UNIVERSIDADE DE SÃO PAULO
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Association of genetic polymorphisms with resistance and susceptibility phenotypes to chronic inflammatory osteolytic periapical and periodontal lesions

Associação de polimorfismos genéticos com os fenótipos de resistência e susceptibilidade à lesões osteolíticas periodontais e periapicais

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‘If something is true, no amount of wishful thinking will change it.’

Richard Dawkins

ABSTRACT

Association of genetic polymorphisms with resistance and susceptibility phenotypes to chronic inflammatory osteolytic periapical and periodontal lesions

Chronic periodontitis and apical periodontitis are infectious diseases characterized by the inflammatory destruction of teeth-supporting tissues. The clinical presentation of these diseases is the result of the interaction between the infecting microorganisms and the host's defense mechanisms, constituting the host/pathogen barrier. Genetic variations are associated with differential susceptibility profiles, modulating simultaneously the patterns of infection and immune response. Therefore, we investigated the association of selected genetic variations with phenotypes of resistance or susceptibility to periodontal and periapical inflammatory bone resorption, as well as with changes in the sub gingival microbial profile and host's response biomarkers. The polymorphism rs4794067 (gene *TBX21*) proved significantly associated with increased risk to suffer periodontitis. Polymorphic allele-carriers demonstrated increased expression of T-bet. IFN-γ expression and bacterial load proved unaltered by genotype differences. The mutation rs333 (a.k.a. CCR5Δ32, in gene CCR5) demonstrated a protective effect against chronic periodontitis. Heterozygous subjects exhibited decreased TNF-α expression. The genetic mutation was unrelated to changes in the bacterial load of putative periodontal pathogens. The polymorphisms rs2521634 (gene *NPY*), rs10010758 (gene *TBC1D*), rs6667202 (gene *IL10*), and rs10043775 (gene *TBXO38*) proved associated with significant changes in the composition of the subgingival biofilm in chronic periodontitis patients. For apical periodontitis we employed an unbiased biomarker screening strategy based in 2D diferential electrophoresis in tandem with mass spectrometry. Among the biomarkers that proved significantly modulated, we discover a substantial upregulation of HSP27 and SERPINB1. Both proteins were preferentially localized in the cytoplasm of epithelial cells in the epithelium lining the cystic cavity and the epithelial chords of epithelized granulomas. Additionally, SERPINB1 was expressed in infiltrating polymorph nuclear neutrophils. The expression of HSP27 and SERPINB1 demonstrated a negative correlation with acute inflammation biomarkers. Overall, these genes and protein biomarkers may be promissory targets to predict the risk profile for periodontal and periapical inflammatory bone resorption.

Keywords: Chronic periodontitis. Apical periodontitis. Polymorphism. Host/pathogen barrier. Proteomics.

RESUMO

A periodontite crônica e a periodontite apical são doenças infecciosas caraterizadas pela destruição inflamatória dos tecidos de suporte dentários. O fenótipo clínico de ambas as doenças é o resultado da interação entre os microrganismos infectantes e os mecanismos de defesa do hospedeiro (barreira hospedeiro/patógeno). Ainda, variações genéticas podem conferir níveis diferenciais de susceptibilidade a tais doenças, teoricamente modulando tanto os padrões de infecção como de resposta do hospedeiro. Neste contexto, investigamos a associação de variações genéticas selecionadas com fenótipos de resistência e susceptibilidade a lesões osteolíticas periodontais e periapicais, assim como possíveis associações com mudanças no perfil microbiológico sub gengival e em marcadores de resposta do hospedeiro. O polimorfismo rs4794067 (no gene *TBX21*) demonstrou uma associação significativa com risco aumentado de sofrer periodontite crônica. Os portadores do alelo polimórfico apresentaram uma expressão significativamente aumentada de T-bet. No entanto, a expressão de IFN-γ e a carga bacteriana mostraram-se independentes do perfil genético para rs4794067. O polimorfismo rs333 (também conhecido como CCR5Δ32, no gene *CCR5*) demonstrou um efeito protetor para periodontite crônica. Os pacientes heterozigotos exibiram níveis de expressão significativamente diminuídos de TNF-α, porém, os níveis bacterianos mostraram-se independentes do perfil genético para rs333. Os polimorfismos rs2521634 (no gene *NPY*), rs10010758 (no gene *TBC1D*), rs6667202 (no gene *IL10*) e rs10043775 (no gene *TBXO38*) demostraram uma associação significativa com mudanças no perfil microbiológico sub gengival em pacientes com periodontite crônica. No caso da periodontite apical, escolhemos uma metodologia de seleção de marcadores baseada no uso consecutivo de eletroforeses diferencial bidimensional e espectrometria de massa. Dentre os marcadores que apresentaram uma modulação significativa, as lesões de periodontite apical demostraram uma supraregulação de HSP27 e SERPINB1. Ambas as proteínas foram preferencialmente imunomarcadas nas ilhas epiteliais dentro das lesões. A expressão de HSP27 e SERPINB1 apresentou uma correlação negativa com os marcadores de inflamação aguda. Assim sendo, estes genes e biomarcadores proteicos mostram-se como alvos promissórios para a determinação do perfil de risco de lesões osteolíticas periodontais e periapicais.

Palavras-chave: Periodontite crônica. Periodontite apical. Polimorfismo. Barreira hospede/patógeno. Proteômica.

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1 INTRODUCTION

1 INTRODUCTION

The infection of the periodontal tissues at the gingival margin or periapical area leads to the inflammatory destruction of periodontal ligament, radicular cement and alveolar bone. Chronic periodontitis and apical periodontitis are the most common forms of inflammatory periodontal destruction and share a plethora of pathological features, including the initiating stimulus (bacterial infection), the pathological mechanism of tissue destruction (chronic and exacerbated immune response), the susceptibility traits (environmental or genetic factors) and the treatment approach (eradication of the infecting microorganisms) (SILVA et al., 2007; HAJISHENGALLIS, 2014a; AMINOSHARIAE; KULILD, 2015; AW, 2016).

From a purely taxonomic point of view, one may argue that the point of entry of the infectious agents fundamentally determines the clinical presentation of the disease, its evolution, prognosis and treatment; while from a pathologic point of view, the essential processes underlying the clinical presentation of chronic periodontitis and apical periodontitis possesses striking similarities (GRAVES; OATES; GARLET, 2011; CAVALLA et al., 2014). Indeed, epidemiological data suggest that there is a correlation between the occurrence of apical periodontitis and marginal bone loss characteristic of periodontitis (JANSSON, 2015), implying that subjects suffering from these conditions share a susceptibility profile that makes them prone to inflammatory periodontal destruction.

Generally, there are two possible infection routes of periodontal tissues: through the gingival sulcus or through the root canal system. In the former case, the microorganism or their products gain access to the sub epithelial gingival connective tissue due to the disruption of the gingival epithelial barrier integrity, specifically the sulcular and junctional epithelium (KORNMAN et al., 1997b). In the latter case, the infectious agents gain access to the pulp chamber and root canal system after surpassing the physical barrier comprised by dental enamel and dentin, usually as a consequence of the progression of dental caries or trauma (AW, 2016; HAJISHENGALLIS; KOROSTOFF, 2017). Independent of the point of entry, the microflora that establishes and colonize the root canal system or deep periodontal pockets are extraordinarily similar (KEREKES; OLSEN, 1990). Once the microbes or

their products become in contact with periodontal connective tissue, a robust immune response is triggered. The succeeding sequence of immune/inflammatory events is the main responsible for the alterations in tissue metabolism that leads to the destruction of the teeth supporting tissues (GRAVES; OATES; GARLET, 2011).

Both chronic periodontitis and apical periodontitis are undoubtedly infectious diseases. Extensive evidence demonstrates that the presence of bacteria is the necessary stimulus for disease initiation (RICUCCI; SIQUEIRA, 2010; WANG et al., 2012; HAJISHENGALLIS, 2014a; LOURENCO et al., 2014). Moreover, over the past five decades a comprehensive field of research has exclusively dedicated to unveiling the intricate microbiological aspects of both diseases (MELVILLE; BIRCH, 1967; OSHRAIN; SALKIND; MANDEL, 1972). It is not this thesis' propose to argue contrary to this well accepted and founded microbial-etiology paradigm, but instead to focus on the host inflammatory immune response to the infectious challenge, understanding that once the response is initiated, the pathological changes that occur in the periodontal tissue are almost entirely caused by the immune and inflammatory mechanisms of infection control (HAJISHENGALLIS, 2014a; HAJISHENGALLIS, 2015).

As already pointed out, although the microorganisms are the essential and necessary stimuli for disease initiation, their presence is not sufficient to explain the pathologic phenomena that generate periodontal tissues' inflammatory destruction (SILVA et al., 2015). The inability of the immune system to eradicate the infecting pathogens, which are protected in anatomically inaccessible sites for the immune effector mechanisms (deep periodontal pockets or pulpal chamber/root canal system), generates a loop of constant activation and amplification of the immune response. In absence of active regulatory or suppressive mechanisms, this amplification loop of the immune response results in an unremitting and exacerbated response, and as a result in the progressive destruction of periodontal support (CAVALLA et al., 2014).

At this point it is essential to unequivocally ascertain that the immune response, independently of its nature, degree, extension and regulation will always be incapable to eradicate the infecting periodontal microorganism located in the sub gingival periodontal biofilm or in the root canal system (RICUCCI; SIQUEIRA, 2010; EBERSOLE et al., 2017). It is this fundamental fact that explains the pathologic developments of both diseases. The differential phenotypic presentations of the diseases are not a result of a more or less efficient immune mechanism in terms of

infection eradication, but emerge from inter individual differences in immune response regulatory mechanisms (GARLET et al., 2010; GARLET; SFEIR; LITTLE, 2014; FRANCISCONI et al., 2016). Having said that, the central theme of this thesis (and of the articles presented in it) is the analysis of determinants of resistance and susceptibility to chronic inflammatory osteolytic periapical and periodontal lesions, and more specifically, of the genetic traits that to a certain degree determine the fate of these inflammatory immune processes, and consequently the lesions' outcome.

The capacity of the host to cope with the bacterial challenge and to organize an efficient response without extensive tissue damage is the principal mechanism underlying the pathogenesis of inflammatory periodontal destruction (GARLET, 2010). A competent and regulated immune response is theoretically capable to control the spread and multiplication of the infecting microorganisms, while maintaining the metabolism, functions, and structure of periodontal tissues. However, during the progression of inflammatory periodontal destruction, the continuous presence of the microbes and their products results in an amplification loop of the immune/inflammatory response, which inevitably leads to periodontal tissue destruction (GARLET et al., 2006; GLOWACKI et al., 2015; FRANCISCONI et al., 2016).

Several environmental and host intrinsic factors can allegedly modulate the nature of the immune response, and in turn dictate the fate of the immune reaction and the course of the disease. In this process, there is a two-way regulation, where the nature of the response determines the nature of the biofilm and vice versa (HAJISHENGALLIS, 2014b; HAJISHENGALLIS; LAMONT, 2016).

Abundant evidence supports the notion that genetic factors are strongly implicated in the susceptibility to inflammatory destruction of periodontal tissues, but despite extensive efforts, the identification of specific genetic risk factors remains elusive (NIKOLOPOULOS et al., 2008; DA SILVA et al., 2017). The causes for this elusiveness are multiple, among them: the complexity of immune regulation and the multilayered and redundant nature of most immune pathways (CAVALLA et al., 2014); the incomplete understanding of the genetic regulatory mechanism of transcription (LARSSON; CASTILHO; GIANNOBILE, 2015); the existence of several checkpoints of regulation over the transcriptional level (DURAN-PINEDO et al., 2015); and the

intrinsically multigenic nature of immune mechanisms, where several genes interact to produce distinct phenotypes of susceptibility or resistance (DA SILVA et al., 2017).

Genetic polymorphic variants in key checkpoints of the immune response have been implicated as susceptibility traits for chronic and apical periodontitis, modulating the host's response and their capacity to adequately respond to infection (KORNMAN et al., 1997a; MORSANI et al., 2011; AMINOSHARIAE; KULILD, 2015; AARABI et al., 2017).

From this paradigm, it is possible to lay out a strategy to identify the key factors implicated in producing differential susceptibility to inflammatory periodontal destruction. The first possibility (and the most common in the current literature) is the selection of 'candidate genes' based on their theoretical involvement in key steps of the immune response and/or the periodontal tissue metabolism. Once the candidate gene is selected, a case/control study is used to establish the association of particular gene mutations with the disease phenotype (KORNMAN et al., 1997a). This 'genome-first' approach represents the greater part of the genetic research in periodontics for the past 20 years, and it has fundamentally contributed to the understanding of the pathogenesis of the disease. Nevertheless, it suffers from the essential flaw of studying only the small proportion of genes that have been theoretically involved in periodontitis' pathogenesis, neglecting the majority of the genome (LAINE; LOOS; CRIELAARD, 2010; LAINE; CRIELAARD; LOOS, 2012).

A second possibility is the genome-wide association study (GWAS), where a large affected population is recruited and genotyped for all known single nucleotide polymorphisms (SNPs) and compared with a suitable control population. This 'phenotype-first' approach offers the advantage of the unbiased discovery of polymorphisms associated with the trait, even if they lack a theoretical involvement with the disease (MUNZ et al., 2017). This newer approach has gained popularity over the last decade, and it is significantly expanding the catalogue of genes associated with differential susceptibility profiles for periodontitis, far beyond the classical immune response-related genes (DIVARIS et al., 2012; RHODIN et al., 2014; OFFENBACHER et al., 2016; MUNZ et al., 2017). Nevertheless, GWAS suffers from several limitations, most notably they are underpowered to detect genotypic-phenotypic associations for SNPs that simultaneously have a small effect and low frequency (<5%) (GIBSON, 2012; MAGI et al., 2012). Additionally, GWAS pose a logistical problem since they

require a large sample population (normally >5000 subjects) that needs to be phenotypically characterized (NSENGIMANA; BISHOP, 2012).

A third possibility, somewhat analog to the GWAS approach, is the screening of the protein products of diseased tissues and the comparison with appropriate control tissues. This approach has the advantages of being unbiased and to focus on the end product of genetic regulation, thus the differentially expressed protein products are the consequence of successive layers of regulation and represent a functional outcome in itself (BOSTANCI; BAO, 2017).

In our efforts to investigate the genetic factors involved in the pathogenesis of inflammatory periodontal destruction we used the former three approaches and/or combinations of them. We employed the ‘candidate gene’ strategy in a large sample population to perform genetic association studies complemented with the analysis of several secondary outcomes. We also performed a genetic association study using the topmost risk-associated SNPs discovered in three recent periodontitis GWAS, including disease phenotype and the subgingival microbial profile as dependent variables (DIVARIS et al., 2012; DIVARIS et al., 2013; SHANG et al., 2015). Finally, we conducted a proteomic analysis of diseased periodontal tissues and healthy periodontal tissue controls. After the identification of the differentially regulated proteins, we executed additional characterization including levels of expression and tissue localization.

Accordingly, here we present five articles about periodontitis pathogenesis and differential risk profiles. The diverse approaches employed are threaded together by the common objective of finding out significant targets implicated in the pathogenic chain leading to periodontal inflammatory destruction. In the future, these targets could be utilized as risk markers, diagnostic aides and/or treatment response molecular surrogates.

The first article (published) serves as an extended introduction to the subject and reviews the immune mechanisms of response to periodontal infection from the perspective of the key signaling molecules that coordinate the immune response, cytokines and chemokines. In doing so, it covers the historical development of the adaptive immune response paradigms and offers a perspective on how to approach immune research of oral inflammatory pathology (CAVALLA et al., 2014).

The second article (published) investigates the effect of a polymorphic variant of a key determinant of T cell polarization (T-bet). The methodology employed in this article is archetypal of the approach we pursued in this subject, including a classical association analysis complemented by secondary molecular and microbiological outcomes (CAVALLA et al., 2015).

The third article (under review) follows the pattern laid on the second article, this time investigating the effect of the genetic deletion of 32 base pairs from the CCR5 gene (a.k.a. CCR5Δ32) in the susceptibility to suffer chronic and aggressive periodontitis, complemented by association analysis of secondary outcomes (signaling biomolecules and putative periodontal pathogens).

The fourth article (in preparation) presents the results of a novel approach to conduct genetic association studies, focusing on candidate genes selected by unbiased tools (GWAS) and relying on the use of a surrogate variable to characterize the disease (*i.e.* subgingival microbiological pattern). In this case, we associated the occurrence of 19 selected polymorphism with quantitative changes in the composition of the subgingival microbiota, which was monitored using DNA-DNA checkerboard for 40 species of bacteria.

The fifth article (published) presents an unbiased approach to discover proteins and pathways implicated in the pathogenesis of apical periodontitis. We used two-dimensional difference gel electrophoresis (2D-DIGE) in tandem with mass spectrometry to detect and identify differentially expressed proteins in healthy and diseased apical tissues. After the exploratory phase (detecting differentially expressed proteins), we performed complementary analysis to localize and further characterize the putative role of these differentially expressed proteins in the chain of events leading to apical periodontal inflammatory destruction (CAVALLA et al., 2017).

2 ARTICLES

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The articles presented in this thesis were written according to the instructions and guidelines for article submission of the corresponding journals.

- ARTICLE 1 – Cytokine Networks Regulating Inflammation and Immune Defense in the Oral Cavity. Current Oral Health Reports.
- ARTICLE 2 –TBX21-1993T/C (rs4794067) polymorphism is associated with increased risk of chronic periodontitis and increased T-bet expression in periodontal lesions, but does not significantly impact the IFN-g transcriptional level or the pattern of periodontophatic bacterial infection. Virulence.
- ARTICLE 3 – CCR5Δ32 (rs333) polymorphism is associated with decreased risk of chronic and aggressive periodontitis: a case-control analysis based in disease resistance and susceptibility phenotypes. Cytokine. (Submitted)
- ARTICLE 4 – Association of genetic polymorphisms with subgingival microbial colonization patterns in chronic. (In preparation)
- ARTICLE 5 –Proteomic Profiling and Differential Messenger RNA Expression Correlate HSP27 and Serpin Family B Member 1 to Apical Periodontitis Outcomes.J Endod. (In press)

2.1 ARTICLE 1 – Cytokine Networks Regulating Inflammation and Immune Defense in the Oral Cavity*

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Cytokine Networks Regulating Inflammation and Immune Defense in the Oral Cavity

Franco Cavalla · Ana Claudia Araujo-Pires · Claudia C. Biguetti · Gustavo P. Garlet

Abstract The host/pathogen interaction in infectious oral diseases is characterized by complex and precisely orchestrated host response mechanisms aimed to protect the host against the microbial challenge with minimal collateral damage to host cells and tissues. Central to the host response in this battlefront is the expression of cytokines. The resulting cytokine networks, which ultimately regulate the host response at multiple levels, thereby determine the clinical outcome of the disease and explain most of its defining features from a mechanistic viewpoint. This review intends to present a structured view of the intricate cytokine networks that regulate inflammation and immune defense in the oral cavity, guiding the reader throughout the evolving paradigms that describe how the simultaneous action of multiple cytokines shapes the nature of the immune response to oral infection.

Keywords Cytokine networks · Inflammation · Immune defense · Oral cavity · Immunology

Introduction

Periodontal and periapical diseases are the most common forms of destructive infectious/inflammatory diseases of the tooth-supporting tissues, and represent the most prevalent form of bone pathology. Periodontitis is initiated by bacteria harbored in the tooth-attached biofilm invading the surrounding epithelial and connective periodontal tissues, triggering a host immune/inflammatory response and the subsequent lesion development. Similarly, lesions of endodontic origin initiate as an immune/inflammatory response to a bacterial insult of the dental pulp, which can lead to necrosis, allowing the spread of the infection front to the periapical region and the consequent lesion formation. Although both conditions share a quite common etiology, it is noteworthy that based on bacterial infection alone, it is not possible to explain these complex pathological processes [1, 2, 3••]. Indeed, while any bacteria can essentially trigger a host response, the current paradigm of periodontal and periapical disease etiology states that specific bacteria (gram negative anaerobic rods) initiate and chronically sustain the host immune/inflammatory response, the nature and extent of which are ultimately responsible for the degree of irreversible tissue destruction and disease severity [4].

In this scenario, the interplay between the challenging microorganisms and resident and inflammatory host cells is thought to determine the disease outcome. Theoretically, if the host response is efficient in keeping the microorganisms spatially confined and in limited number, the tissue homeostasis will then be preserved. Conversely, if the host response is incapable of counteracting the bacterial challenge (where the presence of specific pathogens presenting virulence factors that allow tissue invasion or interfere with host's defense mechanisms is supposed to be a critical event; as well the rupture of physical barriers that limit bacterial infiltration – such as enamel/dentin destruction), the result will be conversion to chronic inflammation, with destruction of the surrounding tissues as a consequence [5, 6].

The initial host cells' innate response involves the recognition of microbial components (i.e., LPS, bacterial DNA,

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diacyl lipopeptides, peptidoglycan, etc.) as "danger signals" by pattern recognition receptors, such as the toll-like receptors (TLRs) [7]. TLRs are expressed by both resident cells and leukocytes in the periodontal/periapical environment, and upon their activation, an intracellular signaling cascade is stimulated, leading to activation of the transcription factors that mediate the cellular response and the subsequent production of inflammatory mediators, such as cytokines [8].

Cytokines exert their functions through binding to specific cellular receptors, therefore both cytokine and receptor expression can determine the nature of an individual cell response, and in a broader context, can impact on the intensity and duration of the inflammatory response, and ultimately the clinical outcome of periodontal/periapical disease. Over the last few decades we have accumulated a broad but somewhat superficial knowledge on how individual cytokines contribute to the development of these diseases. Recently, the application of new technology and analytical tools have revealed the multitude and complexity of cytokines, especially regarding the selective synthesis and secretion of effector cytokines by specific $CD4^+$ T-lymphocyte subsets, and their participation in complex regulatory networks.

Numerous studies have intended to characterize the host response to infection in periodontal/periapical diseases; providing valuable information on the interactions between the bacterial biofilm and the responding host cells [9–11]. With the progress of our knowledge from purely descriptive observations towards more mechanistic findings, we are beginning to get a reasonable glimpse of the whole picture. In this context, the present review intends to present a chronological perspective on the development of the current hypothesis on the etiology of periodontal and periapical diseases, focusing on the specific contribution of cytokines and cytokine networks, and on how our increasing knowledge and insight into the immune processes is producing a more complete account of the extremely complex phenomena that characterizes these destructive diseases (Table 1, Figure 1).

Pro-inflammatory and Anti-inflammatory Cytokines in the Host Innate Immune Response

The initial investigations of immune response to bacterial infection in periodontal/periapical diseases demonstrated that both resident and infiltrating cells were able to recognize oral bacteria (and its antigens), and respond to them in a pro-inflammatory manner. In the case of periodontitis, the gingival epithelium is the first line of defense to infection and is routinely challenged by oral bacteria, constitutively secreting cytokines that attract a moderated but constant influx of immune cells (predominantly neutrophils), which arrest the bacterial growth and prevent infiltration [4, 12]. Epithelial cells respond to the periodontal infection by up-regulating pro-inflammatory cytokine/chemokine expression, thereby providing the stimuli for the leukocyte recruitment that will lead to the latter disease stages [13].

After the initial response of epithelial cells to the microbes, the permeability of the junctional epithelium is increased by the inflammatory mediators, and allows the contact of bacteria (and/or their products) with gingival connective tissue, where most of the host/microbe interaction events occur. Indeed, gingival fibroblasts (GF), the predominant cell type in gingival connective tissue, possess the capacity to respond to infection with the secretion of pro-inflammatory mediators that trigger, and to some extent sustain, the initial steps of the immune response [14].

In the periapical lesion, the spread of the infection front across the root apex/periodontium results in the direct contact of the infecting microbes with the periodontal ligament fibroblasts, that although not routinely exposed to bacterial challenge, also have the capability to respond to infection with cytokine production, participating in the initial recruitment phase of the host response similar to GFs [3••]. It is important to note that leukocytes can also play a role in the initial steps of host response to oral infections, since neutrophils and resident macrophages can be found in clinically healthy connective periodontal, pulpal and periapical tissues.

Within the pro-inflammatory mediators thought to coordinate the inflammatory cell migration in periodontal/periapical tissues, TNF-α, IL-1, and IL-8 have been extensively investigated. IL-1 and TNF-α share several pro-inflammatory properties, paramount among them the capacity to activate endothelial cells to provide the signals required for leukocyte diapedesis [3••]. Indeed, these pro-inflammatory cytokines induce the production of chemokines. These selectively recruit leukocytes from the peripheral circulation to the site of infection via specific ligand-receptor interaction that ultimately triggers integrin-dependent adhesion; cytoskeletal rearrangement to facilitate extravasation and migration, as well as the binding and detachment of cells from their substrate [5]. In the cell migration framework, IL-8 (CXCL8) is the prototypical and firstly identified member of the chemokine family [15]; specifically acting in the chemoattraction of neutrophils, which form a sub-epithelial barrier that exerts a potent microbicidal action by means of its secretory functions (reactive oxygen species and bactericidal proteins), and acts as a unified phagocytic apparatus [16]. IL-8 production can be directly induced by TLR stimulation, or indirectly via TNF-α and/or IL-1 [17]. In periodontitis patients, IL-8 level is significantly increased compared with gingivitis-affected and healthy subjects [18], and seems to correlate with the periodontal status of the patients before and after treatment [19, 20]. While the early neutrophil migration is under the control of IL-8, the developing chronic inflammatory response also involves the migration of macrophages, where CCL2 (MCP-1) appears as a master regulator of monocyte

Table 1 Cytokine networks of pro-inflammatory cells and T lymphocyte helper subsets

	Pro-inflammatory	Th1	Th2	Th17	Treg	Th9	Th22	Tfh
Pro-inflammatory		Th1 cytokines amplify pro-inflammatory response	Th2 cytokines dampen pro-inflammatory response	Th17 cytokines interact with pro-inflammatory cytokines in osteolytic networks	Tregs suppress pro-inflammatory response	Conflicting evidence on amplification of pro-inflammatory response	Conflicting evidence on amplification of pro-inflammatory response	Unknown
Th1	Pro-inflammatory cytokines favor Th1 responses		Th2 cytokines inhibit Th1 polarization	Conflicting evidence on reciprocal inhibition or activation	Tregs suppress Th1-mediated responses	Th9 amplifies Th1 cytokine production (IFN-γ)	Unknown	Unknown
Th2	Pro-inflammatory cytokines favor Th2 responses	Th2 cytokines inhibit Th1 polarization		Th17 cytokines inhibit Th2 polarization	Tregs suppress Th2-mediated responses	Unknown	Unknown	Tfh cells contribute to B-cell mediate antibody response
Th17	Pro-inflammatory cytokines induce Th17 polarization	Conflicting evidence on reciprocal inhibition or activation	Th2 cytokines inhibit Th17 polarization		Tregs suppress Th17-mediated responses	Th9 regulates Th17 polarization	Th22 cytokines amplify IL-17 mediated response	Tfh cytokines induce Th17 polarization
Treg	Pro-inflammatory cytokines suppress Treg polarization	Tregs suppress Th1-mediated responses	Tregs suppress Th2-mediated responses	Tregs suppress Th17-mediated responses		Th9 enhances the suppressive functions of Tregs	Unknown	Tfh cytokines inhibit Tregs polarization
Th9	Pro-inflammatory cytokines amplify Th9 cytokine production	Unknown	Unknown	Th9 up-regulates Th17 cytokines in skin diseases	Unknown		Unknown	Unknown
Th22	Synergic effects of Th22 and pro-inflammatory cytokines	Scarce evidence suggests down-regulatory properties of Th22	Unknown	Synergistic action of Th17 and Th22 cytokines in osteoclastogenesis	Unknown	Unknown		Unknown
Tfh	Unknown	Unknown	Tfh contributes to Th2/B-cell axis activation	Tfh induces Th17 polarization and activation	Tfh suppresses Tregs in low-grade inflammation conditions	Unknown	Unknown	

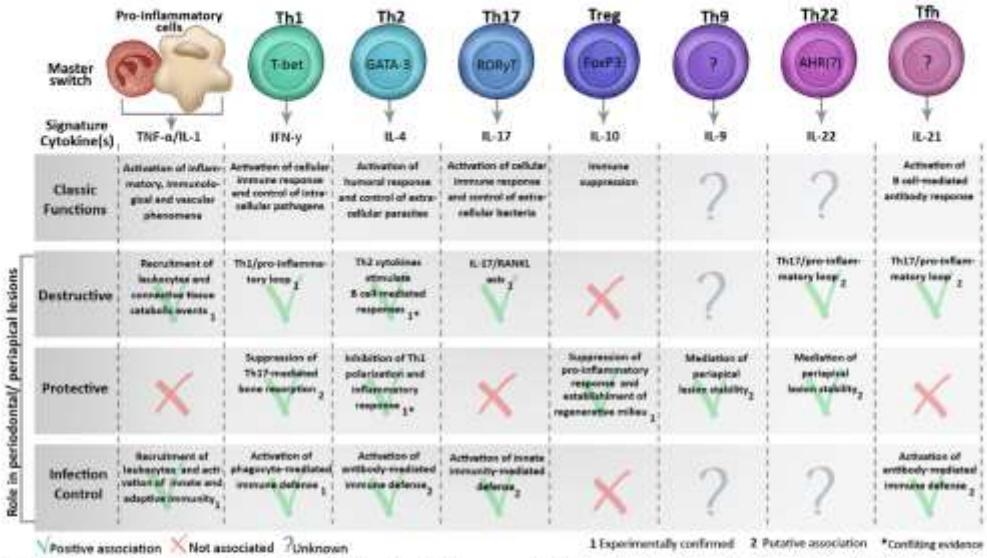

Fig. 1 Defining features of pro-inflammatory and T lymphocyte helper cell subsets in periodontal and periapical lesions. Pro-inflammatory and T lymphocyte helper cell subsets present characteristic features (such as master switches, signature cytokines and classic functions) that define such cell subset in phenotypic and functional terms. When these cells are considered in the context of periodontal and periapical lesions, they have putative roles in the tissue destructive/protective responses, as well in the control of infection

mobilization [21, 22]. The level of CCL2 is increased in the saliva and gingival crevicular fluid (GCF) of periodontitis patients, and correlates with periodontitis severity [23]. It has also been reported that CCL2 levels are increased in the GCF and serum of periodontitis-affected smokers compared with non-smoking patients, suggesting that this chemokine could be involved in the worsening of the periodontal condition characteristic of the former [24]. Similar to periodontitis, IL-8 and CCL2 can be described as coordinating the respective migration of neutrophils and macrophages during the development of periapical lesions [25, 26].

Once in the periodontal/periapical tissues, neutrophils and macrophages seem to act along epithelial and connective tissue cells towards the maintenance and amplification of the inflammatory/immune response. Interestingly, both neutrophils and macrophages can be a significant source of TNF-α and IL-1, generating a positive loop towards response amplification [27].

Taken together, the pro-inflammatory cytokines can account for a significant extent of the tissue destruction associated with periodontal/periapical diseases, since they induce matrix metalloproteinases (MMPs) and receptor activators of nuclear factor kappa-B ligand (RANKL), the main mediators of soft and hard tissue destruction, respectively. Indeed, both soft connective tissue and bone integrity depend on the balance between resorption/deposition. The overexpression of pro-inflammatory cytokines in periodontal and periapical chronic inflammation interferes with the physiologic equilibrium, leading to pathologic tissue loss [2].

Even though pro-inflammatory cytokines have been classically related to destructive events, they also exert pivotal roles in the control of infection. Surprisingly, only recently has the "protective" nature of cytokines against infection been systematically investigated. TNF-α has a central role in the control of periodontal/periapical infections, and its deficiency generates a severe pathogen clearance impairment, increasing the systemic acute phase response. Indeed, TNF-α is a prototypical phagocyte activator, mediating both myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS) expression, respectively the major antimicrobial effectors of neutrophils and macrophages [28]. Accordingly, the lack of iNOS results in an increased periapical abscess formation following endodontic infection [29].

While the pro-inflammatory response reveals a dual nature (destructive while protective against infection), it is also important to remember that there are other cytokines involved in the overall cytokine network to prevent/attenuate the side effects of an exacerbated response. In this context, it is worthwhile to highlight the functions of IL-10, that antagonizes pro-inflammatory cytokine secretion and signaling, down-regulates MMP activity (directly and indirectly via tissue inhibitors of MMPs), and suppresses RANKL-mediated

osteoclastogenesis (*via* osteoprotegerin) [30, 31]. These properties point to IL-10 as a "protective" mediator in periodontal/periapical lesions, although we must bear in mind that the protective/destructive dichotomy is a partial and oversimplified interpretation (albeit instructive) of a more complex phenomenon, given the dual role of host response previously discussed [2].

After the initial innate response characterized by the balance between pro-inflammatory vs. anti-inflammatory cytokines, the selective migration of specific T lymphocyte subsets drives the transition to the adaptive immune response; where T helper lymphocytes (Th) play a critical role in orchestrating the response. As described in the sequence, the discovery of Th subpopulations dramatically changed the understanding of cytokine networks beyond the pro-inflammatory vs. anti-inflammatory framework.

The Th1/Th2 Balance Paradigm

The recognition in the late 1980s of two distinct (Th1 and Th2) clonal linages of Th lymphocytes, defined by unique cytokine secretory patterns, was a cornerstone in the understanding of immunological responses and the development of the current paradigms of immune/inflammatory disease etiology [32, 33]. Th1 cells mediate immune responses against intracellular pathogens and are particularly important in infection control. A dendritic cell-derived cytokine, IL-12, is the principal cytokine responsible for Th1 lineage commitment and differentiation, which involves the activation of the key transcription factor T-bet [34]. The prototypic cytokine product of Th1 lymphocytes is IFN-γ, involved in the activation of macrophage microbicidal functions [35, 36]. In parallel with Th1 cell discovery, their opposing mates Th2 were also unraveled. Th2 cells mediate the host defense against extracellular parasites and secrete a wide array of cytokines, including IL-4, IL-5, IL-9, IL-13, and IL-25. IL-4 is the main positive feedback signal for Th2 lineage commitment via induction of the GATA-3 transcription factor [37]. The selective polarization to Th1 or Th2 lineage is dependent upon the cytokine milieu in which the antigens are presented to the naïve T $CD4^+$ cells, in that the polarization is a mutually exclusive event that also generates subsequent positive feedback to the respective lineage polarization [38].

The Th1/Th2 dichotomy paradigm was quickly replaced by a more realistic Th1/Th2 balance hypothesis, where the distinctive features of immune response could be explained by the predominance of one clonal Th subset over the other, comprising a theoretical framework to investigate T cell-mediated immunological responses and related diseases, including periodontal/periapical diseases. The Th1 predominance hypothesis in periodontal/periapical diseases was supported by experimental evidence of the overexpression of IFN-γ in gingival mononuclear cells isolated from periodontitis patients [39], and by the quantitative dominance of IFN-γ over IL-4 in the lesional tissue/fluid of periodontal/periapical disease patients [21, 40]. The inner rationale for the Th1 predominance hypothesis of periodontal destruction was that the exacerbated activation of neutrophils and macrophages by Th1 cytokines interfered with, and amplified, the pro-inflammatory activity of phagocytes and even of resident fibroblasts, and ultimately increased local levels of MMPs and RANKL, leading to augmented soft tissue and bone destruction [41–43]. While potentially destructive, IFN-γ-mediated Th1 responses are essential to avoid pathogen dissemination. Indeed, IFN-KO mice challenged by A. actinomycetemcomitans oral infection presented a less severe periodontitis phenotype, but on the other hand suffered a widespread (and often lethal) systemic infection, associated with lower MPO and iNOS production [44].

Conversely, other studies supported the Th2 predominance hypothesis in periodontal/periapical diseases, based on the fact that Th lymphocytes isolated from inflamed gingival tissues predominantly produced IL-4 over IFN-γ, after non-antigen specific stimulation [45]. Also, the initial descriptions of established periodontal lesions as a "B-cell lesion" [46] were used to support the possible Th2 role in periodontitis progression in the view of the Th2/B-cell cooperative axis. In this framework, the Th2-stimulated local production of antibodies would be a protective factor, albeit not fully supported by experimental evidence. However, some authors consider that Th2 polarization may indicate an impaired adaptive immune response, in which Th2 cytokines inhibit Th1 polarization (and consequently the phagocytic immune mechanisms), due to inherent host characteristics, or by evasion strategies mediated by virulence factors of periodontopathic bacteria [47–49]. When the pro-inflammatory versus anti-inflammatory and Th1 vs. Th2 frameworks are considered in a global context, evidence points to a cooperative role of Th1 and pro-inflammatory cytokines in the amplification of tissue destructive events in gingival/periapical tissues; while Th2 mediators seem to cooperate with anti-inflammatory cytokines, given the similarity between the properties of IL-10 and IL-4 [50, 51].

Interestingly, conflicting data were readily available in the literature to support either the Th1 or Th2 predominance hypotheses. The heterogeneity of experimental design, disease definitions, time of sample collection, stimulation protocols, and analytical methods used in the different studies could explain some of these differences in outcome [51]. Additionally, the discovery and recognition of new T helper cell lineages overcame the traditional, and quite limited, Th1/Th2 balance hypothesis that restrained the full understanding of the cytokine networks operating in periodontal/periapical lesion development [50]. It will be discussed below how the expansion of the Th family has led to a new level of

complexity of cytokine networks, where multiple sets of T cells can reciprocally inhibit or stimulate the functions of another Th set and by doing so, orchestrate the outcome of adaptive immune responses.

Th17 and Treg

When the relatively simple Th1/Th2 dichotomy and balance theories proved insufficient to fully explain the pathogenesis of a series of immune response-related conditions, the identification of additional Th subsets brought a new perspective, and additional complexity, to the evolving cytokine networks. In 2003, it was demonstrated that IL-23 could drive a population of T cells to express the transcription factor RORγT and to secrete IL-17 [52, 53]. These cells were later named Th17 and are able to secrete IL-17, IL-21 and IL-22, with IL-17 being the cytokine which characterizes the effector functions. IL-17 was found to be largely expressed in inflamed periodontal/periapical lesions [43, 54, 55]. Our group reported overexpression of IL-17, IL-23, IL-6, TGF-β and IL-1β in the gingival tissues of periodontitis patients compared to healthy controls, as well as augmented levels of both IL-17 and RANKL in alveolar bone from periodontitis-affected subjects [56]. These results provided evidence that the inflammatory context characteristic of periodontal disease (i.e., IL-1β, IL-6, and TGF-β) could drive the polarization of the Th17 subset in human periodontitis. Th17 cells are supposed to contribute to the development of periodontal/periapical lesions directly upregulating MMPs, and indirectly by an inflammation amplification loop stimulating the secretion of the classic pro-inflammatory cytokines by other cell types [57, 58].

Interestingly, shortly after the discovery of Th17 cells, studies characterized their interplay with the previously known Th1 and Th2 subsets, since both Th1 and Th2 cytokines were recognized to antagonize IL-17 secretion and Th17 polarization [59]. Most notably, it was demonstrated that IFN-γ neutralization worsened the tissue damage in a delayed-type hypersensitivity model, providing the first evidence that the Th1 subset could arrest the tissue destruction mediated by IL-17, reversing the classical pro-inflammatory and pro-destructive stereotype assigned to Th1 lymphocytes [52, 60]. When a possible Th1/Th17 interplay is considered in the periodontal context, it was observed that progressive periodontal sites demonstrated overexpression of RANKL, IL-1β, IL-17 and IFN-γ compared with inactive sites, and positive correlations exist between RANKL/IL-17, RORγT/RANKL, and RORγT/IL-17. These studies indicate the putative involvement of the Th17/IL-17/RANKL axis in periodontitis progression [61, 62]. Interestingly, unpublished data from our group derived from molecular analysis of active periapical lesions demonstrate an inverse correlation between IFN-γ and IL-17 levels, suggesting that Th1 and Th17 pathways may operate independently (and not cooperatively) in the evolution of periapical lesions.

Switching to the immunosuppressive front, a new Th lineage with broad immunosuppressive properties was characterized, including the inhibition of Th1, Th2 and Th17 polarization [63]. This subset was named T regulatory (Tregs) and it was defined by the expression of the phenotypic markers CTLA-4, IL-10, TGF-β, GITR, CD103, and CD45RO; and Foxp3 was shown to be the master transcriptional regulator for Tregs differentiation [38]. Tregs were associated with high expression of the regulatory cytokines IL-10 and TGF-β in inflamed periodontal tissues [31], and their presence was associated with decreased disease severity [64]. Conversely, some studies have reported an overexpression of Foxp3, T-bet, RANKL, IL-17, IL-1β, and IFN-γ in human active periodontal lesions, compared with inactive ones [61, 62]. Other studies have shown that IL-10-expressing cells outnumber the pro-inflammatory cytokine-expressing cells in human periapical lesions [65], and that the proportion of $Foxp3^+$ cells are more prevalent in periapical granulomas and less prevalent in residual radicular cysts [66]. While data from human samples is conflicting regarding the role of Tregs in periodontal/periapical lesion pathogenesis, experimental data links the presence of Tregs with the attenuation of disease progression rate, such findings being confirmed in a cause-effect manner by disabling Tregs with anti-GITR antibodies [64]. Accordingly, in a recent report we demonstrated that the selective recruitment of Tregs with a controlled release system of the chemokine CCL22 (a known Treg chemoattractant) is effective in reducing the clinical measures of inflammation and bone loss in canine/murine models of periodontitis. Most interestingly, while Tregs have been associated with the impairment of protective immunity in certain diseases, the Tregs local enrichment treatment did not increase the bacterial load in gingival tissues, and did not raise the levels of serum inflammation markers [67••]. This new approach to the periodontal therapy by immune modulation is an excellent example of how immune research could translate to the clinical setting in the near future.

Th9, Th22 and T Follicular Helper

Even more recently, Th9, Th22 cells, and T follicular helper subsets (Tfh) were discovered and shown to interact with the previously known Th subpopulations in the modulation of inflammatory/immune responses [68].

Th9 cells characteristically produce IL-9, initially designated as a Th2 cytokine, which exerts pro-inflammatory or anti-inflammatory activities by modulating Tregs and/or Th17 cell development and function [69]. Th9 cells produce and secrete TNF-α and are involved in pro-inflammatory amplifying loops in many skin diseases [70]. Th22 cells produce IL-22,

which can exert pro-inflammatory effects by a synergistic action with classic pro-inflammatory mediators such as TNF-α and IL-17 [71, 72]. IL-22 can also directly up-regulate RANKL expression and therefore induce osteoclastogenesis [73]. We showed that both Th9 and Th22 cytokines are expressed in human and experimental periapical lesions, where they supposedly contribute to the lesion stability [74••]. However, no mention of the role of these cytokines in periodontal disease can be found in the literature.

Finally, it is important to mention Tfh, a $CD4^+$ T cell subset found in the B-cell follicles of secondary (and feasibly tertiary) lymphoid organs [75], described as a major contributor to B cell-mediated antibody responses and an important source of IL-21 [76]. IL-21 is a pleiotropic cytokine highly expressed in gingival biopsies of chronic periodontitis [77, 78], and it has been implicated in osteoclastogenesis and bone resorption [79], as well as in the development of Th17 cells [80]. It has also been implicated in the inhibition of Tregs polarization in low-grade chronic inflammatory disorders, such as obesity [81]. Nevertheless, no mentions regarding the possible role of Tfh in either periapical lesions or periodontal disease can be found in the literature so far. Indeed, due to the relative novelty surrounding Th9, Th22, and Tfh subsets, along with the lack of specific studies focused on their patterns of expression (and possible role) in periodontal/periapical lesions pathogenesis, it is not possible to make any definitive conclusion regarding their individual and collective role in the overall cytokine network.

Cytokine Networks: from Classic T-helper Prototypes to the T-cell Plasticity Concept

Conventionally, the differentiation of naive $CD4^+$ T cells into subsets has been considered as an irreversible event, defined by the expression of selective signature cytokines and a single master transcription factor [38]. However, with the discovery of new Th subsets our understanding of the fate of T-cell identities has changed. It has been recently established that the same cytokines can be expressed by more than one polarized Th subset, and that they can also change their phenotype, characterizing a phenomenon described as plasticity [82, 83••]. As an example, IL-10 was once thought to be a Th2 exclusive cytokine, but it is now clear that IL-10 can be produced by multiple Th subsets, such as Th1, Th2, Tregs, and Th17 cells [83••, 84]. Similarly, IL-9 is preferentially produced by the Th9 subset [85], but it can also can be produced by Th2, Th17, and Tregs [69, 86]. Another interesting plastic characteristic of Th cells is the recently discovered possibility of simultaneous expression of the Th17 and Tregs transcription factors (RORγT and Foxp3) in the same cell [87]. There are many other examples of Th plastic features and the complexity of these findings calls into question whether Th cell subsets are more appropriately viewed as a "work in progress", rather than a terminally differentiated cell [83••]. Nevertheless, it is noteworthy that the majority of the experimental data regarding Th cell differentiation and plasticity is derived from in vitro studies employing extreme (and possibly artificial) polarizing stimuli to obtain homogenous populations [88], so extrapolations to the clinical setting must be made carefully.

With the perspective of the plastic capabilities of Th lymphocytes in mind, it is possible to hypothesize that the controversies that regularly arise, regarding the functions of all Th subsets in the pathogenesis of periodontal/periapical diseases could merely be the expression of the continuous metamorphosis of the Th subpopulations along disease stages, possibly as an adaptive reflex to the changing environmental conditions and immunological necessities, in the battlefront of periodontal infection. More powerful analytical tools will be required in order to comprehensibly reappraise our current data and understandings in light of this new viewpoint.

Concluding Remarks

Currently, great efforts are being made to integrate the overwhelming amount of new data regarding cytokine networks and their involvement in the overall regulation of inflammatory immune responses to periodontal/periapical infection. The discovery and characterization of Th subsets provided the conceptual framework to evaluate complicated data, helping in the development of explanatory models for the intricate immunological process underlying the pathogenesis of periodontal/periapical diseases. As ingeniously portrayed by John Conway in his famous cellular automaton "Game of Life", complex systems could emerge from a limited set of simple rules, and the rules are often impossible to infer from the observation of the working system [89]; the focus of our efforts should therefore be to unveil the underlying rules that govern the biological systems, and not to get caught in the trap of using advanced technology simply to further describe these complex phenomena. For the last 25 years, the Th1/Th2 and the Th17/Tregs paradigms have provided researchers an invaluable intellectual framework to interpret experimental data. With the refinement of research techniques our apparently solid models are changing faster than ever before, and the scope of our understanding should expand accordingly. In trying to keep up with this growing knowledge we must apply the lessons learned and integrate the new concepts and ideas emerging from research data in more complex and holistic models, understanding our current notions and paradigms as flexible ones. The unveiling of Th plasticity opens a new opportunity for revisiting our ideas of cytokine networks in immunological processes; instead of rejecting the fresh notions and attempting to protect the established paradigms, we

should embrace them and be willing to reinterpret all our accumulated data under this new light.

Compliance with Ethics Guidelines

Conflict of Interest Dr. Franco Cavalla, Dr. Ana Claudia Araujo-Pires, Dr. Claudia C. Biguetti, and Dr. Gustavo P. Garlet each declare no potential conflicts of interest relevant to this article.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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2.2 ARTICLE 2 – TBX21-1993T/C (rs4794067) polymorphism is associated with increased risk of chronic periodontitis and increased T-bet expression in periodontal lesions, but does not significantly impact the IFN-g transcriptional level or the pattern of periodontophatic bacterial infection*

RESEARCH PAPER

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TBX21-1993T/C (rs4794067) polymorphism is associated with increased risk of chronic periodontitis and increased T-bet expression in periodontal lesions, but does not significantly impact the IFN-g transcriptional level or the pattern of periodontophatic bacterial infection

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Th1-polarized host response, mediated by IFN-γ, has been associated with increased severity of periodontal disease as well as control of periodontal infection. The functional polymorphism TBX21-1993T/C (rs4794067) increases the transcriptional activity of the TBX21 gene (essential for Th1 polarization) resulting in a predisposition to a Th-1 biased immune response. Thus, we conducted a case-control study, including a population of healthy controls (H, n = 218), chronic periodontitis (CP, n = 197), and chronic gingivitis patients (CG, n = 193), to investigate if genetic variations in TBX21 could impact the development of Th1 responses, and consequently influence the pattern of bacterial infection and periodontitis outcome. We observed that the polymorphic allele T was significantly enriched in the CP patients compared to CG subjects, while the H controls demonstrated and intermediate genotype. Also, investigating the putative functionality TBX21-1993T/C in the modulation of local response, we observed that the transcripts levels of T-bet, but not of IFN-γ, were upregulated in homozygote and heterozygote polymorphic subjects. In addition, TBX21-1993T/C did not influence the pattern of bacterial infection or the clinical parameters of disease severity, being the presence/absence of *red complex* bacteria the main factor associated with the disease status and the subrogate variable probing depth (PD) in the logistic regression analysis.

Introduction

Periodontitis is a chronic infectious diseases characterized by the progressive and irreversible destruction of teeth-supporting structures. Periodontitis is initiated by the bacteria harbored in the teeth-attached biofilm that infiltrates the surrounding epithelial and connective periodontal tissues, triggering a host inflammatory immune response that leads to the subsequent lesion development.[1]

While any bacteria can essentially trigger a host response, specific Gram negative anaerobic rods have a special capability to elicit and chronically sustain the host inflammatory immune response that mediates periodontal tissue destruction. Most prominent among periodontitis-associated bacteria are the so called *red complex* cluster (*Porphyromonas gingivalis, Tanerella forsytia* and *Treponema denticola*), which has been consistently associated with the occurrence, severity and negative response to treatment of the disease.[2] As previously mentioned, it is noteworthy that the sole bacterial infection is not sufficient to explain the complex pathological processes of periodontitis, being the nature and extent of host's response ultimately responsible for the disease occurrence and outcome.[3]

While microbial and environmental factors (lifestyle factors [as smoking and stress] or acquired diseases [as diabetes]) characteristically modulate host responses (and consequently periodontitis outcome), studies suggest that as much as 50% of the risk of disease can be determined by genetic factors,[4-6] and that numerous disease modifying genes may be involved in the pathogenesis

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of periodontal diseases by modulating the host's response and his susceptibility to infection.[3,7,8] Recently, the interaction between host genetic factors that can impact the ability of pathogens to invade and proliferate on host's tissues has been termed infectogenomics.[9] This concept highlights the close and intimate dependence of the host's immune mechanisms and the commensal and/or pathogenic microflora, which co-evolved in a mutually dependent fashion.[9]

The Th1-polarized immune responses have long been implicated in the pathogenesis of periodontal diseases, since their characteristic cytokine products are overexpressed in the diseased tissues and in mononuclear cells isolated from periodontitis patients.[10] Also, from a mechanistic viewpoint, the prototypic Th1-cytokine IFN-γ amplify and exacerbate the pro-inflammatory activity of infiltrating phagocytes and resident cells, ultimately increasing matrix metalloproteinase (MMP) and receptor activator of NFκB ligand (RANKL) levels, leading to augmented tissue destruction.[11-15] While recent evidence points to the participation of other T helper subsets (such as Th17 and Thf) in the pathogenesis of osteolytic inflammatory lesions, numerous studies demonstrate that the Th1 subset can be independently responsible for the establishment and progression of periodontitis.[16]

Th1 lineage commitment and differentiation involves the activation of the key transcription factor T-bet (a member of the T-box family of transcription factors)[15] and the secretion of the hallmark cytokine IFN-γ, involved in the activation of macrophage's microbicide functions.[16] Indeed, in addition of modulating the periodontitis' outcome from a tissue destruction perspective, it is also important to mention that Th1 responses have a fundamental role in the control of experimental periodontal infection.[15] Taking into consideration the essential role of T-bet in Th1 responses development, the *TBX21* (17q21.32) -1993T/C polymorphism (rs4794067) of the promoter region of T-bet gene has been associated with an increased binding affinity to Sp1 transcription factor and increased transcriptional activity of the TBX21 gene, resulting in a predisposition to a Th-1 biased immune response characterized by increased IFN-γ secretion.[17,18] Indeed, numerous studies have associated the TBX21-1993T/C SNP with increased risk or early onset of different diseases, such as type I autoimmune hepatitis,[19] systemic lupus erythematosus,[18] childhood asthma[20] and aspirin-induced asthma.[17] Despite the previous association of Th1-type responses with periodontitis development, no study has investigated the possible influence of this SNP in periodontitis, although it has a potential functional influence in Th1-polarization.

Therefore, it is possible to hypothesize that individual variations in TBX21 could impact the development of Th1 responses, and consequently could influence the pattern of bacterial infection and the outcome of chronic periodontitis. In this context, we conducted a case-control study to investigate whether the TBX21-1993T/C single nucleotide polymorphism (rs4794067) is associated with increased chronic periodontitis risk; and we also investigated the putative functionality of this SNP in the modulation of T-bet and IFN-γ levels *in situ*, as well as its potential impact in the susceptibility of developing infection caused by classic periodontal pathogens.

Results

TBX21-1993T/C SNP (rs4794067) frequency analysis

There were significant differences in the inflammatory and clinical periodontal parameters between all groups, as previously reported in detail.[21] A summary of the population's demographics, clinical characteristics, genotype, and allele distribution is provided in **Tables 1 and 2**. The CP and H subpopulations selected for microbiological analysis and quantification of T-bet and IFN-γ expression in gingival tissue were not significantly different from the total population sample (data not shown).

Genotype and allele frequencies of the studied polymorphism were in Hardy-Weinberg equilibrium in the H, CG and CP sample populations ($P > 0.05$) (**Table 2**). The allele distribution of the sample was T = 78.2% and C = 21.8%, resembling almost exactly the average allele distribution of the sample of the phase I "*1000 Genomes Project*" (polymorphic T-allele = 78%; ancestral C-allele = 22%).[22] The frequency of the polymorphic allele homozygote genotype (TT) was significantly higher in the CP group, compared with the CG group (70.6% vs 59.1%, p = 0.0087). Similarly, the allele frequency of the polymorphic T-allele was significantly higher in the CP group compared with the CG group (18% vs 25.6%, p = 0.0051). Interestingly, the

Table 1. Demographic, clinical and microbiological characteristics in healthy controls (H), chronic periodontitis (CP) and chronic gingivitis (CG) patients

	Healthy (N = 218)	Chronic Periodontitis (N = 197)	Chronic Gingivitis (n = 193)	Healthy *versus* Cheonic Periodontitis	Healthy *versus* Chronic Gingivitis	Chronic Gingivitis *versus* Chronic Periodontitis
N and gender distribution*	**105 f / 113 m**	**96 f / 101 m**	**97 f / 96m**	**0.921**	**0.839**	**0.958**
Age	47.48 ± 5.96	46.63 ± 7.34	49.54 ± 6.47	0.190	0.009	<0.001
Clinical parameters	***value ± SD***	***value ± SD***	***value ± SD***			
Probing depth	2.25 ± 0.62	4.29 ± 0.75	2.72 ± 0.52	<0.001	<0.001	<0.001
Clinical Attachment Loss	0.62 ± 0.22	3.92 ± 0.64	0.69 ± 0.25	<0.001	0.002	<0.001
% BOP	4.86 ± 2.86	62.99 ± 8.71	61.85 ± 11.49	<0.001	<0.001	0.269
Plaque index	34.71 ± 8.26	51.26 ± 9.78	53.47 ± 10.05	<0.001	<0.001	0.028
16S DNA ($x10^6$)	0.09 ± 0.20	14.58 ± 34.54	15.51 ± 33.18	<0.001	<0.001	0.786

Table 2. Genotype and allele distribution of healthy controls (H), chronic periodontitis (CP) and chronic gingivitis (CG) patients. Fisher´s exact test and equality of proportions test were performed to evaluate the risk of suffering periodontitis associated with the genotype/allele

				Fischer´s exact test			Equality of proportions test		
TBX21 SNP (rs4794067)	H (N = 218)	CP (N = 197)	CG (N = 193)	H vs CP	H vs CG	CP vs CG	H vs CP	H vs CG	CP vs CG
Genotype	n (%)	n (%)	n (%)						
CC *	17 (7.8)	13 (6.6)	20 (10.4)						
CT *	61 (28)	45 (22.8)	59 (30.6)	p = 1.000 OR = 0.9647 CI = 0.4255 to 2.187	p = 0.707 OR = 0.8221 CI = 0.3926 to 1.722	p = 0.840 OR = 0.8522 CI = 0.3833 to 1.895	0.225	0.563	**0.040**
TT *	140 (64.2)	139 (70.6)	114 (59.1)	p = 0.566 OR = 1.298 CI = 0.6075 to 2.775	p = 0.377 OR = 0.6921 CI = 0.3463 to 1.383	p = 0.098 OR = 0.5331 CI = 0.2541 to 1.119	0.165	0.288	**0.008**
CT+TT	201 (92.2)	184 (93.4)	173 (89.6)	p = 0.706 OR = 1.197 CI = 0.5658 to 2.533	p = 0.391 OR = 0.7316 CI = 0.3714 to 1.441	p = 0.205 OR = 0.6111 CI = 0.2949 to 1.266	0.637	0.358	0.178
Allele									
C *	95 (21.8)	71 (18)	99 (25.6)						
T *	341 (78.2)	323 (82)	287 (74.4)	p = 0.840 OR = 0.8522 CI = 0.3833 to 1.895	p = 0.840 OR = 0.8522 CI = 0.3833 to 1.895	**p = 0.011** **OR = 1.569** **CI = 1.113 to 2.213**	0.171	0.200	**0.005**

healthy control group (classically used as a control in periodontitis genetic studies) demonstrated an intermediary genotype, which was not significantly different from the CG or CP groups, as previously described for other SNPs.[21]

When the H and CP groups where compared, the recessive model rendered the lower Akaike information criteria (AIC), demonstrating the best fit with the data (**Table 3**), although failing to demonstrate any association between the genetic variants with the disease status, which was somewhat predictable taking into consideration the intermediate genotype of the H group. On the other hand, when the CG and CP groups where compared, theoretically representing the dichotomy between resistant and susceptible phenotypes,[21] the log-additive model demonstrated the best fit with the data and both the recessive and log-additive model provided the framework for a significant association between the genetic variants with the disease status, supporting the association of the T allele with the increased susceptibility to chronic periodontitis (**Table 3**). Power calculation demonstrated 97%, 57%, 86% and 16% power with tolerance for 0.001 false positives in the multiplicative, additive, recessive and dominant models, respectively.

TBX21 genotype vs T-bet, IFN-γ levels and clinical parameters

We compared the profiles of T-bet and IFN-γ expression levels with the clinical parameters of periodontitis among the different genotypes of TBX21-1993T/C SNP. The expression of T-bet and IFN-γ was significantly higher in the CP in relation to the H group (≈15-fold, $P < 0.001$ and ≈2-fold, $P < 0.001$; respectively) [**Fig. 1**]. In the CP and H groups the expression of T-bet was significantly higher in the polymorphic homozygotes (TT genotype) in comparison with both the heterozygotes (CT)

Table 3. Inheritance models for TBX21 SNP (rs4794067) and association of genotype with disease. Lower AIC demonstrates best fit of the data with inheritance model. OR (95% CI) and associated p-value represent the risk of disease for subjects carrying the risk allele (polymorphic T allele) compared to ancestral allele (C) carriers according to each inheritance model

		Healthy vs Chronic periodontitis			Healthy vs Chronic gingivitis			Chronic gingivitis vs chronic periodontitis		
Model	Genotype	OR (95% CI)	*P*-value	AIC	OR (95% CI)	*P*-value	AIC	OR (95% CI)	*P*-value	AIC
Codominant	CC									
	CT	1.07(0.28–4.16)	0.681	245.8	1.61(0.49–5.3)	0.590	290.7	1.5(0.57–3.94)	0.101	429.5
	TT	0.8(0.22–2.87)			1.75(0.57–5.44)			2.21(0.89–5.45)		
Dominant	CC									
	CT/TT	0.87(0.24–3.08)	0.830	244.6	1.71(0.56–5.19	0.321	288.7	1.97(0.81–4.79)	0.121	429.7
Recessive	CT/CC									
	TT	0.75(0.40–1.42)	0.384	243.8	1.21(0.67–2.17)	0.532	289.3	1.6(1.01–2.58)	0.049	428.2
Over dominant	CC/TT									
	CT	1.32(0.67–2.6)	0.420	243.9	0.98(0.53–1.82)	0.951	289.7	0.74(0.45–1.23)	0.243	430.7
Log additive		0.82(0.5–1.35)	0.443	244	1.22(0.78–1.91)	0.370	288.9	1.48(1.02–2.13)	0.033	427.5

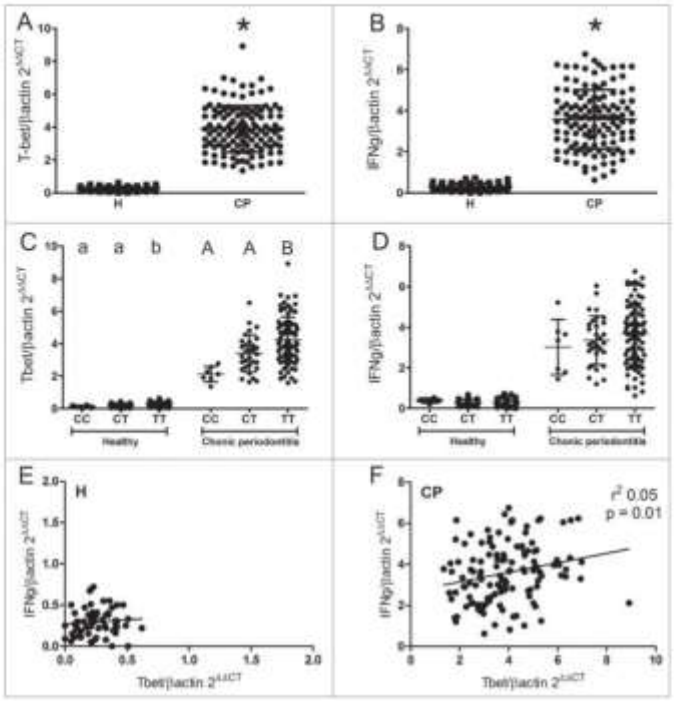

Figure 1. T-bet and IFN-γ expression levels in gingival tissue from chronic periodontitis patients and healthy controls. Total RNA was extracted from gingival tissues of controls (H, n = 63) and chronic periodontitis (CP, N = 123) patients, and levels of T-bet and IFN-γ mRNA were determined by RealTimePCR (with normalization to β-actin using the Ct method). (**A**) T-bet expression in healthy controls and chronic periodontitis patients. * = $P < 0.05$. (**B**) IFN-γ expression in healthy controls and chronic periodontitis patients. * = $P < 0.05$. (**C**) T-bet expression in healthy controls and chronic periodontitis patients according to their genotype for TBX21 SNP (rs4794067). Different letters represent statistically significant difference between the groups. H and CP groups were tested independently. (**D**) IFN-γ expression in healthy controls and chronic periodontitis patients according to their genotype for TBX21 SNP (rs4794067). (**E**) Linear correlation of T-bet and IFN-γ expression levels in healthy subjects. (**F**) Linear correlation of T-bet and IFN-γ expression levels in chronic periodontitis patients (r^2 = correlation coefficient).

and the ancient-allele homozygotes (CC) (p = 0.0128 and p = 0.021; respectively). Nevertheless, the IFN-γ expression levels only demonstrated a non-significant trend for increased expression in polymorphic homozygotes [**Fig. 1**]. When T-bet and IFN-γ expression levels were compared irrespectively of the TBX21 genotypes, no correlation between T-bet and IFN-γ expression levels was observed in healthy control subjects, while a weak, but statistically significant correlation (r^2 = 0.05, p = 0.01) was observed in chronic periodontitis patients [**Fig. 1**]. Regarding the analysis of the possible impact of the TBX21-1993T/C SNP in chronic periodontitis development, no significant variations were observed in the clinical parameters of periodontitis (probing depth, mean probing depth, attachment loss and bleeding on probing) in the different TBX21 genotype carriers (**Fig. 2**). When T-bet and IFN-γ expression levels were correlated with the clinical parameters irrespectively of the TBX21 genotypes, no positive correlations were observed (data not shown).

TBX21 genotype vs the pattern of bacterial infection, modulation of T-bet and IFN-γ levels, and clinical parameters

The detection frequency of the red complex's bacteria (**Table 4**), as well the bacterial load was significantly higher in the CP group in comparison to the H controls ($P < 0.001$). The dichotomic analysis of detection frequency (positive or negative) of *P. gingivalis*, *T. forsythia*, and *T. denticola* in H and CP groups demonstrated no influence of TBX21 genotype in the odds to suffer periodontal infection [**Table 4**]. Since the groups/microbes stratification resulted in 'zero' subjects with CC genotype positive for *T. forsythia* and *T. denticola* (making the Fischer's exact test analysis impossible) the comparative analysis was carried out based in the TBX21 C and T alleles instead of the genotypes [**Table 4**]. Notwithstanding the lack of association of genotype with the red complex bacteria detection, we evaluated the possible variation in the bacterial load in the distinct TBX21 genotype carriers [**Fig. 3**]. In this case, despite a trend toward higher bacterial levels in association with the T allele in CP patients, no statistically significant differences were observed [**Fig. 3**]. Also, no significant association was observed in the load of red complex bacteria within the different TBX21 genotype carriers in the H group (data not shown). We then investigated if the presence and load of the pathogen bacteria could impact the gingival expression levels of T-bet and IFN-γ among the different genotypes [**Fig. 4**], dichotomously subdividing the samples in positive/negative for the detection of *P. gingivalis*, *T. forsythia*, and *T. denticola*. The analysis demonstrated no significant difference in T-bet and IFN-γ expression levels according to infection status, remaining the TBX21 genotype as the main factor associated with increased T-bet expression in the lesions [**Fig. 4**]. Also, the bacterial load of *P. gingivalis*, *T. forsythia, and T. denticola* was not correlated with T-bet nor IFN-γ expression levels in the periodontal lesions (data not shown).

Finally, since T-bet genetic variants were not associated with CP clinical parameters [Fig. 2], we tested the possible association of the red complex bacteria with CP clinical parameters [Fig. 5]. Our results demonstrated a strong monotonic correlation between the absolute load of each individual red complex bacteria with the disease subrogate PD mean [Fig. 5]. The correlation coefficients (rho) were 0.53 for *P. gingivalis* ($P < 0.0001$); 0.48 for *T. forsytia* ($P < 0.0001$); and 0.55 for *T. denticola* ($P < 0.0001$). In the logistic regression model, when the genetic data was analyzed in interaction with the presence/absence of red complex bacteria as environmental co-variable, the combined effect on disease status was significant for each bacteria species individually: *P. gingivalis* showed and odds ratio of 9.44 (CI 3.58-24.89) and 10.32 (CI 2.03-52.52) in association with TT and CT genotypes, respectively; *T. forsytia* showed and odds ratio of 12.41 (CI 4.06-37.88) and 24.94 (CI 2.94-211.06) in association with TT and CT genotypes, respectively; while *T. denticola* showed and odds ratio of 12.41 (CI 4.06-37.88) and 8.44 (CI 2.02-35.26) in association with TT and CT genotypes, respectively. Nevertheless, the Cochrane-Armitage test for interaction in the trend for the genetic/microbiologic association failed to detect a significant interaction (0.84; 0.29 and 0.86 for *P. gingivalis, T. forsytia* and *T. denticola*, respectively).

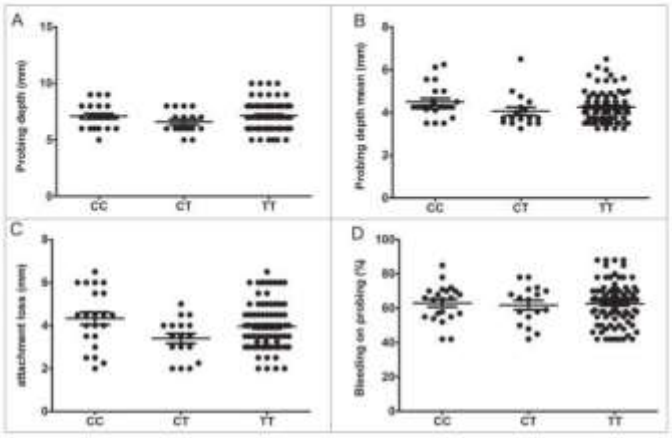

Figure 2. Clinical parameters of periodontal disease according to genotype for TBX21 SNP (rs4794067) in chronic periodontitis patients. Chronic periodontitis (CP) patients were subjected to periodontal examination, and the genotype of TBX21 SNP (rs4794067) was determined using Taqman® chemistry. (**A**) Probing depth of deepest site according to genotype for TBX21 SNP (rs4794067). (**B**) Probing depth mean according to genotype for TBX21 SNP (rs4794067). (**C**) Attachment loss mean according to genotype for TBX21 SNP (rs4794067). D) Bleeding on probing according to genotype for TBX21 SNP (rs4794067).

The ordinary least squared (OLS) regression model including simultaneously the detection of red complex bacteria and genotype (with PD as response variable) failed to detect a significant contribution of the genetic variants in the changes of the dependent variable, while the microbiological data proved significant to explain the variations of PD ($P < 0.0001$; R-squared 0.3531). When the genotype was fitted in the OLS regression and the logistic regression as the sole explanatory variable it did not demonstrate a significant effect in the outcome (data not shown).

Discussion

Periodontitis is a complex disease in which microbial, environmental and genetic factors interact increasing or decreasing the predisposition to periodontal tissue inflammatory destruction. The principal causative factor recognized of the disease is the periodontal infection by pathogen bacteria, specifically the so called red complex bacteria (*P. gingivalis, T. forsytia* and *T. denticola*), which are characterized by their ability to elicit and chronically sustain the host's inflammatory immune response that mediates tissue destruction. Th1-type immune responses have been mechanistically associated with tissue destruction and with the control of experimental periodontal infection.[11-13] Since the functional TBX21-1993T/C SNP directly impacts Th1-type response development, we conducted parallel case-control and functional studies to address the possible influence of this genetic variant in the susceptibility of developing chronic periodontitis.

We employed a previously validated strategy to increase the power of the case-control study, selecting a "resistant" control population opposed to the "susceptible" subjects presenting chronic periodontitis.[21] The "resistant" control population, represented by chronic gingivitis patients (exposed to microbial challenge without developing the clinical signs of periodontitis) proved significantly different from the "susceptible" chronic periodontitis sample, both in allele frequencies and allele distribution, while the "classical" healthy controls (individuals presetting periodontal health but with a low exposure to chronic microbial challenge due to proper oral hygiene methods) did not prove genetically different from the CP or the CG subjects. These results further support the notion that the "classical" healthy control population usually enrolled in case-control studies in periodontitis is unsuitable for direct comparison with the susceptible patients, since it is arguably composed of a heterogeneous mixture of genetically "susceptible" and "resistant" subjects, whose real phenotype remains unrevealed by the relatively low microbial challenge to which they are exposed.[21] An analogous alternative strategy that has been recently proposed is the enrolment of the "highly susceptible" phenotype as the cases, clinically characterized as aggressive periodontitis patients, increasing the power of

Table 4. Frequencies of detection of the studied pathogens in healthy control subjects and chronic periodontitis patients according to their TBX21 genotype. The values of Fisher's exact test in the right column represent the increased risk of positive detection in chronic periodontitis subject compared to healthy controls (CC genotype vs TT genotype in the grey row and among alleles in the white rows). The values of the Fisher's exact text in the healthy and chronic periodontitis columns represent the risk of positive detection of pathogens among T and C allele carriers; calculations were based on alleles and not in genotypes due to the existence of categories with zero subjects

	Healthy (n = 63)		Chronic Periodontitis (n = 123)		
	Negative (n/%)	Positive (n/%)	Negative (n/%)	Positive (n/%)	
P gingivalis	54 (85.7)	9 (14.3)	45 (36.6)	78 (63.4)	p = < 0.0001 OR= 10.400 CI= 4.694 to 23.043
CC	3 (75)	1 (25)	1 (14.3)	6 (85.7)	C
CT	17 (86.5)	2 (10.5)	14 (45.2)	17 (54.8)	p= < 0.001
TT	34 (85)	6 (15)	30 (35.3)	55 (64.7)	OR= 10.422
	p= 1.00		p= 1.000		CI= 3.061 to 35.479
	OR= 0.9471		OR= 0.9469		
	CI= 0.2843 to 3.154		CI= 0.4824 to 1.859		
T forsythia	58 (92)	5 (8)	49 (39.8)	74 (60.2)	p = < 0.001 OR= 17.518 CI= 6.558 to 46.794
CC	4 (100)	0 (0)	2 (28.6)	5 (71.4)	C
CT	18 (94.7)	1 (5.3)	15 (48.4)	16 (51.6)	p = < 0.001
TT	36 (90)	4 (10)	32 (37.6)	53 (62.4)	OR= 35.579
	p = 0.688		p = 0.738		CI= 4.429 to 285.81
	OR= 2.600		OR= 1.129		
	CI= 0.3146 to 21.490		CI= 0.5857 to 2.175		
T denticola	56 (88.9)	7 (11.1)	49 (39.8)	74 (60.2)	p = < 0.001 OR= 12.082 CI= 3.061 to 35.479
CC	4 (100)	0 (0)	4 (57.1)	3 (42.9)	C
CT	16 (84.2)	3 (15.8)	11 (35.5)	20 (64.5)	p = < 0.001
TT	36 (90)	4 (10)	34 (40)	51 (60)	OR= 10.947
	p = 1.00		p = 0.738		CI= 2.871 to 41.742
	OR= 1.000		OR= 1.129		
	CI= 0.2581 to 3.874		CI= 0.5857 to 2.175		

the study, but without addressing the issue of the relatively low exposure of the healthy control group.[25]

Our results demonstrated that TBX21-1993 polymorphic T allele carriers were more prevalent in the disease group, proving that the TBX21 SNP polymorphism exerts some influence in disease susceptibility. Previous studies demonstrated that the increased expression of T-bet in polymorphic subjects predispose to a Th1-biased adaptive immune response, which is characterized by a robust and stronger production of pro-inflammatory mediators.[24] Accordingly, the Th1-polarized immune responses have long been implicated in the pathogenesis of periodontal diseases, linked with increased disease severity owing to the pro-inflammatory and catabolic activities mediated by the prototypic Th1 cytokine IFN-γ.[11-13] Indeed, the expression of both T-bet and IFN-γ was significantly higher in diseased than in healthy tissues, reinforcing the assumed association of Th1 response with periodontitis development.

While the case-control data from TBX21 SNP genotypes/alleles frequency in "susceptible" and "resistant" subjects suggests its involvement in periodontitis susceptibility/resistance, additional experimental approaches were conducted to support such potential association from the functional and mechanistic viewpoints. In this context, our data demonstrate that polymorphic homozygote subjects (TT) exhibited significantly increased expression of T-bet (2-fold change compared to ancestral homozygotes), providing supporting evidence of the functionality of the investigated SNP. In this aspect our result are in accordance with published evidence.[25,26] However, it is important to notice that despite the strong influence of T allele/genotypes over T-bet expression (≈2 fold), the subsequent impact over IFN-γ expression was significantly lower (1.21-fold), and proved statistically non-significant.

The weak influence of TBX21 genetic variance in the Th1-response expression (represented by IFN-γ mRNA levels) reinforces the concept of the complex nature of the leukocyte polarization. Indeed, the local Th1 response (as measured in this study in the gingival biopsies) is the result of leukocyte polarization, probably in the lymph nodes, followed by their migration to periodontal tissues, local stimulation by antigen presenting cells under the influence of a complex local cytokine milieu, followed by the continuous regulation of Th1 cells by the local cytokine networks.[27,28] As a matter of fact, while Th1 cells exhibit epigenetic stability at signature transcription factor loci level, a number of other factors such as chromatin modifications and intergenic noncoding RNAs (such as Tmevpg1) can decisively impact IFN-γ production.[29,30]

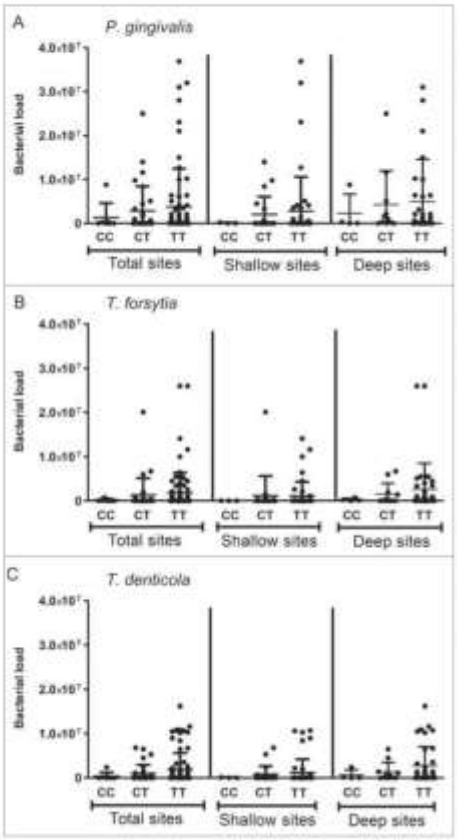

Figure 3. Bacterial load of *P. gingivalis*, *T. forsythia* and *T. denticola* in total periodontal sites and shallow/deep sites (above and below the patient's median) of chronic periodontitis patients (n = 123), according to TBX21 SNP (rs4794067) genotype. The presence and load of the periodontopathogens were determined by RealTimePCR. (**A**) *P. gingivalis*. (**B**) *T. forsythia*. (**C**) *T. denticola*.

Nevertheless, it is worth noting that regardless of the TBX21 genotype, CP subjects exhibited an overall ≈10-fold increase in IFN-γ expression compared with the H group, and T-bet and IFN-γ levels presented a significant statistical correlation (even though such correlation was relatively weak) in periodontal lesions, reinforcing the association of T-bet with Th1 responses *in situ*. However, no clear association was observed between the TBX21 genotype with the clinical parameters of chronic periodontitis, or between T-bet and IFN-γ expression levels and the clinical parameters of periodontitis. Such lack of a significant association between clinical phenotype and genotype (and T-bet and IFN-γ levels) could be explained by the multifarious and overlapping layers of finely tuned processes which intervene in the pathogenesis of the disease. A single SNP (even one accounting for a major change in an important pathway of immune response, such as TBX21-1993T/C) is unlikely to produce such a large phenotype change to be evident in a routine clinical periodontal examination.[4] Accordingly, despite the proven association between pro-inflammatory cytokines (such as IL-1β and TNF-α) with periodontitis onset and progression,[31] previous studies have failed to provide a strong correlation between the levels of such mediators and the clinical parameters of periodontitis.[32-34]

Interestingly, in spite of the demonstrated association between IFN-γ and the control of experimental periodontal infection,[13] our results indicated a lack of significant interaction in the trend between microbiological data and genotype. One possible explanation derives from the diluted impact of TBX21 variants on the Th1 response outcome, as measured by the local IFN-γ levels. Additionally, we must consider that other cytokines, such as TNF-α and IL-6, are supposed to contribute to the control of periodontal infection, as suggested by experimental models and human studies data.[33-35] It is also important to consider that the experimental evidence linking IFN-γ with the control of periodontal infection derives from IFN-KO mice, which obviously represent an extreme situation that does not reflect the variation in IFN-γ levels observed in humans.[13] Correspondingly, published evidence from our group demonstrated that after the host's immune response reach a minimal threshold that confers protection, the increase of host responsiveness in degree/intensity does not provide additional protection and, conversely result in heightened tissue damage.[36]

It is also important to mention that, contrary to previously described for TNF-α and IL-1β, the local levels of T-bet (and in a lower extent, the IFN-γ levels) are not influenced by the presence/absence or load of the red complex pathogens.[33,34] While TNF-α and IL-1β production is supposed to be derived by immediate stimulation of leukocytes and resident cells by microbial components in the lesions, our results suggests that Th1 polarization (represented by T-bet expression) is probably a process centered in the lymph nodes, with a minor influence from the gingival environment *per se*. Accordingly, modification in the lymph nodes environment by co-morbidities, such as arthritis, can trigger or exacerbate periodontitis.[37-39] On the other hand, the local expression of Th1 markers in the lesions (represented by IFN-γ levels), as previously discussed, is influenced by numerous other factors, including the complex local cytokine milieu.[27,28]

Indeed, the complexity of local cytokine milieu in infectious chronic inflammatory osteolytic lesions, such as periodontitis and periapical lesions, is far from being completely understood. In a recent study that investigated various Th expression markers, we demonstrated that multiple cytokine clusters are accountable for the activity of osteolytic lesions, including Th1-, Th17- and Thf-biased clusters.[14]

While the immune response pattern associated with a clear disease outcome seems to vary significantly, the common point among progressive lesions (in both periodontitis and periapical lesions) seems to be the presence of recognized pathogens.[40-43]

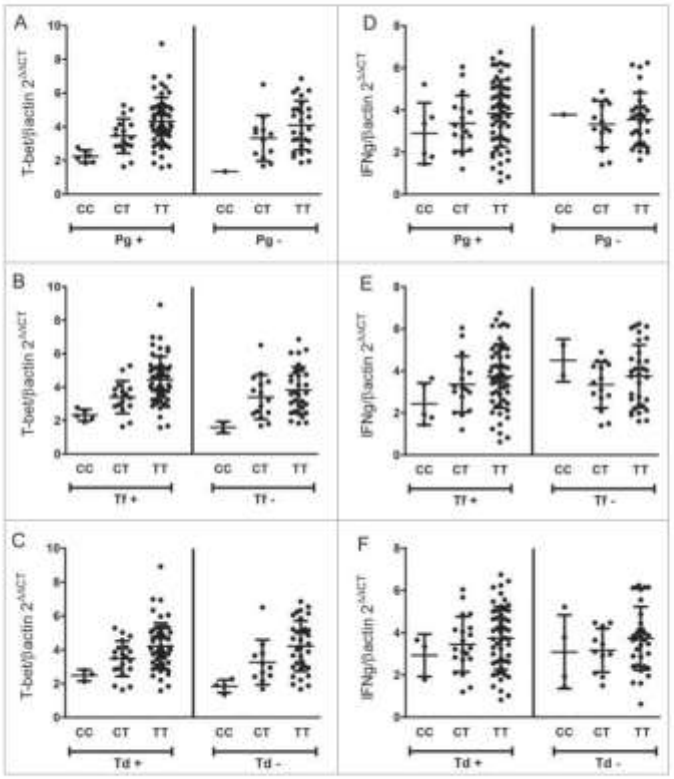

Figure 4. Quantitative assessment of T-bet and IFN-γ mRNA expression in the presence or absence of periodonto pathogens associated with the genotypes of TBX21 SNP (rs4794067) in CP patients. Total RNA was extracted from gingival tissues, and levels of T-bet and IFN-γ mRNA were measured quantitatively by RealTimePCR, and the results are presented as expression of the individual mRNAs, with normalization to β-actin. The presence of the periodontopathogens *P. gingivalis*, *T. forsythia* and *T. denticola* was investigated by RealTimePCR. The graphs depict the expression of T-bet and IFN-γ in CP patients regarding their TBX21 SNP (rs4794067) genotype concomitantly with their positiveness or not to the detection of each periodonto pathogen. (**A**) T-bet mRNA expression in positive/negative subjects for *P. gingivalis*. (**B**) T-bet mRNA expression in positive/negative subjects for *T. forsythia*. (**C**) T-bet mRNA expression in positive/negative subjects for *T. denticola*. (**D**) IFNγ mRNA expression in positive/negative subjects for *P. gingivalis*. (**E**) IFN-γ mRNA expression in positive/negative subjects for *T. forsythia*. (**F**) IFN-γ mRNA expression in positive/negative subjects for *T. denticola*.

microbiological data accounted for a 35% of the variation of the response variable PD, and the genetic variance proved insignificant when fitted into the model. It is also worth noting that the generic data failed to demonstrate a significant effect in the OLS regression (PD as outcome) and in the logistic regression (disease as outcome) when tested as the sole explanatory variable.

In summary, the data presented supports the previous indicatives that TBX21-1993T/C (rs4794067) polymorphism is in fact functional, and that genotypes carrying the T allele are associated with a noticeable increase in T-bet transcription as well as a significant (while modest) increase in the risk to develop chronic periodontitis. Interestingly, the marked impact of TBX21-1993 T allele is not reflected in a similar magnitude in the IFN-γ transcription, and does not influence the pattern of pathogen bacterial infection or the clinical parameters of disease severity, being the presence/absence of red complex bacteria the main factor associated with the disease status and subrogate variable PD in the logistic regression analysis. However, it is important to consider that the cross-sectional nature of the study design poses limitations on the conclusions that can be inferred from the data. Further, it is possible that uncontrolled variables in the recruitment phase of the study could have influenced the results. Particularly, although the voluntaries were age and gender matched, no socio-economical information was recorded. Since socio-economic factors can impact the development of periodontitis, it is possible that this uncontrolled confounder exerted some impact in the reported results. Nevertheless, it is worth considering that the population that uses the services from the dental clinic of the Ribeirão Preto Dentistry School is fairly homogeneous in socio-economic terms, so the effect of this confounder is arguably limited.

Also, it is possible that the single time point sampling caused the lack of association between genotype and microbiological data and that a time-series sampling strategy could overcome this issue. Indeed, the very nature of the pathogenesis of periodontal disease impedes a simple straightforward association between a particular genotype and disease phenotype, requiring for its

Therefore, the data presented herein demonstrates a disproportionate large effect of the presence/absence of red complex bacteria with the disease status and the periodontitis subrogate variable PD. Even though the T-allele carriers were enriched in the diseased group, and the TT and CT genotypes were overrepresented in the CP patients group (indicating an association of the SNP with the disease status); the environmental microbiological covariate obscures this association in the logistic regression model. This is also true for the OLS regression model, where the

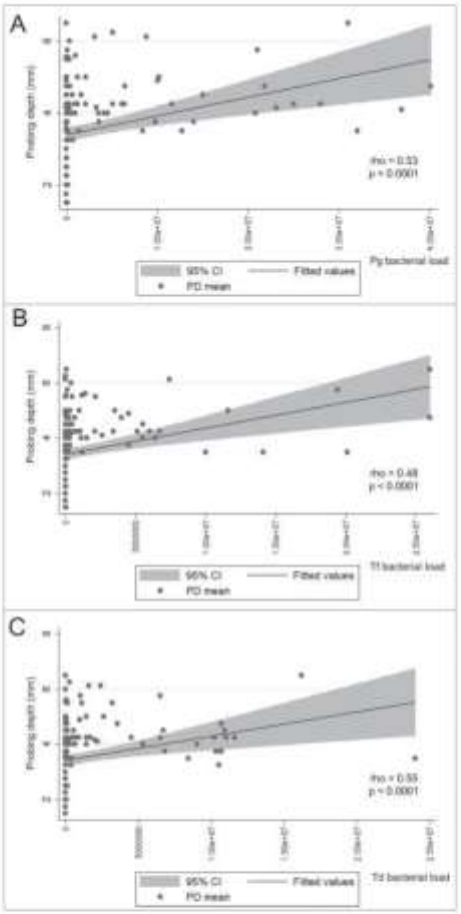

Figure 5. Correlation between the load of each pathogen and the disease subrogate clinical measure probing depth in chronic periodontitis patients. Quantitative assessment of bacterial load was performed by RealTimePCR. The graphs depict the lineal regression and the 95% confidence interval (in gray) for the bacterial load and the PD. Regression coefficients (rho) and corresponding p-values were calculated. (**A**) *P. gingivalis* bacterial load correlation to PD (rho = 0.53, *P*-value < 0.0001). (**B**) *T. forsythia* bacterial load correlation to PD (rho = 0.48, *P*-value < 0.0001). (**C**) *T. denticola* bacterial load correlation to PD (rho = 0.53, *P*-value < 0.0001).

development the cumulative effect of multiple genes and environmental causal factors, each of which are neither necessary nor sufficient to individually cause the disease.[44,45] The simultaneous analysis of numerous functional SNPs and multiple microbiological, metabolic and immune markers through hypothesis-free tools (such as cluster analysis) becomes necessary in order to unveil the complicated and intermingled paths that tip the balance from health to disease. The way to future discovery and insight in the paths that leads to disease must contemplate the simultaneous association of known causative factors (such as red complex infection), with multiple putative disease markers (genetic, environmental, metabolic, inflammatory, etc.) in order to construct valid explanatory and prognosis models. The knowledge gained from these kinds of approaches will provide the tools for the development of more specific diagnostic protocols and therapeutic interventions.

Materials and Methods

Study population and sample collection

Patients and controls from the São Paulo state, south-eastern region of Brazil, scheduled for treatment at the Dentistry School of University of Ribeirão Preto (UNAERP), were submitted to anamnesis, radiographic study and clinical examination by an experienced periodontist (scored for bleeding on probing, probing depth and clinical attachment loss). All enrolled subjects signed an informed consent form that was approved by the Institutional Review Board, and received supra gingival prophylaxis. Exclusion criteria was applied as follows: not providing informed consent; medical history indicating evidence of known systemic modifiers of periodontal disease, and having received periodontal therapy in the previous 2 years. Current and former smokers were specifically excluded. No strategy was used to identify subpopulations (population stratification) or population relatedness among the recruited subjects. After the diagnostic phase, patients were subsequently categorized into healthy (H; n = 218) (the classic control), chronic gingivitis (CG; n = 193)(the 'resistant' phenotype control) or chronic periodontitis (CP; n = 197)(the susceptible subjects, the case-study group) groups, as previously described.[21,33]

Epithelial buccal cells were sampled scrapping the inner cheek buccal mucosa after a mouthwash with 3% glucose in all groups; while biopsies of gingival tissue and microbiological samples were collected from fractions of CP and H groups as previously described in detail.[21,33] Patients in the chronic periodontitis group (CP; n = 197) presenting moderate to advanced periodontitis (at least one teeth per sextant with probing depth > 6mm and clinical attachment loss >3 mm plus radiographic evidence of extensive bone loss [>30% alveolar bone height in at least 50% of teeth]), received basic periodontal therapy. Biopsies of gingival tissue (one sample from each patient) were obtained during surgical therapy of the sites that exhibited no improvement in clinical condition (i.e. persistent bleeding on probing and increased probing depth) 3–4 weeks after the basic periodontal therapy (n = 123). Gingival biopsies were taken during surgical therapy as previously described[34] and comprised junctional epithelium, pocket epithelium and gingival connective tissue. The healthy control group (H; n = 218) included subjects with clinically healthy gingival tissues (<10% of bleeding on probing; no sites with probing depth >3 mm, no clinical attachment loss and

no radiographic evidence of alveolar bone loss) scheduled to undergo restorative dentistry procedures. A representative fraction of the control group (n = 63) was also scheduled to surgical procedures for restorative/prosthetic reasons, when biopsies of healthy gingival tissue were taken. Gingival biopsies were taken from sites showing no bleeding on probing and probing depth <3 mm during surgical procedures due to esthetics, orthodontic and/or prosthetic reasons, and also comprised junctional epithelium, gingival crevicular epithelium and gingival connective tissue, as previously described.[34] The chronic gingivitis group (CG; n = 193), representing a 'resistance phenotype' to case-control analysis,[21] was composed of subjects with clinical history of poor oral hygiene, bleeding on probing >70% of periodontal sites and no clinical attachment loss (CAL) or radiographic evidence of alveolar bone loss. Due to ethical restrictions (lack of indication of surgical therapy) no gingival samples were collected from CG group. The clinical features of the groups are summarized in **Table 1**.

In order detect and quantify *P. gingivalis*, *T. forsythia* and *T. denticola*, periodontal crevice/pocket biofilm samples were collected with a sterile paper point ISO #40 from the same site biopsied immediately before the periodontal surgical procedure (H n = 63; CP n = 123), as described elsewhere.[46] Since no surgical procedures were conducted in CG group due to ethical restrictions as previously mentioned, no samples for microbiological analysis were collected from CG group.

Analysis of TBX21-1993T/C SNP (rs4794067)

DNA was extracted from epithelial buccal cells with sequential phenol/chloroform solution and precipitated with salt/ethanol solution, as described elsewhere.[47] Extracted DNA was immediately used for genotyping. DNA integrity was checked and the allelic discrimination of TBX21-1993T/C SNP (rs4794067) variants was performed in 3 μL reactions using Taqman (Applied Biosystems, Warrington, UK) chemistry as previously described.[48,49] Genotyping was performed blinded to group status. For reaction quality control, a sample of known genotype was included in the plate and a no DNA template sample was included as negative control. Only genotypes with an automatic call rate >95% were considered, error rate was <3%. Samples that failed to provide a genotype were repeated in additional reactions. Genoryping procedures, from isolation to allelic discrimination and data analysis was performed in the laboratory of Osteoinmunology of the Bauru Faculty of Dentistry of the University of Sao Paulo.

Real-Time PCR reactions – IFN-γ and T-bet mRNA quantification

The extraction of total RNA from periodontal tissues samples was performed with Trizol reagent (Invitrogen), and the cDNA synthesis were accomplished as previously described.[34] Real-Time-PCR mRNA or DNA analyses were performed in an Mini-Opticon system (BioRad, Hercules, CA, USA), using SybrGreen MasterMix (Invitrogen), specific primers (IFN-γ: sense ATGAAATATACAAGTTATATCATGC, antisense TGTTTCGAGGTCGAAGAGCATCCCAGTAA; T-bet: sense CCTCTTCTATCCAACCAGTAT, antisense CTCCGCTTCATAACTGTG; Beta-actin: sense ATGTTTGAGACCTTCAACAC, antisense CACGTCADACTTCATGATGG) and 2.5ng of cDNA in each reaction, as previously described.[32,33] The standard PCR conditions were 95°C (10 minutes), and then 40 cycles of 94°C (1 minute), 56°C (1 minute), and 72°C (2 minutes), followed by a standard denaturation curve. Negative controls without cDNA and without the primer/probe sets were also performed. Calculations for determining the relative levels of gene expression were made from triplicate measurements of the target gene, with normalization to β-actin in the sample, using the cycle threshold (Ct) method and the $2^{-\Delta\Delta ct}$ equation, as previously described.[33]

Real-Time PCR reactions – bacterial DNA quantification

In order to detect *P. gingivalis*, *T. forsythia* and *T. denticola*, periodontal crevice/pocket biofilm samples were collected with a sterile paper point ISO #40 from the same site biopsied immediately before the periodontal surgical procedure (H n = 63 and CP n = 123), as described elsewhere.[46] Bacterial DNAs were extracted from plaque samples by DNA Purification System (Promega).[34] RealTime-PCR mRNA or DNA analyses were performed in a MiniOpticon system (BioRad), using SybrGreen MasterMix (Invitrogen), specific primers (*P. gingivalis*: sense TGCAACTTGCCTTACAGAGGG, antisense ACTCGTATCGCCCGTTATTC; *T. forsythia*: sense GGGTGAGTAACGCGTATGTAACCT, antisense ACCCATCCGCAACCAATAAA; *T. denticola*: sense AGAGCAAGCTCTCCCTTACCGT, antisense TAAGGGCGGCTTGAAATAATGA), and 5ng of DNA in each reaction.[35] The standard PCR conditions were 95°C (10 minutes), and then 40 cycles of 94°C (1 minute), 56°C (1 minute), and 72°C (2 minutes), followed by a standard denaturation curve. The positivity to bacteria detection and the bacterial counts in each sample were determined based on the comparison with a standard curve comprised by specific bacterial DNA (10^9 to 10^2 bacteria) and negative controls, similar to previously described,[21,35] and then adjusted for sample dilution in the assay to obtain the copy numbers in each site (i.e., absolute load). The sensibility range of bacteria detection and quantification of our RealTime-PCR technique was of 10^5 to 10^8 bacteria to each of the 3 putative periodontal pathogens tested.

Statistical analysis

The Shapiro-Wilk test was performed to test the distribution of all test groups prior to comparative analysis; *P*-value >0.05 was considered indicative of normal distribution. The differences in the demographic and clinical data for the study populations were tested with the Fisher's exact test, rank sum Wilcoxon test (Mann-Whitney U test) and one-way ANOVA or Kruskal-Wallis test, depending of the data distribution. The intra examiner agreement was tested by Cohen's kappa 4 times a year during the recruiting phase of the project using the repeated measures strategy, a kappa value >0.81 was considered the critical value (regarded as almost perfect agreement). Genotype and allele distribution among groups was tested by Fisher's exact test and the

equality of proportions test. The bacterial load between CP and H groups was compared by the rank sum Wilcoxon test (Mann-Whitney U test); the microbiological data was compared between genotype groups inside the CP and H groups by the Kruskal-Wallis test. The T-bet and IFN-γ expression levels were compared by the unpaired t test and rank sum Wilcoxon test (Mann-Whitney U test). All tests were performed in Stata11.1 (College Station, TX USA) or GraphPad Prism 5.01 (San Diego, CA USA). Power calculation of the study was performed using CaTS software (Department of Biostatistics and Center for Statistical Genetics, University of Michigan) as elsewhere described[50] using Multiplicative, Additive, Dominant and Recessive models. Chi-squared test with 1 degree of freedom was performed to test the Hardy-Weinberg equilibrium in the allele frequencies of the study populations, using the *Hardy-Weinberg equilibrium calculator including analysis for ascertainment bias* online-based tool.[51] The association between genetic and microbiological data was tested by logistic regression and Cochrane-Armitage interaction in the trend analysis using the *SNPStats* web-based tool.[52] A *P*-value < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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2.3 ARTICLE 3 – CCR5Δ32 (rs333) polymorphism is associated with decreased risk of chronic and aggressive periodontitis: a case-control analysis based in disease resistance and susceptibility phenotypes*

Abstract

Chronic and aggressive periodontitis are infectious diseases characterized by the irreversible destruction of periodontal tissues, which is mediated by the host inflammatory immune response triggered by periodontal infection. The chemokine receptor CCR5 play an important role in disease pathogenesis, contributing to pro-inflammatory response and osteoclastogenesis. CCR5Δ32 (rs333) is a loss-of-function mutation in the *CCR5* gene, which can potentially modulate the host response and, consequently periodontitis outcome. Thus, we investigated the effect of the CCR5Δ32 mutation over the risk to suffer periodontitis in a cohort of Brazilian patients (total n=699), representative of disease susceptibility (chronic periodontitis, n=197; and aggressive periodontitis, n=91) or resistance (chronic gingivitis, n=193) phenotypes, and healthy subjects (n=218). Additionally, we assayed the influence of CCR5Δ32 in the expression of the biomarkers TNFα, IL-1β, IL-10, IL-6, IFN-γ and T-bet, and key periodontal pathogens *P. gingivalis, T. forsythia,* and *T. denticola*. In the association analysis of resistant versus susceptible subjects, CCR5Δ32 mutant allele-carriers proved significantly protected against chronic (OR 0.49; 95% CI 0.29-0.83; p-value 0.01) and aggressive (OR 0.46; 95% CI 0.22-0.94; p-value 0.03) periodontitis. Further, heterozygous subjects exhibited significantly decreased expression of TNFα in periodontal tissues, proving a functional effect of the mutation in periodontal tissues during the progression of the disease. Conversely, no significant changes were observed in the presence or quantity of the periodontal pathogens *P. gingivalis, T. forsythia,* and *T. denticola* in the subgingival biofilm that could be attributable to the mutant genotype.

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1 Introduction

Periodontitis is a chronic infectious disease characterized by the irreversible and progressive destruction of teeth-supporting structures. Periodontitis is initiated by the bacteria harbored in the teeth-attached biofilm that activate the host's inflammatory immune response, which provides protection against the infecting agents. However, host response mediators also modulate local proteolytic and osteoclastogenic activities, leading to soft and mineralized tissue destruction as collateral damage (1).

In this context, destructive and protective host immune responses have been described to present specific molecular patterns in response to periodontal infection. Pro-inflammatory responses mediated by IL-1, IL-6 and TNFα, along with Th1 and/or Th17 cells, have been associated with the establishment and progression of periodontal destruction, while Th2 and Treg-associated response patterns have been described to attenuate/arrest tissue destruction (2, 3). While the exact mechanisms underlying the host response polarization in periodontium remains to be elucidated, the chemokine system has been described as an important player in the determination of the nature of the host response via the selective recruitment of immune cells subsets, which can amplify the inflammation or suppress it (4).

The system comprised by the receptor CCR5 and its ligands CCL3, CCL4 and CCL5 has proved important in the inflammatory periodontal destruction associated with periodontitis (5, 6). CCR5 is a 352-amino acid G protein-coupled chemokine receptor which is involved in the selective recruitment of multiple leukocytes subsets, including neutrophils, monocytes, NK cells and lymphocytes (7, 8). Indeed, CCR5 mediates the recruitment of CCR5+F4/80+ and CCR5+CD4+ leukocytes, regarded as pre- and pro-osteoclastogenic cells involved in the bone resorption that characterizes periodontitis (9). The activation of the transduction signals triggered by CCR5 coupling with ligands such as CCL3, CCL4 and CCL5 stimulates not only cell migration, but also influences proliferation, cytokine expression and activation of effector functions (10). In fact, pharmacological inhibition of CCR5 using met-RANTES had proved sufficient to arrest inflammatory cytokine expression, and consequently to suppress alveolar bone loss in mice (8). Similarly, CCR5 deficient mice (CCR5KO) present a decreased infiltration of leukocytes and decreased bone loss upon experimental periodontal infection (9).

Importantly, CCR5+ cells also contribute to the protective responses against periodontal infection (8, 9).

Interestingly, the human counterpart of the CCR5KO mice strain may be represented by carriers of the CCR5Δ32 mutation. CCR5Δ32 (rs333) is a 32-base pair deletion causing a frameshift variant in the *CCR5* gene leading to a loss-of-function due by the abolition of the receptor's cell-surface expression (11, 12). Indeed, this truncated variant has been demonstrated to be clinically relevant since it has been associated with various phenotypes, including resistance to HIV infection, decreased risk for type 1 diabetes, increased risk for diabetic retinopathy in type 1 diabetes patients and increased risk of multiple sclerosis (13-15). Thus, based on the relevance of CCR5 to experimental periodontitis pathogenesis, it is reasonable to hypothesize that CCR5Δ32 could be a relevant risk factor for human periodontitis. Two previous studies had found no association between CCR5Δ32 and chronic periodontitis, but methodological shortcomings in their designs warrant a reappraisal of the possible contribution of the mutation to periodontitis' risk profile (16, 17).

Indeed, the absence of proper susceptibility and resistance phenotypes definition critically dampen such studies power and odds of identification of genetic factors potentially involved in periodontitis pathogenesis (1, 18). Chronic periodontitis is the most common form of the disease, which is characterized by destruction of teeth-supporting tissues over a long period of time, comprising progressive/active and stable/inactive periods (2, 19). A less common variant is the aggressive form of the disease, characterized by rapid progression of the periodontal destruction (20). Conversely, chronic gingivitis is a periodontal disease characterized by widespread gingival inflammation without irreversible destruction of the teeth-supporting tissues, even over long periods of time (21). Despite some differences in the microbial biofilm associated with the different forms of periodontal disease, such conditions are basically initiated by the same stimuli, Gram-negative periodontal bacteria (22, 23). However, the clinical course of each entity reflects inherent differences in the host's capacity to cope with the presence of periodontophatic bacteria (2, 3). Consequently, chronic and aggressive periodontitis can be considered the phenotypic expression of supposed susceptibility genotypes, while chronic gingivitis allegedly represents a resistant phenotype/genotype (18, 24). Indeed, the analysis of opposing resistant and susceptible phenotypes, determined

by the observance the outcome of exposure to the microbial challenge, increases the possibilities of finding a true association between genetic markers and disease phenotype (1, 18).

Therefore, it is plausible to hypothesize that CCR5Δ32 (rs333) may account for the differential expression of disease susceptibility (such as chronic and aggressive periodontitis) or resistance (such as chronic gingivitis). Also, it is possible that the hypofunctional CCR5 variant could affect the expression of host response factors in periodontal tissues, as well as influence the pattern of bacterial infection in periodontitis.

Therefore, we conducted a case-control study to investigate whether the CCR5Δ32 (rs333) is associated with periodontitis risk; performing for the first time an analysis in groups defined as susceptible and resistant, and also investigated the putative functionality of this SNP in the modulation of CCR5-related host mediators (TNFα, IL-1β, IL-6, IL-10, IFN-γ, and T-bet) *in situ*, as well as its possible impact in the composition of subgingival biofilm, in an attempt to unveil a conceivable mechanism linking the gene variant with the disease phenotype.

2 Materials and methods

2.1 Participants

The sample was recruited at the São Paulo state, south-eastern region of Brazil, from patients scheduled for treatment at the Dentistry School University of Ribeirão Preto. Patients were examined by an experienced periodontist and scored for bleeding on probing (BOP), probing depth (PD) and clinical attachment loss (CAL). Enrolled subjects provided informed consent that was approved by the Institutional Review Board. Subjects were excluded from the study if they were tobacco smokers (including former smokers), had medical history indicating evidence of known systemic modifiers of periodontal disease, and/or had received periodontal therapy in the previous 2 years. No strategy was used to identify subpopulations (population stratification) or population relatedness among the recruited subjects. After the diagnostic phase, patients were subsequently categorized into healthy (H; n=218) [classic control], chronic gingivitis (CG; n=193) [‘resistant’ phenotype control], chronic periodontitis (CP;

n=197) [susceptible subjects] and aggressive periodontitis (AP; n=91) [highly susceptible subjects], as previously described (1, 18).

The control group (H; n=218) included subjects with clinically healthy gingival tissues scheduled to undergo dental restorative procedures. The chronic gingivitis group (CG; n=193) corresponded to subjects with history of poor oral hygiene, BOP >70% of periodontal sites and no CAL or radiographic evidence of alveolar bone loss. Patients in the chronic periodontitis group (CP; n=197) included subjects diagnosed with moderate to severe periodontitis (at least one teeth per sextant with probing depth >6 mm and clinical attachment loss >3 mm plus radiographic evidence of extensive bone loss [>30% alveolar bone height in at least 50% of teeth]), scheduled to receive periodontal therapy. The aggressive periodontitis group (AP; n=91) included subject with moderate to severe periodontitis (at least one teeth per sextant with probing depth >6 mm and clinical attachment loss >3 mm plus radiographic evidence of extensive bone loss [>30% alveolar bone height in at least 50% of teeth]) under the age of 35 years. Table 1 summarize all relevant clinical and demographical information of each sample population.

2.2 DNA sampling and CCR5 genotyping (rs333)

Oral mucosa epithelial cells from each participant (n=699) were obtained by scrapping the inner cheek after a mouthwash with 3% glucose. DNA was extracted from epithelial cells with sequential phenol/chloroform solution and precipitated with salt/ethanol solution. Extracted DNA was immediately used for genotyping. We genotyped CCR5Δ32 (rs333) by polymerase chain reaction (PCR) with forward primer 5′ – ATGCTGTGTTTGCTTTAAAAGCCAGG – 3′ and reverse primer 5′ – AGGACCAGCCCCAAGATGACTA – 3′ flanking the 32 nucleotide deletion in the CCR5 gene. Amplification was performed in 35 cycles as follows: denaturation step 94 °C for 30 seconds, annealing 56 °C for 30 seconds, and extension 72 °C for 30 seconds. The DNA amplicons were visualized under UV light on 2% agarose gel stained with ethidium bromide. The wild-type amplicon corresponded to a 220-bp band while the CCR5Δ32 allele corresponded to a 188-bp band. Negative and positive controls were included in each reaction.

2.3 Gingival tissue sampling, subgingival biofilm sampling, and quantitative real-time PCR

Gingival biopsies were obtained during surgical procedures and comprised epithelium and underlying connective tissue from a fraction of the healthy (n=63) and chronic periodontitis samples (n=123). For the healthy sample, gingival biopsies were taken from a representative fraction of the control group (n=63) when they underwent surgical procedures for restorative/prosthetic reasons. For the chronic periodontitis group (n=123), gingival biopsies were taken during periodontal surgical therapy as previously described (25). Briefly, biopsies of gingival tissue were obtained during surgical therapy of the sites that exhibited no improvement in clinical condition 4-6 weeks after the basic periodontal therapy. Aggressive periodontitis subjects were not biopsied, since the treatment protocol required a 14 days antibiotic treatment previous to the surgical therapy. Ethical considerations (lack of indication of surgical therapy) prohibited tissue sampling from the chronic gingivitis group.

Periodontal crevice/pocket biofilm samples were collected with a sterile paper point ISO #40 from the same site biopsied immediately before surgical procedure (H n=63; CP n=123) to quantify the bacterial load of the pathogens *P. gingivalis*, *T. forsythia* and *T. denticola*. The bacterial DNA quantification procedures has been extensively described elsewhere (1, 26). Briefly, bacterial DNA from periodontal biofilm samples was extracted by DNA Purification System (Promega) (25). RT-PCR analyses were performed in a MiniOpticon system (BioRad), using SybrGreen MasterMix (Invitrogen), specific primers (*P. gingivalis*: sense TGCAACTTGCCTTACAGAGGG, antisense ACTCGTATCGCCCGTTATTC; *T. forsythia*: sense GGGTGAGTAACGCGTATGTAACCT, antisense ACCCATCCGCAACCAATAAA; *T. denticola*: sense AGAGCAAGCTCTCCCTTACCGT, antisense TAAGGGCGGCTTGAAATAATGA), and 5ng of DNA in each reaction (27). The standard PCR conditions were 95°C (10 minutes), and then 40 cycles of 94°C (1 minute), 56°C (1 minute), and 72°C (2 minutes), followed by a standard denaturation curve. A standard of specific bacterial DNA (10^9 to 10^2 bacteria) was used to quantify the bacterial load of the tested samples.

RNA was extracted from gingival biopsies using TRIzol (ThermoFisher Scientific) reagent following the manufacturer's instructions. Reverse transcription was

accomplished with RevertAid First Strand cDNA synthesis kit (ThermoFisher Scientific) following the manufacturer's instructions. Quantitative RT-PCR were performed in an MiniOpticon system (BioRad, Hercules, CA, USA), using SybrGreen MasterMix (Invitrogen), specific primers (TNFα: sense AAGCCTGTAGCCCATGTTGT, antisense CAGATAGATGGGCTCATACC; IL-1β: sense GGAACCCCAGAGCGAAATACA, antisense CCTGAAGAATGCCTCCTCACA; IL-10: sense AGATCTCCGAGATGCCTTCA, antisense CCGTGGAGCAGGTGAAGAAT; IL-6: sense AAATTCGGTACATCCTCGAC, antisense CAGGAACTGGATCAGGACTT; IFN-γ: sense ATGAAATATACAAGTTATATCATGC, antisense TGTTTCGAGGTCGAAGAGCATCCCAGTAA; T-bet: sense CCTCTTCTATCCAACCAGTAT, antisense CTCCGCTTCATAACTGTG; Beta-actin: sense ATGTTTGAGACCTTCAACAC, antisense CACGTCADACTTCATGATGG) and 2.5ng of cDNA in each reaction, as previously described in detail (28, 29). The standard PCR conditions were 95°C (10 minutes), 40 cycles of 94°C (1 minute), 56°C (1 minute), and 72°C (2 minutes), followed by a standard denaturation curve. Negative controls without cDNA and without the primer/probe sets were also performed. Calculations for determining the relative levels of gene expression were made from triplicate measurements of the target gene normalized to β-actin, using the $2^{-\Delta\Delta ct}$ method.

2.4 Statistical analysis

Differences among clinical and demographical data was tested with chi-squared test and Student's t test. Differences in allele frequency between the sample population and reference populations were tested by Yates's corrected chi-square test. Standard and allelic case/control association analysis was performed using chi-squared test and Fisher's exact test. Data distribution of continuous variables was tested with Shapiro-Wilk test. Differences between continuous data were tested with Student's t-test or Mann-Whitney u test. A p-value < 0.05 was considered significant. Analyses were performed in GraphPad Prism v7.02 (GraphPad software, La Jolla CA) and PLINK v1.07 (30).

3 Results

3.1 Clinical characteristics, genotyping and case/control association

There were significant differences in demographics and clinical parameters among patient's groups that had been extensively described elsewhere (1, 18). The AP group presented similar periodontal destruction to the CP group (represented by attachment loss) at a significantly lower age (Table 1).

	Healthy [H] (N=218)	Chronic Periodontitis [CP] (N=197)	Aggressive periodontitis [AP] (n=91)	Chronic Gingivitis [CG] (n=193)	H v/s CP	H v/s CG	CG v/s CP	CP v/s AP
N and gender distribution	***105 f / 113 m***	***96 f / 101 m***	***49 f / 42 m***	***97 f / 96m***	***0.9219***	***0.8395***	***0.9583***	***0.4485***
Age	47.48 ± 5.96	46.63 ± 7.34	26.34 ± 4.72	49.54 ± 6.47	0.19	0.0093	<0.001	<0.001
Clinical parameters	***value ± SD***	***value ± SD***	***value ± SD***	***value ± SD***				
Probing depth	2.25 ± 0.62	4.29 ± 0.75	3.75 ± 1.38	2.72 ± 0.52	<0.001	<0.001	<0.001	<0.001
Clinical Attachment Loss	0.62 ± 0.22	3.92 ± 0.64	3.88 ± 1.51	0.69 ± 0.25	<0.001	0.0027	<0.001	0.7523
% BOP	4.86 ± 2.86	62.99 ± 8.71	51.14 ± 7.92	61.85 ± 11.49	<0.001	<0.001	0.2696	<0.001
Plaque index	34.71 ± 8.26	51.26 ± 9.78	46.41 ± 8.51	53.47 ± 10.05	<0.001	<0.001	0.0284	<0.001
16S DNA ($x10^9$)	0.09 ± 0.20	14.58 ± 34.54	11.87 ± 31.26	15.51 ± 33.18	<0.001	<0.001	0.7865	0.5244

Table 1. Demographical information and clinical parameters of H, CP, AP, and CG groups.

Genotype and allele frequencies of the studied polymorphism were in Hardy-Weinberg equilibrium in the H, CG, CP, and AP sample populations ($p>0.05$).

The allele frequency for the minor allele was 0.078 [1289/109], resembling the average allele frequency described in the latest release of the Exome Aggregation Consortium (ExAC) (12) 0.073 [112599/8813], but significantly different from the allele frequency reported for the Latino sample of ExAC [11257/321] (Yates's corrected chi-square 96.72; p-value <0.001) (31).

The polymorphic allele proved significantly more common in the chronic gingivitis group than in the chronic periodontitis (p-value 0.01) and the aggressive periodontitis groups (p-value 0.04). All other allele frequency comparisons among groups proved non-significant (Table 2).

CCR5d32 (rs333)	**Healthy (n=218)**		**CP (n=197)**		**AP (n=91)**		**CG (n=193)**	
GENOTYPE	N	%	N	%	N	%	N	%
WT/WT	186	85.3	175	88.8	81	89.0	156	80.8
WT/D32	31	14.2	21	10.7	10	11.0	31	16.1
D32/D32	1	0.5	1	0.5	0	0.0	6	3.1
ALELLE	N	%	N	%	N	%	N	%
WT	403	92.4	371	94.2	172	94.5	343	88.9
D32	33	7.6	23	5.8	10	5.5	43	11.1

Table 2. Genotype and allele frequencies for H, CP, CG and AP groups.

The association analysis demonstrated a significant protective effect of the polymorphic allele when the chronic gingivitis ['resistant' phenotype] sample was compared with both the chronic (OR 0.49; CI 0.29-0.83; p-value 0.01) and aggressive (OR 0.46; CI 0.22-0.94; p-value 0.03) periodontitis groups ['susceptible' phenotypes] (Table 3). The polymorphic allele was significantly more prevalent in unaffected/control subjects than in patients/cases. The case/control association tests using healthy patients as controls failed to prove any significant effect of rs333.

	Frequency affected	Frequency unaffected	chi^2	P	OR	SE	95% CI
Healthy vs Gingivitis	0.11	0.08	3.11	0.08	1.53	0.24	0.95-2.46
Healthy vs Chronic periodontitis	0.06	0.08	0.99	0.32	0.76	0.28	0.43-1.31
Healthy vs Aggressive periodontitis	0.05	0.08	0.85	0.36	0.71	0.37	0.34-1.47
Gingivitis vs Chronic periodontitis	0.06	0.11	7.08	**0.01**	0.49	0.27	0.29-0.83
Gingivitis vs Aggressive periodontitis	0.05	0.11	4.66	**0.03**	0.46	0.36	0.22-0.94

Table 3. Allelic association analysis for rs333 and the phenotypes H, CG, CP and AP. OR = odds ratio. SE = standard error. 95% CI = 95% confidence interval.

3.2 Inflammatory biomarkers

As expected, all inflammatory biomarkers where significantly upregulated in chronic periodontitis patients irrespective of their genotype. There was a significant decrease in the expression level of TNFα in heterozygous subjects compared with ancestral homozygous in the chronic periodontitis group (p-value 0.01). There were no significant differences in IL-1β, IL-10, IL-6, T-bet, and IFN-γ mRNA expression between wild type and heterozygous subjects in the chronic periodontitis group (Figure 1).

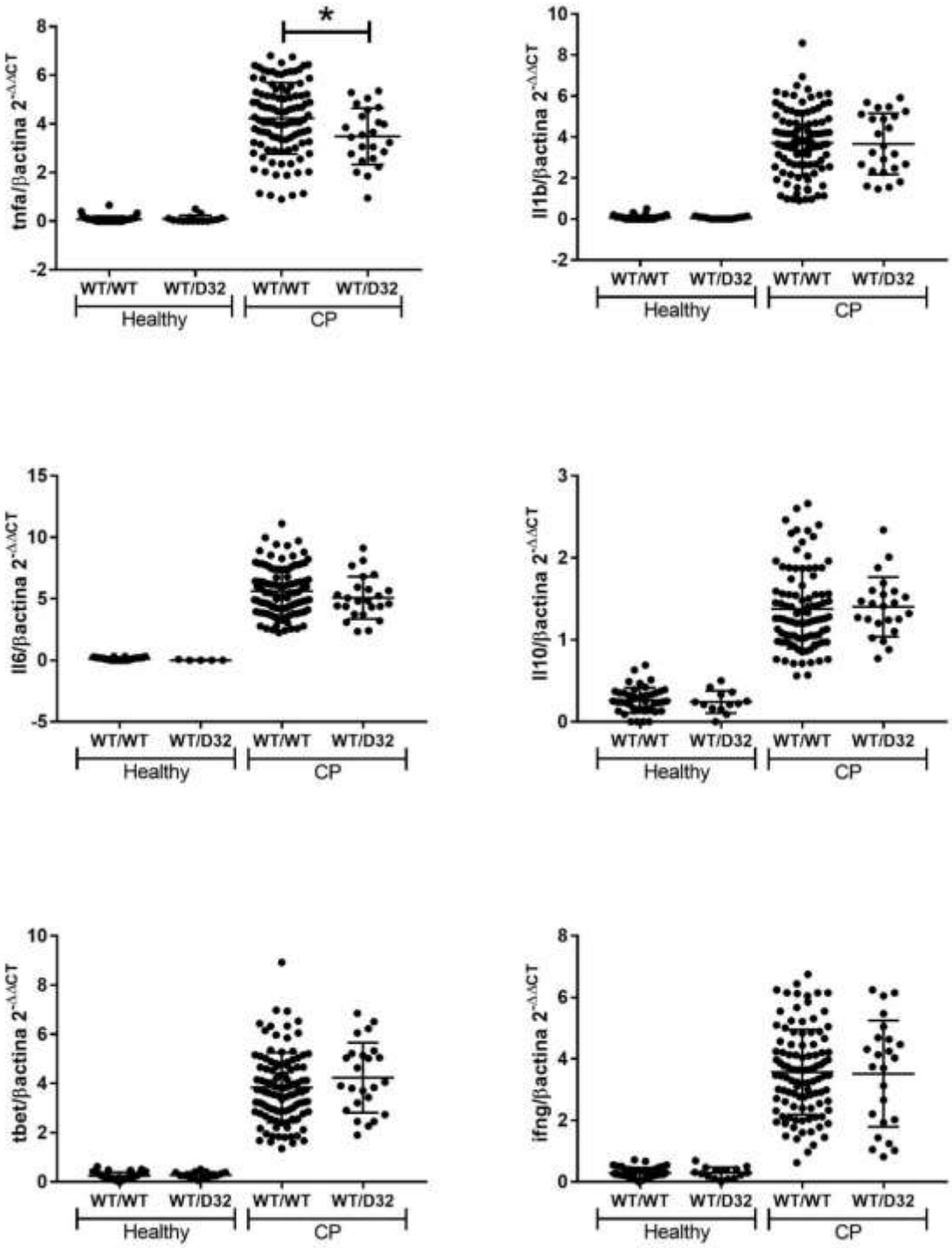

Figure 1. Relative expression of TNFα, IL-1β, IL-6, IL-10, T-bet, and IFN-γ in H and CP subjects according to their genotype for CCR5Δ32. * = p-value < 0.05.

3.3 Subgingival biofilm profile

There were no significant differences in the bacterial load for the tested putative periodontal pathogens (*P. gingivalis, T. forsythia, and T. denticola*) between the wild type (WT) and WT/CCR5Δ32 heterozygous chronic periodontitis patients (Figure 2).

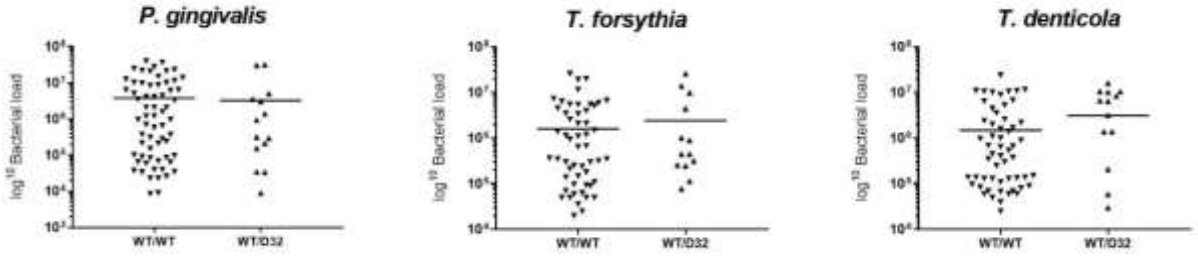

Figure 2. Bacterial load of *P. gingivalis, T. forsythia, and T. denticola* on periodontal biofilm from chronic periodontitis patients arranged by CCR5Δ32 genotype.

Similarly, there were not significant differences for the frequency of detection of the three putative periodontal pathogens tested (*P. gingivalis, T. forsythia, and T. denticola*) between the WT and WT/CCR5Δ32 heterozygous chronic periodontitis patients (Table 4).

	P. gingivalis				T. forsythia				T. denticola			
	WT/WT		WT/D32		WT/WT		WT/D32		WT/WT		WT/D32	
	N	%	N	%	N	%	N	%	N	%	N	%
Positive	64	62.1	14	58.3	61	59.2	13	54.2	61	59.2	13	54.2
Negative	39	37.9	10	41.7	42	40.8	11	45.8	42	40.8	11	45.8

Table 4. Frequency of detection of *P. gingivalis, T. forsythia, and T. denticola* on periodontal biofilm from chronic periodontitis patients.

4 Discussion

Periodontitis is characterized by the inflammatory destruction of periodontal tissues concomitant to the immune response triggered by periodontal infection. The chemokine system is a critical determinant of host response by directing the selective infiltration of distinct leukocyte subsets into periodontal tissues, and consequently influences the outcome of the disease. The chemokine receptor CCR5 has a putative crucial role in periodontitis' pathogenesis, since it directs the migration of pro-inflammatory and pro-osteoclastogenic cells. Taking in consideration that CCRΔ32 (rs333) directly impacts CCR5 function, we conducted a genetic association study coupled with the analysis of secondary functional variables to address the possible influence of this genetic variant in the susceptibility to develop periodontitis.

The allele frequency of the polymorphic allele CCR5Δ32 in the studied population was similar to the average reported frequency in the ExAC (Exome Aggregation Consortium) (31), nevertheless it was significantly different to the frequency reported

for Latino ancestry in ExAC. This is arguably due to the methodological discrepancy between our sampling and the ancestry definition strategy used by ExAC. Briefly, ExAC uses a principal component analysis to distinguish the major axes of geographic ancestry and then clusters the individuals according with their putative origin. It is possible that the Latino definition of the ExAC data base encompass a significantly broader and diverse population than the one recruited for this study, which is exclusively constituted by south-eastern Brazilians that are characterized by significant European (86.1%) and African (12.0%) heritage contributions (31, 32). Still, due to the distinctive high genetic admixture observed in Brazilian population, the ancestry cannot be inferred based on phenotypic characteristics (33-35).

The association analysis demonstrated a protective effect of the CCR5Δ32 polymorphism, which was significantly more prevalent in the resistant subjects presenting chronic gingivitis (CG) than in the two susceptible samples represented by aggressive and chronic periodontitis (section 3.1). Interestingly, when the association analysis was performed with the traditional design used in genetic association periodontitis studies, with healthy individuals as the control population, no significant associations were observed. As previously demonstrated by our group, CG subjects are chronically exposed to the bacterial challenge to periodontal tissues (evidenced by a bacterial load similar to that of CP patients), but suffer no irreversible inflammatory destruction of the periodontium. In this sense, the CG group is a truly control population, because they share the exposure to the causal factor of the disease with the CP and AP patients. Contrariwise, the healthy sample is not equally exposed to the bacterial challenge as the diseased individuals and it is arguable composed of a heterogeneous population from the susceptibility point of view, ranging from highly resistant to highly susceptible individuals (1, 18). Even though the putative risk genetic profile for human periodontitis is extremely complex, the increased frequency of CCR5Δ32 in ‘resistant’ subjects (CG) is in accordance with our data from experimental periodontitis in CCR5KO mice, where CCR5 null mice proved resistant to periodontitis induction when compared to wild type mice (8, 36).

Indeed, CCR5Δ32 analysis reinforces the mixed nature of healthy subjects, which presents an intermediary frequency of Δ32 allele/genotype, located exactly in between resistant and susceptible groups. At this point, it is important to mention that the two previous studies, which did not find significant association between CCR5Δ32 and

aggressive or chronic periodontitis (16, 17) used the ‘healthy’ group as control, which in fact decrease the odds of finding possible associations in view of the unclear phenotypic/genotypic nature of heathy subjects. In addition, the sample size of both cited studies was insufficient to perform a solid genetic association analysis, since in a complex disease such as periodontitis, the effect of any mutation is expected to be small (37). In the first case (16), the study was most probably under powered due to the small sample recruited (total sample n=175). This can also be said of the second study (17), which recruited only 213 subjects. In comparison, our sample consisted of 699 subjects. Moreover, the inclusion of ‘resistant’ (CG) and a ‘highly susceptible’ sample population (AP) further strengthen our results, because even with a relatively small sample size our strategy detected a significant contribution of the polymorphism in the susceptibility to the disease. This strategy has been already proposed by other groups and it has proven valuable to reveal genotype/phenotype associations without the need of recruiting extremely large sample populations (38-40). In this sense, the careful clinical characterization of the subjects provides a homogenous sample population from the phenotypic point of view, that it is arguably also homogenous from a genetic perspective in terms of prevalence of risk mutations. Our group already demonstrated that this strategy increases the power and odds of genetic studies and that the classic healthy control used in most genetic association periodontal studies has an intermediate genotype for several polymorphisms, occupying an intermediate position between resistant and susceptible subjects (1, 18).

Furthermore, the requirement for screening and exclusion of major disease co-factors during the recruitment stage is critical. Tobacco smoking is the most widely recognized behavioral risk factor for periodontitis (41), thus we explicitly excluded smokers and former smokers in the recruitment phase. This basic precaution wasn’t practiced in the previous studies investigating the role of CCR5Δ32 in periodontitis (16, 17). It is of paramount importance to emphasize that tobacco smoking is the most important modifiable risk factor for periodontitis (42-45) and that failing to control for this factor completely invalidates any genetic association analysis. The confounding effect of tobacco smoking cannot be adequately corrected in small sample populations as the ones used in both cited studies (16, 17), and the only sensible study design alternative is the exclusion of smokers from the sample, as we did. Further, the minor allele frequency for CCRΔ32 (0.13) reported in one of the studies (17) is more than 10-fold

higher than the frequency reported in ExAC for East Asian and Southern Asian populations (0.0002 and 0.014, respectively), probably caused by a biased recruitment strategy which predisposed to a type II error due to population stratification.

Aiming to complement our association results and reveal a possible mechanism of action of CCRΔ32 in conferring resistance to periodontitis, we performed additional experiments characterizing the inflammatory expression pattern of key biomarkers and quantified the bacterial load of selected periodontal pathogens. The analysis of inflammatory biomarkers (section 3.2) revealed a slight, yet significant reduction of TNFα expression levels in heterozygous subjects. Accordingly, previous results in animal models have demonstrated that CCR5 genetic deficiency or pharmacological inhibition results in decreased TNFα levels, and more importantly, reduced periodontal destruction (9). However, while CCR5KO mice presents a broad disruption in the cytokine production pattern during experimental periodontitis, CCR5Δ32 mutation produced no significant effect in the expression levels of IL-1β, IL-10, IL-6, T-bet, or IFN-γ. The redundancy in the functions of the chemokine receptors CCR1 and CCR5 could be partially responsible for the minor effect the mutation exerted over the expression level of inflammatory biomarkers. Our group has already demonstrated that there is a compensatory effect in the CC chemokine system, where the absence of CCR3 in an experimental periodontitis model generates no phenotypic alteration by the compensatory functions of CCR1 and CCR5 (8). Further, we previously demonstrated that the combined pharmacological inhibition of CCR1 and CCR5 with met-RANTES has a more pronounced phenotypic effect in the progression of experimental periodontitis than the congenital absence of CCR5 receptor in a KO mice model system (9, 36).

Additionally, it is important to take into consideration that the density of expression of CCR5 in the surface of leukocytes is highly variable by factors other than the underlying genotype. T cells isolated from WT homozygous individuals can express low levels of CCR5 not significantly different from those of WT/CCR5Δ32 heterozygous subjects (46). A possible explanation for this phenomenon is the epigenetic regulation of CCR5 expression via methylation of the *cis* regulatory region of the *CCR5* gene, which can be accountable for 20-fold variations in the density of CCR5 surface expression, effectively causing WT homozygous subjects to have levels of CCR5 expression comparable to WT/CCR5Δ32 heterozygous counterparts (47).

Despite the expectation of a more prominent association between CCR5Δ32 and cytokine mRNA levels (based in our previous experimental data) the effect on TNFα expression reveals that CCR5Δ32 heterozygosity has a measurable effect in the expression pattern of effector molecules produced in response to periodontal infection, and provides support for the mutation's functionality. We must also consider that the expression levels were only compared between chronic periodontitis patients and healthy subjects, since ethical considerations impeded us to harvest gingival tissue from chronic gingivitis ('resistant') subjects, which could have provided the same resistance/susceptibility phenotypes comparison performed in our genetic association analysis. Such results also highlight the complexity of the regulatory mechanisms of inflammation during the course of periodontitis. Indeed, we encountered an equivalent result in a recently analysis of TBX21-1993T/C (rs4794067) and chronic periodontitis (1). In the cited study, we were unable to find an upregulation of IFN-γ in subjects carrying the TBX21-1993 polymorphic allele, despite the significant effect of the polymorphism in the expression levels of the Th1-differentiation key master switch T-bet (1).

Indeed, it is important to consider that the individual contribution of single mutation to a complex disease like periodontitis is expected to be relatively small. Accordingly, previous studies demonstrated that the presence of specific pathogens, such as *P. gingivalis, T. forsythia,* and *T. denticola* have a stronger influence in the levels of pro-inflammatory cytokines than functional SNPs in their respective genes. Repeated and persistent stimulation from a constant influx of antigens derived from a pathogenic biofilm can overcome the genetic predisposition for a milder inflammatory response caused by a genetic variant (48). In this sense, our data shows a strong and significant correlation between the bacterial load of *P. gingivalis, T. forsythia,* and *T. denticola* and the expression of the tested inflammatory biomarkers, irrespective of the genotype (data not shown).

Similarly, we did not find any effect of the CCR5Δ32 in the bacterial load of classic putative periodontal pathogens *P. gingivalis, T. forsythia, T. denticola* (section 3.3). Comparably, in our experiments with CCR5KO mice, the reduced leukocyte chemoattraction to periodontal tissues did not result in increased bacterial burden and systemic biomarkers of acute response to infection remained unaltered (9, 36). However, a dose response analysis of the effects of Met-RANTES revealed a distinct

scenario. While low and intermediate doses reduce inflammatory cell infiltration and bone loss and did not compromise the infection control, increased doses seemed to trespass a critical threshold, interfering with the production of the antimicrobial markers myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and IgG, resulting in increased bacterial loads (36). In this case, the impaired protective response is possibly due the lack of the compensatory role of CCR1, also blocked by Met-RANTES (8).

In conclusion, the strategy employed in our genetic association analysis proved useful to detect the effect of CCR5Δ32 (rs333) on the risk to suffer periodontitis. Furthermore, we were able to correlate the genotype for rs333 with significant changes in the expression pattern of a key inflammatory cytokine (TNFα), providing a probable mechanistic link between the genetic background and the risk profile. These results, coupled with our previous results *in vivo* (8, 36), point to a significant contribution of CCR5 in the pathogenesis of periodontitis. Thus, future studies employing a more sensitive and broad sampling strategy of inflammatory biomarkers and microbiological profile are required to unequivocally ascertain the pathway by which rs333 protects against periodontal inflammatory destruction. Also, it is possible that the single time point sampling limits the strength of the association between genotype and biomarkers microbiological data and that a time-series sampling strategy could overcome this issue. In the future, these kind of evidence will contribute to develop tailored periodontal interventions shaped to the individual risk profile of patients and will also help to direct preventive interventions at risk population before the occurrence of irreversible periodontal damage.

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2.4 ARTICLE 4 – Association of genetic polymorphisms with subgingival microbial colonization patterns in chronic*

Introduction

Chronic periodontitis is an infectious disease characterized by the progressive and irreversible damage of teeth-supporting structures. The initiating stimulus for chronic periodontitis is the teeth-attached subgingival biofilm that cause the activation of host immune response (1). While the host inflammatory immune response provides the protection against the infecting agents dissemination, response mediators also stimulates local proteolytic and bone resorptive activities, which leads to inflammatory periodontal tissue destruction (2, 3). Periodontitis is a complex disease, with etiologic factors acting at numerous levels: at the microbial level, based on the presence of dysbiotic microbial communities; at the host level, based on host response variations and genetic factors that may predispose to or protect from disease; and at the environmental, factors that modify the host response in either protective or destructive direction (4).

The subgingival biofilm is a complex community of several bacterial species that are capable of stablishing intricate ecologic relationships, organized in recognizable mutually supportive communities or complexes (5). Recent analyses have indicated that periodontitis is associated with a shift in the normal commensal oral microbiome resulting in different microbial colonization patterns that represent distinct, but stable, dysbiotic communities associated with disease (6). Thus, disease results not from individual pathogens but rather from polymicrobial synergy and dysbiosis, which disrupts the homeostasis of the ecologically balanced biofilm and the homeostatic host response (7, 8).

Indeed, even though periodontitis is regarded as an infectious disease, the extent and nature of the immune response against the subgingival biofilm is definitive determinant of the clinical disease outcome (2). In this sense, when the response triggered by the infecting microorganisms is biased towards a Th1, Th17 or pro-inflammatory phenotypes, the effector mechanisms of the immune system tamper with the

* Association of genetic polymorphisms with subgingival microbial colonization patterns in chronic. (In preparation)

homeostatic balance of periodontal tissues, leading to the progressive destruction of tooth-supporting structures. Conversely, when the response is biased towards a regulatory phenotype, the tissue metabolism remains unhampered and the periodontium is preserved (1, 9). Noteworthy, the nature of the immune response is at least partially under genetic control (10, 11). While microbial and environmental factors characteristically modulate host responses in chronic periodontitis, evidence suggest that as much as 50% of the risk to suffer periodontitis can be determined by genetic factors (12), and that numerous disease modifying genes may be involved in the pathogenesis of periodontitis by modulating the host's response and his susceptibility to infection (2).

Over the past decades, the vast majority of genetic association studies for periodontitis susceptibility have been based in the strategy of 'candidate genes', selected based on their theoretical involvement in key steps of the immune response or periodontal tissue metabolism (11, 13). Nevertheless, generally this approach has proved inconclusive and most SNP associations lack the necessary replication in different populations (14). A new strategy based on the results of large Genome Wide Association Studies (GWAS) (15-18) overcomes some limitation of the classic studies by offering the crucial advantage of hypothesis-free gene selection criteria.

All the same, GWAS of chronic periodontitis relying exclusively in clinical criteria to perform the association analysis have had modest success in identify risk genetic markers (19, 20). One possible cause for this difficulty is the selection of adequate cases and controls to perform the association study. We have previously argued that the classic approach in genetic association studies using a healthy population as controls violates the principle of equal exposure to the causative factor that is of paramount importance in case/control studies (21, 22). Additionally, most published GWAS in periodontitis have used sample populations presenting known confounding risk factors, such as smoking and diabetes, contributing to lower the statistical power of the analysis (23, 24).

A recent GWAS analyzing a total sample of 6458 subjects, including secondary/surrogate microbiological outcomes has proven a more successful strategy, since changes in the subgingival biofilm structure could be considered proxy outcomes of the disease phenotype, and offer a more sensitive screening strategy (24). Despite

the limited microbiologic screening used in this study, the rationale behind the use of proxy outcomes of periodontitis is sound and promising.

Therefore, it is possible to hypothesize that genetic risk determinants of disease could modify the capacity of the host to cope with the bacterial challenge posed by subgingival biofilm, altering the host/pathogen barrier and facilitating the establishment of dysbiotic subgingival communities (7). These microbiological changes could in turn modify the susceptibility to suffer from periodontitis, and can be monitored using highly sensible molecular tools.

In this context, we selected 19 candidate genes based on the top hits of GWAS that included clinical and microbiological outcomes (16, 17, 24), as well as SNPs located in strong linkage disequilibrium with those SNPs identified in the cited studies, but with a higher minor allele frequency. These selected SNPs were associated with the occurrence of chronic periodontitis and with the presence and counts of 40 subgingival bacteria belonging to the classical subgingival microbiological complexes (5) in a sample of Brazilian population. We deliberately excluded smokers (including former smokers) and subjects presenting diabetes and other metabolic diseases, in an effort to avoid the effect of confounding factors. We also employed a broad and highly sensible microbiologic screening, including putative pathogens and commensal species.

Material and methods

Sample population

The chronic periodontitis (CP) sample (n=76) was recruited at the São Paulo state, south-eastern region of Brazil, from patients referred to the Periodontal Clinic University of Guarulhos (UnG). Patients were examined by an experienced periodontist and scored for bleeding on probing (BOP), probing depth (PD) and clinical attachment loss (CAL). The chronic periodontitis diagnosis was based on the current classification of the American Academy of Periodontology (25). The inclusion criteria were as follows: ≥30 years of age and a minimum of six teeth with at least one site each with PD and clinical attachment level (CAL) ≥5 mm, as well as at least 30% of the sites with PD and CAL ≥4 mm and bleeding on probing (BOP) (26). Enrolled subjects provided informed consent that was approved by the Institutional Review

Board. Subjects were excluded from the study if they were tobacco smokers (including former smokers), had medical history indicating evidence of known systemic modifiers of periodontal disease, were pregnant or nursing, were under or require treatment with antibiotics or anti-inflammatory drugs, and/or had received periodontal therapy in the previous 2 years. Subpopulations (population stratification) or population relatedness were not investigated among the recruited subjects. Clinical and demographical information of the sample population is summarized in table 1. Age/gender matched healthy controls (n=91) were recruited at the School of Dentistry University of Guarulhos.

Table 1. Clinical and demographical data of the recruited cases (ChP) and healthy controls.

	Healthy (n=91)	ChP (n=76)
gender distribution	***50 f / 41 m***	***39 f / 37 m***
Age	45.1 ± 5.9	46.8 ± 6.1
Clinical parameters	***value ± SD***	***value ± SD***
Probing depth	2.2 ± 0.6	4.5 ± 0.7
Clinical Attachment Loss	0.6 ± 0.2	4.2 ± 0.7
% BOP	4.5 ± 2.7	66.2 ± 8.9
Plaque index	30.2 ± 6.2	56.9 ± 9.1

Genotyping

Saliva was collected from all the participants at the enrollment session using a DNA Oragene OG-500 kit (DNA Genotek, USA) following the manufacturer's instructions. DNA was extracted from participant´s saliva using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's guidelines. A spectrophotometer (Nanodrop 1000, Thermo Scientific, USA) was used to quantify and qualify the DNA samples. All isolated DNA samples were between 1.7 and 1.9 (260/280 nm ratio) and 1.9 and 2.1 (260/230 nm ratio). Genotyping was performed using TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA), containing a 20X mix of unlabeled PCR forward and reverse primers as well as a VIC and FAM labeled allele discrimination probes. Table 2 details all 19 SNP assayed.

Table 2. 19 SNPs assayed in the sample population. Association refers to previously published evidence linking the polymorphism with an outcome or phenotype. MAF* = minor allele frequency, latest release of phase 2 1000 genome project. NCTEV = non-coding transcript exon variant.

Code	Neaby Gene	Allele	Ancestral	Chrm.	Position	Association	MAF*	Most severe consequence
rs2521634	NPY	G/A	G	7	37614913	Severe ChP	0.24	Intergenic variant
rs7762544	NCR2	G/A	G	6	147785313	Severe ChP	0.15	Intergenic variant
rs12032672	PKN2	A/C	A	1	74883781	Red complex	0.44	Intergenic variant
rs10010758	TBC1D1	T/C	T	4	1842012	Red complex	0.29	Intron variant
rs1932040	RUNX2	A/G	A	6	88398224	Orange complex	0.4	Intergenic variant
rs9942773	CSMD3	A/C	C	8	22115347	Orange complex	0.19	Intergenic variant
rs1616122	VAMP3	C/T	C	1	137669543	Orange complex	0.42	Intron variant
rs11621969	FOS	T/C	T	14	7444172	Aa	0.15	Intergenic variant
rs9287989	WAPAL	T/C	C	2	45804766	Aa	0.5	Regulatory region variant
rs8080364	KIAA0753	C/T	T	17	24344565	Control	0.27	NCTEV
rs2836981	BRWD1-IT2	G/A	G	21	39610235	Control	0.39	Intron variant
rs11695297	MYT1L	T/A	A	2	22088619	Control	0.47	Intron variant
rs1537415	GTL6D1	G/C	G	9	6838736	AgP	0.31	Intron variant
rs1333048	CDKN2A/2B	A/C	A	9	43163827	AgP; ChP	0.45	Downstream gene variant
rs6667202	IL10	C/A	C	1	205023715	AgP	0.31	Intergenic variant
rs3826782	VAV1	G/A	G	19	41487293	Severe ChP	0.15	Intron variant
rs10043775	FBXO38	T/C	C	5	6478800	Red complex	0.26	Missense variant
rs4794067	TBX21	T/C	C	17	176425987	ChP	0.25	Upstream gene variant
rs2891168	CDKN2B-AS1	A/G	A	9	115190203	AgP	0.42	Intron variant

Quantitative polymerase chain reaction (qPCR) was carried out in a 5 µL reaction mixture with 4 ng of genomic DNA and 2.5 µL of the Taqman genotyping PCR master mix (Applied Biosystems, Foster City, California, USA). Amplification and detection were performed using the ViiA 7 platform (Applied Biosystems, Foster City, California, USA).

Thermal cycling conditions were 10 minutes at 95°C followed by 50 two-step cycles, including 15 seconds of denaturation at 92°C and 60 seconds of annealing/extension at 60°C. All reactions were performed in duplicate, and allele calling was done using QuantiStudio software. Allele calling was double checked manually in the raw data plot comparing the amplitude of fluoresce patterns. All genotyping experiments, including

DNA isolation, DNA quantification/quality control, and genotyping were performed at the OSTEOimmunology laboratory Bauru School of Dentistry University of Sao Paulo (FOB/USP).

DNA-DNA Checkerboard

Subgingival biofilm samples were collected from 9 subgingival sites of a fraction of our total sample (N=146; 69 cases and 77 controls), and were assayed for the presence and quantity of 40 bacterial species (5, 27) (Figure 1), as extensively described elsewhere (5, 28, 29). In brief, collected samples were stored in 150 µL buffer TE, then 100 µL NaOH 0.5 M were added and the sample was agitated for 1 minute. Later, the samples were boiled for 10 minutes and 800 µL of fresh 5 M NH_4 acetate were added. 1 mL of the mixture was placed in each individual lane on a nylon membrane using a Minislot device (Immunetics, Cambridge, MA). After fixation of the DNA to the membrane, the membrane was placed in a Miniblotter 45 (Immunetics), with the lanes of DNA at a 90-degree angle with respect to the lanes of the device. Digoxigenin-labeled whole-genome DNA probes for 40 subgingival species were hybridized in individual lanes of the Miniblotter 45. After hybridization, the membranes were washed at high stringency, and the DNA probes were detected using a digoxigenin-specific antibody conjugated with alkaline phosphatase. Signals were detected using AttoPhos substrate (Amersham Life Sciences, Arlington Heights, IL), and results were read using a Typhoon Trio Plus variable mode imager (Molecular Dynamics, Sunnyvale, CA). Two lanes in each run contained standards with 10^5 or 10^6 cells of each species. Signals evaluated using the Typhoon Trio Plus variable mode imager were converted to absolute counts by comparison with the standards on the same membrane. Failure to detect a signal was recorded as zero.

A.gerencseriae	S.sanguinis	F.nucleatum.ssp.nucleatum	T.denticola
A.israelii	Aa	F.nucleatum.ssp.polymorphum	E.saburreum
A.naeslundii	C.gingivalis	F.nucleatum.ssp.vincentii	G.morbillorum
A.oris	C.ochracea	F.periodonticum	L.buccalis
A.odontolyticus	C.sputigena	P.micra	P.acnes
V.parvulla	E.corrodens	P.intermedia	P.melaninogenica
S.gordonii	C.gracilis	P.nigrescens	N.mucosa
S.intermedius	C.rectus	S.constellatus	S.anginosus
S.mitis	C.showae	T.forsythia	S.noxia
S.oralis	E.nodatum	P.gingivalis	T.socranskii

Figure 1. Bacterial species assayed by DNA-DNA hybridization checkerboard.

Statistical analysis

Compliance with Hardy-Weinberg equilibrium for each SNP was tested by chi-square test. Standard and allelic case/control association analysis with disease phenotype were performed using chi-square test and Fisher's exact test. Significant associations between phenotype/genotype and bacterial counts were stablished by the two-stage step-up adaptive method (30). Briefly, in the first stage, the genotype (represented as a binary variable by allele-carrying) was sequentially tested against the counts for each bacterial species, obtaining a series of p-values. The distribution of these p-values was used to estimate the fraction of null hypothesis that were actually true. In the second stage, a reductive iterative process determined which p-values were low enough to be considered discoveries. A Q value of 5% was accepted as the maximum false positive rate. This adaptive procedure greatly diminishes the probability of false positives in repetitive testing.

A p-value <0.05 was considered significant. Analyses were performed in GraphPad Prism v7.02 (GraphPad software, La Jolla CA), Stata14 (Stata Corp, College Station, USA) and PLINK v1.07 (31).

Results

All 19 SNPs were successfully genotyped with call rates >95% (data not shown). SNPs rs7762544 and rs11695297 failed the Hardy-Weinberg equilibrium test and were excluded from subsequent analysis (Table 3). There were no mutant homozygous subjects in the control group of rs3826782, thus it was excluded from the case/control association analysis (Table 3).

The case/control association test did not demonstrate a significant effect of any of the 16 assessed SNPs with the disease phenotype (Table 3). No further association analysis with disease phenotype were performed, since the focus of our study was in the interaction between genetic polymorphisms and subgingival microbiological profile.

Table 3. Genotype count and frequencies for all tested SNPs. HZ= homozygous; AF= allele frequency; H-W= Hardy-Weinberg equilibrium chi-square test; Chi-square/p-value= case/control association test with disease phenotype.

	Control [n (%)]				ChP [n (%)]				Data analysis		
	Ancestral HZ	HetZ	Mutant HZ	Mutant AF	Ancestral HZ	HetZ	Mutant HZ	Mutant AF	H-W	Chi-square	p-value
rs2521634	46 (51.7)	31 (34.8)	12 (13.4)	36 (25)	39 (54.1)	30 (41.6)	3 (4.1)	55 (30.8)	0.443	4.245	0.12
rs7762544	7 (9.6)	20 (27.3)	46 (63)	112 (76.7)	0 (0)	22 (33.3)	44 (66.6)	110 (83.3)	**0.048**	NA	NA
rs12032672	25 (40.3)	30 (48.3)	7 (11.3)	44 (35.5)	20 (31.2)	35 (54.7)	9 (14.1)	53 (41.4)	0.65	1.159	0.56
rs10010758	22 (62.8)	11 (31.4)	2 (5.7)	15 (21.4)	32 (57.1)	16 (28.6)	8 (14.3)	32 (28.5)	0.33	1.168	0.44
rs1932040	3 (3.6)	33 (39.3)	48 (57.1)	129 (76.7)	6 (8.2)	36 (49.3)	31 (42.5)	98 (67.1)	0.44	4.038	0.13
rs9942773	4 (5)	27 (33.7)	49 (61.2)	125 (78.1)	6 (9.4)	19 (29.7)	39 (60.9)	97 (75.7)	0.6	1.164	0.55
rs1616122	27 (31.8)	35 (41.1)	23 (27)	81 (47.6)	14 (18.4)	43 (56.6)	19 (25)	81 (53.2)	0.057	4.835	0.08
rs11621969	60 (71.4)	22 (26.2)	2 (2.4)	26 (15.4)	49 (64.5)	24 (31.5)	3 (3.9)	30 (19.7)	0.75	0.999	0.61
rs9287989	28 (32.5)	47 (54.6)	11 (12.7)	69 (40)	22 (29.7)	37 (50)	15 (20.2)	67 (45.2)	0.111	1.635	0.44
rs8080364	37 (44)	39 (46.4)	8 (9.5)	55 (32.7)	46 (60.5)	27 (35.5)	3 (3.9)	33 (21.7)	0.282	5.043	0.08
rs2836981	14 (16.1)	42 (48.3)	31 (35.6)	104 (59.7)	7 (9.2)	40 (52.6)	29 (38.1)	98 (64.4)	0.808	1.714	0.42
rs11695297	43 (53.7)	25 (31.2)	12 (15)	49 (30.6)	20 (27.4)	38 (52)	15 (20.5)	68 (46.5)	**0.029**	NA	NA
rs1537415	12 (14.1)	37 (43.5)	36 (42.3)	109 (64.1)	6 (7.9)	39 (51.3)	31 (40.8)	101 (66.4)	0.97	1.929	0.38
rs1333048	17 (25)	33 (48.5)	18 (26.4)	69 (50.7)	16 (25.8)	28 (45.1)	18 (29)	64 (51.6)	0.993	0.1636	0.92
rs6667202	12 (13.6)	30 (34.1)	46 (52.2)	122 (69.3)	10 (13.1)	35 (46)	31 (40.8)	97 (63.8)	0.191	3.365	0.18
rs3826782	64 (78)	18 (21.9)	0 (0)	18 (10.9)	57 (76)	16 (21.3)	2 (2.6)	20 (13.3)	0.204	NA	NA
rs10043775	5 (6)	24 (28.9)	54 (65.1)	132 (79.5)	4 (5.4)	26 (35.1)	44 (59.4)	114 (77)	0.57	1.159	0.56
rs4794067	8 (9.3)	31 (36)	47 (54.6)	125 (72.6)	3 (3.9)	28 (36.8)	45 (59.2)	118 (77.6)	0.367	3.216	0.2
rs2891168	20 (29.8)	31 (46.2)	16 (23.9)	63 (47)	18 (28.6)	33 (52.3)	12 (19)	57 (45.2)	0.998	0.761	0.68

The two-stage linear set-up procedure (30) discovered 36 significantly different bacterial counts between control and ChP individuals (Figure 2 and Table 4).

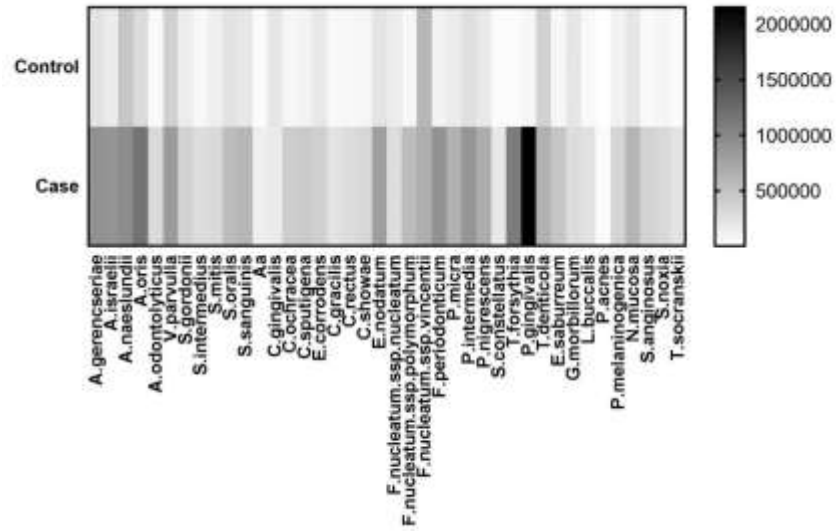

Figure 2. Heat map of bacterial count for 40 subgingival species belonging to the classic subgingival microbial complexes in controls (n=77) and cases (chronic periodontitis) (n=69).

Table 4. Significantly altered bacterial counts of subgingival microorganisms between control and ChP subjects. Adjusted p-value= Discovery by two-stage linear set-up procedure Benjamini, Krieger, and Yekutieli (Q=5%), sorted from the lowest to highest significant p-value.

Microorganism	Control	ChP	Adjusted p-value
A.israelii	160345	893511	<0.001
P.gingivalis	68876	2150036	<0.001
A.oris	302836	1171655	<0.001
T.forsythia	26506	1104569	<0.001
F.periodonticum	98231	910910	<0.001
A.gerencseriae	235808	913498	<0.001
P.intermedia	259762	882319	<0.001
E.nodatum	228546	795966	<0.001
P.nigrescens	188205	692758	<0.001
P.micra	161019	659696	<0.001
A.naeslundii	468479	957118	<0.001
F.nucleatum.ssp.polymorphum	67973	552734	<0.001
V.parvulla	385381	837456	<0.001
E.saburreum	61402	480620	<0.001
N.mucosa	200501	615542	<0.001
S.sanguinis	189625	603031	<0.001
C.ochracea	68674	423281	<0.001
C.sputigena	99803	433313	<0.001
S.anginosus	39551	368112	<0.001
S.oralis	232754	552347	<0.001
T.denticola	374527	648599	<0.001
A.odontolyticus	42836	311538	<0.001
C.showae	73988	325362	<0.001
P.melaninogenica	111172	353281	0.001
S.noxia	75718	308615	0.001
S.intermedius	54897	285286	0.001
C.rectus	59601	289257	0.001
S.gordonii	146660	368106	0.001
E.corrodens	183801	388124	0.002
T.socranskii	33894	226699	0.003
L.buccalis	52565	244344	0.003
C.gracilis	48808	224658	0.005
S.mitis	137170	306687	0.006
S.constellatus	23077	190087	0.007
F.nucleatum.ssp.nucleatum	162832	280366	0.025
Aa	32715	135463	0.034

SNPs in Hardy-Weinberg equilibrium (n=17) were tested for discovery of significant changes in subgingival microbiological pattern in the ChP group.

The mutant allele for the polymorphisms rs2521634 proved significantly associated with decreased counts of *T. forsythia, A. gerencseriae, F. periodonticum,* and *P. nigrescens* (Figure 3, A). Discoveries were *T. forsythia* p-value = 0.001; *A. gerencseriae* p-value = 0.02; *F. periodonticum* p-value = 0.03; and *P. nigrescens* p-value = 0.03.

The mutant allele for the polymorphism rs10010758 proved significantly associated with increased counts of *P. gingivalis* (Figure 3, B). Discovery was *P. gingivalis* p-value = 0.01.

The mutant allele for the polymorphism rs6667202 proved significantly associated with increased counts of *P. gingivalis* (Figure 3, C). Discovery was *P. gingivalis* p-value = 0.005.

The mutant allele for the polymorphism rs10043775 proved significantly associated with decreased counts of *P. intermedia* (Figure 3, D). Discovery was *P. intermedia* p-value = 0.02.

The remaining 13 SNPs failed to pass the discovery threshold for p-value adjustment of the two-stage linear set-up procedure and were considered not associated with changes in the subgingival microbiological pattern (data not shown).

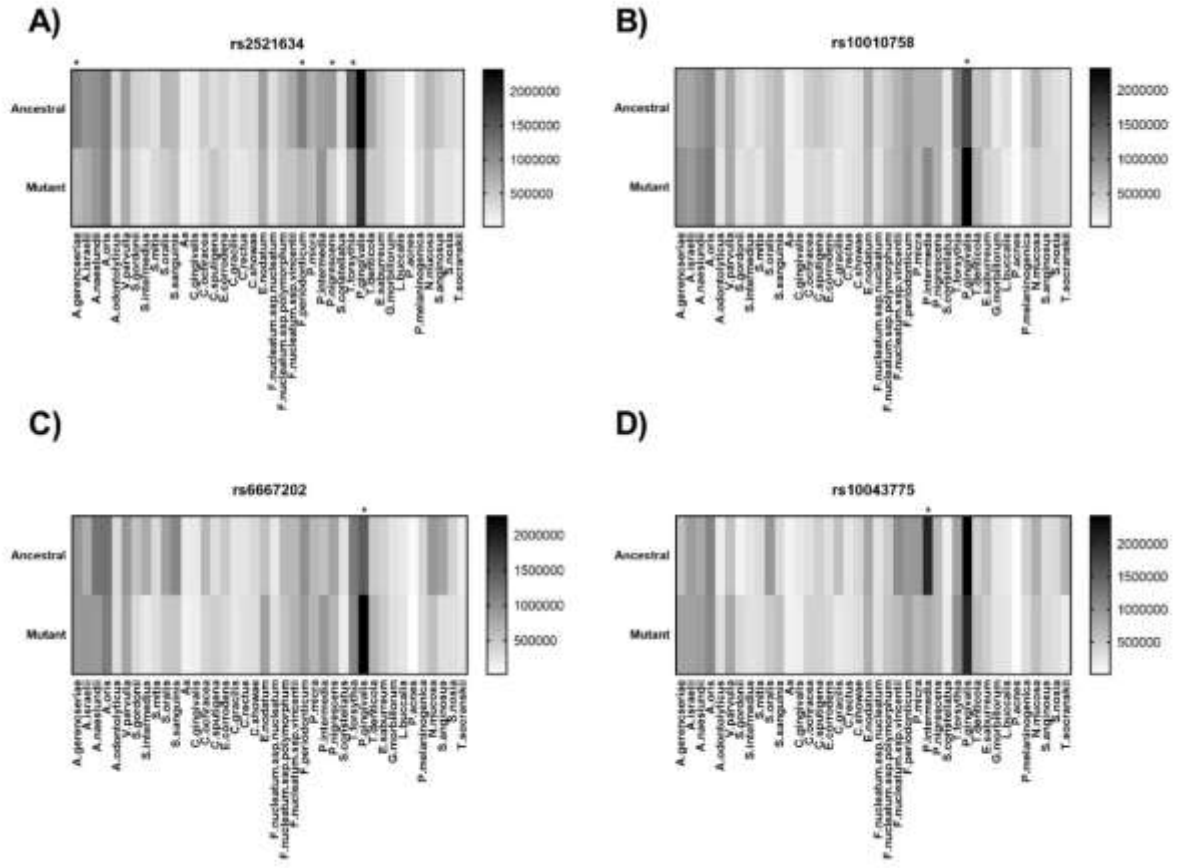

Figure 3. Heatmaps of bacterial count for 40 subgingival species in ancestral and mutant allele-carrying ChP subjects. A) rs2521634. B) 10010758. C) rs6667202. D) rs10043775. *= discovery by two-stage linear set-up procedure Benjamini, Krieger, and Yekutieli (Q=5%).

Discussion

The SNPs selected for this study had been previously associated with the occurrence of periodontitis or with changes on the subgingival biofilm in GWAS (16, 19).

Our results are in line with the published evidence, since four of the tested SNPs (rs2521634, rs10010758, rs6667202, and rs10043775) proved significantly associated with changes in the subgingival microbiological pattern. As already stated, the strategy used to characterize the subgingival biofilm in our study was more broad and sensitive than the strategy used in the cited GWAS (16, 19). We tested for 40 subgingival species, including all species belonging to the classic subgingival microbial complexes (5). This approach allows us to gain an insight to the effect of the polymorphism over the subgingival microbiological pattern as a whole.

Our case/control association test failed to demonstrate any significant effect of the tested SNPs on periodontitis risk. This is somewhat expected, since our sample is underpowered to detect small genetic effects over the disease phenotype, as these SNPs probably exert (32). It is noteworthy, that from a design perspective, our study was intended to test the interaction between the selected SNP and the subgingival microbiological pattern. Testing for disease phenotype association requires a larger sample, in which it would be impractical to perform a comprehensive microbiological profiling as the one carried out in this study.

The focus of our study was to unveil the possible effect of the mutations in the subgingival microbiological pattern in the chronic periodontitis sample, and to this aim we performed a broad and sensitive quantification of 40 subgingival bacterial species. This strategy, coupled with a statistical analysis designed to adaptively adjust for false positives (30, 33), proved powerful enough to detect significant microbiological effects of the polymorphic variations. Even though the unit of observation for the statistical analysis remained the subject, it is important to bear in mind that each subject was genotyped for 19 SNPs and that the microbiological profiling was the result of sampling 9 different subgingival sites, each one tested independently for 40 species. The amount of independent data included in each analysis strengthened our results, even in a relatively small sample (34). Also important, the clinical characterization of the subjects and the clinical diagnosis was performed by a trained and experienced periodontist, assuring that in phenotypic terms our sample is extremely homogenous.

SNP rs2521634 (located nearby *NPY* gene) has been previously associated with the occurrence of severe chronic periodontitis (16, 19). Our results demonstrated that mutant allele-carriers were at decreased risk of harboring *T. forsythia, A. gerencseriae, F. periodonticum,* and *P. nigrescens*, regarded as disease-associated bacteria (5, 29). This result is coherent with GWAS results that demonstrated a pooled estimate effect for severe chronic periodontitis of OR 1.49, 95% CI = 1.28-1.73, P = 3.5×10^{-7} for the ancestral allele (G). In other words, the mutant allele for rs2521634 (A) is protective for severe chronic periodontitis, and arguably mutant allele-carrier subjects would be at decreased risk of harboring periodontal pathogens. Although it is impossible to trace a complete parallel between our results and the GWAS results, the fact that the direction of the association is the same increases our confidence in the reality of the association. It is noteworthy, that our population and the European population tested on the cited GWAS have completely different genetic backgrounds (35, 36). The fact that the association was replicated in our population supports the notion of an important role of this polymorphism conferring a susceptibility genetic profile for periodontitis.

At this point, the mechanism linking mutations in NPY and differential subgingival microbial patterns is purely speculative. Recent evidence has demonstrated immune modulatory functions for NPY, inhibiting the recruitment of monocytes in severe infections of the central nervous system (37). Similarly, *in vivo* experiments suggest that NPY agonists are effective in diminishing the blood title of TNFα in endotoxin-induced septic shock (38). Hypothetically, it is plausible to argue that NPY has a regulatory effect in the response against subgingival microbes, and that the mutant-allele for rs2521634 confers protection against periodontal pathogens by a mechanism associated with the infiltration of monocytes and TNFα secretion in periodontal tissues.

Our results demonstrated an association of the mutant allele (C) for rs10010758 (located in an intronic region of *TBC1D1* gene) with significantly increased counts of the ‘red complex’ pathogen *P. gingivalis.* This result is in line with previous evidence demonstrating that SNP rs10010758 is associated with increased risk of harboring ‘red complex’ disease-associated bacteria (OR 1.91, 95% CI 1.45-2.51, P = 3.7×10^{-6}) for the mutant allele (16). Again, the fact that the result was replicated with identical direction of association is suggestive of a real effect of the polymorphism over the composition of the subgingival microbiota. Further, the effect seems to be conserved in populations with different ethnic backgrounds. The possible mechanism of action of

this association is uncertain, since there is no a single evidence of a possible link between TBCD1 (Rab GTPase activating protein) and immune functions. In this context, there are two discrete possibilities to explain the association with changes in the subgingival microbiota. First, the mutation may be involved in conferring differential expression or functional properties to TBCD1, which may be linked to changes in the host/pathogen barrier by a mechanism that has not yet been described. Second, the mutation could be in linkage disequilibrium with another mutation that is the true responsible for the changes in the subgingival microbiota (20).

SNP rs6667202 (located nearby *IL10* gene) mutant allele-carriers (A) demonstrated a significant increase in the counts of *P. gingivalis*. This result is concordant with previous evidence associating ancestral allele-carriers (C) for rs6667202 with decreased risk of aggressive periodontitis (OR=0.77, 95% CI=0.6-0.95, P=0.016) in a German/Austrian population (17). As previously stated, it is not possible to establish a direct parallel between increased risk to suffer periodontitis and increased counts of pathogenic bacteria, nevertheless the correspondence in the direction of the association and the strong evidence linking *P. gingivalis* with the characteristic dysbiotic changes that occur in periodontitis (39) provide suggestive evidence of an important role for rs6667202 in the pathogenesis of the disease.

IL-10 is a key regulatory cytokine involved in the suppression of inflammation and return to homeostatic state (40), and extensive evidence links increased levels of IL-10 with resistance to inflammatory bone loss in experimental periodontitis (41, 42). Therefore, the link with differential patterns of subgingival infection appears straightforward. It is plausible to hypothesize that the mutant allele for rs6667202 generates an impaired regulatory function of IL-10 (by a direct or indirect mechanism) causing a detrimental effect on the host's immune regulatory capacity, leading to the establishment of a dysbiotic subgingival microflora.

SNP rs10043775 (missense variant of *TBXO38* gene) ancestral allele-carriers exhibited a 3-fold increase in the counts of *P. intermedia*, which is an 'orange complex' bacteria and regarded as a periodontal pathogen (5, 43, 44). Our results are in concordance with previous reports associating a Han Chinese population of ancestral allele-carriers with increased risk to suffer severe chronic periodontitis (OR 1.24, P=0.0009) (18). The possible mechanism of association between rs10043775 and changes in the subgingival microbiological pattern is completely uncertain, since there

is no available evidence suggesting any functional pathway that could be theoretically linked with changes in the host/pathogen barrier. As previously stated for rs10010758, it is possible that a yet unknown mechanism is responsible for the differential susceptibility, or alternatively that rs10043775 is in linkage disequilibrium with another variant that is truly responsible for the changes in the subgingival microbiological pattern.

Finally, it is worth nothing that in all four cases of significant association, the direction of association was concordant with previously published evidence. Further, our sample population and the European population used in three cases (16, 17, 19) and Han Chinese population used in one case (18), possess very distinct genetic backgrounds (35, 36, 45). The fact that the associations were maintained is indicative of an important and conserved role for these four polymorphisms in the host/microbe barrier.

Conclusion

The polymorphisms rs2521634, rs10010758, rs6667202, and rs10043775 demonstrated significant association with changes in the subgingival biofilm. In all four cases, the direction of the association was coherent with previous evidence associating these SNPs either with risk to suffer periodontitis or increased risk to suffer pathogenic bacterial colonization. Taken together, these results highlight the importance of genetic factors in defining the effectiveness of host/pathogen barrier during periodontitis.

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2.5 ARTICLE 5 – Proteomic Profiling and Differential Messenger RNA Expression Correlate HSP27 and Serpin Family B Member 1 to Apical Periodontitis Outcomes*

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Basic Research—Biology

Proteomic Profiling and Differential Messenger RNA Expression Correlate HSP27 and Serpin Family B Member 1 to Apical Periodontitis Outcomes

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Abstract

Introduction: Understanding protein expression profiles of apical periodontitis may contribute to the discovery of novel diagnostic or therapeutic molecular targets. **Methods:** Periapical tissue samples ($n = 5$) of patients with lesions characterized as nonhealing were submitted for proteomic analysis. Two differentially expressed proteins (heat shock protein 27 [HSP27] and serpin family B member 1 [SERPINB1]) were selected for characterization, localization by immunofluorescence, and association with known biomarkers of acute inflammatory response in human apical periodontitis ($n = 110$) and healthy periodontal ligaments ($n = 26$). Apical periodontitis samples were categorized as stable/inactive ($n = 70$) or progressive/active ($n = 40$) based on the ratio of expression of receptor activator of nuclear factor kappa-B ligand (*RANKL*)/osteoprotegerin (*OPG*). Next, the expression of *HSP27*, *SERPINB1*, C-X-C motif Chemokine Receptor 1 (*CXCR1*), matrix metalloproteinase 8 (*MMP8*), myeloperoxidase (*MPO*), and cathepsin G (*CTSG*) messenger RNA was evaluated using real-time polymerase chain reaction. Data analysis was performed using the Shapiro-Wilk test, analysis of variance, and the Pearson test. P values <.05 were considered statistically significant. **Results:** Proteomic analysis revealed 48 proteins as differentially expressed in apical periodontitis compared with a healthy periodontium, with 30 of these proteins found to be expressed in all 4 lesions. The expression of HSP27 and SERPINB1 was ~2-fold higher in apical periodontitis. Next, an increased expression of HSP27 was detected in epithelial cells, whereas SERPINB1 expression was noted in neutrophils and epithelial cells. *HSP27* and *SERPINB1* transcripts were highly expressed in stable/inactive lesions ($P < .05$). Significant negative correlations were found between the expression of *HSP27* and *SERPINB1* with biomarkers of acute inflammation including *CXCR1*, *MPO*, and *CTSG*. **Conclusions:** Our data suggest HSP27 and SERPINB1 as potential regulators of the inflammatory response in apical periodontitis. Additional functional studies should be performed to further characterize the role of these molecules during the development/progression of apical periodontitis. (*J Endod 2017;■:1–8*)

Key Words

Apical periodontitis, gene expression, heat shock protein 27, protein expression, SERPINB1

Significance

Understanding protein expression profiles of periapical lesions may contribute to the discovery of novel diagnostic or therapeutic molecular targets.

Apical periodontitis is the inflammatory destruction of periradicular tissues caused by persistent microbial infection within the root canal system. Factors contributing to persistent apical periodontitis include inadequate aseptic control, poor access cavity design, missed canals, inadequate instrumentation, and defective temporary or permanent restorations (1). However, it has been recently proposed that an imbalance in the expression of genes involved in healing and genetic predisposition can also contribute to persistent apical periodontitis (2).

Chronic inflammation is the hallmark of apical periodontitis and drives the pathological changes that lead to tissue destruction in the periapical environment as well as the progression of the lesion. Even after conventional endodontic treatment, the persistence of infectious stimuli from residual microorganisms or their antigens can perpetuate the inflammatory process, precluding the return to homeostasis, regression, and healing (3).

From a clinical perspective, the ability to distinguish between a progressive lesion and a stable lesion that is undergoing regression and healing is critical to determine the need of additional treatment interventions, including periapical surgery. Currently, the decision of additional treatment is based on previous treatment history along with clin-

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ical and radiologic signs and symptoms evaluated during follow-up visits. Additional diagnostic tools such as the use of a molecular signature of apical periodontitis would provide clinical benefits in terms of determining when complex interventions are needed (4).

Proteomic analysis of apical periodontitis tissues represents a powerful and unbiased approach to identify key molecules that are differentially expressed and produced within the lesions (5). These molecules might be key components of the biological pathways involved in lesion resolution or progression.

In the present study, we performed proteomic analysis of apical periodontitis and healthy periodontal tissue samples using 2-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry. Selected differentially expressed proteins were further investigated using quantitative real-time polymerase chain reaction (PCR) and immunofluorescence. We also verified the association of selected protein targets with the expression of known biomarkers of acute inflammatory response.

Material and Methods

Sample Population

This study was approved by the Institutional Review Boards at the University of Texas Health Science Center at Houston, Houston, TX, and the University of São Paulo (Faculdade de Odontologia de Bauru Universidade de São Paulo), Bauru, São Paulo, Brazil. Patients were recruited from the endodontics and oral surgery clinics at these institutions. The subjects were patients with apical periodontitis (N = 114, 19–61 years of age) presenting with periapical rarefaction characterized radiographically by the disappearance of the periodontal ligament space and discontinuity of the lamina dura. All patients were referred for endodontic surgery after conventional root canal treatment had failed. Patients with medical conditions requiring the use of systemic modifiers of bone metabolism or other assisted drug therapy (ie, systemic antibiotics, anti-inflammatory drugs, and hormone therapy) 6 months before initiation of the study, patients with preexisting conditions such as periodontal disease, and pregnant or lactating women were excluded from the study. At the time of surgery, periapical biopsies were collected, and each specimen was divided into 2 fragments of similar size and stored in 10% formalin or rinsed in phosphate-buffered saline (PBS) and immediately frozen at −80°C. Samples stored in 10% formalin were submitted to routine histologic processing, serial sectioning for hematoxylin-eosin staining, and histopathological analysis. All experimental samples were histologically diagnosed as apical granulomas with or without epithelium (72%) and radicular cysts (28%). The clinical diagnosis included previously endodontically treated teeth (100%) with asymptomatic apical periodontitis (43%), symptomatic apical periodontitis (19%), and chronic apical abscess (38%).

Healthy periodontal ligament tissues were used as control tissues and were collected from individuals referred for the extraction of premolars for orthodontic reasons (n = 27, aged 19–24 years). In total, 114 human apical periodontitis samples and 27 healthy periodontal ligaments were collected postoperatively and used in this study.

2D-DIGE

Five tissue samples were submitted to 2D-DIGE. Of these, 4 periapical tissue samples were from cases with a histopathological diagnosis of a periapical granuloma and a clinical diagnosis of a previously endodontically treated tooth with a chronic apical abscess (n = 2) and a previously endodontically treated tooth with asymptomatic apical periodontitis (n = 2). One healthy periodontal ligament tissue obtained from a premolar extracted for orthodontic purposes was used as the control specimen.

Samples were incubated in 2-dimensional cell lysis buffer (30 mmol/L Tris-HCl (ThermoFisher, Waltham, MA), pH = 8.8, containing 7 mol/L urea, 2 mol/L thiourea, and 4% CHAPS [ThermoFisher]), sonicated at 4°C, and kept in a shaker for 30 minutes at room temperature. Samples were then centrifuged at 25,000*g* for 30 minutes at 4°C, and the supernatants were collected. Protein concentration was measured using the Bradford method (6). Each sample was diluted in 2-dimensional cell lysis buffer to a protein concentration of 8 mg/mL. We incubated 30 ng protein concentrate from each sample with 1 μL size/charge-matched CyDye (GE Healthcare Life Sciences, Marlborough, MA) at 0.2 nmol/μL for 30 minutes in ice inside a dark chamber. We added 1 μL lysine 10 mmol/L to the mixture and incubated for additional 15 minutes. Samples were labeled, mixed with buffer, and actively rehydrated as described elsewhere (7). The conditions for isoelectric focusing were as follows: 300 V for 2 hours, 500 V for 2 hours, 1000 V for 2 hours, and 8000 V for 8 hours. We equilibrated the focused strips, rinsed them in running buffer, and performed the second-dimension electrophoresis in a 12% agarose sodium dodecyl sulfate gel at 10 mA for approximately 30 minutes.

Image scans were performed immediately using Typhoon TRIO (GE Healthcare Life Sciences). Scanned images were analyzed using Image QuantTL software (GE Healthcare Life Sciences) and subjected to in-gel and cross-gel analysis using DeCyder software version 6.5 (GE Healthcare Life Sciences). Changes in protein expression between lesions and control tissue and between individual lesions were obtained from cross-gel DeCyder software analysis. Protein spots of interest were picked up by an Ettan Spot Picker (GE Healthcare Life Sciences) based on the in-gel analysis and spot-picking design using DeCyder software. The protein spots were washed and digested in gel with Trypsin Gold–modified porcine trypsin protease (Promega, Madison, WI). The digested tryptic peptides were further desalted by Zip-tip C18 (Millipore, Billerica, MA); peptides were eluted with 0.5 μL matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid, and 25 mmol/L ammonium bicarbonate) and spotted on a matrix-assisted laser desorption ionization-time of flight mass spectrometry.

Mass Spectrometry and Database Search

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry and time-of-flight/time-of-flight tandem mass spectrometry were performed on a 5800 mass spectrometer (AB Sciex, Framingham, MA). Matrix-assisted laser desorption ionization–time-of-flight mass spectra were acquired in the reflectron positive ion mode (8). Time-of-flight/time-of-flight tandem mass spectrometry fragmentation spectra were acquired for each sample, averaging 2000 laser shots per fragmentation spectrum on each of the 5 to 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Both the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer (version 3.5; Matrix Science, Boston, MA) equipped with the MASCOT search engine (Matrix Science) to search the database of the National Center for Biotechnology Information nonredundant (9) or the Swiss Protein database (10). Searches were performed without constraining the protein molecular weight or isoelectric point, with variable carbamidomethylation of cysteine and oxidation of methionine residues, and with 1 missed cleavage allowed in the search parameters. Only candidates with either a protein score confidence interval or an ion confidence interval greater than 95% were considered.

Microscopic Analysis

Heat shock protein 27 (HSP27) and serpin family B member 1 (SERPINB1) were selected as targets for additional investigation based

on their ~2-fold overexpression in apical periodontitis compared with healthy tissue and considering their putative regulatory role in inflammation.

Formalin-fixed paraffin-embedded tissue samples of nonepithelialized apical granulomas, epithelialized apical granulomas, and apical cysts were obtained from our in-house tissue collection and sectioned at 6 μm for standard hematoxylin-eosin staining and immunolocalization of HSP27 and SERPINB1. Formalin-fixed paraffin-embedded sections were cleared in xylene and rehydrated in graduated alcohol baths. Antigen retrieval was performed in 10 mmol/L sodium citrate buffer (pH = 6) at 96°C for 5 minutes. Sections were then permeabilized with 0.5% Triton X-100 (ThermoFisher) in PBS, blocked with 1% bovine serum albumin in 10% goat serum, and incubated with either rabbit polyclonal antihuman HSP27 (cat no. AB5579; Abcam, Cambridge, UK) or rabbit monoclonal antihuman SERPINB1 (cat no. AB181084, Abcam) antibody overnight at 4°C. Sections were washed in PBS and incubated with Alexa 488 goat antirabbit secondary antibody (ThermoFisher), counterstained with 4′,6-diamidino-2-phenylindole, and mounted. Images were acquired using an Eclipse Ni-U upright fluorescence microscope (Nikon, Tokyo, Japan) equipped with a Zyla 5.5 sCMOS camera (Andor Technologies, Belfast, UK).

Quantitative Real-time PCR

We measured the messenger RNA (mRNA) levels of *HSP27*, *SERPINB1*, receptor activator of nuclear factor kappa-B ligand (*RANKL*), osteoprotegerin (*OPG*), C-X-C motif chemokine receptor 1 (*CXCR1*), matrix metalloproteinase 8 (*MMP8*), myeloperoxidase (*MPO*), and cathepsin G (*CTSG*) in 110 periapical granulomas and 26 healthy periodontal ligament tissue samples as previously described (11). The total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). RNA quality was analyzed using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), and complementary DNA synthesis was achieved using 3 μg RNA/sample as a template in a reverse transcription reaction (QuantiTectRT Kit, Qiagen).

Samples were categorized as either progressive/active ($n = 40$) or stable/inactive ($n = 70$) based on the ratio of *RANKL/OPG* expression as previously described (12). Next, mRNA levels of *HSP27*, *SERPINB1*, *CXCR1*, *MMP8*, *MPO*, and *CTSG* were evaluated using on-demand TaqMan gene expression assays (Invitrogen, Carlsbad, CA) in a Viia7 Real Time PCR instrument (LifeTechnologies, Carlsbad, CA). The reaction conditions were as follows: 40 cycles at 95°C for 10 minutes, 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes. Results are depicted as the expression levels of target genes relative to the expression of housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase [*GAPDH*] and β-actin) in each sample using the $2^{-\Delta\Delta Ct}$ method (13).

Statistical Analysis

The Shapiro-Wilk test was used to test the distribution of the data, whereas analysis of variance was used to test the differences in the relative expression of the investigated genes between lesions and control tissues and between progressive and stable lesions. We also used the Pearson correlation to analyze the association between selected variables. All statistical analyses were performed in Stata/IC 14.1 (Stata Corp, College Station, TX) and GraphPad Prism 7.0 (GraphPad, La Jolla, CA). Depending on the analysis, fold changes greater than or around 2 or P values < .05 were considered statistically significant.

Results

2D-DIGE

The complete data obtained from the database search including the top-ranking protein name, accession number, molecular weight, isoelectric point, peptide count, and Mascot scores are provided as Supplementary Material.

We found 48 differentially expressed proteins between the control tissue and the 4 apical periodontitis samples (Supplemental Table S1 is available online at www.jendodon.com). Of these, 30 were present in all 4 lesions; 19 of 30 (63.3%) proteins were down-regulated, whereas 11 of 30 (36.6%) were up-regulated (Table 1). These peptides were further categorized based on the 12 putative functional pathways in which they belong as follows: 15 (50%) were cytoskeleton proteins (actin cytoplasmic 2, annexin A1, gelsolin, involucrin, keratin type I cytoskeletal 13, keratin type I cytoskeletal 14, keratin type I cytoskeletal 16, keratin type II cytoskeletal 1, keratin type II cytoskeletal 2 oral, keratin type II cytoskeletal 3, keratin type II cytoskeletal 4, keratin type II cytoskeletal 5, keratin type II cytoskeletal 6A, tropomyosin alpha-4 chain, and vimentin), 3 (10%) were blood plasma proteins (apolipoprotein A-I, serotransferrin, and serum albumin), 2 (6.6%) were antioxidant defense system enzymes (superoxide dismutase mitochondrial and thioredoxin), and 2 (6.6%) were cell cycle regulating proteins (14-3-3 protein sigma and protein S100-A9); the remaining 8 peptides included a Ca^{+2} signaling protein (calreticulin), a protein biosynthesis molecule (elongation factor 2), a metabolic enzyme (alpha-enolase), a transport protein (fatty acid–binding protein, epidermal), a serine protease inhibitor family member (SERPINB1), a stress response protein (HSP27), a coagulation cascade protein (protein-glutamine gamma-glutamyltransferase E), and an immune response protein (immunoglobulin gamma-1 chain C region).

HSP27 and SERPINB1 expression was up-regulated in apical periodontitis tissues by 3.79-fold and 1.93-fold, respectively, when compared with the control. Because of their previously reported role in inflammation and apical periodontitis (14–16), these proteins were selected for further characterization and association analysis with known acute inflammatory response markers (*CXCR1*, *MMP8*, *MPO*, and *CTSG*) (17–20).

Microscopic Analysis

Microscopic analysis of the hematoxylin-eosin–stained apical periodontitis sections revealed the presence of granulomatous tissue with a dense fibrous capsule, fibroblasts, and a large area filled with chronic and acute inflammatory infiltrate. This infiltrate was composed by lymphocytes intermixed with neutrophils, macrophages, and plasma cells (Fig. 1*A–L*). Three different patterns of apical periodontitis were noted: nonepithelialized (Fig. 1*A–D*) and epithelialized (Fig. 1*E–H*) granulomas and apical cysts (Fig. 1*I–L*). Highly epithelialized granulomas contained granulation tissue infiltrated by scattered and nonorganized epithelium cords. An apical cyst exhibited a typical cavity-lining epithelium (ie, a hyperplasic, nonkeratinized, and stratified epithelium surrounded by a thick granulation tissue) (21).

Immunofluorescence assays revealed the expression of HSP27 and SERPINB1 in apical periodontitis (Fig. 2). HSP27 expression was not detected in nonepithelialized apical granulomas (Fig. 2*A–D*); however, an increased expression was noted in the epithelia of epithelialized granulomas and apical cysts (Fig. 2*E–L*, respectively). In the case of epithelialized granulomas, the expression of HSP27 was confined to the hyperplasic nonkeratinized epithelial cords infiltrating the granulomatous tissue (Fig. 2*E–H*), with epithelial cells showing a homogenous cytoplasmic immune staining in all layers. Apical cysts showed a similar pattern of immune staining, with a strong cytoplasmic signal in all layers of the epithelium lining the cystic cavity (Fig. 2*K* and *L*).

Similarly, SERPINB1 was expressed by the epithelial cells of epithelialized granulomas and apical cysts (Fig. 2*F* and *J*, respectively). The staining pattern was cytoplasmic and homogeneous in all layers

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TABLE 1. Pathways Regulated in Apical Periodontitis Compared with Control Samples

Pathway	*N* (%)	*N* (%) up-regulated	*N* (%) down-regulated
Antioxidant defense system	2 (6.6)	1 (50)	1 (50)
Blood plasma protein	3 (10)	3 (100)	0
Ca+2 signaling	1 (3.3)	1 (100)	0
Cell cycle regulator	2 (6.6)	0	2 (100)
Coagulation cascade	1 (3.3)	0	1 (100)
Cytoskeleton	15 (50)	3 (18.75)	12 (81.25)
Immune response	1 (3.3)	1 (100)	0
Metabolic enzyme	1 (3.3)	0	1 (100)
Protein biosynthesis	1 (3.3)	0	1 (100)
Serpin family member	1 (3.3)	1 (100)	0
Stress response	1 (3.3)	1 (100)	0
Transport protein	1 (3.3)	0	1 (100)
	Total	11 (36.6)	19 (63.3)

of the hyperplasic epithelia. In the epithelialized granuloma, strong staining was evident in the scattered islets and cords of epithelia (Fig. 2*F*′ and *G*′). In the apical cyst, the immune staining followed the same pattern, and the signal was evident in all layers of the epithelia lining the cystic cavity (Fig. 2*I*′–*L*′).

SERPINB1 was also expressed by the infiltrating polymorph nuclear neutrophils (PMNs) in the nonepithelialized granuloma, epithelialized granuloma, and apical cyst (Fig. 2*D*′, *H*′, and *L*′). The staining was very specific to PMNs, and no other infiltrating cell showed positive staining. In the nonepithelialized granuloma, the PMNs were clustered in defined sections of the lesion surrounded by other infiltrating leukocytes and granulomatous tissue (Fig. 2*A*′–*D*′). In the epithelialized granuloma, the infiltrating PMNs were located preferentially in the central part of the lesion (Fig. 2*G*′ and *H*′). The apical cyst also showed clustered infiltration of PMNs in the central portion of the lesion (Fig. 2*I*′). Some PMNs transmigrated through the epithelia into the cystic cavity (Fig. 2, *arrow* in *L*′).

Expression Analyses

Increased levels of *HSP27* and *SERPINB1* were detected by quantitative real-time PCR in inactive lesions when compared with the control

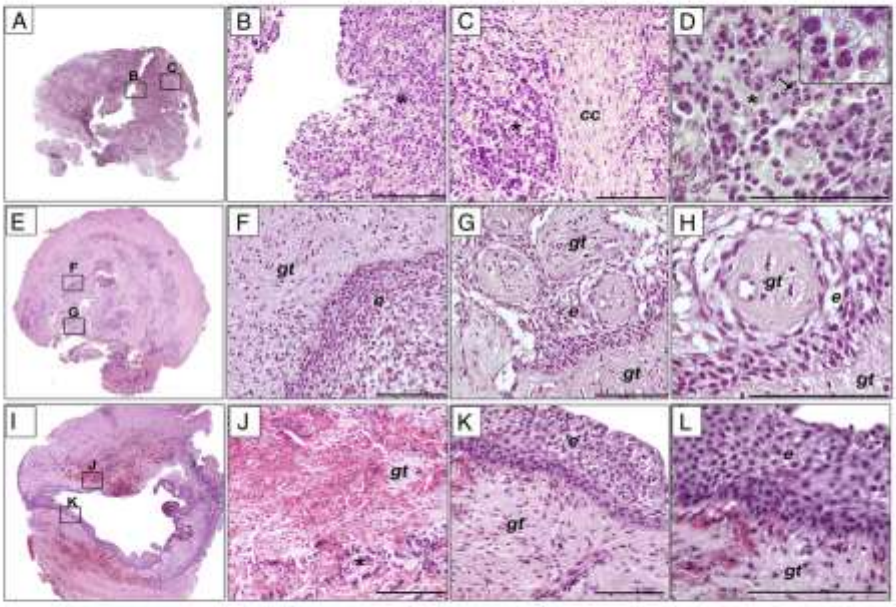

Figure 1. Histopathological characterization of inflammatory apical periodontitis. (*A–L*) A mix of inflammatory cells (*) including neutrophils (*arrows*) were predominant in all lesions. (*A–D*) A nonepithelialized granuloma with a clear decrease of inflammatory infiltrate into the thick collagenous capsule (cc). (*E–H*) A highly epithelialized granuloma containing granulation tissue (gt) enclosed by nonkeratinized epithelium (e) cords. (*I–L*) An apical cyst lined by a hyperplasic, nonkeratinized, stratified epithelium and a granulation tissue (gt) surrounding the lesion. Hematoxylin-eosin stain. Scale bar = 250 μm.

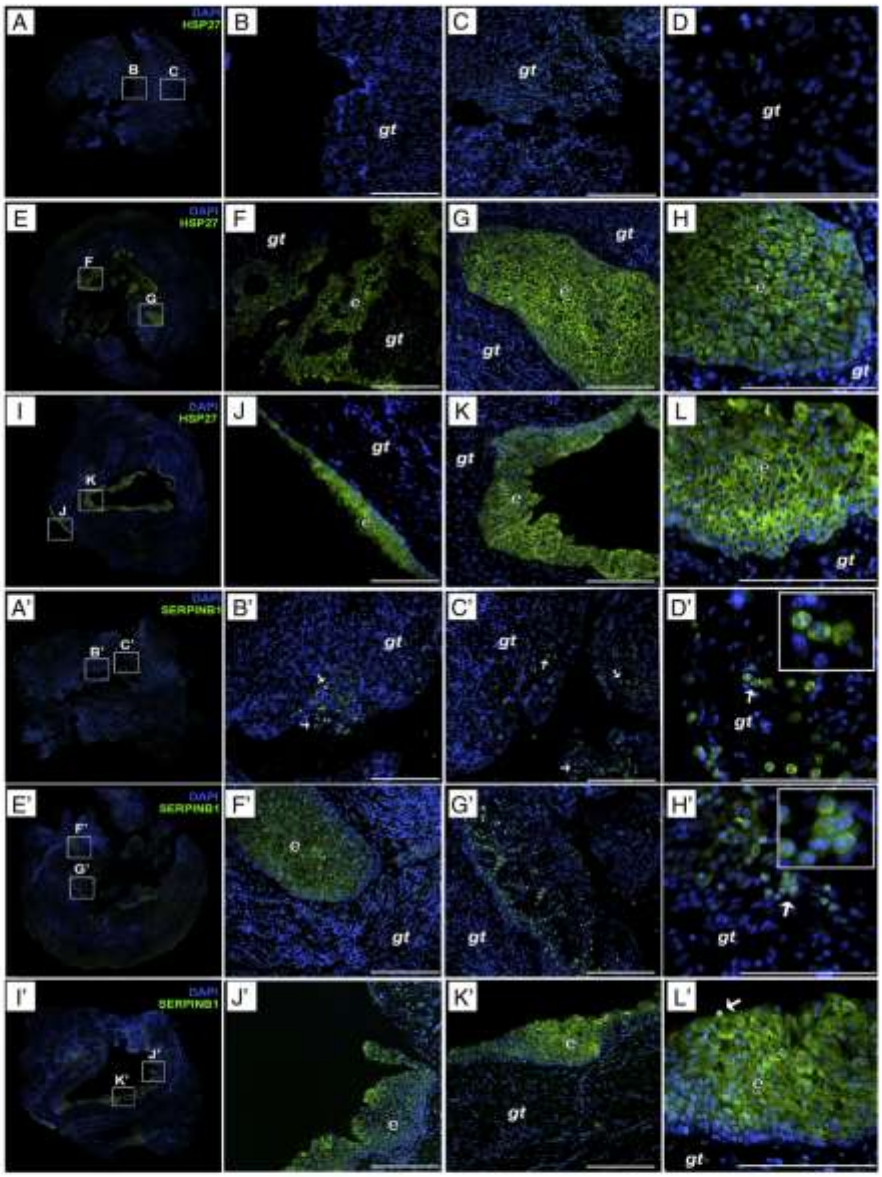

Figure 2. The expression of HSP27 and SERPINB1 in inflammatory apical periodontitis (*A–D* and *A'–D'*): nonepithelialized granuloma, *E–H* and *E'–H'*: epithelialized granuloma, and *I–L* and *I'–L'*: apical cyst). (*A–I*) For HSP27, there was a high expression of HSP27 in the cytoplasm of epithelial cells (e). (*A'–I'*) For SERPINB1, there was a high expression in the cytoplasm of neutrophils (*arrows*) and the epithelial cells (e). All cells present in the granulation tissue (gt) and epithelium (e) were identified using DAPI nuclear staining (blue). Scale bar = 250 μm.

tissue ($P < .05$) (Fig. 3*A*). A significantly higher expression of *HSP27* and *SERPINB1* was also detected in inactive lesions in comparison with active lesions ($P < .05$) (Fig. 3*B*). Moreover, a significant positive correlation was noted between *HSP27* and *SERPINB1* expression levels in apical periodontitis ($r^2 = 0.12$, $P < .001$) (Fig. 3*C*).

We then tested the potential association between *HSP27* expression levels with the expression of known acute inflammation biomarkers and detected significant negative correlations with *CXCR1* ($r^2 = 0.12$, $P < .001$), *MPO* ($r^2 = 0.06$, $P = .007$), and *CTSG* ($r^2 = 0.08$, $P = .002$) (Fig. 3*D*). Likewise, *SERPINB1* showed significant negative correlations with *CXCR1* ($r^2 = 0.09$, $P = .001$), *MMP8* ($r^2 = 0.12$, $P < .001$), and *MPO* ($r^2 = 0.15$, $P < .001$) and a significant moderate (−) correlation with *CTSG* ($r^2 = 0.52$, $P < .001$) (Fig. 3*E*).

Discussion

In this study, we used proteomic analysis as an unbiased approach to identify differentially expressed proteins in apical periodontitis in comparison with healthy control tissue. Overall, of the 48 proteins identified as differentially expressed, 30 were found to be expressed in all 4 lesions. Of these, 19 of 30 (63.3%) were down-regulated, whereas 11 of 30 (36.6%) were up-regulated. Most of these peptides belonged to biological pathways with functions related to the cytoskeleton, antioxidant defense system, and cell cycle, whereas a few peptides have suggested functions in calcium signaling, biosynthesis and metabolism, protein transport, inhibition of proteases, stress response, immune response, and coagulation cascade.

Interestingly, most of the differentially expressed proteins in apical periodontitis appeared down-regulated, and these were mainly related to cytoskeleton assembly and cell-cell interaction pathways. Although speculative, we may argue that the down-regulation of cytoskeleton proteins might reflect the tissue collapse and disorganization resultant of the chronic inflammatory process affecting the apical tissue during the progression of apical periodontitis. Recently, chronic inflammation has been linked to cytoskeletal changes, and direct links between cytoskeletal proteins and proinflammatory signaling are beginning to emerge in the literature (22).

The up-regulated proteins included an antioxidant enzyme, 3 blood plasma proteins, a calcium signaling protein, 2 cytoskeleton proteins, an immune response protein, a serpin family member, and a stress response protein. We focused our analysis on 2 of the significantly up-regulated proteins, HSP27 and SERPINB1, because of their putative roles as key

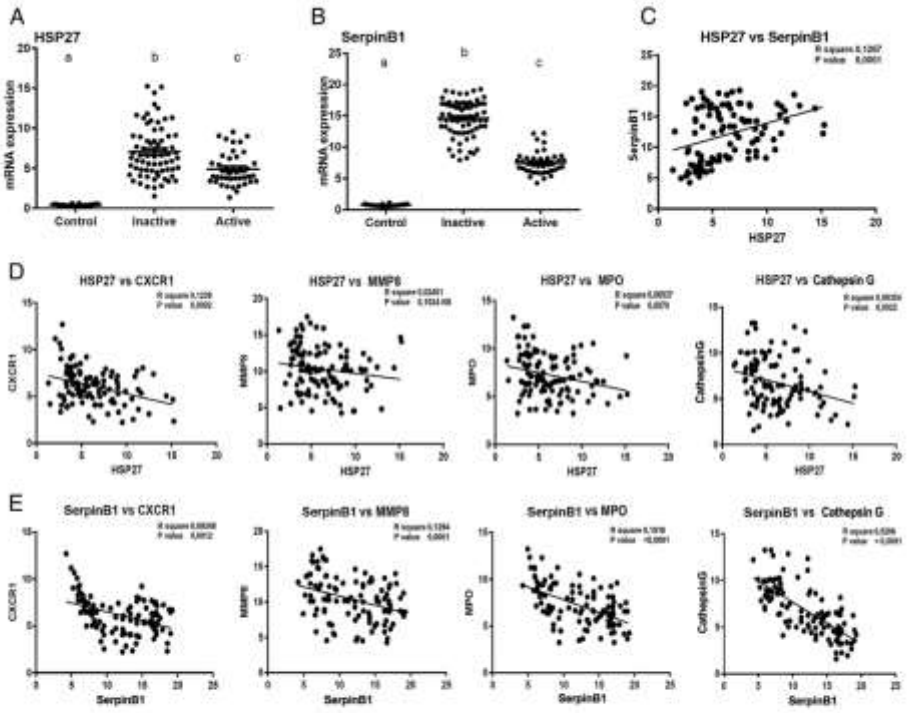

Figure 3. The expression of HSP27 and SERPINB1 in apical periodontitis and correlation analysis with additional immune inflammatory markers. (*A*) The relative expression of HSP27 in healthy apical tissues (controls) and active and inactive apical periodontitis. (*B*) The relative expression of SERPINB1 in healthy apical tissues (controls) and active and inactive apical periodontitis. In *A* and *B*, different letters represent statistically significant differences ($P < .05$). Pearson correlation analysis between (*C*) the relative expression of *HSP27* and *SERPINB1* in apical periodontitis; between (*D*) *HSP27* and *CXCR1*, *MMP8*, *MPO*, and *CTSG*, and between (*E*) *SERPINB1* and *CXCR1*, *MMP8*, *MPO*, and *CTSG*. $n = 110$ apical granulomas and $n = 26$ healthy periapical tissues.

regulators of tissue metabolism and their tissue protective roles during various chronic inflammatory conditions and animal models (23, 24). HSP27 was 3.79-fold up-regulated in apical periodontitis. This protein belongs to the heat shock protein gene family and has an important role in the inhibition of apoptosis in thermal and chemical stress, protecting the cells from injury in hostile environments (25, 26). In contrast, *HSP27*-deficient mice display impaired wound healing (27). Additional evidence suggests that HSP27 can modulate neutrophil chemotaxis and activity (28, 29), which could account for the local immune regulation of apical periodontitis. The results of our immunofluorescence assays revealed the expression of HSP27 predominantly in the epithelial cells of both epithelialized granulomas and apical cysts. These results corroborate previous reports in which HSP27 expression was found in odontogenic epithelial cells, dental lamina, and the enamel organ of tooth germs but not in fibroblasts or other stromal cells (30). Furthermore, the observed overexpression of the HSP27 protein in periapical granulomas is in line with our previously published findings in which HSP27 mRNA was significantly up-regulated in periapical granulomas and in lipopolysaccharide-stimulated macrophages (15). HSP27 expression was significantly higher in inactive lesions, implicating a potential role for this molecule in regulating lesion progression (15). Nevertheless, additional mechanistic studies are needed to determine a functional role for HSP27 in the pathogenesis of apical periodontitis.

SERPINB1 expression was also higher (~2-fold) in apical periodontitis. SERPINB1 is a potent inhibitor of neutrophil serine proteases (elastase and cathepsin G) and commonly up-regulated in ulcerative colitis (31). This protein also plays a crucial role in tissue protection against neutrophil-induced damage, acting as an apoptosis inhibitor in a caspase-independent pathway mediated by cathepsin G (32). We have previously shown the overexpression of another serpin family member (*SERPINE1*) in inactive apical periodontitis (2); the current findings of SERPINB1 up-regulation in apical periodontitis reinforce the evidence for a role of this gene family in the pathogenesis of the condition. Our results showed that SERPINB1 is exclusively expressed in the epithelium and in infiltrating PMNs present in the granulation tissue. We may speculate that whether in the epithelium or in the granulation tissue, SERPINB1 is serving a protective role, inhibiting the cathepsin G–mediated apoptosis of neutrophils and epithelial cells and thereby limiting the damage derived from the diffuse and uncontrolled secretion of neutrophil elastase and cathepsin G. The results of our quantitative PCR support this hypothesis because *SERPINB1* appears significantly more up-regulated in stable lesions than in progressive lesions. Interestingly, the negative correlation between *CTSG* and *SERPINB1* in apical periodontitis suggests the involvement of this protein in neutrophil-mediated inflammation. Recent evidence suggests that neutrophils can exert a regulatory role during the acute phase of the immune response, partially because of the release of neutrophil extracellular traps (NETs). These NETs contain DNA and up to 20 different inflammatory mediators and effector molecules, such as cathepsin G and elastase. The up-regulation of SERPINB1 might regulate and reduce the destructive potential of NET release, inhibiting the direct degradation of the tissue and helping to stabilize the lesion (33).

Interestingly, the results of our correlation analyses suggest dynamic and interactive roles for *SERPINB1* and *HSP27* in the regulation of apical periodontitis through their modulation of the expression of additional biomarkers of acute inflammation. Of note, significant negative correlations were identified for both *SERPINB1* and *HSP27* with *CXCR1*, *MMP8*, *MPO*, and *CTSG*. We observed a strong negative correlation was observed between the expression of *HSP27* and *CXCR1*, the receptor for interleukin 8 (CXCL8) and the main mediator of neutrophil infiltration in periapical inflammation (17). Additionally, significant negative correlations were observed between *HSP27* with *MPO* and *CTSG*, suggesting that its expression is correlated with decreased neutrophil infiltration and activity. Likewise, significant negative correlations were observed between the expression of *SERPINB1* with all the selected biomarkers of acute inflammation. The strongest negative correlation was noted for *CTSG* ($r^2 = 0.52$), which possibly suggests a direct regulatory link between the expression of *SERPINB1* and the inhibition of the expression of cathepsin G, which is 1 of the main proteolytic effector molecules released by neutrophils and responsible for direct tissue degradation and proinflammatory signaling (34, 35).

In summary, using an unbiased proteomic analysis, we identified HSP27 and SERPINB1 as differentially expressed proteins in apical periodontitis and confirmed their predominant epithelial localization. We also observed interesting correlations between the expression of these proteins with other inflammatory molecules that may provide insights into the functional role of HSP27 and SERPINB1 as regulators of apical periodontitis.

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Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (http://dx.doi.org/10.1016/j.joen.2017.03.014).

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3 DISCUSSION

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The common features of chronic periodontitis and apical periodontitis suggest the existence of common pathologic mechanisms, and supports the idea that they may share genetic risk factors. In this thesis, we have approached the association of genetic polymorphisms with resistance and susceptibility phenotypes to inflammatory osteolytic periapical and periodontal lesions under this frame of reference. Moreover, the articles presented in this thesis are a partial representation of a larger genetic association study including more than 1400 subjects and various surrogate markers of disease, which in all practical terms regards chronic and apical periodontitis as anatomically distinct representations of a similar pathology.

Still, our approach is susceptible of critical appraise from practitioners of periodontics and endodontics, the disciplines that deal with chronic periodontitis and apical periodontitis at the core of their practice. Probably, the first argument against a common handling of both pathologies will follow the lines of the extent to which both diseases are clinically different, present distinct histopathological features, and even affect a significantly different demographic cohort of the population. To these possible critics, we clarify that our approach does not intend to equal chronic and apical periodontitis from a clinical, histological or demographical point of view, but rather from an etiopathogenic perspective.

It is our main argument that chronic and apical periodontitis display an astonishingly similar pathologic behavior once the host's response is activated. Whatever differences both diseases possess in their initiation, after the immune system becomes activated and henceforth responsible for most of the destruction of periodontal tissues, they appear to be undistinguishable (SILVA et al., 2007; GARLET, 2010; MARTON; KISS, 2014). Other than anatomical (and hence histological) differences, the progressive inflammatory destruction of periodontal tissues in chronic and apical periodontitis seems to follow a common route. From our perspective, this alone justifies the clustering of both diseases as a single outcome ('susceptibility to chronic inflammatory osteolytic periodontal destruction') in genetic association studies. From this paradigm follows that risk factors discovered in this manner could be linked indistinctly to the causal chain of both diseases. Nevertheless, it is mandatory to

reinforce that we are just completing the exploratory phase of our investigations and these assertions must be confronted with experimental evidence to establish their plausibility. Future articles will address these issues in depth, thus at this point it seems adequate to enunciate the principal theoretical foundations of our current research line to contextualize the system of reference under which the articles presented in this thesis were written.

Whatever arguments could be raised against the treatment of chronic and apical periodontitis as distinct ‘flavors’ of the same underlying disorder, it is undoubtedly clear that both diseases require as a prerequisite the existence of a susceptible host. The evidence to support this understanding is abundant and follows from the self-evident realization that not every individual challenged by identical initiating stimuli develops the disease (MORSANI et al., 2011; KARABUCAK et al., 2016; HUUMONEN; SUOMINEN; VEHKALAHTI, 2017; RAMSEIER et al., 2017).

Our proposal is that the ‘disorder’ that underlies the pathogenesis of chronic and apical periodontitis is an inflammatory/immunological one. At the risk of oversimplification, we argue that at the very core of both diseases’ pathogenesis are impaired or limited immune suppression mechanisms, allowing for an uncontested and unremitting immune response for extended periods of time.

To support this point of view, we included in this thesis a review that extensively presents our perspective in the subject matter, thus an extended repetition of our arguments at this point appears redundant. Nevertheless, it is fundamental to ascertain that currently we lack solid evidence to establish the exact nature of the ‘disorder’ that predispose to chronic and apical periodontitis. Our efforts (and those from others) have failed to establish a hierarchical organization of risk factors, and most of our knowledge is an unorderly collection of facts with high internal coherence but little external consistency. At present, this is true for almost any discipline in the biological sciences.

Having said that, the internal coherence of our supporting evidence is a required starting point from which a firm theoretical etiologic paradigm can arise. It is our conviction that in the future, a coherent hierarchical organization of the current evidence will produce a solid explanatory model for the etiology of periodontal inflammatory destruction, providing us with reliable predictive tools to guide the prevention, diagnosis, treatment and follow up of these diseases.

The genetic susceptibility traits underlying the clinical phenotype of periodontitis had caused lengthy debates and controversies on the periodontal literature (KORNMAN et al., 1997a; FIEBIG et al., 2008). Although there is virtually universal agreement on the concept of genetic contribution to the susceptibility of periodontitis, the adequate strategy to unveil this association is less clear (LAINE; LOOS; CRIELAARD, 2010; SCHAFER; JEPSEN; LOOS, 2011). As a research group, we have developed the concept that the inclusion of a 'resistant' control group enhances the power and odds of our genetic association studies (GARLET et al., 2012b). Other groups have followed a similar rationale using an analog strategy, including a 'highly susceptible' (aggressive periodontitis) population to increase the power and odds of genetic association studies (DE JONG et al., 2014; VAITHILINGAM et al., 2014; VIEIRA; ALBANDAR, 2014). We have also followed this path in recent researches.

In brief, the reasoning underlying our approach is that the recruitment of diseased and healthy subjects for periodontitis genetic studies violates the principle of equal exposure to the causal factor, that it is the corner stone of the case/control study design. While this concept seems to be very clear and widely applied to the study of infectious diseases, where subjects to an endemic area (i.e. exposed to the disease-causing pathogen) express susceptibility or resistance phenotypes, it is less straightforward for periodontitis association studies. The inexistence of prototypical endemic areas for periodontal diseases, along with the generally widespread application of oral hygiene methods, results in a unique situation, which has blurred the phenotypic nature of the 'control' group in the genetics studies over the years. Arguably, in a healthy sample population could exist a random mixture of highly susceptible, average and highly resistant subjects, their genetic susceptibility obscured by the systematic removal of the initiating stimulus by oral hygiene procedures. Follows from this, that when compared to the diseased sample, the risk contribution of any given polymorphism is diluted and undetectable by standard statistical analysis (GARLET et al., 2012b). The inclusion of a 'resistant' group (chronic gingivitis patients) offers and elegant solution to this challenge. By being equally exposed to the initiating stimulus (oral bacteria), but not displaying inflammatory periodontal destruction, they provide the 'real' control to periodontitis patients. Both 'candidate gene' genetic

association articles included in this thesis were conducted including the chronic gingivitis ‘resistant’ group as control.

Following this reasoning, our results regarding *TBX21* polymorphism (rs4794067) demonstrated that polymorphic allele-carrier subjects were more likely to suffer from chronic periodontitis. Nevertheless, the association analysis rendered significant results only at allele level, and when ‘resistant’ subjects (chronic gingivitis) were compared to ‘susceptible’ subjects (chronic periodontitis). These two facts point to a small effect of the polymorphic allele in the disease phenotype. This is a common finding in genetic association studies, and probably one of the main causes of the lack of replication that most polymorphic markers exhibit in the literature (DA SILVA et al., 2017).

The polymorphic T-allele of *TBX21* was associated with increased expression of T-bet, demonstrating a clear cause-effect relationship. Arguably, T-allele carriers are biased towards a Th1 polarization phenotype, predisposing them to a hyper inflammatory response (IWATA, et al., 2017). Unfortunately, we were unable to demonstrate this cause-effect relationship at IFN-γ expression level, which could have offered us a mechanistic connection with the disease phenotype. The failure to demonstrate a connection between *TBX21* genotype and expression of the signature cytokine of the Th1 profile (IFN-γ) is the best possible illustration of the complex and poorly understood regulatory mechanism of immune response.

Previous studies have found no effect of periodontal treatment on the levels of expression of T-bet in peripheral blood, and concluded somewhat extraordinarily, that T-bet and the Th1 polarization phenotype are insignificant for chronic periodontitis pathogenesis (ZHAO et al., 2011). We distance ourselves from those exaggerated and unsubstantiated claims, and simply conclude that the effect that we could demonstrate is a small contributor in an extremely intricate pathogenic chain of events.

On the other hand, we couldn’t prove a relationship between genotype and clinical parameters or microbiological profile. Possibly, the genotype’s effect is too mild to cause detectable changes in these parameters at the resolution level that they were assayed. A recent systematic review and meta-analysis including 43 studies (our *TBX21* article among them), has concluded that no single polymorphism is capable of explaining quantifiable changes in the subgingival microbiota (NIBALI et al., 2016). To overcome this issue, we are already using a broader and highly sensible microbiologic

assay (DNA-DNA checkerboard), and we present an article in preparation with those results in this thesis (article 2.4).

Our article regarding CCR5Δ32 mutation rs333 (under review) follows a similar structure than the previous one. In this instance, we expanded the inflammatory biomarkers assayed by quantitative RT-PCR to assess an across-the-board range of possible effects of the genotype in effector immune markers. We proved that the polymorphic allele was significantly more common in the chronic gingivitis ('resistant') group than in chronic periodontitis and aggressive periodontitis groups. The inclusion of the aggressive periodontitis group was an attempt to further increase the power and odds of the study, nevertheless the low incidence of this form of periodontitis and the intrinsic difficulty of stablishing a precise diagnosis seriously limited the sample size (ALBANDAR, 2014, VIEIRA; ALBANDAR, 2014). We believe that our approach using a 'resistant' phenotype is a more practical one, since the prevalence and incidence of this condition in adult population are extremely high (ALBANDAR; KINGMAN, 1999; GARLET et al., 2012b; JIN et al., 2016).

The Δ32 allele proved protective for chronic periodontitis (OR 0.49; CI 0.29-0.83; p-value 0.01) and for aggressive periodontitis (OR 0.46; CI 0.22-0.94; p-value 0.04). In a similar fashion to our results for *TBX21* polymorphism (rs4794067), the association tests using the classic control group (healthy patients) failed to prove significant association. It is our opinion that these results and previous published evidence from our group validates our strategy to conduct genetic association studies. Importantly, our strategy allows for the use of relatively small samples (± 600 subjects), given that the clinical characterization is carefully performed including clearly distinct (and extreme) phenotypes.

For CCR5Δ32 mutation (rs333) we expanded the number of inflammatory biomarkers assayed as secondary outcomes, including TNFα, IL-1β, IL-10, IL-6, T-bet, and IFN-γ. This was an effort to portray a wider spectrum of biomarkers characteristic of different polarization profiles of the immune response. Our results demonstrated only a slight effect, with a reduced expression of TNFα in heterozygous subjects. Again, this is testimony of the complex and redundant regulatory mechanism of the immune response, as well as of the overlapping layers of controlling mechanism characteristic of immune homeostasis. As previously stated, the risk genotype for periodontitis is probably composed of numerous risk alleles interacting with each other

and with overlapped layers of regulation, with no individual allele being responsible for a large portion of the risk, and thus the phenotype (NIBALI et al., 2017).

In our 'Association of genetic polymorphisms with subgingival microbial colonization patterns in chronic' article (manuscript in preparation) we explore a different approach from our previous genetic association studies. Rather than recruit a large sample of subjects and correlate the occurrence of a candidate polymorphic variant with the phenotype of disease, we employed a broad and sensitive microbiological profiling tool (DNA-DNA checkerboard) in tandem with multiple genotyping in a smaller, but extremely well characterized sample. The rationale behind this approach was to associate the periodontitis surrogate outcome ‘dysbiotic changes of the subgingival microbiota’ (HAJISHENGALLIS, 2014b) with a pool of polymorphisms previously associated with these changes in genome-wide association studies (GWAS) (DIVARIS et al., 2012; DIVARIS et al., 2013; SCHAEFER et al., 2013; SHANG et al., 2015). This strategy required the use of non-conventional statistical analysis to adjust for the necessary multiple testing. Instead of the usual chi-square or Fisher’s exact tests, we employed a two-stage step-up adaptive method (BENJAMINI; KRIEGER; YEKUTIELI, 2006) to detect the association between the polymorphic alleles and changes in the counts of any of the 40 bacteria species tested simultaneously.

The traditional concern when testing multiple hypotheses simultaneously is to control the family-wise error rate (*i.e.* the probability of making any false discovery). The problem of multiple testing is one of balance; in the one hand, there is the possibility of adjusting to a level were testing procedures are not powerful in the sense that the probability of rejecting null hypotheses that are false becomes unpractically small (e.g. Bonferroni correction). In the other hand, there is the temptation of ignoring the multiplicity issues altogether, increasing the possibility of type I error.

The fundamental advantage of the two-stage step-up adaptive method (BENJAMINI; KRIEGER; YEKUTIELI, 2006) is that uses the plotted distribution of p-values of the multiple hypothesis testing to estimate the slope of a regression line that is used in the second stage to discriminate between the null hypothesis that are true from those that are false. This strategy has an elegant internal consistency, since the number of hypothesis tested, minus the number of hypothesis rejected, less the proportion of hypothesis erroneously rejected (Q), is also the number used as the

denominator in the linear regression used to estimate the number of true null hypothesis. The resulting ‘discoveries’ are both highly sensitive and suffer from a small risk of being false positives (BENJAMINI; KRIEGER; YEKUTIELI, 2006).

Following this strategy, we identified four polymorphic variants: rs2521634 (nearby *NPY* gene), rs10010758 (nearby *TBC1D1* gene), rs6667202 (nearby *IL10* gene), and rs10043775 (missense variant of *TBXO38* gene) as being associated with significant changes in the subgingival ecology that are compatible with periodontal disease (SOCRANSKY et al., 1998; HAJISHENGALLIS, 2014b; HAJISHENGALLIS; LAMONT, 2016).

The inherent flaw of this strategy is that such comprehensive and sensitive microbiological profiling is unpractical for use in large samples. Thereby, the external validity of our results is somewhat limited. Nevertheless, the fact that in all four cases the direction of the association was concordant with previous evidence from independent populations increases our confidence in the legitimacy of this strategy (DIVARIS et al., 2012; DIVARIS et al., 2013; SCHAEFER et al., 2013; SHANG et al., 2015).

Finally, for apical periodontitis we employed an unbiased proteomic approach to discover differentially expressed proteins from diseased periapical tissues. This strategy is conceptually analog to the use of genes selected from GWAS to perform association studies, since the selection was made based on the natural discrepancy of protein profile of diseased tissues, rather than based on biased theoretical pre conceptions (BOSTANCI; BAO, 2017). This strategy identified 30 differentially expressed proteins in periapical granulomas, being 19 down-regulated and 11 up-regulated. We selected two up-regulated proteins (HSP27 and SERPINB1) for further analysis by immune localization and correlation with markers of acute inflammation.

Both HSP27 and SERPINB1 were strongly localized in the cytoplasm of epithelial cells in the epithelium lining the cystic cavity and the epithelial chords of epithelized granulomas. Additionally, SERPINB1 was expressed in infiltrating polymorph nuclear neutrophils in non-epithelized granulomas, epithelized granulomas and apical cysts.

The expression analysis demonstrated significantly increased expression of HSP27 and SERPINB1 in inactive lesions (RANKL/OPG <1) compared to active lesions (RANKL/OPG >1) (GARLET et al., 2012a).

The categorization of the lesions in active and inactive based exclusively on the RANKL/OPG ratio could be considered an oversimplification, but it is a useful tool to gain additional insights into the molecular basis of periapical pathogenesis, and we have been using this approach for several years (ARANHA et al., 2013, ARAUJO-PIRES et al., 2014). The fact that HSP27 and SERPINB1 present a differential expression pattern in active and inactive apical periodontitis further supports the use of our categorization system.

We found significant negative correlations between the expression of HSP27 and the acute inflammation biomarkers CXCR1, MPO and Cathepsin G. Furthermore, SERPINB1 was negatively correlated with all the former biomarkers, plus MMP8. Altogether, this evidence points to a protective effect of HSP27 and SERPINB1 during the progression of apical periodontitis, and suggest that they could be useful biomarkers of apical periodontitis regression/improvement.

In sum, the articles presented follow the common theme that the host's response to periodontal infection is at least partially determined by genetic traits. Follows from this concept that those genetic traits interact with environmental factors to outline the secretome of periodontal tissues and shape the composition of the microbiome during the progression of the disease. The interaction between the infecting microorganisms and the host's defense mechanisms (host/pathogen barrier) is what generates a risk profile that is the final determinant of the clinical presentation of apical and chronic periodontitis. We presented three different but complementary approaches to gain insight into this process. These articles are part of a larger endeavor including more than 1400 subjects suffering from apical and chronic periodontitis and their controls, which are currently being analyzed to discover novel genetic traits associated with the phenotype of inflammatory periodontal destruction.

In future studies, we will include the molecular targets and genetic risk markers discovered in these articles to characterize the common susceptibility profile to inflammatory periodontal destruction. Each molecule or gene that we successfully associate with the occurrence of the disease will be eventually integrated into a wide-

ranging explanatory model capable of accurately predicting the behavior of periodontitis.

The final goal of our investigations is to discover molecular and genetic markers that could be used to accurately predict the individual risk profile to suffer from inflammatory periodontal destruction. Gaining this predictive ability will allow for precisely directing preventive measures and resources to risk populations, and help in the decision-making process during the therapy. In the future, these efforts will permit clinicians to tailor their patients' treatment based in a comprehensive assertion of their real risk profile, avoiding the threat of over or under treatment.

4 CONCLUSIONS

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Regarding the role genetic polymorphism and protein biomarkers in the pathogenesis of chronic and apical periodontitis, we conclude:

- polymorphic variations of the gene *TBX21* (rs4794067) increase the risk to suffer chronic periodontitis, probably by biasing the immune response towards a pro-inflammatory Th1 response profile;
- the mutation CCR5Δ32 (rs333) elicits resistance to inflammatory periodontal destruction, probably by impairing the recruitment of specific populations of leukocytes that amplify the inflammatory response in the first steps of immune response;
- both rs4794067 and rs333, although conferring differential susceptibility profiles, produce only slight variations in inflammatory markers and had no measurable effect in microbiologic markers;
- mutant allele-carriers of rs2521634 presented decreased counts of disease-associated bacteria *T. forsythia, A. gerencseriae, F. periodonticum,* and *P. nigrescens*. Thus, this mutation could be protective for chronic periodontitis;
- mutant allele-carriers of rs10010758 and rs6667202 presented increased counts of disease-associated bacteria *P. gingivalis*. Thus, these mutations could be risk indicators for chronic periodontitis;
- mutant allele-carriers of rs10043775 exhibited decreased counts of disease-associated bacteria *P. intermedia*. Thus, this mutation could be protective for chronic periodontitis;
- proteomic profiling identified HSP27 and SERPINB1 as key risk factors in the development of apical periodontitis. Their expression was negatively correlated with the expression of acute inflammation markers, suggesting a protective role mediated by suppression of inflammation.

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APPENDIXES

APPENDIX A

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article **Cytokine networks regulating inflammation and immune defense in the oral cavity** will be included in the Thesis of the student Ian Franco Cavalla Ruiz and was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

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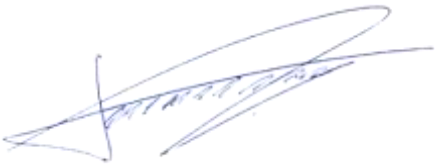

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Claudia C. Biguetti

Ana Claudia Araujo-Pires

Gustavo Pompermaier Garlet

APPENDIX B

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article **TBX21-1993T/C (rs4794067) polymorphism is associated with increased risk of chronic periodontitis and increased T-bet expression in periodontal lesions, but does not significantly impact the IFN-g transcriptional level or the pattern of periodontophatic bacterial infection** will be included in the Thesis of the student Ian Franco Cavalla Ruiz and was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

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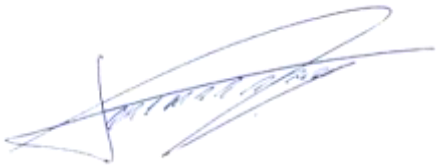

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APPENDIX C

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article **CCR5Δ32 (rs333) polymorphism is associated with decreased risk of chronic and aggressive periodontitis: a case-control analysis based in disease resistance and susceptibility phenotypes** will be included in the Thesis of the student Ian Franco Cavalla Ruiz and was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

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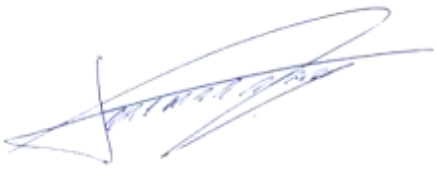

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APPENDIX D

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THESIS

We hereby declare that we are aware of the article **Proteomic profiling and differential messenger RNA expression correlate HSP27 and Serpin family B member 1 to apical periodontitis outcomes** will be included in the Thesis of the student Ian Franco Cavalla Ruiz and was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

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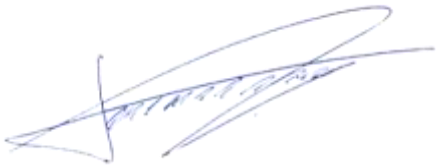

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ANNEXES

ANNEX A

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ANNEX C

Cover letter and submission receipt for article ‘CCR5Δ32 (rs333) polymorphism is associated with decreased risk of chronic and aggressive periodontitis: a case-control analysis based in disease resistance and susceptibility phenotypes’.

Universidade de São Paulo
Faculdade de Odontologia de Bauru

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Bauru, August 9 2017

Dear Prof. Dr. D. Kalvakolanu,

We wish to submit an original research article entitled **“CCR5Δ32 (rs333) polymorphism is associated with decreased risk of chronic and aggressive periodontitis: a case-control analysis based in disease resistance and susceptibility phenotypes”** for consideration by Cytokine. We confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication somewhere else.

In this paper, we report that CCR5Δ32 mutation is protective against chronic and aggressive periodontitis, and present evidence of a possible mechanistic link between the genotype and the effector immune response. This is noteworthy because previous evidence had failed to demonstrate a significant effect of CCR5Δ32 in periodontitis pathogenesis. In this report, we employed an alternative approach to conduct the association analysis that our group had been using successfully in the past few years.

We believe that this manuscript is appropriate for publication by Cytokine because it report the effect of a loss-of-function mutation of a well-known chemokine receptor over the phenotype of a highly prevalent human disease. We also present results from additional surrogate variables of disease, including expression analysis of several pro-inflammatory cytokines *in situ*.

We strongly believe that our approach to this subject is novel, presenting an alternative strategy for increasing the power of genetic association studies by including extreme phenotypes. Using this approach, we provide the first evidence linking CCR5Δ32 with periodontitis, and suggested a putative mechanism of action via diminished inflammatory response in the periodontal tissue.

We declare that we have no conflicts of interest to disclose. Please address all correspondence concerning this manuscript to me at garletgp@usp.br. Thank you for your consideration of this manuscript.

Sincerely,

Gustavo Pompermaier Garlet

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Manuscript Number: CYTO-17-397

Title: CCR5Δ32 (rs333) polymorphism is associated with decreased risk of chronic and aggressive periodontitis: a case-control analysis based in disease resistance and susceptibility phenotypes

Article Type: Full length article

Keywords: Periodontitis, CCR5Δ32, inflammation, pathogens

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Abstract: Chronic and aggressive periodontitis are infectious diseases characterized by the irreversible destruction of periodontal tissues, which is mediated by the host inflammatory immune response triggered by periodontal infection. The chemokine receptor CCR5 play an important role in disease pathogenesis, contributing to pro-inflammatory response and osteoclastogenesis. CCR5Δ32 (rs333) is a loss-of-function mutation in the CCR5 gene, which can potentially modulate the host response and, consequently periodontitis outcome. Thus, we investigated the effect of the CCR5Δ32 mutation over the risk to suffer periodontitis in a cohort of Brazilian patients (total N=699), representative of disease susceptibility (chronic periodontitis, N=197; and aggressive periodontitis, N=91) or resistance (chronic gingivitis, N=193) phenotypes, and healthy subjects (N=218). Additionally, we assayed the influence of CCR5Δ32 in the expression of the biomarkers TNFα, IL-1β, IL-10, IL-6, IFN-γ and T-bet, and key periodontal pathogens P. gingivalis, T. forsythia, and T. denticola. In the association analysis of resistant versus susceptible subjects, CCR5Δ32 mutant allele-carriers proved significantly protected against chronic (OR 0.49; 95% CI 0.29-0.83; p-value 0.01) and aggressive (OR 0.46; 95% CI 0.22-0.94; p-value 0.03) periodontitis. Further, heterozygous subjects exhibited significantly decreased expression of TNFα in periodontal tissues, pointing to a functional effect of the mutation in periodontal tissues during the progression of the disease. Conversely, no significant changes were observed in the presence or quantity of the periodontal pathogens P. gingivalis, T. forsythia, and T. denticola in the subgingival biofilm that could be attributable to the mutant genotype.