the lack of long-term clinical studies for this comparison, the bleeding reduction should be considered short term (a year or less). Even with long-term follow-up, it can be difficult to discern treatment effects from other factors, such as level of oral hygiene. Furthermore, no statistical difference relative to probing depth reduction was attained for meta-analysis of adjunctive use of lasers when treating peri-implantitis. Of note, in the same review and meta-analysis, a significant but slight mean bone level loss with nonsurgical laser treatment was reported [161]. Thus, other than bleeding on probing and mean bone level, adjunctive nonsurgical laser treatment results in a nonsignificant change in the clinical outcomes of probing depth, plaque levels, and recession relative to the net changes of mechanical debridement alone.

15.6.2.2 Surgical Treatment Outcomes for Peri-implantitis

The surgical treatment of peri-implantitis has shown potential for clinical benefit; however, the predictability of treatment approaches remains unclear [165]. In a meta-analysis of nonsurgical and surgical treatment, it was concluded that regenerative surgical treatment of peri-implantitis was most effective when compared to nonsurgical and resective surgical techniques [166]. Recent comparative summative reviews have not included the evaluation of laser surgery as a modality for peri-implantitis treatment.

Early application of laser therapy by Romanos et al. [167] reported that decontamination of implant surfaces with a CO_2 laser, in combination with augmentative techniques, was effective for establishing radiographic bone fill. The CO_2 laser did not harm the implant surface and presumptively aided in clot formation. Interestingly, a surgical flap was elevated and then the implant surface laser treated in this report. As such, there are currently no controlled clinical trials with the use of lasers as a monotherapy [161]. In a best-evidence review of laser therapy for peri-implantitis [161], nine studies were identified with lasers as a surgical intervention. Analysis of only Er:YAG, CO_2, and diode lasers was possible since no controlled studies were available for other laser types. When compared with debridement by hand with curettes and antiseptics in combination with a surgical flap access, the addition of laser treatment showed only minimal to no benefit in reduction of probing depth and bleeding on probing or gain in clinical attachment level. Meta-analysis of long-term (greater than 48 months) outcomes following surgical treatment, with and without the addition of laser treatment, yielded weighted mean differences of 7.26% for bleeding on probing reduction, 0.22 mm for clinical attachment level gain, and 0.45 mm of probing depth reduction.

Although minimal additive benefit may be realized with the addition of laser treatment to surgical treatment of peri-implantitis, in a report that factored analysis of cost-effectiveness of combinations of therapy for peri-implantitis into the assessment of varied treatment modalities, it was concluded the most effective treatment combination includes bone grafts, barrier, and laser treatment [168].

The incorporation of lasers remains an effective application for peri-implantitis treatment, including regenerative therapies (Figs. 15.13, 15.14, 15.15, 15.16, 15.17, and 15.18). Systematic reviews indicate that although peri-implantitis treatments can produce successful outcomes, no strong evidence is available to suggest the most effective treatment intervention [7]. A more recent case series [169] utilized an Er:YAG laser for implant surface and defect debridement of the granulomatous tissue prior to grafting the peri-implant defects. Probing depths of 6 mm or greater were reduced on average to 3.5 mm and presented with radiographic defect fill 1 year following treatment. Unfortunately, this report did not have standard-

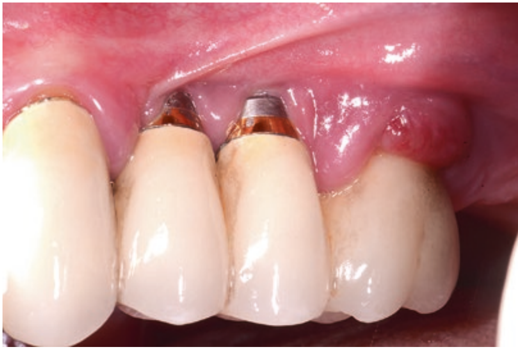

Fig. 15.13 Pretreatment clinical presentation of a 59-year-old Caucasian man (treatment was provided by PSR). Medical history includes cigarette smoking (approximately one pack of cigarettes per day) and arthritis. Dental implants were placed in another office 7 years prior, with cemented restorations. Purulence and bleeding upon probing are present. Significant swelling and erythema of the soft tissues are evident posteriorly

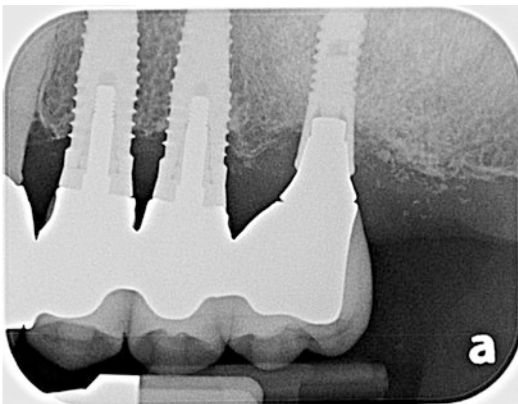

Fig. 15.14 Pretreatment radiograph suggests early to moderate bone loss around the dental implants. A gap appears in the crown-abutment interface of the posterior implant

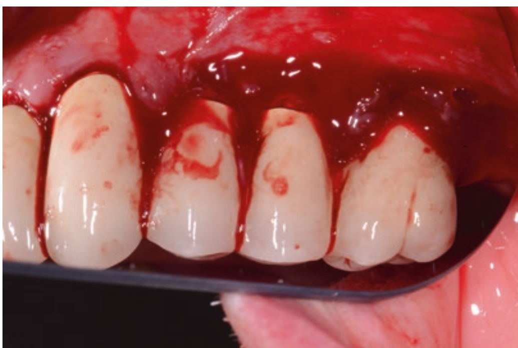

Fig. 15.15 The dental implants can be seen after the second pass using the laser-assisted peri-implantitis procedure with the Nd:YAG laser (3.8 W for each pass). Postoperative management included amoxicillin (500 mg t.i.d.) for 1 week and a botanical rinse twice daily (PeriActive, Izun Pharma) for 2 weeks

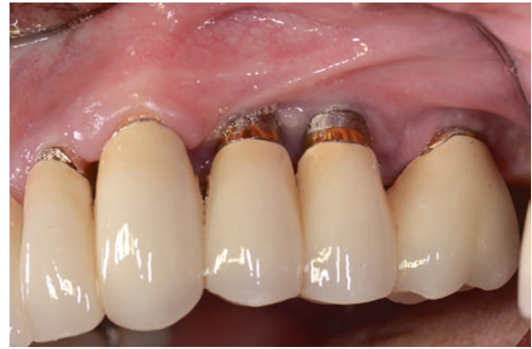

Fig. 15.16 Buccal view of the implants 6 weeks following the laser treatment. Note the significant clinical resolution of inflammation, with minimal soft tissue recession. Bleeding on light probing is absent

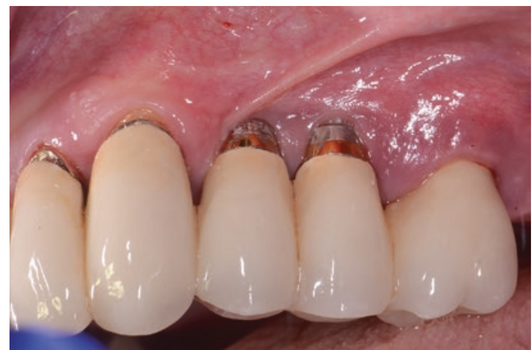

Fig. 15.17 Buccal view of the implants 1 year following the laser treatment. Initial improvements in soft tissue appearance remain stable. Supportive maintenance care was performed on a 3-month interval

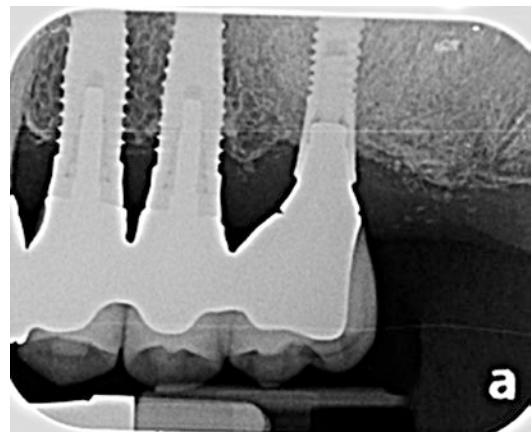

Fig. 15.18 Radiographic appearance at 1 year suggests no change in crestal bone levels or possible improvement (distal of implant #15)

ized repeatable measurements or a control group to ascertain the impact of laser versus curettes for bone debridement. Currently, in the absence of histologic data, the effect of lasers on the re-osseointegration of dental implants remains unclear.

15.7 Patient Preferences

Patient preferences in treatment decisions are an important consideration in the overall assessment of laser therapies, especially given the factors commonly influencing treatment selection, such as cost, convenience, comfort, and clinical outcomes, among others. Laser therapies often achieve clinical improvements comparable to conventional periodontal/peri-implant treatment, as reviewed earlier, and can be associated with less discomfort and pain [170, 171]. The adoption of lasers into clinical practice continues to rapidly increase, and practitioners incorporating lasers into clinical care continue to report high levels of satisfaction [171]. Patient preferences, therefore, must be taken into consideration when reviewing treatment options.

15.8 Conclusions

Lasers have the capacity to stimulate cellular activity, reduce inflammation, and promote wound healing. Surgical treatment with lasers is generally associated with less pain than conventional therapy. Lasers have been shown to provide an effective and safe alternative to conventional therapeutic approaches for biofilm disruption and calculus removal. Laser treatment as a monotherapy or adjunct to conventional therapy has been generally associated with improvements in clinical attachment level and probing depth comparable or marginally superior to conventional therapy. Nevertheless, systematic reviews conclude that clinical outcomes of laser treatment are similar or slightly better than reference nonsurgical or surgical therapies; however, any differential benefits remain short term. Moreover, there is limited human histologic evidence that is consistent with the potential for periodontal regeneration following laser-assisted therapy in patients with moderate to severe periodontitis. The ability of lasers to interact with the periodontium and surrounding peri-implant tissues warrants the continued development and evaluation of laser treatment protocols that capture the cellular, biochemical, and molecular potential of laser energy.

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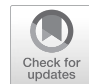

Spatiotemporal Controls of Tooth-Supportive Structure Neogenesis by 3D Printing Technology

16

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16.1 Periodontal Disease: Causes and Effects

Periodontitis is a common inflammatory infectious disease with high prevalence to periodontal complexes [1]. The periodontal disease or the destruction of periodontal constructs is generally caused by bacteria and their products like lipopolysaccharides (LPS), which physiologically activate osteoclastogenesis or differentiate osteoclastic cells [2]. Periodontal diseases or traumatic injuries can particularly lead to destruction of tooth-supportive structures such as alveolar bone and periodontal ligaments (PDLs) and loss of teeth [3]. Over 40% of over 30-year-old adults in the USA have periodontal disease, and approximately 7% possess the most severe form [4, 5]. Moreover, periodontal disease is suspected as a risk cofactor in systematic diseases [6].

16.2 Anatomy of Periodontal Tissue Complexes with Systematic Functions

The periodontium is composed of four different tissues (Fig. 16.1): gingiva, cementum (mineralized layer on the root surface), PDL (micron-scaled space with perivasculature and Sharpey's fibers), and alveolar bone (mineralized tissue to support tooth structures). The PDL is a fibrous connective tissue bundle containing a heterogeneous cell population and obliquely/perpendicularly oriented to the tooth-root surface with structural integration between the bone surface and cementum to generate biomechanical responses against mastication or occlusion. As shown in Fig. 16.1, the periodontal ligament of a single-rooted tooth is subdivided into four different groups in order to resist vertical and intrusive forces: the alveolar crest (radiated bundles), horizontal (perpendicularly oriented bundles), oblique (obliquely oriented bundles coronally attached to the bone), and apical PDL (radiated bundles) groups (Fig. 16.1). This ligamentous tissue has a spatial angular organization and high vascularity that provide a nutritive function for the periodontium. For the tissue anchorage, Sharpey's fibers associated with the PDL interface facilitate to insert ligamentous bundles to the mineralized surface for biomechanical responsiveness.

16.3 Traditional Strategies for the Periodontal Regeneration Procedure

Conventional therapeutic strategies for periodontal tissues have been mainly focused to eliminate periodontitis-associated bacteria and

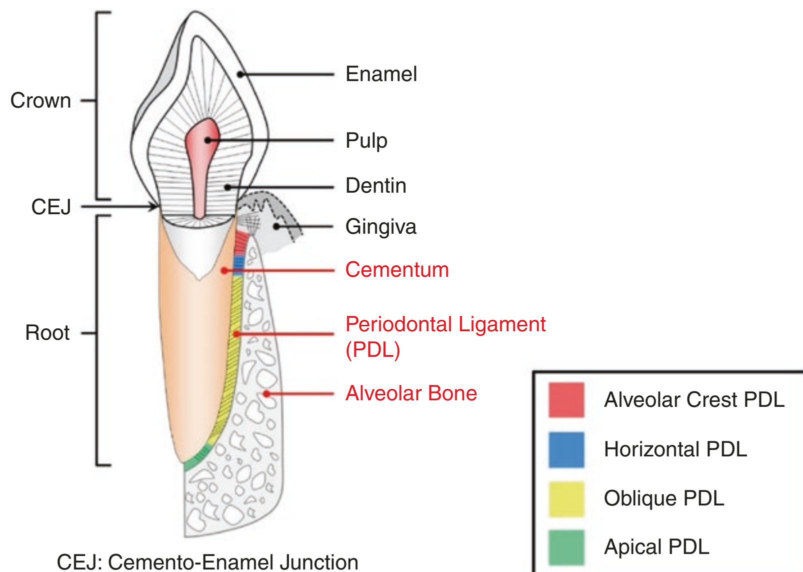

Fig. 16.1 Schematic illustration of periodontal tissue complex and periodontal ligament (PDL) fiber arrangements between the tooth and the alveolar bone. *Adapted from Park* et al. *J Dent Res 2014;93(12):1304–1312 with permission of SAGE Publications* [7]

biofilm which can induce periodontitis or gingivitis. Such procedures include scaling and root planing and respective surgery or bone regenerative surgery in combination with guiding tissue members or biologics. Although nonsurgical or surgical periodontal therapies have been developed for periodontal tissue regeneration after periodontal destruction by diseases or traumatic injuries, it is still challenging to encourage multiple tissue (cementum-PDL-alveolar bone) and structural integration of regenerated complexes within micron-scaled interfaces [3, 8]. Traditional strategies are relatively unpredictable and uncontrollable to spatiotemporally organize and compartmentalize periodontia with micron-scaled interfaces as well as orientations of PDLs [8]. In particular, because angular coordination of PDLs in specifically categorized regions (Fig. 16.1) is critical for functionalization of nascent tooth-supportive constructs, 3D micro-architecture designs using biopolymeric materials for scaffolding systems have been recently developed to control perpendicular or oblique organizations of PDLs using the 3D printing technology to created micro-patterned structures [9–11] including the freeze-casting method to control longitudinal pore angulations [7].

16.4 Biomaterial-Based Approaches for Periodontal Tissue Regeneration

Research on periodontal tissue regeneration such as guided tissue regeneration (GTR) and guided bone regeneration (GBR) is still ongoing with significant evidence at the preclinical and clinical studies. The primary goal of periodontal tissue regeneration is to remove the inflammatory factor or infection source and then to provide a space by which neighboring cells can grow and regenerate the new cementum, PDL, and alveolar bone [12]. To achieve the periodontal regeneration, various types of biomaterials have been developed and applied in the clinical field. Biomaterials used in periodontal tissue regeneration are divided by two main categories: biodegradable and nonbiodegradable the biodegradable materials are also subdivided according to their components—natural and synthetic polymeric materials—and nonbiodegradable materials include metallic and polymeric materials (Table 16.1). GTR and GBR are representative dental regenerative therapies, and barrier membranes and/or bone graft materials are essential for the procedure. Nonbiodegradable membranes have developed at first. Despite advantages with their

Table 16.1 The uses of biodegradable and nonbiodegradable biomaterials for guided tissue and bone regeneration

	Type	Material	Property	Periodontal application (References)
Nonbiodegradable	Metallic	Titanium mesh	Corrosion resistance, high strength-to-weight ratio, and biocompatibility	Alveolar ridge augmentation [12–14]
	Polymeric	e-PTFE	Good space maintainer; relatively stiff; handling	Bone augmentation and GBR [15, 16]
		d-PTFE	Less than 0.3 μm pores as a barrier	GBR [17, 18]
Biodegradable	Natural	Collagen	High biocompatibility, rapid biodegradability, and no antigenicity	Collagen-based barrier membranes for periodontal regeneration and implant therapy [19–23]
	Synthetic	PLA	Hydrophilicity and relatively rapid hydrolysis	Ridge or socket preservation [24]
		PLGA	Control of hydrolysis rates with the ratio of PLA and PGA	Socket preservation [25] Scaffold for tissue regeneration [26, 27]
		PCL	Hydrophobicity and slow hydrolysis	Tissue engineering [28, 29]

solidity and formability to maintain the space for ingrowth of cells, unfavorable risks such as interruption of healing process, structural collapse of newly formed tissues, and alveolar bone resorption caused by exposure of membrane, gingival flap elevation, and removal of membrane have changed the attention to the biodegradable membranes to prevent these problems [30–32].

16.4.1 Nonbiodegradable Materials in Periodontal Tissue Regeneration

Most nonbiodegradable materials for periodontal tissue regeneration contribute as 2D barrier membranes to prevention of epithelial downgrowth, preservation of bone crest structures, or spatial secure for volumetric bone formation around defects after tooth extractions. Metallic materials such as titanium and polymers as expanded polytetrafluoroethylene (e-PTFE) or dense polytetrafluoroethylene (d-PTFE) are typical nonbiodegradable materials for preclinical and clinical applications in periodontal tissue engineering.

16.4.1.1 Titanium or Titanium Alloy

Typically, titanium (Ti) alloy is a widely used metallic material with its excellent mechanical, chemical, and biological properties, corrosion resistance, high strength-to-weight ratio, and biocompatibility [33] even though the pure Ti material is relatively softer for dental implant applications [34]. In particular, Ti alloy with specific surface modifications has the prominent osseointegration properties. Therefore, Ti alloy has been used as an implantable medical device and for tooth replacement dental implants since the 1960s. In addition to dental replacements, biocompatible Ti-membrane (or Ti-mesh) is also developed as a barrier membrane for prevention of downgrowth of epithelia into defects and preservation of alveolar bone ridges after tooth removal and prior to dental implant installation [15]. In the preclinical study, mechanical advantages enabled Ti-based products to show great bone regeneration and maturity [15], and it was well maintained if early exposure does not occur in the clinical application [18, 35, 36]. Her et al. showed substantial bone augmentation using Ti-membrane in conjunction with bone grafting [17], and Chan et al. reported successful outcomes with Ti-mesh and particulate allograft in the vertical augmentation [20].

16.4.1.2 Polytetrafluoroethylene (PTFE)

Biologically inert and chemically stable PTFE has two major types: e-PTFE as a gold standard for vertical/horizontal GBR treatments and d-PTFE with low porosity but high mechanical/biological stabilities [37]. The non-resorbable membrane generally has two different sides: an open microstructure which is suitable for collagen fiber attachment with blood clot formation for the stabilization of the membrane placement and an occlusive part which prevents the ingrowth of epithelial cells and tissues [22]. Although the longest clinical experience with e-PTFE has proven the success of bone regeneration from stable space maintenance and easy handling [22, 38, 39], the porous structures of e-PTFE can allow the adhesion of bacteria or biologics on the membrane surface, bacterial biofilm formation, and severe infection by the membrane exposure in the oral environment [37]. Therefore, d-PTFE as alternative of e-PTFE is also featured because of a high density and small pore size that protects the grafting materials from oral contamination [19, 23] and easy clinical manageability [37].

16.4.2 Biodegradable Materials in Periodontal Tissue Regeneration

Biodegradability of implantable biomaterials depends on the hydrolysis in individual polymers including the enzymatic degradation process as the catalysis in physiological environments [40]. In particular, biopolymeric materials critically categorize as natural or synthetic materials [40] and clinically applicable biodegradable materials in periodontal tissue engineering are relatively limited such as collagen material as the

natural and poly (lactide-co-glycolide) (PLGA), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(ε-caprolactone) (PCL) as the synthetic [3].

16.4.2.1 Natural Materials: Collagen and Gelatin

Of the diverse structural components that comprise periodontal complexes, type I collagen is the major in periodontal connective tissues and mineralized structures. Due to proteins and biologics in the collagen structures, collagens strongly contribute for wound healing and various tissue formations with tissue maturation to restore their functions [41, 42]. Moreover, high biocompatibility, rapid biodegradability, and no antigenicity are the key properties to generate appropriate cell responses like proliferation, differentiation, or apoptosis for target tissue regenerations [21, 43]. In particular, type I collagen as a major organic component of the bone is commonly used as collagen-derived membranes from different sources such as bovine or porcine [43]. In general, highly porous collagen scaffolds could have the volumetric shrinkage by cell-mediated contraction, and tissue infiltration into the collagen construct could be difficult [44, 45]. To prevent unpredictable volume changes and improve the mechanical properties of micro-/macro-architectures, various cross-linking treatments with biocompatibility are investigated for the development of biodegradable membranes for alveolar bone regeneration [46]. In particular, the treatments can manage biodegradation rates in physiological environments, cell-material interaction by biologics in membranes, or mechanical improvements [45].

Many studies evaluating GTR using collagen membranes in intrabony defect have shown the greater reduction of periodontal probing depth, increases in clinical attachment gain, and bone regeneration than open flap debridement (OFD) [43]. Many trials have been performed to enhance the regenerative potential by combining with bone graft materials to strengthen the space maintenance and provide osteo-conductive or inductive capacity [47, 48]. Although the collagen has many great advantages in biological and physiological aspects for tissue regenerations, it is limited to manufacture of micron-scaled constructs with sufficient mechanical properties for periodontal complex regeneration and anatomically adaptable architectures using 3D printing systems.

16.4.2.2 Synthetic Materials: PLGA, PLA, and PCL

Different biopolymer-based scaffolding systems have been developed to facilitate tissue regeneration with different biologics, but the temporal control of tissue regeneration rates and spatial provisions for tissue infiltration and periodontal compartmentalization to damaged tissue sites are still challenging with conventional scaffolding systems [3]. Therefore, 3D printing strategy can be one promising approach to promote multiple tissue formations securing specific dimension for individual tissues and their functioning restorations with structural systemic integrations in specific characterized architectures [8, 49]. In particular, periodontal tissues have the complicated spatial geometry with specific functions to support the teeth under mastication or occlusion [49], so spatial compartmentalization and provision are critical to optimize tissue regeneration with functioning restorations.

Polylactide (or polylactic acid; PLA) is a biodegradable polyester with the stereocomplexation between poly (L-lactic acid) (PLLA) and poly (D-lactic acid) (PDLA) [50]. It has excellent mechanical and thermal properties with significant hydrophilicity without any chemical or physical treatments of material surfaces [51, 52]. In dental applications, PLA is utilized as a tooth-extraction socket filler (or space filler) for the ridge preservation and bone regeneration with dimensional maintenance [53]. PLA has the relatively slower hydrolytic degradation rate than polyglycolide (or polyglycolic acid; PGA), which has the rapid biodegradability by hydrolytic and enzymatic degradation in physiological conditions. However, acidic products from rapidly biodegraded PGA can significantly have the acute inflammatory reactions [54], so poly (lactide-co-glycolide) (PLGA) is investigated as a resorbable polymer which is derived from

a random copolymerization of PLA and PGA with certain ratios (PLA/PGA) [27]. In particular, depending on the ratio, engineered PLGA can control degradation rates and regulate to produce acidic environments [27]. Moreover, PLGA has a great cellular affinity such as adhesion and proliferation; therefore, it is widely used in the medical or dental application such as the scaffolds for drug delivery [55–58]. Of the various biopolymer-based materials considered for biomedical applications, poly(ε-caprolactone) (PCL) is an aliphatic polyester which has good solubility to organic solvents, is nontoxic, and has low melting temperature (55–65 °C) [40, 59]. In addition, the ductility in physical or mechanical properties can provide the high fracture resistance and the processing of PCL-based products to improve adaptation to defects during surgery [60, 61]. Due to the high biocompatibility and the slower degradability (or hydrolysis) as compared to other biopolymers, PCL is more widely used for long-term implantable devices like controlled-release systems and cardiovascular, nerve, bone, or tracheal tissue regenerative procedures [61, 62]. In particular, PCL has been clinically approved by the US Food and Drug Administration (US FDA) for humanitarian indications (i.e., selective laser sintering (SLS) periodontal scaffolds or tracheal splints) in clinical situations [63–65].

16.5 3D Printable Biopolymeric Materials for Periodontal Complex Regeneration

Different biologics could accelerate the formation and maturation of mineralized tissues around the teeth or dental implants, but excessive bone formations could also induce the ankylosis or bone fusion to tooth-root surface excluding periodontal ligaments (PDLs) which play a critical role to generate biomechanical responses against mastication or occlusion. In the results, it could make a damage to the teeth or supportive bone structures after masticatory stimulations. Therefore, it is significantly required to preserve spatially compartmentalized regions to generate individual tissue formations like PDL, alveolar bone, and cementum as well as design architectural interconnectivity for tissue integrations for functioning restorations [66]. Of technologies, 3D printing technique has been recently highlighted to consequently overcome the limitations [3, 49, 67].

Prior to designing and manufacturing scaffolds for periodontal tissue regeneration by material-direct printing systems, the properties of biomaterials could be important to manufacture micron-scaled features with complicated interiors of scaffolds to guide regeneration of periodontal complexes [70]. In the aspect of biocompatibility or biological responsiveness, natural materials or collagen-based biomaterials have significant advantages compared with synthetic polymers. However, it is quite difficult to have adjustments of mechanical properties to target tissues like the bone as well as preserve their bioactivity during manufacturing of 3D features [70]. Recently, decellularized matrices have been studied and investigated as bioink materials for the bioprinting in musculoskeletal tissue engineering and regenerative medicine [71, 72]. However, due to the easy fabrication with more predictable properties, synthetic polymer materials have been widely utilized with different manufacturing systems: selective laser sintering (SLS) and fused deposition modeling (FDM) or periodontal tissue engineering [73, 74]. In addition, 3D wax printer can manufacture material-casting wax molds with diverse sophisticated architectures, and it is more beneficial to select various materials for biological scaffolding systems [73, 74].

PLGA and PCA have been applied as some of the most common polymeric materials for GTR and GBR membranes as a barrier to prevent gingival tissue invasion and collapse into the bony defect sites [75]. The materials can be rapidly degraded by physiological immune responses and subsequent acidic products. However, because of their brittleness, it is more advantageous to create PLGA, PLA, or PGA membranes rather than 3D constructs, which are more challenging to fabricate. Recently, PCL biopolymer has been commonly utilized because it is ductile and easily post-fabricated before transplantations, even though it has slower biodegradation rates than

Table 16.2 3D printing systems categorized by resolutions and accuracy for periodontal engineering applications

3D printing systems	Applicable biomaterials	Resolutions and accuracy	Applications in periodontal engineering
Fused deposition modeling (FDM)	PCL	Low resolution Low accuracy	– Multiple dimensions of microchannels for periodontal complex formation [68]
3D wax printing	Paraffin materials to cast biopolymers (PCL, PLGA, PGA, etc.)	High resolution High accuracy	– Rat fenestration defect model with geometric adaptation to surgically created periodontal defects [69] – PDL scaffolds to control angular orientations [9]
Selective laser sintering (SLS)	PCL	Low resolution High accuracy	– First clinical trials for PDL and bone regeneration [64]

PLGA and PLA. In particular, bone scaffolds were manufactured by fused deposition modeling (FDM) using PCL and assembled to electrospun PCL membrane for PDL cell sheets for periodontal complex neogenesis [76] (Table 16.2). Lee et al. investigated the multiphasic scaffold to design microchannels using FDM for cementum, PDL, and alveolar bone with different dimensions: 100 μm, 600 μm, and 300 μm, respectively [68] (Table 16.2). The three different phases could contribute to tissue compartmentalization within micron-scaled interfaces as well as periodontal tissue regenerations [68].

16.5.1 3D Printing Architectures for Preclinical Periodontal Complex Regeneration

For preclinical studies, Park et al. investigated PCL-based scaffolding system to guide periodontal tissue regeneration like PDL, alveolar bone, and limited cementum in the rodent fenestration defect model [66, 69] (Table 16.2). After surgical creation of periodontal defects with denuded tooth-root surface and exposed dentinal tubules, cadaveric defects were scanned using micro-computed tomography (micro-CT) to generate the medical image dataset. Based on the image data, defect-adaptable scaffolds were designed by computer-aided design (CAD) program, and the 3D wax printer manufactured wax molds to cast 25% PCL solution. In particular, it is significantly advantageous that 3D-printed wax molds could allow casting of various polymeric materials in selective solvents like acetone, 1,4-dioxane, or chloroform. At this point, because sacrificial wax materials have different solubility to solvents, the wax molds should be carefully designed with casting polymer solutions [69].

After transplantations of scaffolds in preclinical models, different tissues (bone-PDL-cementum) could be formed with spatial compartmentalization and their integrations in a single scaffold architecture [69]. Interestingly, ligamentous tissues were specifically aligned with orientation similarity to native PDL structures. Despite the limited formation, mineralized layers could be found on denuded tooth-root dentin surfaces and adjacent to regenerated PDLs [66, 69]. To validate PDL integrations to the teeth with cementum-like tissues, functioning restorations of PDLs were investigated with periostin expressions, and 3D-printed, fiber-guiding scaffolds could promote periodontal complex formation with multiple tissue types such as bone, PDL, and cementum [69]. Therefore, the 3D printing strategy for periodontal tissue engineering facilitated to regulate micron-scaled periodontal tissue formations and lead to functioning integrations. Based on the design for periodontal regeneration, 3D customized fiber-guiding scaffolds were recently developed with geometric adaptation to the one-wall defect using the canine model (Fig. 16.2).

After designing and manufacturing PCL scaffolds using a 3D wax printer and the solvent casting method, PCL scaffolds were examined for adaptations to tooth-root structures using the 3D-printed models and quantitatively analyzed (Fig. 16.2) [9]. For the adaptation analysis, the gap distance (d^{gap}) between individual PDL archi-

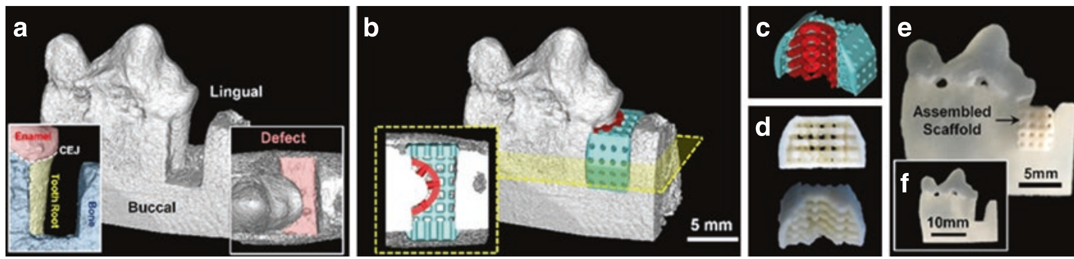

Fig. 16.2 (**a**) The one-wall defect model of a harvested canine mandible was surgically scanned using micro-CT to generate the 3D digital image dataset. Using the reverse-engineering technology, (**b**, **c**) the fiber-guiding scaffold for PDL and bone regeneration compartments was designed and (**d**, **e**) manufactured a PCL scaffold. (**f**) The periodontal defect prototype was manufactured using the 3D printer. *Adapted from Park* et al. *Int J Mol Sci 2017;18(9):1927 with permission of MDPI under CC BY 4.0* [9]

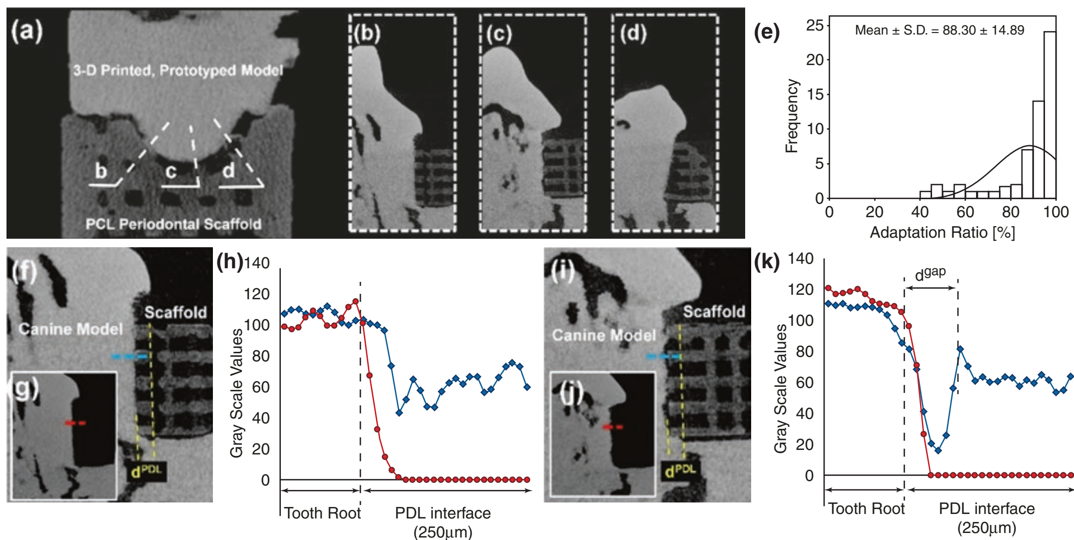

Fig. 16.3 PDL structures of the scaffold were created with perpendicular directionalities with geometrical adaptations to the tooth-root surface. Every analysis was performed with micro-computed tomography (micro-CT) with 2D coronal (**a**) and sagittal section images (**b**–**d**, **f**, **g**, **i**, **j**). (**e**) The gap distance (d^{gap}) between individual PDL architecture and root surface was measured, and the high adaptation ratio (88.30% ± 14.89) was calculated. Scaffold adaptability was measured and calculated using the grayscale-based histograms from the part of tooth-root (**h**, **k**). *Adapted from Park* et al. *Int J Mol Sci 2017;18(9):1927 with permission of MDPI under CC BY 4.0* [9]

tecture and modeled toot-root surface was measured (Fig. 16.3). Using the histogram from the micro-CT image datasets, the adaptation ratio could be calculated with d^{gap} and the length of PDL (200 μm < length of PDL (d^{PDL}) < 300 μm; Fig. 16.3).

In addition, the use of a 3D wax printer would generate predictable surface errors called stair-stepping errors with programed intervals which were controlled by the slicing step before the 3D printing. By positioning designed PDL scaffolds with three different angles, different micro-groove patterns on PDL architecture surfaces could be created with predictably controlled pattern intervals. In results, micron-scaled surface topography played a critical role to regulate PDL angulations, and specific intervals could make more predictable angular organizations of ligaments (Fig. 16.4). In general, surface patterns could be represented as the manufacturing artifacts, and people remove them to have smooth surface features. However, Park et al. reinter-

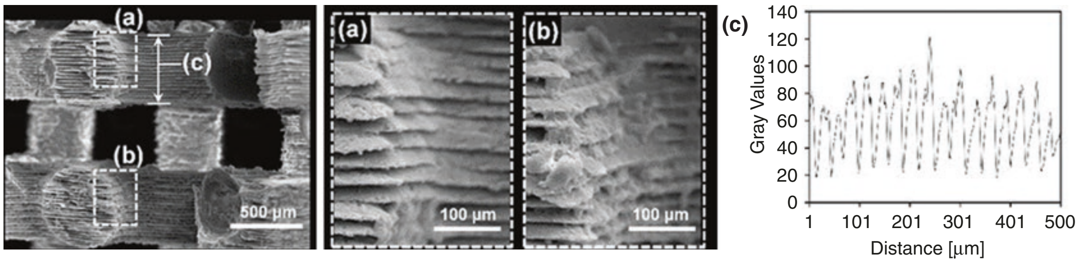

Fig. 16.4 SEM images demonstrated the parallel microgroove patterns to PDL architectures of the fiber-guiding scaffold with geometric adaptation to periodontal one-wall defect. *Adapted from Park* et al. *Int J Mol Sci 2017;18(9):1927 with permission of MDPI under CC BY 4.0* [9]

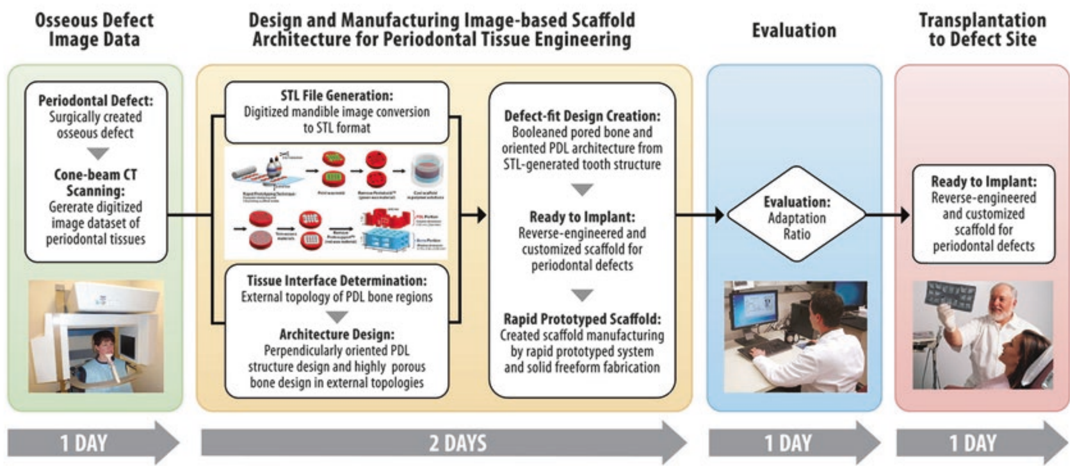

Fig. 16.5 (**a**, **b**) The high magnification images of PDL architectures to show the microgroove patterns on the surface. The patterns could provide the key micro-structures to guide PDL cells and tissues with perpendicular angulations to the tooth-root surfaces. (**c**) The microgroove pattern intervals were quantitatively analyzed and the 3D printing system could predictably and accurately create 25.40 mm-intervals of microgroove patterns the PDL-guidable architectures

preted the artifacts as the microgroove patterns to angularly control ligament cells and tissues with high populations for 3 weeks in vitro [9].

16.5.2 3D Printing Architectures for Clinical Periodontal Complex Regeneration

For periodontal tissue regeneration, the first clinical report using the SLS system to manufacture the periodontal scaffolds addressed that the approach facilitated to design and manufacture complicated, unpredictable geometries with micron-scaled architecture dimensions. Following the flowchart, the customized fiber-guiding scaffolds were manufactured by 3D printing system (SLS) and clinically transplanted the construct to the labial defect (Fig. 16.5) [66].

After the cone-beam CT scan of the patient periodontal defect, digitized 3D image datasets were utilized to create fiber-guiding scaffolds (Fig. 16.6), and the SLS manufactured PCL constructs with high adaptability to the defect (adaptation ratio: 82 ± 7%) [64]. The SLS-manufactured PCL scaffold covered the destructive labial defect region and was secured with biodegradable screws. Scaffold transplantation site had no clinical signs like acute/chronic inflammation, infectious symptoms, and dehiscence for 12 months [64].

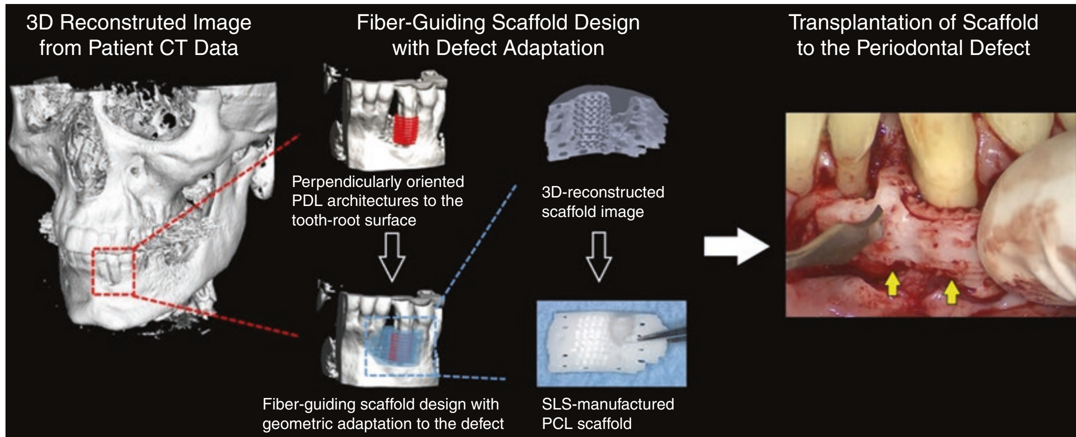

Fig. 16.6 The customized periodontal scaffolding system design and manufacture using CT scanned image dataset and SLS technique for a PCL scaffold. *Modified from Rasperini* et al. *J Dent Res 2015;94(9 Suppl):153S–157S with permission of SAGE Publications* [64]

16.6 Future Prospects for the Use of Novel 3D Scaffolding Technologies for Periodontal Regenerative Medicine

This chapter highlights the many advances ongoing in periodontal regenerative medicine for the development of more predictable clinical therapies. Biomaterials that can be better controlled for biomechanical, defect adaptation and bioresorption characteristics will have many greater applications in the clinical arena. These new designs will allow more rapid chairside, intraoperative applications by 3D printing of scaffold constructs to periodontal defects. Further, the use of combination therapies (cells, genes, and/or biologics) with these new materials will make for more bioactive constructs that can improve regenerative outcomes. The use of 3D bioprinting can serve to immobilize these reparative entities onto material surfaces to aid in the better programming of cells for the promotion of new tissue formation.

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