UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Impact of *Slc11a1* gene variants on the host response patterns and in the determination of periodontal diseases resistance and susceptibility phenotypes

Impacto das variantes do gene *Slc11a1* nos padrões de resposta do hospedeiro e na determinação de fenótipos de resistência e susceptibilidade às doenças periodontais

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Orientador: Prof. Dr. Gustavo Pompermaier Garlet

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Albert Einstein

RESUMO

Estudos em humanos e em modelos experimentais tem demonstrado a influência de múltiplos *loci* genéticos na determinação de fenótipos de susceptibilidade/resistência à periodontite. Dentre estes genes, o *Slc11a1*, cujas funções pleiotrópicas incluem a regulação da atividade macrófagos e linfócitos, tem potencial papel na modulação da resistência/susceptibilidade às doenças periodontais. Nesse contexto, nosso grupo demonstrou que camundongos das linhagens AIRmax e AIRmin, caracterizados pela predominância de variantes distintas de alelos do *Slc11a1*, associadas a diferentes padrões de resposta imune e inflamatória, apresentam fenótipos distintos de resistência/susceptibilidade à periodontite experimental. Ainda, variantes genéticas (SNPs) no *Slc11a1* se mostram associadas a diferentes doenças infecciosas em humanos. Neste contexto, este estudo teve como objetivo correlacionar os polimorfismos genéticos rs17228995, rs17235409, rs2290708, rs2695343, rs3731865 do gene *Slc11a1* com perfis de resistência/susceptibilidade às doenças periodontais em humanos, assim avaliar o impacto de variantes hipo/hiperresponsivas na periodontite experimental em camundongos. Para tanto, foram analisados 444 pacientes com periodontite crônica (CP), 476 indivíduos saudáveis (H) e 207 indivíduos com gengivite crônica (CG) para as análises de associação, e subgrupos para análise de possíveis correlações entre expressão/genótipo (CP=127, H=63) e para ensaios *in vitro* (H=29). Na análise dos genótipos, apenas os ensaios para a caracterização dos SNPs rs2290708 e rs37371865 se mostraram efetivos na descriminação alélica, os demais ensaios foram considerados tecnicamente inefetivos. Os polimorfismos rs2290708 e rs37371865 se mostraram associados ao risco de periodontite, sendo os genótipos CT+TT e GC+CC e alelos T e C (respectivamente) mais frequentes no grupo CP. Além da associação na abordagem caso controle, observamos que a presença dos alelos polimórficos T (rs2290708) e C (rs37371865) se mostrou associada ao aumento de expressão de TNF-α, IL-1β, IL-6, RANKL e RANKL/OPG nas lesões periodontais. Não foram observadas diferenças no padrão de colonização microbiológica de sítios com periodontite crônica com relação aos SNPs rs2290708 e rs37371865. A análise *in vitro* reforça a natureza hiper-reativa dos alelos polimórficos T (rs2290708) e C (rs37371865), uma vez que a produção de citocinas inflamatórias por macrófagos portadores de tais alelos se mostra aumentada frente ao estímulo por LPS. Finalmente, observamos que variantes hipereativas do *Slc11a1*, caracterizadas nas linhagens murinas AIRmin e AIRmax, se mostram associadas ao aumento de perda óssea

alveolar, influxo de leucócitos e maior produção de citocinas pró-inflamatórias na periodontite experimental. Dessa forma, é possível concluir que polimorfismos funcionais no gene *Slc11a1* associados ao aumento da responsividade inflamatória, e influenciam o risco ao desenvolvimento de periodontite em humanos e em modelo experimental.

Palavras-chave: Doença Periodontal. Gene *Slc11a1*. SNPs.

ABSTRACT

Impact of *Slc11a1* gene variants on the host response patterns and in the determination of periodontal diseases resistance and susceptibility phenotypes

Studies in humans and experimental models have demonstrated the influence of multiple genetic loci on the determination of susceptibility/resistance phenotypes to periodontitis. Among these genes, *Slc11a1*, whose pleiotropic functions include the regulation of macrophages and lymphocyte activity, has a potential role in the modulation of resistance/susceptibility to periodontal diseases. In this context, our group demonstrated that AIRmax and AIRmin mice, characterized by the predominance of distinct variants of *Slc11a1* alleles, associated to different patterns of immune and inflammatory response, present distinct phenotypes of resistance/susceptibility to experimental periodontitis. Furthermore, genetic variants (SNPs) in *Slc11a1* are shown to be associated with different infectious diseases in humans. In this context, this study aimed to correlate the genetic polymorphisms rs17228995, rs17235409, rs2290708, rs2695343, rs3731865 of the gene *Slc11a1* with profiles of resistance/susceptibility to periodontal diseases in humans, thus to evaluate the impact of hypo/hyperresponsive variants on experimental periodontitis in mice. Forty-five patients with chronic periodontitis (CP), 476 healthy individuals (H) and 207 individuals with chronic gingivitis (CG) were analyzed for association analysis, and subgroups were analyzed for analysis of possible correlations between expression / genotype (CP = 127, H = 63) and for *in vitro* tests (H = 29). In the analysis of the genotypes, only the assays for the characterization of the SNPs rs2290708 and rs3731865 were effective in the allelic discrimination, the other tests were considered technically ineffective. The polymorphisms rs2290708 and rs3731865 were shown to be associated with the risk of periodontitis, with a higher frequency of the genotypes CT+TT and GC+CC and the alleles T and C (respectively) in the CP group. In addition to the association in the control case approach, the presence of the polymorphic T (rs2290708) and C (rs37371865) alleles was shown to be associated with increased TNF-α, IL-1β, IL-6, RANKL and RANKL/OPG periodontal lesions. No differences were observed in the pattern of microbiological colonization of sites with chronic periodontitis with respect to the SNPs rs2290708 and rs37371865. *In vitro* analysis reinforces the hyper-reactive nature of the polymorphic alleles T (rs2290708) and C (rs37371865), since the production of inflammatory cytokines by macrophages bearing such alleles is shown to be increased in response to LPS

stimulation. Finally, we observed that hyperreactive variants of *Slc11a1*, characterized in the AIRmin and AIRmax murine are associated with increased alveolar bone loss, leukocyte influx, and increased production of proinflammatory cytokines in experimental periodontitis. Thus, it is possible to conclude that functional polymorphisms in the *Slc11a1* gene, associated with increased inflammatory responsiveness, influence the risk to the development of periodontitis in humans and in mice experimental model.

Keywords: Periodontal diseases. *Slc11a1* Gene. SNPs.

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LIST OF ABREVIATIONS

AIRmax	Maximal Inflammatory Reactions
AIRmin	Minimal Inflammatory Reactions
BOP	Bleeding on Probing
CAL	Clinical Attachment Loss
CG	Chronic Gingivitis
CP	Chronic Periodontitis
DNA	Desoxyribonucleic acid
H	Healthy
IL	Interleukin
LPS	Lypopolysaccharide
M1	Macrophages exhibit high levels of pro-inflammatory
M2	Macrophages exhibit high levels of anti-inflammatory
mRNA	messenger RNA
OPG	Osteoprotegerin
PD	Probing Depth
PDs	Periodontal Diseases
R	Resistance
RANKL	Receptor activator of nuclear factor kappa-B ligand
S	Susceptibility
SNP	Single Nucleotide Polymorphism
TNF	Tumor Necrosis Factor

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

Periodontal diseases (PDs) are chronic and multifactorial in nature, involving in its pathogenesis microbial, inflammatory and immunological factors, that together can be modulated by local, environmental, genetic and epigenetic factors, which ultimately result in alterations of the teeth protection and sustentation tissues (MARTINEZ and HOLT, 1999; KINANE and LAPPIN, 2001; LALLA *et al.*, 2003). Even in clinical health conditions, periodontal tissues are in close proximity with potentially pathogenic microorganisms, and to some extent coexist successfully with such microbes without deleterious consequences. However, qualitative and quantitative changes occur with the development of the bacterial biofilm, generating and intense antigenic load on the gingival sulcus, usually associated to significant changes in the pattern of host response, with in turn are associated with the appearance of clinical signs of the disease (KINANE and LAPPIN, 2001; LALLA *et al.*, 2003).

In this context, the understanding of the interrelationship between the host inflammatory immune response and the bacterial biofilm is fundamental to explain the pathogenesis of PDs. The complexity of the subgingival biofilm has been noticed from the primary Van Leeuwenhoek's microscopic observation of and evolved with modern microbial culture and identification techniques. Using the DNA-DNA hybridization checkerboard technique, five fundamental bacterial complexes were identified. The red complex, composed of *Porfiromonas gingivalis*, *Treponema denticola* and *Tenerella forsytia*; the orange complex, composed of *Fusobacterium nucleatum*, *Prevotella intermedia*, *Prevotella nigricens*, *Micromonas micros*, *Campilobacter rectus* e *Campilobacter showae*, are strongly associated with clinical parameters of periodontitis. Unlike the red and orange complex, the green complex (*Capnocytophaga spp*, *Aggregatibacter actinomycetemcomitans* serotype a and *Eikenella corrodens*), yellow (species of streptococci) and purple (*Actinomyces odontolyticus* and *Veillonella parvula*) are usually associated with periodontal health. The recognized periodontal pathogen *Aggragatibacter actinomycetemcomitans* serotype b is not routinely isolated in association with any other pathogen and is not classified in any of the clusters (SOCRANSKY *et al.*, 1998).

It is important to note that although the presence of pathogenic microorganisms is associated with PD occurrence and severity, their presence is not mandatorily associated with the occurrence of periodontitis (such microorganisms can be isolated from healthy periodontal

sites), as it is not obligatory for the development of periodontitis. In fact, it is believed that periodontitis is associated with ecological modifications in oral microflora, where the lack of beneficial or health-associated microorganisms may be as or more important than the presence of pathological microorganisms. This qualitative change is the relative proportions within the microbial biofilm, known as dysbiosis, has been associated with multiple pathologies in addition to periodontitis (SOCRANSKY and HAFFAJEE, 2005) such as gastroesophageal reflux (YANG *et al.*, 2009), otitis media (USVIATSOV *et al.*, 2000) and ulcerative colitis (FRANK *et al.*, 2007), among others (MARSH, 1994). In a general context, such changes in of proportions of microorganisms in the subgingival biofilm (dysbiosis) result in the induction of a chronic inflammatory and immunological response by the host, such response being an important factor in the establishment of pathological changes associated with periodontitis (PEYYALA e EBERSOLE, 2013; PEYYALA *et al.*, 2013).

Indeed, although the association of different microbial complex with the occurrence and severity of PDs, it has been shown that the amplification and progression of these processes are highly dependent of the host's immune and inflammatory response in response to bacteria or their products (GENCO *et al.*, 1998; MATRICARDI and RONCHETTI, 2001; GIBSON *et al.*, 2006). Healthy tissues maintain a mild and permanent inflammatory state known as subclinical inflammation, characterized by selective (and slight) expression of chemokines and cytokines, which guide the constant infiltration of neutrophils through the junctional epithelium, supposed to control the growth and tissue invasion of the biofilm. On the other hand, sites with periodontitis are characterized by increased expression of multiple inflammatory mediators, infiltration of different leukocyte subpopulations, and disruption of the normal architecture (BEREZOW and DARVEAU, 2011). In this way, the presence of the microbial stimulus associated with host responsiveness, which involves the intensity and nature of the developed response and the self-regulation capacity of the system, are determinants of the development of periodontitis (KORNMAN *et al.*, 1997).

In other words, while the presence of specific microorganisms is required to the establishment and progression of periodontitis, the pattern of host response triggered by the microbial stimuli seems to be the major determinant of the development of the disease. Generally, host inflammatory mediators have been associated with tissue destruction, while anti-inflammatory mediators counteract and attenuate disease progression. With the discovery of several T-cell subsets bearing distinct immunoregulatory properties, this pro- vs. anti-inflammatory scenario became more complex, and a series of studies has hypothesized

protective or destructive roles for Th1, Th2, Th17, and Treg subpopulations of polarized lymphocytes. Interestingly, the "protective vs. destructive" archetype is usually considered in a framework related to tissue destruction and disease progression. However, it is important to remember that periodontal diseases are infectious inflammatory conditions, and recent studies have demonstrated that cytokines (TNF-α and IFN-γ) considered harmful in the context of tissue destruction play important roles in the control of periodontal infection. On the other hand, immunoregulatory elements such as Th2 and Treg subsets seems to limit the tissue damage without critically impairing the protection against the infection. Therefore, a delicate balance between anti-microbial defenses and immunoregulatory circuits seems to operate towards the protection of the host with a minimal collateral damage to the periodontal tissues (GARLET, 2010).

In this context, extrinsic (i.e. environmental) and intrinsic factors that can modulate the nature and intensity of inflammatory immune response can contribute to the modulation of disease severity. Basically, intrinsic factors correspond to genetic variants, the most common being the ones that affect single nucleotides, called single nucleotide polymorphism (SNP). The SNPs may occur in a coding region of the DNA and entail the substitution, addition or deletion of an amino acid, altering the protein structure with profound biological effects or engaging in a promoter region, resulting in the inhibition or stimulation of gene expression, as well as also may not generate any changes when affected in non-coding regions (SCHORK *et al.*, 2000).

Despite controversies in the literature (considered in the sequence) (KORNMAN *et al.*, 1997; KORNMAN, 2008; BRUNNER *et al.*, 2010; LAINE and CRIELAARD, 2012; LAINE *et al.*, 2013) periodontitis has been associated with SNPs in multiple genes, each of which allegedly contributes in a small proportion to the increased relative risk of the disease, by which can be considered modifying genes (HART *et al.*, 2000; BRUNNER *et al.*, 2010; LAINE e CRIELAARD, 2012; LAINE *et al.*, 2013). It is believed that genetic risk factors can influence the natural history of periodontitis, increasing the probability of suffering the disease, being part of the causal chain and relating and increasing the effect of other risk factors (LOOS *et al.*, 2005). Studies in twins has shown that from 32 to 82% of individual variation in clinical parameters of periodontitis (CAL, PD, BOP, GI and PI) could be attributed to genetic factors, and even considering statistical adjustments for known environmental risk factors, genetic variations would be explain up to 50% of the variability of chronic periodontitis in adults (MICHALOWICZ *et al.*, 1991; MICHALOWICZ *et al.*, 2000).

To date, the possible genetic influence on the development of PDs has been generally studied through case-control approaches focusing on candidate genes, in which a "case" group (patients with periodontitis) is compared to a "control" group (patients presenting periodontal health) to determine the frequency of the polymorphic alleles in the different groups. Such approaches have identified a multiplicity of associations between the presence of polymorphisms in genes related to initiation, effector response and regulation of immune response to infection, and an increased risk of presenting periodontitis, although most of the associations describe are inconsistent and/or controversial (KORNMAN *et al.*, 2002; LAINE *et al.*, 2002). In this context, the case-control definitions (or lack of proper definitions) used in this approach can be considered the major factor responsible for the literature controversies previously mentioned. Indeed, limitations in the experimental design generally used in such studies may make it difficult to identify the possible risk factors in their real magnitude (GARLET *et al.*, 2012). Using an alternative case-control definitions approach, which primarily considers the exposure to etiological/microbial factors, our group demonstrated that the use of a control group of patients presenting periodontal health with adequate microbial control, and therefore not exposed to bacterial challenge (such as the control group "classic" traditionally used in these studies), limits both the correct identification of the *odds ratio* of a given SNP and the sampling power by up to 60% (GARLET *et al.*, 2012). Alternatively, the use of a control group exposed to the risk factor in an equivalent manner, but which does not present a destructive inflammatory manifestation of the periodontal tissues unlike to the "case" group, and consequently can be considered as are "resistant", can fulfill the requirements of a proper case-control design to study the genetic basis of an infectious disease. In this approach, in periodontitis context 'resistant' subjects are represented by chronic gingivitis, which are exposed to a microbial challenge but in longitudinal evaluations does not experience progression to periodontitis, and theoretically demonstrates the existence of a "resistant" genotype more appropriate to the case-control analysis (GARLET *et al.*, 2012).

Additionally, in order to overcome the limitations of the classic case-control studies usually performed in humans, supplementary approaches have been successfully used in studies in the field. Indeed, in addition to simply test the possible association between a given genotype and susceptibility clinical phenotype, it is possible to perform functional host response evaluations to understand the genetic influence on specific elements of host inflammatory and immune elements, and its subsequent impact on expression of the disease phenotype (GEMMELL *et al.*, 2000; TROMBONE, CARDOSO*, et al.*, 2009). In this context, previous

studies from our research group identified that certain gene variants, regardless of the modest genetic association in the classic case-control approach, were associated with different expression profiles of inflammatory and immunological mediators in the periodontal tissues. As examples, we can mention IL10-592 SNP whose AA/CA polymorphic genotype is shown to be associated with lower levels of IL-10 anti-inflammatory cytokine expression in periodontal tissues (CLAUDINO *et al.*, 2008); and IL1B-954 and TNFA-308 SNPs, which are respectively related to higher levels of expression of IL-1β and TNF-γ and with clinical parameters of periodontitis (FERREIRA *et al.*, 2008; TROMBONE, CARDOSO*, et al.*, 2009). It is important to highlight that such functional analysis also showed that even when considering gene variants specifically related to inflammatory cytokines, the presence of certain microorganisms (i.e. red complex) had a more significant and evident association with increased transcript levels (mRNA) for cytokine inflammatory conditions than the SNPs investigated (FERREIRA *et al.*, 2008; TROMBONE, CARDOSO*, et al.*, 2009). Indeed, our research group has previously shown that although MMP1-1607 SNP was proven to be functional, being related increased MMP-1 expression in periodontal tissues, the presence of classic periodontopathogens has a preponderant effect on the levels of expression of MMP-1 mRNA *(*REPEKE *et al., 2009)*. Such study also demonstrated that the strong and persistent microbial and inflammatory stimuli overcome the genetic predisposition to MMP-1 expression (REPEKE *et al., 2009)*. In another study, Tbet-related SNP TBX21-1993 T/C (rs4794067), which is essential for lymphocyte polarization to the Th1 pattern, was found to have an immediate relation to Tbet expression levels in gingival tissue, but not with the prototypical cytokine IFNg (CAVALLA *et al.*, 2015), adding some complexity to the genetic influence over host response patterns in periodontal environment. Despite the lack of a direct correlation with Th1-type response, TBX21-1993 T/C (rs4794067) was found to be associated with increased risk for the development of periodontitis, independently of the pattern of periodontal infection (CAVALLA *et al.*, 2015).

It is also important to consider that certain genetic variants may account for the risk of periodontitis by modulating the patterns of microbial colonization of periodontal tissues. In a recent study, our group demonstrated that the polymorphism rs2521634 proved significantly associated with *Tannerella. forsythia, Actinomyces gerencseriae, Fusobacterium periodonticum, and Prevotella nigrescens*; rs10010758 and rs6667202 were associated with increased counts of *Porphyromonas gingivalis*; and rs10043775 proved significantly associated with decreased counts of *Prevotella intermedia*; comprising therefore a strong connection

between the host's genetic profile and the occurrence of chronic periodontitis-associated bacteria (CAVALLA *et al.*, 2018).

Thus, independent studies suggest that both microbial and genetic factors may play a significant role in determining the host response associated with periodontitis, and consequently, may influence the outcome of the disease. Still, it is imperative to highlight the complexity of the disease model. From the genetic viewpoint, we must consider the potential participation of multiple genes in the determination of resistance and susceptibility phenotypes to periodontal diseases. Additionally, it is mandatory to consider the additional complexity underlying periodontitis pathogenesis, which involves of microorganism-host interaction, possible systemic and environmental cofactors (or modifying factors) that increase the complexity of human studies.

In this context, experimental models using mouse strains with known genetic backgrounds and well characterized host response phenotypes may comprise interesting tools for studying the genetic basis of resistance and susceptibility to periodontal diseases, pointing to candidate genes that regulate such phenotype, and whose homologous genes in humans could have similar role. In fact, studies comparing different classical strains of isogenic mice (BAKER, 2005; SHUSTERMAN, DURRANT*, et al.*, 2013; SHUSTERMAN, SALYMA*, et al.*, 2013; HIYARI *et al.*, 2015) demonstrate in a cause and effect manner the influence of the genetic background on host response and susceptibility to experimental periodontitis. Indeed, experimental studies performed in mice strains with different genetic backgrounds, support the genetic influence in the determination of resistance/susceptibility phenotypes to periodontitis. Early reports, still in the 1960´s, described opposite profiles for isogenic strains STR/N and DBA/2JN regarding the susceptibility to experimental periodontitis (BAKER *et al.,* 1961). Subsequent studies involving other strains, such as BALB/c, C57Bl/6, AKR/J, A/J,129/J and SJL/J, also reported the existence of resistance and susceptibility genotypes (BAKER *et al.*, 2000; BAKER e ROOPENIAN, 2002; HART *et al.*, 2004; BAKER, 2005). Furthermore, studies demonstrated the heritability of susceptibility to alveolar bone loss by generating recombinant lines derived from 5 standard isogenic lines, reinforcing the genetic aspect of susceptibility to experimental periodontitis (HIYARI *et al.*, 2015). However, despite the clear influence of the genetic background in the resistance/susceptibility to experimental periodontitis, the number and identity of such genetic determinants remains unclear.

In this context, our group demonstrated that mice genetically selected for maximum (AIRmax) our minimum (AIRmin) inflammatory reaction also had different phenotypes when submitted to the induction of periodontitis (TROMBONE, CARDOSO*, et al.*, 2009). Importantly, such variation was associated with different patterns of immune and inflammatory response presented by such strains. These mice strains were established by the Laboratory of Immunogenetics of the Butantan Institute by selective bi-directional reproduction in in order to understand the genetic basis of the immunological and inflammatory response, and in order to study the effect of different genotypes and phenotypes in relation to the immune and inflammatory response, (IBANEZ *et al.*, 1992; ARAUJO *et al.*, 1998; VIGAR *et al.*, 2000; CARNEIRO *et al.*, 2002; PETERS *et al.*, 2007). Such strains derive from a genetically heterogeneous founding population (F0) produced through the cross-linking of eight lines of isogenic mice of independent origin (A/J, DBA/2J, P/J, SWR/J, SJL/J, CBA/J, BALB/cJ e C57BL/6J) (STIFFEL *et al.*, 1990). The cross-linking of these strains was performed based on the intensity of the inflammatory reaction generated by the injection of the phylogenetic agent Biogel into the subcutaneous tissue of the animal, and mating between animals with higher or lower inflammatory response relative to the normal distribution of the resulting mouse population in each generation. From the 20th generation of selective mating it was mating accepted that the strains reached the maximum of phenotypic separation (called the selection limit), in which the allele(s) conferring the maximum and minimum inflammatory response are fixed in homozygosis AIRmax and AIRmin lines (IBANEZ *et al.*, 1992). In fact, the AIRmax and AIRmin lines present significant differences in the ability of the inflammatory response to various inflammatory agents (VASQUEZ-BRAVO,1996) (CARNEIRO *et al.*, 2002), constituting in a suitable model to study the mechanisms of the inflammatory/immunological response in different infectious models (ARAUJO *et al.*, 1998; BIOZZI *et al.*, 1998; VIGAR *et al.*, 2000; MARIA *et al.*, 2003; PETERS *et al.*, 2007). The AIRmax and AIRmin strains were successfully used in previous studied by our research group (TROMBONE, FERREIRA*, et al.*, 2009; TROMBONE *et al.*, 2010), in a model of experimental periodontitis and arthritis/periodontitis comorbidity models, in which the dichotomous inflammatory phenotypes were confirmed, and the variations of the AIRmax and AIRmin animals are due to a distinct inflammatory profile involving the modulation of the expression of several inflammatory mediators simultaneously, such as TNF-α, IL-1b and IL-6 (VIGAR *et al.*, 2000; DI PACE *et al.*, 2006).

Aiming to explore the mechanisms by which the AIRmax and AIRmin lines have their distinct phenotypes determined, genetic studies were conducted, and identified *Slc11a1* (Solute carrier Family 11a member 1) as one of the genes responsible for the differential response. Located on the chromosome 2q35, the *Slc11a1* gene contains 15 exons and plays a determinant role in the immune and inflammatory response, impacting the host's susceptibility to pathogens and autoimmune diseases. Described for the first time in mice for their functions in regulation of resistance and susceptibility to infectious agents, this gene was previously called Nramp1 (Natural resistance-associated macrophage protein -1) (FORBES and GROS, 2001). *Slc11a1* encode a highly hydrophilic integral membrane protein with 12 transmembrane domains and with a glycosylated extracellular cycle (EJGHAL *et al.*, 2014), and presents pleiotropic functions, such as the transport of essential ions, like Fe^{+2}, protons and other divalent cations (Zn^{+2} and Mn^{+2}) (FRITSCHE *et al.*, 2007). Regarding the modulation of the immune and inflammatory response, *Slc11a1* regulates the activity of macrophages reflecting in its activation and consequently in the production of nitric oxide, TNF-α, IL-1 (KITA *et al.*, 1992; RAMARATHINAM *et al.*, 1993), and also influence the activation of Th1 and Th2 lymphocytes (ARCHER *et al.*, 2015). Therefore, despite the limited information regarding the mechanisms by which *Slc11a1* modulates immune responses, it has been considered an candidate gene to influence the susceptibility to autoimmune and infectious diseases (ARCHER *et al.*, 2015).

In relation to the AIRmin and AIRmax strains, the allelic variants of *Slc11a1* are called R or S alleles since they confer resistance (R) or susceptibility (S) certain infections/diseases (ARAUJO *et al.*, 1998). In fact, subsequent studies demonstrated significant differences in the frequency of these alleles in the AIRmax and AIRmin lineage, with the R allele being predominant in AIRmax animals, while the presence of allele S is characteristic of the AIRmin strain (ARAUJO *et al.*, 1998). In humans, although there are no variations identical to the R and S alleles of *Slc11a1* described in mice, polymorphisms with similar potential immunoregulatory impact have been identified and associated with susceptibility to different infectious diseases (FATTAHI-DOLATABADI *et al.*, 2016). These polymorphisms vary in the location of coding regions, still involving missense mutations or silent substitutions (ABEL *et al.*, 1998; BELLAMY *et al.*, 1998; ALCAIS *et al.*, 2000; GREENWOOD *et al.*, 2000; FATTAHI-DOLATABADI *et al.*, 2016).

Among the SNPs described in the *Slc11a1* gene, rs17228995, rs17235409, rs2290708 rs2695343 and rs3731865 can be considered as SNP tags since are described in the literature as important markers of susceptibility or resistance to various infectious diseases (SAPKOTA *et al.*, 2012; BIBERT *et al.*, 2017). *Slc11a1*SNP rs17235409 was used for the study of susceptibility to cutaneous leishmaniasis, along with eight other SNPs from this same gene. This study demonstrated a strong linkage disequilibrium between the SNPs rs17235409 in exon 15 and the insertion/deletion of the polymorphism rs17235416. However, the polymorphisms of the *Slc11a1* gene did not influence the susceptibility to the development of cutaneous leishmaniasis (SOPHIE *et al.*, 2017). The SNP rs2290708, has little information in the literature. The only study that brings information about it is also unique to a single population, the Pakistani and the susceptibility to cutaneous leishmaniasis, and a mentioned above there was no significant difference between the distribution of alleles and genotypes (SOPHIE *et al.*, 2017). Three previously described polymorphisms point to a statistically significant difference for the susceptibility to tuberculosis in African American and Caucasian individuals for the *Slc11a1* gene, being rs3731865, rs3731863 and rs17221959. And those African American individuals who had the rs3731865 polymorphism with the CG and GG genotypes had a higher risk of developing tuberculosis with CT and TT genotypes presented a decreased risk for the disease when the analysis was performed in *multilocus (*VELEZ *et al., 2009)*. In addition to infectious diseases, type 1 diabetes was also associated with SNP rs3731865 (YANG *et al.*, 2011). The polymorphic allele of the SNPs rs3731865 has been associated with an increased risk of suffering from otitis media in a pediatric population in Australia, but relative to the SNPs rs2276631 and rs2695343 this significant statistical difference does not hold (RYE *et al.*, 2013). Together, the evidence indicates that the polymorphic variants in the *Slc11a1* gene may be associated with a variety of risk phenotypes in infectious diseases, making it an interesting candidate for an association study in chronic periodontitis.

With regard to periodontal diseases, a single recent study investigated the possible association of variants in *Slc11a1* with such conditions. In a genetic association study including 75 patients with chronic periodontitis and 50 healthy controls, the association of the rs17235409 and rs2276631 polymorphisms in the *Slc11a1* gene with the periodontal disease phenotype was described, with the polymorphic allele having a protective effect for chronic periodontitis (KADKHODAZADEH *et al.*, 2016). It is noteworthy that even with relatively small experimental N identified a possible risk variant in the *Slc11a1* gene. However, no other type of correlation (with host response patterns or microbial colonization patterns) were

investigated, as was a single population with a distinct genetic background of the Brazilian population, was tested.

Therefore, this study goal is to correlate the genetic polymorphisms rs17228995, rs17235409, rs2290708, rs2695343, rs3731865 of the gene *Slc11a1* with profiles of resistance/susceptibility to periodontal diseases in humans, thus to evaluate the impact of hypo/hyperresponsive variants on experimental periodontitis in mice.

2 ARTICLE

2 ARTICLE

Journal of Leucocyte Biology

Hiper reactive variants of *Slc11a1* gene are associated with increased inflammatory responsiveness and comprise a risk factor for periodontal diseases: evidences from human and experimental periodontitis

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LIST OF ABREVIATIONS

AIRmax	Maximal Inflammatory Reactions
AIRmin	Minimal Inflammatory Reactions
BOP	Bleeding on Probing
CAL	Clinical Attachment Loss
CG	Chronic Gingivitis
CP	Chronic Periodontitis
DNA	Desoxyribonucleic acid
H	Healthy
IL	Interleukin
LPS	Lypopolysaccharide
M1	Macrophages exhibit high levels of pro-inflammatory
M2	Macrophages exhibit high levels of anti-inflammatory
mRNA	messenger RNA
OPG	Osteoprotegerin
PD	Probing Depth
PDs	Periodontal Diseases
R	Resistance
RANKL	Receptor activator of nuclear factor kappa-B ligand
S	Susceptibility
SNP	Single Nucleotide Polymorphism
TNF	Tumor Necrosis Factor

ABSTRACT

Studies in humans and experimental models have demonstrated the influence of multiple genetic loci on the determination of susceptibility/resistance phenotypes to periodontitis. Among these genes, *Slc11a1*, whose pleiotropic functions include the regulation of macrophages and lymphocyte activity, has a potential role in the modulation of resistance/susceptibility to periodontal diseases. In this context, our group demonstrated that AIRmax and AIRmin mice, characterized by the predominance of distinct variants of *Slc11a1* alleles, associated to different patterns of immune and inflammatory response, present distinct phenotypes of resistance/susceptibility to experimental periodontitis. Furthermore, genetic variants (SNPs) in *Slc11a1* are shown to be associated with different infectious diseases in humans. In this context, this study aimed to correlate the genetic polymorphisms rs17228995, rs17235409, rs2290708, rs2695343, rs3731865 of the gene *Slc11a1* with profiles of resistance/susceptibility to periodontal diseases in humans, thus to evaluate the impact of hypo/hyperresponsive variants on experimental periodontitis in mice. Forty-five patients with chronic periodontitis (CP), 476 healthy individuals (H) and 207 individuals with chronic gingivitis (CG) were analyzed for association analysis, and subgroups were analyzed for analysis of possible correlations between expression / genotype (CP = 127, H = 63) and for *in vitro* tests (H = 29). In the analysis of the genotypes, only the assays for the characterization of the SNPs rs2290708 and rs3731865 were effective in the allelic discrimination, the other tests were considered technically ineffective. The polymorphisms rs2290708 and rs3731865 were shown to be associated with the risk of periodontitis, with a higher frequency of the genotypes CT+TT and GC+CC and the alleles T and C (respectively) in the CP group. In addition to the association in the control case approach, the presence of the polymorphic T (rs2290708) and C (rs37371865) alleles was shown to be associated with increased TNF-α, IL-1β, IL-6, RANKL and RANKL/OPG periodontal lesions. No differences were observed in the pattern of microbiological colonization of sites with chronic periodontitis with respect to the SNPs rs2290708 and rs37371865. *In vitro* analysis reinforces the hyper-reactive nature of the polymorphic alleles T (rs2290708) and C (rs37371865), since the production of inflammatory cytokines by macrophages bearing such alleles is shown to be increased in response to LPS stimulation. Finally, we observed that hyperreactive variants of *Slc11a1*, characterized in the AIRmin and AIRmax murine are associated with increased alveolar bone loss, leukocyte influx, and increased production of proinflammatory cytokines in experimental periodontitis. Thus, it

is possible to conclude that functional polymorphisms in the *Slc11a1* gene, associated with increased inflammatory responsiveness, influence the risk to the development of periodontitis in humans and in mice experimental model.

Keywords: Periodontal diseases, *Slc11a1* Gene and SNPs

1 INTRODUCTION

Periodontal diseases (PDs) are chronic and multifactorial in nature, involving in its pathogenesis microbial, inflammatory and immunological factors, that together can be modulated by local, environmental, genetic and epigenetic factors, which ultimately result in alterations of the teeth protection and sustentation tissues [1-3]. Even in clinical health conditions, periodontal tissues are in close proximity with potentially pathogenic microorganisms, and to some extent coexist successfully with such microbes without deleterious consequences. However, qualitative and quantitative changes occur with the development of the bacterial biofilm, generating and intense antigenic load on the gingival sulcus, usually associated to significant changes in the pattern of host response, with in turn are associated with the appearance of clinical signs of the disease [2, 3].

In this context, the understanding of the interrelationship between the host inflammatory immune response and the bacterial biofilm is fundamental to explain the pathogenesis of PDs. The complexity of the subgingival biofilm has been noticed from the primary Van Leeuwenhoek's microscopic observation of and evolved with modern microbial culture and identification techniques. Using the DNA-DNA hybridization checkerboard technique, five fundamental bacterial complexes were identified. The red complex, composed of *Porfiromonas gingivalis*, *Treponema denticola* and *Tenerella forsytia*; the orange complex, composed of *Fusobacterium nucleatum*, *Prevotella intermedia*, *Prevotella nigricens*, *Micromonas micros*, *Campilobacter rectus* e *Campilobacter showae*, are strongly associated with clinical parameters of periodontitis. Unlike the red and orange complex, the green complex (*Capnocytophaga spp*, *Aggregatibacter actinomycetemcomitans* serotype a and *Eikenella corrodens*), yellow (species of streptococci) and purple (*Actinomyces odontolyticus* and *Veillonella parvula*) are usually associated with periodontal health. The recognized periodontal pathogen *Aggragatibacter actinomycetemcomitans* serotype b is not routinely isolated in association with any other pathogen and is not classified in any of the clusters [4].

It is important to note that although the presence of pathogenic microorganisms is associated with PD occurrence and severity, their presence is not mandatorily associated with the occurrence of periodontitis (such microorganisms can be isolated from healthy periodontal sites), as it is not obligatory for the development of periodontitis. In fact, it is believed that periodontitis is associated with ecological modifications in oral microflora, where the lack of beneficial or health-associated microorganisms may be as or more important than the presence of pathological microorganisms. This qualitative change is the relative proportions within the microbial biofilm, known as dysbiosis, has been associated with multiple pathologies in addition to periodontitis [5] such as gastroesophageal reflux [6], otitis media [7] and ulcerative colitis [8], among others [9]. In a general context, such changes in of proportions of microorganisms in the subgingival biofilm (dysbiosis) result in the induction of a chronic inflammatory and immunological response by the host, such response being an important factor in the establishment of pathological changes associated with periodontitis [10, 11].

Indeed, although the association of different microbial complex with the occurrence and severity of PDs, it has been shown that the amplification and progression of these processes are highly dependent of the host's immune and inflammatory response in response to bacteria or their products [12-14]. Healthy tissues maintain a mild and permanent inflammatory state known as subclinical inflammation, characterized by selective (and slight) expression of chemokines and cytokines, which guide the constant infiltration of neutrophils through the junctional epithelium, supposed to control the growth and tissue invasion of the biofilm. On the other hand, sites with periodontitis are characterized by increased expression of multiple inflammatory mediators, infiltration of different leukocyte subpopulations, and disruption of the normal architecture [15]. In this way, the presence of the microbial stimulus associated with host responsiveness, which involves the intensity and nature of the developed response and the self-regulation capacity of the system, are determinants of the development of periodontitis [16].

In other words, while the presence of specific microorganisms is required to the establishment and progression of periodontitis, the pattern of host response triggered by the microbial stimuli seems to be the major determinant of the development of the disease. Generally, host inflammatory mediators have been associated with tissue destruction, while anti-inflammatory mediators counteract and attenuate disease progression. With the discovery of several T-cell subsets bearing distinct immunoregulatory properties, this pro- vs. anti-inflammatory scenario became more complex, and a series of studies has hypothesized protective or destructive roles

for Th1, Th2, Th17, and Treg subpopulations of polarized lymphocytes. Interestingly, the "protective vs. destructive" archetype is usually considered in a framework related to tissue destruction and disease progression. However, it is important to remember that periodontal diseases are infectious inflammatory conditions, and recent studies have demonstrated that cytokines (TNF-α and IFN-γ) considered harmful in the context of tissue destruction play important roles in the control of periodontal infection. On the other hand, immunoregulatory elements such as Th2 and Treg subsets seems to limit the tissue damage without critically impairing the protection against the infection. Therefore, a delicate balance between anti-microbial defenses and immunoregulatory circuits seems to operate towards the protection of the host with a minimal collateral damage to the periodontal tissues [17].

In this context, extrinsic (i.e. environmental) and intrinsic factors that can modulate the nature and intensity of inflammatory immune response can contribute to the modulation of disease severity. Basically, intrinsic factors correspond to genetic variants, the most common being the ones that affect single nucleotides, called single nucleotide polymorphism (SNP). The SNPs may occur in a coding region of the DNA and entail the substitution, addition or deletion of an amino acid, altering the protein structure with profound biological effects or engaging in a promoter region, resulting in the inhibition or stimulation of gene expression, as well as also may not generate any changes when affected in non-coding regions [18].

Despite controversies in the literature (considered in the sequence) [16, 19-22] periodontitis has been associated with SNPs in multiple genes, each of which allegedly contributes in a small proportion to the increased relative risk of the disease, by which can be considered modifying genes [20-23]. It is believed that genetic risk factors can influence the natural history of periodontitis, increasing the probability of suffering the disease, being part of the causal chain and relating and increasing the effect of other risk factors [24]. Studies in twins has shown that from 32 to 82% of individual variation in clinical parameters of periodontitis (CAL, PD, BOP, GI and PI) could be attributed to genetic factors, and even considering statistical adjustments for known environmental risk factors, genetic variations would be explained up to 50% of the variability of chronic periodontitis in adults [25, 26].

To date, the possible genetic influence on the development of PDs has been generally studied through case-control approaches focusing on candidate genes, in which a “case” group (patients with periodontitis) is compared to a “control” group (patients presenting periodontal health) to determine the frequency of the polymorphic alleles in the different groups. Such approaches

have identified a multiplicity of associations between the presence of polymorphisms in genes related to initiation, effector response and regulation of immune response to infection, and an increased risk of presenting periodontitis, although most of the associations describe are inconsistent and/or controversial [27, 28]. In this context, the case-control definitions (or lack of proper definitions) used in this approach can be considered the major factor responsible for the literature controversies previously mentioned. Indeed, limitations in the experimental design generally used in such studies may make it difficult to identify the possible risk factors in their real magnitude [29]. Using an alternative case-control definitions approach, which primarily considers the exposure to etiological/microbial factors, our group demonstrated that the use of a control group of patients presenting periodontal health with adequate microbial control, and therefore not exposed to bacterial challenge (such as the control group "classic" traditionally used in these studies), limits both the correct identification of the *odds ratio* of a given SNP and the sampling power by up to 60% [29]. Alternatively, the use of a control group exposed to the risk factor in an equivalent manner, but which does not present a destructive inflammatory manifestation of the periodontal tissues unlike to the "case" group, and consequently can be considered as are "resistant", can fulfill the requirements of a proper case-control design to study the genetic basis of an infectious disease. In this approach, in periodontitis context 'resistant' subjects are represented by chronic gingivitis, which are exposed to a microbial challenge but in longitudinal evaluations does not experience progression to periodontitis, and theoretically demonstrates the existence of a "resistant" genotype more appropriate to the case-control analysis [29].

Additionally, in order to overcome the limitations of the classic case-control studies usually performed in humans, supplementary approaches have been successfully used in studies in the field. Indeed, in addition to simply test the possible association between a given genotype and susceptibility clinical phenotype, it is possible to perform functional host response evaluations to understand the genetic influence on specific elements of host inflammatory and immune elements, and its subsequent impact on expression of the disease phenotype [30, 31]. In this context, previous studies from our research group identified that certain gene variants, regardless of the modest genetic association in the classic case-control approach, were associated with different expression profiles of inflammatory and immunological mediators in the periodontal tissues. As examples, we can mention IL10-592 SNP whose AA/CA polymorphic genotype is shown to be associated with lower levels of IL-10 anti-inflammatory cytokine expression in periodontal tissues [32], and IL1β-954 and TNFA-308 SNPs, which are

respectively related to higher levels of expression of IL-1β and TNF-γ and with clinical parameters of periodontitis [31, 33]. It is important to highlight that such functional analysis also showed that even when considering gene variants specifically related to inflammatory cytokines, the presence of certain microorganisms (i.e. red complex) had a more significant and evident association with increased transcript levels (mRNA) for cytokine inflammatory conditions than the SNPs investigated [31, 33]. Indeed, our research group has previously shown that although MMP1-1607 SNP was proven to be functional, being related increased MMP-1 expression in periodontal tissues, the presence of classic periodontopathogens has a preponderant effect on the levels of expression of MMP-1 mRNA [34]. Such study also demonstrated that the strong and persistent microbial and inflammatory stimuli overcome the genetic predisposition to MMP-1 expression [34]. In another study, Tbet-related SNP TBX21-1993 T/C (rs4794067), which is essential for lymphocyte polarization to the Th1 pattern, was found to have an immediate relation to Tbet expression levels in gingival tissue, but not with the prototypical cytokine IFN-γ [35], adding some complexity to the genetic influence over host response patterns in periodontal environment. Despite the lack of a direct correlation with Th1-type response, TBX21-1993 T/C (rs4794067) was found to be associated with increased risk for the development of periodontitis, independently of the pattern of periodontal infection [35].

It is also important to consider that certain genetic variants may account for the risk of periodontitis by modulating the patterns of microbial colonization of periodontal tissues. In a recent study, our group demonstrated that the polymorphism rs2521634 proved significantly associated with *Tannerella. forsythia, Actinomyces gerencseriae, Fusobacterium periodonticum, and Prevotella nigrescens*; rs10010758 and rs6667202 were associated with increased counts of *Porphyromonas gingivalis*; and rs10043775 proved significantly associated with decreased counts of *Prevotella intermedia*; comprising therefore a strong connection between the host's genetic profile and the occurrence of chronic periodontitis-associated bacteria [36].

Thus, independent studies suggest that both microbial and genetic factors may play a significant role in determining the host response associated with periodontitis, and consequently, may influence the outcome of the disease. Still, it is imperative to highlight the complexity of the disease model. From the genetic viewpoint, we must consider the potential participation of multiple genes in the determination of resistance and susceptibility phenotypes to periodontal diseases. Additionally, it is mandatory to consider the additional complexity underlying periodontitis pathogenesis, which involves of microorganism-host interaction, possible

systemic and environmental cofactors (or modifying factors) that increase the complexity of human studies.

In this context, experimental models using mouse strains with known genetic backgrounds and well characterized host response phenotypes may comprise interesting tools for studying the genetic basis of resistance and susceptibility to periodontal diseases, pointing to candidate genes that regulate such phenotype, and whose homologous genes in humans could have similar role. In fact, studies comparing different classical strains of isogenic mice [37-40] demonstrate in a cause and effect manner the influence of the genetic background on host response and susceptibility to experimental periodontitis. Indeed, experimental studies performed in mice strains with different genetic backgrounds, support the genetic influence in the determination of resistance/susceptibility phenotypes to periodontitis. Early reports, still in the 1960´s, described opposite profiles for isogenic strains STR/N and DBA/2JN regarding the susceptibility to experimental periodontitis [37]. Subsequent studies involving other strains, such as BALB/c, C57Bl/6, AKR/J, A/J,129/J and SJL/J, also reported the existence of resistance and susceptibility genotypes [37, 41-43]. Furthermore, studies demonstrated the heritability of susceptibility to alveolar bone loss by generating recombinant lines derived from 5 standard isogenic lines, reinforcing the genetic aspect of susceptibility to experimental periodontitis [40]. However, despite the clear influence of the genetic background in the resistance/susceptibility to experimental periodontitis, the number and identity of such genetic determinants remains unclear.

In this context, our group demonstrated that mice genetically selected for maximum (AIRmax) our minimum (AIRmin) inflammatory reaction also had different phenotypes when submitted to the induction of periodontitis [31]. Importantly, such variation was associated with different patterns of immune and inflammatory response presented by such strains. These mice strains were established by the Laboratory of Immunogenetics of the Butantan Institute by selective bi-directional reproduction in in order to understand the genetic basis of the immunological and inflammatory response, and in order to study the effect of different genotypes and phenotypes in relation to the immune and inflammatory response [44-48]. Such strains derive from a genetically heterogeneous founding population (F0) produced through the cross-linking of eight lines of isogenic mice of independent origin (A/J, DBA/2J, P/J, SWR/J, SJL/J, CBA/J, BALB/cJ e C57BL/6J) [49]. The cross-linking of these strains was performed based on the intensity of the inflammatory reaction generated by the injection of the phylogenetic agent Biogel into the subcutaneous tissue of the animal, and mating between animals with higher or

lower inflammatory response relative to the normal distribution of the resulting mouse population in each generation. From the 20th generation of selective mating it was mating accepted that the strains reached the maximum of phenotypic separation (called the selection limit), in which the allele(s) conferring the maximum and minimum inflammatory response are fixed in homozygosis AIRmax and AIRmin lines [44]. In fact, the AIRmax and AIRmin lines present significant differences in the ability of the inflammatory response to various inflammatory agents [47] constituting in a suitable model to study the mechanisms of the inflammatory/immunological response in different infectious models [45, 46, 48, 50, 51]. The AIRmax and AIRmin strains were successfully used in previous studied by our research group [52, 53], in a model of experimental periodontitis and arthritis/periodontitis comorbidity models, in which the dichotomous inflammatory phenotypes were confirmed, and the variations of the AIRmax and AIRmin animals are due to a distinct inflammatory profile involving the modulation of the expression of several inflammatory mediators simultaneously, such as TNF-α, IL-1β and IL-6 [46, 54].

Aiming to explore the mechanisms by which the AIRmax and AIRmin lines have their distinct phenotypes determined, genetic studies were conducted, and identified *Slc11a1* (Solute carrier Family 11a member 1) as one of the genes responsible for the differential response. Located on the chromosome 2q35, the *Slc11a1* gene contains 15 exons and plays a determinant role in the immune and inflammatory response, impacting the host's susceptibility to pathogens and autoimmune diseases. Described for the first time in mice for their functions in regulation of resistance and susceptibility to infectious agents, this gene was previously called Nramp1 (Natural resistance-associated macrophage protein -1) [55]. *Slc11a1* encode a highly hydrophilic integral membrane protein with 12 transmembrane domains and with a glycosylated extracellular cycle [56], and presents pleiotropic functions, such as the transport of essential ions, like Fe^{+2}, protons and other divalent cations (Zn^{+2} and Mn^{+2}) [57]. Regarding the modulation of the immune and inflammatory response, *Slc11a1* regulates the activity of macrophages reflecting in its activation and consequently in the production of nitric oxide, TNF-α, IL-1 [58, 59], and also influence the activation of Th1 and Th2 lymphocytes [60]. Therefore, despite the limited information regarding the mechanisms by which *Slc11a1* modulates immune responses, it has been considered a candidate gene to influence the susceptibility to autoimmune and infectious diseases [60].

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fact, subsequent studies demonstrated significant differences in the frequency of these alleles in the AIRmax and AIRmin lineage, with the R allele being predominant in AIRmax animals, while the presence of allele S is characteristic of the AIRmin strain [45]. In humans, although there are no variations identical to the R and S alleles of *Slc11a1* described in mice, polymorphisms with similar potential immunoregulatory impact have been identified and associated with susceptibility to different infectious diseases [61]. These polymorphisms vary in the location of coding regions, still involving missense mutations or silent substitutions [61-65].

Among the SNPs described in the *Slc11a1* gene, rs17228995, rs17235409, rs2290708 rs2695343 and rs3731865 can be considered as SNP tags since are described in the literature as important markers of susceptibility or resistance to various infectious diseases [66, 67]. *Slc11a1* SNP rs17235409 was used for the study of susceptibility to cutaneous leishmaniasis, along with eight other SNPs from this same gene. This study demonstrated a strong linkage disequilibrium between the SNPs rs17235409 in exon 15 and the insertion/deletion of the polymorphism rs17235416. However, the polymorphisms of the *Slc11a1* gene did not influence the susceptibility to the development of cutaneous leishmaniasis [68]. The SNP rs2290708, has little information in the literature. The only study that brings information about it is also unique to a single population, the Pakistani and the susceptibility to cutaneous leishmaniasis, and a mentioned above there was no significant difference between the distribution of alleles and genotypes [68] . Three previously described polymorphisms point to a statistically significant difference for the susceptibility to tuberculosis in African American and Caucasian individuals for the *Slc11a1* gene, being rs3731865, rs3731863 and rs17221959. And those African American individuals who had the rs3731865 polymorphism with the CG and GG genotypes had a higher risk of developing tuberculosis with CT and TT genotypes presented a decreased risk for the disease when the analysis was performed in *multilocus* [69]. In addition to infectious diseases, type 1 diabetes was also associated with SNP rs3731865 [70]. The polymorphic allele of the SNPs rs3731865 has been associated with an increased risk of suffering from otitis media in a pediatric population in Australia, but relative to the SNPs rs2276631 and rs2695343 this significant statistical difference does not hold [71]. Together, the evidence indicates that the polymorphic variants in the *Slc11a1* gene may be associated with a variety of risk phenotypes in infectious diseases, making it an interesting candidate for an association study in chronic periodontitis.

With regard to periodontal diseases, a single recent study investigated the possible association of variants in *Slc11a1* with such conditions. In a genetic association study including 75 patients with chronic periodontitis and 50 healthy controls, the association of the rs17235409 and rs2276631 polymorphisms in the *Slc11a1* gene with the periodontal disease phenotype was described, with the polymorphic allele having a protective effect for chronic periodontitis [72]. It is noteworthy that even with relatively small experimental N identified a possible risk variant in the *Slc11a1* gene. However, no other type of correlation (with host response patterns or microbial colonization patterns) were investigated, as was a single population with a distinct genetic background of the Brazilian population, was tested.

Therefore, this study goal is to correlate the genetic polymorphisms rs17228995, rs17235409, rs2290708, rs2695343, rs3731865 of the gene *Slc11a1* with profiles of resistance/susceptibility to periodontal diseases in humans, thus to evaluate the impact of hypo/hyperresponsive variants on experimental periodontitis in mice

2 MATERIAL AND METHODS

2.1. Subjects and samples

Patients and controls were recruited in São Paulo state, southeastern Brazil, scheduled for treatment at the Dentistry School of University of Ribeirão Preto (UNAERP) or University of Guarulhos (UnG), after institutional review board approval (CAAE: 32465714.4.1001.5506). All the subjects were examined by experienced periodontists and score for bleeding on probing (BOP), probing depth (PD) and clinical attachment loss (CAL). 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. After the diagnostic phase, patients were subsequently categorized into healthy (H; n=476), chronic gingivitis (CG; n=207) or chronic periodontitis (CP; n=444), as previously described 21, 33; following the classification of the American Academy of Periodontology [73]. The healthy control group (H; n=476) included subjects with clinically healthy gingival tissues (<10% of bleeding on probing; no sites with probing depth >3mm, no clinical attachment loss and no radiographic evidence of alveolar bone loss)

scheduled to undergo restorative dentistry procedures. Patients in the chronic periodontitis group (CP; n=444) presenting moderate to advanced periodontitis (at least one teeth per sextant with probing depth >6mm and clinical attachment loss >3mm plus radiographic evidence of extensive bone loss [>30% alveolar bone height in at least 50% of teeth]), which received basic periodontal therapy. The chronic gingivitis group (CG; n=193), 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, which received basic periodontal therapy. The clinical features of the groups are summarized in Table 1.

Saliva was collected from all controls and patients at the enrollment session using a DNA Oragene OG-500 kit (DNA Genotek, Ottawa, ON, Canada), following the manufacturer's instructions. 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 <3mm 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]. A representative fraction of the chronic periodontitis group (n=127) was also scheduled to surgical procedures; biopsies of gingival tissue (one sample from each patient, comprising junctional epithelium, pocket epithelium and gingival connective tissue) 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=127), as previously described [34]. The gingival biopsies were obtained during surgery procedures and comprised epithelium and underlying connective tissues from a fraction of the healthy sample (n=63) and chronic periodontitis samples (n=127), gingival biopsies were taken from a representative fraction of the control group when they underwent surgical procedures for restorative/prosthetic reasons. Due to ethical restrictions (lack of indication of surgical therapy) no gingival samples were collected from CG group. Subgingival biofilm samples were collected from a representative fraction of the chronic periodontitis group (n=69), from 9 subgingival sites/patients to microbiological analysis.

2.2. Genotyping of *Slc11a1* 639+22C/T (rs2290708) and -469+14G/C (rs3731865)

Saliva was collected from all the participants at the enrollment session using a DNA Oragene OG-500 kit (DNA Genotek, Ottawa, ON, Canada), following the manufacturer's instructions. DNA was extracted from participants saliva using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's guidelines. A spectrophotometer (Nanodrop 1000, Thermo Scientific, Waltham, MA, 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, CA, USA), containing a 40x mix of unlabeled PCR forward and reverse primers as well as a VIC- and FAM-labeled allele discrimination probes. For, rs 17228995 (Assay ID: C65978820), rs17235409 (Assay ID:C2563529610), rs2290708 (Assay ID:C15885305.10) rs2695343 (Assay ID:C165978910) and rs3731865 (Assay ID:C1659793.10). Quantitative polymerase chain reaction (qPCR) was carried out in a 5µL reaction mixture with 4ng of genomic DNA and 2.5µL of the TaqMan genotyping PCR master mix (Applied Biosystems). Amplification and detection were performed using the ViiA 7 platform (Applied Biosystems). Thermal cycling conditions were 10 min at 95°C followed by 50 two-step cycles, including 15s of denaturation at 92°C and 60s of annealing/extension at 60°C. All reactions were performed in duplicate and allele calling was done using QuantiStudio software; only genotypes with an automatic call rate >95% were considered, error rate was <3%. Allele calling was double-checked manually in the raw data plot, comparing the amplitude and kinetics of fluoresce patterns. Samples that failed to provide a genotype were repeated in additional reactions.

2.3. RNA extraction and RealTime-PCR

Total RNA was extracted from samples with RNeasyKIT (Qiagen Inc, Valencia, CA) according to the manufacturers' instructions [74]. The integrity of RNA samples was checked with 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturers' instructions. After RNA extraction, complementary DNA was synthesized by using 3µg of RNA through a reverse transcription reaction using QuantiTectRTkit (Qiagen Inc, Valencia, CA). All targets mRNA levels were measured by means of RealTimePCR using inventoried optimized TaqMan primers/probes sets (Invitrogen, Carlsbad, CA) in a Viia7 instrument (LifeTechnologies, Carlsbad, CA). The results are depicted as the relative level of gene expression; calculated in reference to internal controls GAPDH and β-actin expression in each sample using the $2^{-\Delta\Delta}$Ct method.

2.4. Subgingival biofilm sampling and analysis

Subgingival biofilm samples were collected from 9 subgingival sites of a fraction of chronic periodontitis group (N=69), and were assayed for the presence and quantity of 40 bacterial species [4, 75] (Supplementary Material, Table 1), as extensively described elsewhere [4, 76, 77]. In brief, after periodontal examination three deep (>5 mm), three medium (4-5 mm) and three shallow (1-3 mm) periodontal sites were selected for microbiological sampling. After relative isolation with gauze and cotton rolls a sterile Gracey curette (Hu-Friedy, Chicago USA) was gently introduced into the bottom of the periodontal pocket and then rinsed in a tube containing 150 μL of TE buffer, then 100 μL NaOH 0.5 M were added and the sample was agitated for 1 minute. The nine samples per subject were pooled together. 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.

2.5. Cell culture

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood from 29 systemically and periodontally healthy subjects by density gradient centrifugation with Ficoll (Invitrogen), as described previously [78]. CD14+ monocytes were purified from fresh PBMCs with anti-CD14 magnetic beads (MiltenyiBiotec, Bergisch, Gladbach, Germany), as recommended by the manufacturer and as described previously [78]. The purity of monocytes was between 95% and 97% as verified by FACS. Monocytes were cultured at 5 x 10^5 cells/mL in RPMI medium (Invitrogen) supplemented with 10% FBS (HyClone, Waltham, MA, USA), and subjected to *P. gingivalis* LPS stimulation (0, 1, 10 or 100 ng/mL) (Sigma-Aldrich, St.

Louis, MO, USA). The concentrations were defined based on previous studies [79-81] and preliminary experiments, to achieve a low, intermediate and intense degree of stimuli to the cells. After 12-h post stimulation, the cells supernatants were colleted to posterior ELISA analysis. N=29; presenting *Slc11a1* rs2290708 CC (N=16) or CT+TT (N=13), or *Slc11a1* rs3731865 GG (N=15) or GC+CC (N=14) genotypes.

2.6. Experimental periodontitis

Experimental groups comprised 8-week-old AIRmin (previously described to present a low susceptibility to experimental periodontitis, and to be characterized by the predominance of 'S' hipo-responsive *Slc11a1* allele), AIRmax (previously described to present a high susceptibility to experimental periodontitis, and to be characterized by the predominance of 'R' hiper-responsive *Slc11a1* allele), AIRmaxRR and AIRmaxSS (strains presenting the AIRmax background but homozygous for R and S *Slc11a1* alleles)[44, 49], bred at Butantan Institute (São Paulo, Brazil) and maintained during the experimental period in the animal facilities of FOB/USP. Experimental periodontitis was induced as previously described [82]. In brief, the animals received an oral delivery of $1x10^9$ colony-forming units (CFU) of a diluted culture of *A. actinomycetemcomitans* JP2 (grown anaerobically in supplemented agar medium, TSBV), in 100 µl of phosphate-buffered saline (PBS) with 2% of carboxymethylcellulose, placed/inoculated (not directly injected into the gingival tissue) in the oral cavity of mice with a micropipette. After 48 and 96 h, this procedure was repeated. Negative controls comprised sham-infected mice, which received PBS with carboxymethylcellulose in solution without *A. actinomycetemcomitans*. The evaluation of the extent of alveolar bone loss (the increase in CEJ–ABC area in comparison with control mice) was performed as described previously[82], and involved the measurement of the area between the cement–enamel junction (CEJ) and the alveolar bone crest (ABC) in the three posterior teeth, in arbitrary units of area (AUA), analyzed using ImageTool 2·0 software (University of Texas Health Science Center, San Antonio, TX, USA). The isolation and characterization of leucocytes present in the lesion site were performed as described previously [82], and involved the enzymatic digestion of tissues with Medimachine (BD Biosciences PharMingen, San Diego, CA, USA), according to the manufacturer's instructions. After processing, cell viability was assessed by Trypan blue exclusion, and the cell count was performed in a Neubauer chamber; with this data depicted in the manuscript as the total inflammatory cell count. The concentrations of cytokines in periodontal extracts were determined by ELISA using commercially available kits (R&D Systems, Minneapolis, MN, USA), as follows: interleukin (IL)-1β (sensitivity > 3 pg/mL), TNF-α (sensitivity > 3·4 pg/mL),

interferon (IFN)-γ (sensitivity > 2 pg/mL), IL-6 (sensitivity > 2 pg/mL), IL-17 (sensivity > 5pg/mL and IL-10 (sensitivity> 4 pg/mL). All assays were carried out according to the manufacturer's instructions. The results were expressed as picograms of cytokine (± s.d.) per milligram of periodontal tissue, for one experiment representative of three.

2.7. Data 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 [83]. Molecular data analysis was performed Kruskal-Wallis or ANOVA followed by Bonferroni post-test. A p-value <0.05 was considered statistically significant. All tests were performed in Stata14 (College Station, TX, USA) or GraphPad Prism 6.05 (San Diego, CA, USA).

3 RESULTS

3.1. *Slc11a1* SNPs frequency analysis.

Initially the objective of this study was to correlate the genetic polymorphisms rs17228995, rs17235409, rs2290708, rs2695343, rs3731865 of the gene *Slc11a1*, selected to represent the entire gene in view of the binding imbalance pattern of *Slc11a1*. However, rs17228995, rs17235409, and rs2695343 genotyping assays (despite described as functional and validated by the suppliers) does not present a satisfactory technical performance (Supplementary Fig: 1,2 and 4), and consequently, only the data regarding rs2290708 and rs3731865 (Supplementary Fig: 3 and 5) were considered valid and analyzed.

There were significant differences in the inflammatory and clinical periodontal parameters between all groups, as predicted by the inclusion criteria and the numerous reports regarding the features of each condition. A summary of the population´s demographics, clinical characteristics, genotype, and allele distribution is provided in Table 1,2 and Table 3. The CP and H subpopulations selected for gene expression and microbiological analysis were not significantly different from the total population sample (data not shown). There was no evidence of deviation from Hardy-Weinberg equilibrium between the groups (data not shown).

When the patients presenting chronic periodontitis were compared to healthy controls (Table 2), it was observed that the frequency of SNP rs2290708 CT and TT genotypes (P=0,0276,

OR=0,7404, CI:0.569to0.969; P=0,0043, OR=2,953, CI:1.399to6.443; respectively) was lower in the CP group. When the individuals presenting chronic gingivitis were compared to patients with chronic periodontitis (Table 2), it was observed that the frequency of rs2290708 CT and TT had a significant difference for CP group (P=0,0135, OR=0,654, CI:0.465to0.919; P=0.0104, OR=5.496, CI:1.491to23.78; respectively). When we analyzed the genotype frequencies of SNP rs3731865 (Table 3), the CC genotype (P=0,0052 OR=2,104 CI:1.253 to 3.532) was lower in patients. When the individuals presenting chronic gingivitis were compared to patients with chronic periodontitis (Table 3) CC genotype (P=0,0077 OR=2,946 CI:1.282 to 6.948) was lower in the patient's group. However, the haplotype analysis of the SNPs did not show a significant difference (data not shown). Study power, performed with using cases/controls sample size, disease prevalence, disease allele frequency and genotype relative risk as parameters, resulted in a 98% study power for rs2290708 and 95% for rs3731865 (multiplicative model).

3.2. Association between *Slc11a1* SNPs with mRNA expression.

Our data initially showed a stronger expression of all the molecular targets expression in periodontal lesions than in controls tissues (Fig. 1A), in accordance with previous descriptions. Additionally, our data originally demonstrate an increased expression of *Slc11a1* in periodontal lesions than in healthy tissues. When analyzing the expression levels of the biomarkers in relation to the polymorphism rs2290708, allele T (CT and TT genotypes) carriers presented a significantly lower TNF-α expression in chronic periodontitis when compared with CC (Fig.1A and Fig.2A). The IL-1β and IL-4 biomarker presented a high level of expression in CT genotype when compared to the CC genotype, but the TT genotype presented the same levels of expression as CC and CT in chronic periodontitis group (Fig.1A and Fig.2A). IL-6 expression levels of the T allele (CT and TT) were higher when compared to the CC genotype. Levels of RANKL expression in the CT and TT genotypes were significantly higher than the CC genotype in chronic periodontitis group. In addition, the RANKL/OPG ration also showed a significant difference between the heterozygous and polymorphic homozygous genotypes when compared to the wild genotype in the chronic periodontitis group (Fig.1A and Fig.2A). When analyzing the expression levels of the SNP biomarkers rs37371865, allele C (GC and CC genotypes) carriers presented a significantly lower TNF-α, expression in chronic periodontitis when compared with GG (Fig.1B and Fig.2B). Allele C (GC and CC genotypes) carriers presented a significantly higher IL-1β, expression in chronic periodontitis when compared with GG (Fig.1B and Fig. 2B). IL-6 and RANKL expression levels were the same in GC and CC genotypes, but

expression levels of both differed in CC genotype in the group of patients with chronic periodontitis (Fig. 1B and Fig. 2B). The same observed in the SNP rs2290708 occurred in the SNP rs3731865 where, the RANKL/OPG ratio also presented a statistically significant difference in the heterozygous and polymorphous homozygous genotypes when compared to the genotype GG in the group with chronic periodontitis (Fig. 1B and Fig. 2B).

3.3. Subgingival biofilm sampling

When comparing the ancestral and mutant genotypes for SNPs rs2290708 (Fig.3A) and rs3731865(Fig.3B) taking into account the presence to the classic subgingival microbial complexes, a significant difference was not found.

3.4. *Slc11a1* vs cytokine production *in vitro*.

The stimulation of genotypes of SNP rs2290708 in both CC and CT+TT macrophage cells, derived from healthy donor PBM, resulted in production of TNF-α, IL-1β and IL-6 and IL-10. Only TNF-α and IL-6 levels significantly were higher in CT+TT when compared to CC genotype (Fig. 4A). The SNP rs3731865 in both GG and GC+CC macrophage cells, derived from healthy donor PBM, resulted in production of the same biomarkers cited above. However, TNF-α and IL-1β levels significantly were higher in GC+CC when compared to GG genotype (Fig. 4B). Meanwhile, IL-6 levels significantly were higher CT+TT when compared to GG genotype in two moments with 10 and 100 stimulation of LPS ng/mL (Fig.4B)

3.5. *Slc11a1* vs experimental periodontitis in mice.

In order to test the impact of *Slc11a1* hipo/hiper-responsive variants in periodontitis outcome in a cause-and-effect manner, experimental periodontitis was induced in AIRmin (characterized by the predominance of 'S' hipo-responsive *Slc11a1* allele), AIRmax (characterized by the predominance of 'R' hiper-responsive *Slc11a1* allele), AIRmaxRR and AIRmaxSS (strains presenting the AIRmax background but homozygous for R and S *Slc11a1* alleles). Our results (Fig. 5) demonstrate that AIRmax presented a more pronounced bone loss and inflammatory cell influx into periodontal tissues than AIRmin strain, and that AIRmaxRR presented a more pronounced bone loss and inflammatory cell influx into periodontal tissues than AIRmaxSS strain. Such differential response was also confirmed by the significant variation in the levels of TNF, IL1β, IL-6 and IL-17 when AIRmin/AIRmax and AIRmaxRR/AIRmaxSS strains were compared. No significant differences were observed in the levels of IL-10 and IFNβ.

4 DISCUSSION

Periodontal diseases are chronic and multifactorial involving in this pathogenesis microbial, inflammatory and immunological factors, that together can be modulated by local, environmental, genetic and epigenetic factors [1, 3]. Periodontal disease initiation and propagation are triggered by the dysbiosis of the commensal oral microbiota, which then interacts with the immune defenses of the host, leading to inflammation and subsequently to the development of disease symptoms [84]. In this context, understanding host mechanisms in the face of the microbial challenge are essential for the understanding and treatment of periodontal disease.

One of the factors that interfere in the response of the host that has been much studied are the genetic polymorphisms. Among the genes with potential to modulate periodontitis outcome, the predominance of *Slc11a1* hipo/hiper-responsive variants has been demonstrated to influence the severity of experimental periodontitis in mice. In humans, a recent study suggests that one SNP in *Slc11a1* could account for the risk to periodontitis development. Our results, showed that 639+22C/T (rs2290708) is related the development of chronic periodontal disease, since the CT and TT genotypes presented a statistically significant difference. The same relation was found for the same CT and TT genotypes when comparing the groups with chronic gingivitis and chronic periodontitis. This study comprises the first description of the potential association of such variant with periodontal disease. A previous study described the lack of association between rs2290708 with leishmania in Pakistani individuals [68]. However, we must consider that the pathogenesis of leishmanial significantly differ for periodontitis pathogenesis, and also that Brazilian and Pakistani individuals present distinct genetic backgrounds.

Our results also demonstrate that rs3731865 was also associated with periodontitis. Indeed, allele C and the CT and TT genotypes are related to the presence of periodontal diseases, since its frequency is higher when comparing healthy groups and chronic gingivitis with that of periodontal diseases. Importantly, the frequency of rs3731865 was similar to that previously reported in the Brazilian population [85].

Also, polymorphism rs17235409 quoted initially to be studied is associated with a protective factor against periodontal diseases in the Iranian population. And as mentioned above, due to technical problems this polymorphism was not analyzed [72]. Additionally, rs3731865 was

associated with the risk to other infectious conditions, such as susceptibility in tuberculosis in Turkish patients [86], and in Brazilian populations was associated with the risk of leprosy [85].

While the case-control data from suggests the involvement of *Slc11a1* SNPs in periodontitis susceptibility/resistance, additional experimental approaches were conducted to support such potential association from the functional and mechanistic viewpoints. When analyzing the expression of cytokines taking into account the genotypes of the chronic periodontitis group of both polymorphisms, we noticed an increase in the expression of the cytokines of the innate immune response, TNF-α, IL-1β and IL-6 (Fig. 1), associated the presence of the T allele (rs2290708) and the allele C (rs3731865). To date, no previous studies have been investigated the possible association between *Slc11a1* and host response parameters *in vivo*, being the immunomodulatory effects of *Slc11a1* studied basically in experimental models [87, 88]. In human cells, the expression of *Slcl11a1* have been associated with enhancement of pro-inflammatory responses, promotes efficient resolution of infection, but is associated with autoimmunity and inflammation, such as type 1 diabetes [89].

In periodontitis context, and it is already known that direct effect on the pathogenesis of periodontal diseases, TNF-α up-regulates the production of other classic pro-inflammatory innate cytokines, such IL-1β and IL-6[17, 90, 91]. And both IL-1β and IL-6 also have been characteristically associated with inflammatory cell migration and osteoclast genesis processes [90, 92]. This was also confirmed by our results, since the levels of RANKL expression and the RANKL/OPG ratio (Fig. 2), since in both polymorphisms these markers are with increased expression associated with polymorphic alleles. An earlier study, showed the blockage of RANKL by OPG leads to a reduction in alveolar bone loss throughout experimental periodontal disease in mice [93], and analysis of experimental data supports results from human studies, since RANKL/OPG balance was associated with alveolar bone loss rate and experimental disease progression [94]. Certainly, the absence of an exaggerated inflammatory response decreases bone loss in individuals with periodontal disease.

While a significant association between *Slc11a1* SNPs and host response parameters was observed in periodontal lesions, we must consider the complexity of periodontitis pathogenesis, where multiple factors can modulate host response and periodontitis outcome.

In this scenario, we observed that *Slc11a1* SNPs were not associated with variations in the patterns of periodontal infection. No difference was observed in the pattern of microbiological colonization of sites with chronic periodontitis with respect to SNPs rs2290708 and 3731865

(Fig.3). However, the presence of these microbial agents induces the microbial challenge, because they present lipopolysaccharide (LPS), bacterial DNA, diacyl lipopeptides and peptidoglycan, where there is poor oral hygiene favors the development of periodontal disease [95].But, strong evidence supporting a direct connection between the host's genetic profile, specifically rs2521634, rs10010758, rs666702 and rs10043775 polymorphisms and the occurrence of chronic periodontitis associated bacteria[36]. It shows that there is an association between genetic polymorphisms and the presence of certain microorganisms.

In order to gain further insight into the potential modulation of host response ant periodontal environment, we next performed an *in vitro* analysis of macrophages derived from donors with distinct *Slc11a1* genotypes. Macrophages are considered key cells in periodontitis pathogenesis, in the view of its properties, which can range from pro-inflammatory M1 cells to pro-reparative M2 phenotype. Furthermore, the expression of *Slc11a1* have been associated with macrophage response *in vitro* increase after infection with osteopathogens [71]. *In vitro* analysis confirms the hyper-reactive response of the polymorphic alleles T (rs2290708) and C (rs3731865), because in both the presence of these alleles induced a greater production of TNF-α, IL-1β, IL-6 and IL-10 (Fig. 4). Although IL-6, has been produced by macrophage with increased LPS stimulation in the C (rs3731865) allele. This can be explained because high IL-6 levels are frequently observed in patients with chronic diseases, in addition to there is evidences that IL-6 is capable of mediating both proinflammatory effects. Thus, the increase in the LPS stimulus causes increases of IL-6 to occur as the balance between the proinflammatory and anti-inflammatory effects of IL-6 may influence the development of chronic inflammation and diseases [96].

Finally, in order to test the impact of *Slc11a1* hipo/hiper-responsive variants in periodontitis outcome in a cause-and-effect manner, experimental periodontitis was induced in AIRmin (characterized by the predominance of 'S' hipo-responsive *Slc11a1* allele), AIRmax (characterized by the predominance of 'R' hiper-responsive *Slc11a1* allele), AIRmaxRR and AIRmaxSS (strains presenting the AIRmax background but homozygous for R and S *Slc11a1* alleles). Ultimately, we observed that hyperactivity variants of *Slc11a1*, characterized in the AIRmin and AIRmax murine lines, are associated with increased alveolar bone leukocyte influx and increased production of TNF-α, IL-1β, IL-6 and IL-17 (Fig.5). Accordingly, a previous study demonstrated thatgenetic bases that result the differential phenotypes between the AIRmax and AIRmin strain, demonstrated that the gene *Slc11a1* ("solute carrier family 11a member 1") it is one of the genes responsible for the deferential response between the strains.

Slc11a1 alleles are named alleles R or S once they demonstrate resistance (R) or susceptibility (S) of determined infections/diseases [45, 97]. Also, our data demonstrate that the presence of homozygous R and S *Slc11a1* genotypes in AIRmax background was also associated with a significant modulation of experimental periodontitis severity in mice.

Thus, it is possible to conclude that polymorphisms in the *Slc11a1* gene show to be functional, being associated to the increase of inflammatory responsiveness, and that consequently influence the risk to the development of periodontitis.

AUTHORSHIP

JLM, FC, MCSA, PMC, MF, MF, AB, MDF, GPG were associated with study conception and design. JLM, FC, GPG, performed acquisition of data. JLM, FC, MCSA, PMC, AL, RMDS, RMDS, GPG were associated with analysis and interpretation of data. JLM., FC, GPG were associated with drafting of manuscript. AL., RMDS, MF, MF, GPG performed critical revision. JLM. and FC contributed equally to this work.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

FIGURES & FIGURES LEGENDS

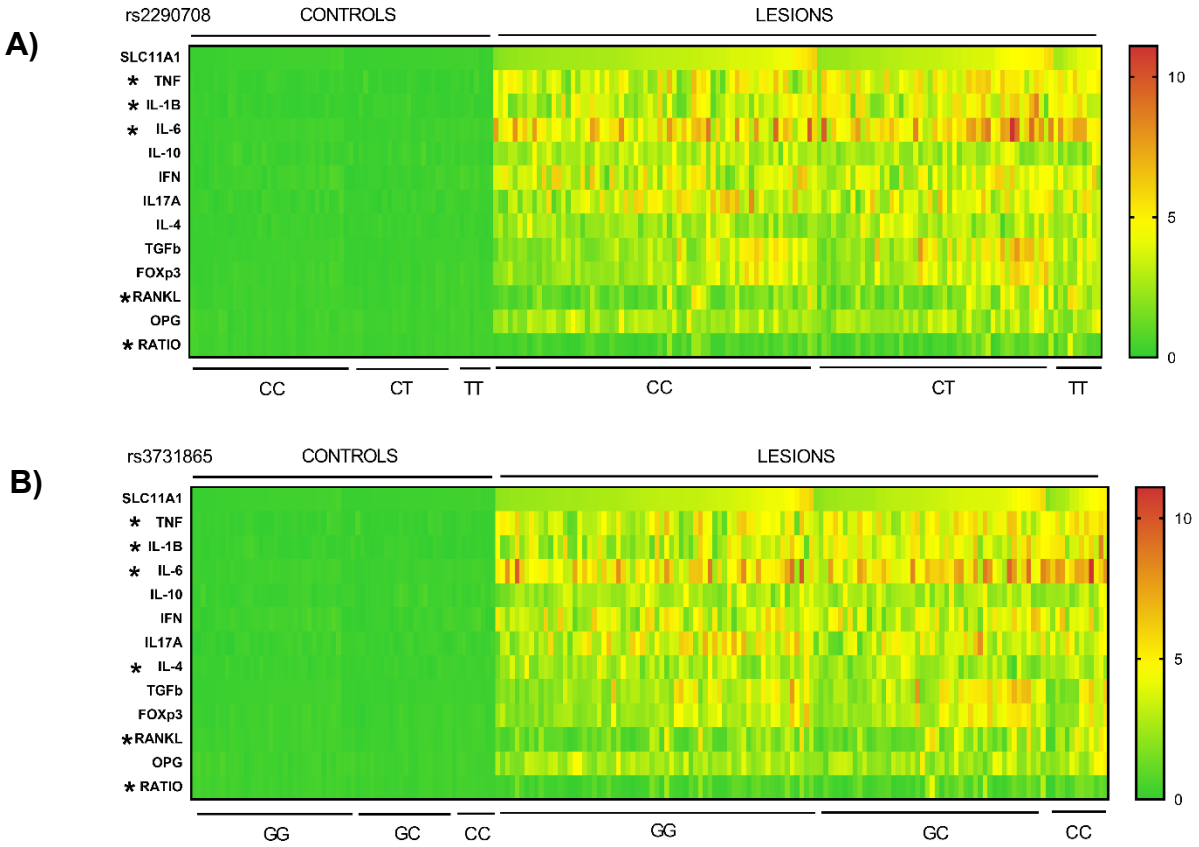


Figure 1: *Slc11a1* rs2290708 and rs3731865 genotypes and its association with inflammatory, immunological and osteoclastogenic factors mRNA levels in periodontal tissues. Total RNA was extracted from healthy periodontal tissues (control, N=63) and chronic periodontal lesions (lesions, N=127) and levels of *Slc11a1*, TNF, IL-1β, IL-6, IL-10, IFNα, IL-17A, IL-4, TGFβ, FOXp3, RANKL and OPG were measured quantitatively by Real Time PCR using TaqMan chemistry; the results are presented as expression of the individual mRNAs (with normalization to beta-actin using the Ct method). (**A**) Gene expression profile in healthy periapical tissues and periapical lesions categorized according to rs2290708 *Slc11a1* SNP; (**B**) gene expression profile in healthy periapical tissues and periapical lesions categorized according to rs3731865 *Slc11a1* SNP. The expression of all targets was significantly different when controls and lesions were compared ($p<0.05$; Kruskal-Wallis); * represent statistically significant differences between *Slc11a1* SNPs genotypes for each marker ($p<0.05$; One-way ANOVA, Bonferroni post-test).

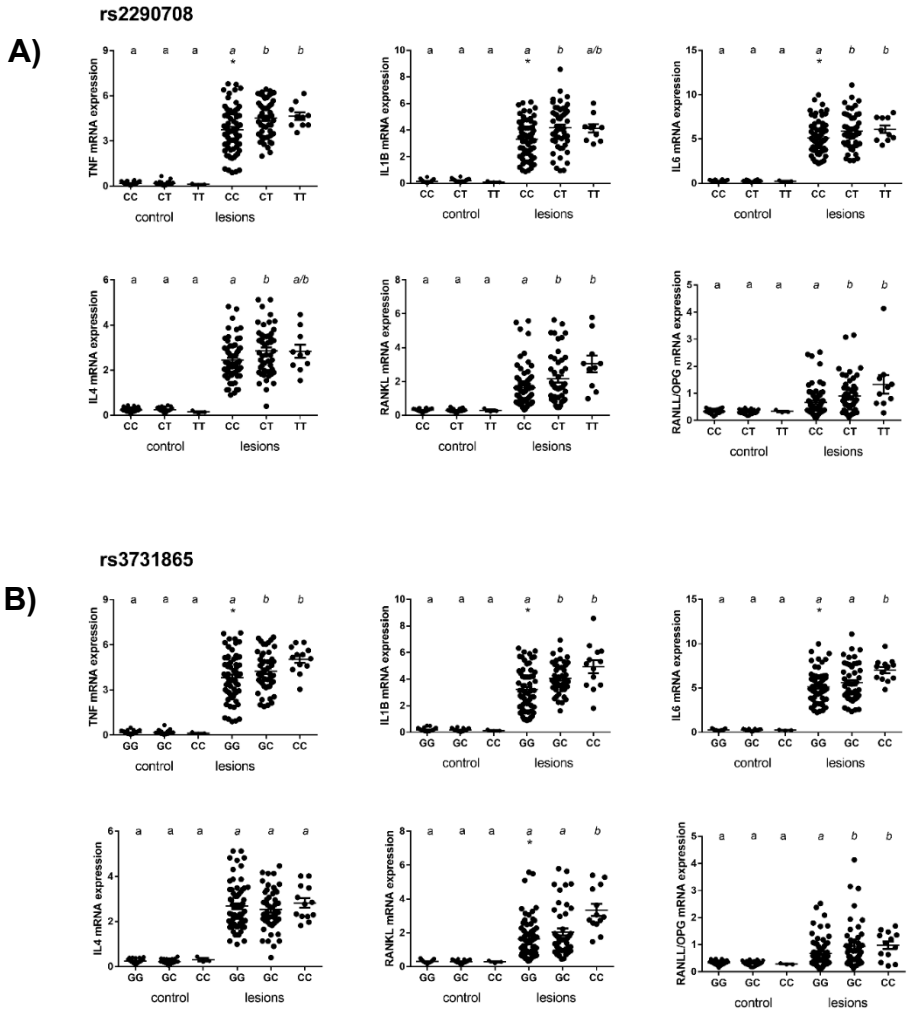


Figure 2: ***Slc11a1*** **rs2290708(A) and rs3731865(B) genotypes and its association with inflammatory, immunological and osteoclastogenic factors mRNA levels in periodontal tissues.** Total RNA was extracted from healthy periodontal tissues (control, N=63) and chronic periodontal lesions (lesions, N=127) and levels of *Scl11a1*, TNF, IL-1β, IL-6, IL-10, IFNγ, IL-17A, IL-4, TGFb, FOXp3, RANKL and OPG were measured quantitatively by RealTimePCR using TaqMan chemistry; the results are presented as expression of the individual mRNAs (with normalization to beta-actin using the Ct method). The targets identified in the initial analysis to be differentially expressed depending on *Slc11a1* rs2290708(A) and rs3731865(B) genotypes were also depicted individually as a dispersion graph to allow additional comparisons visualization. The expression of all targets was significantly different when controls and lesions were compared ($p<0.05$; Kruskal-Wallis); different letters represent statistically significant differences within the genotypes in each group ($p<0.05$; One-way ANOVA, Bonferroni post-test) and * represent statistically significant differences between TT and TC+CC groups ($p<0.05$; unpaired t-test).

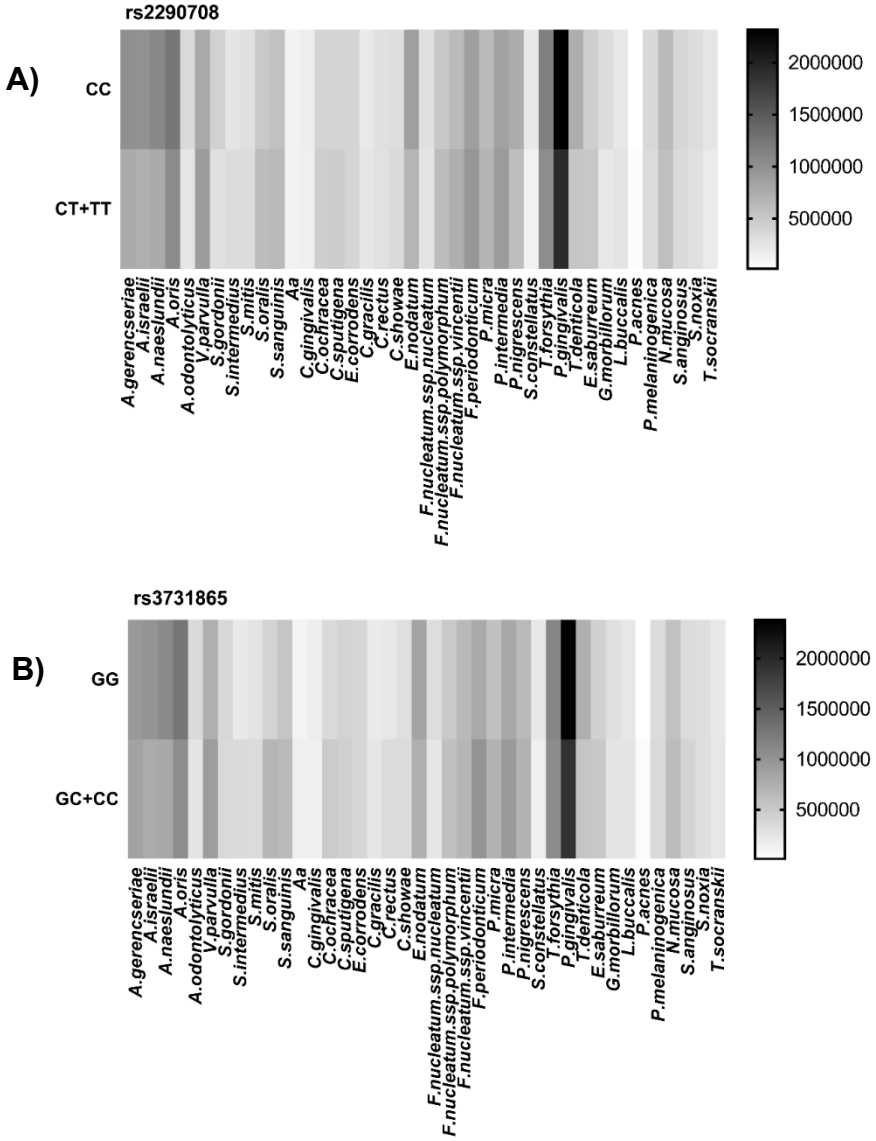


Figure 3: *Slc11a1* rs2290708(A) and rs3731865(B) genotypes and its association with bacterial counts for 40 subgingival species belonging to the classic subgingival microbial complexes taking into account the genetic polymorphisms genotypes rs2290708 and rs3731865. Subgingival biofilm samples were collected from 9 subgingival sites of a fraction of chronic periodontitis group (N=69), and were assayed for the presence and quantity of 40 bacterial species by DNA-DNA checkerboard.

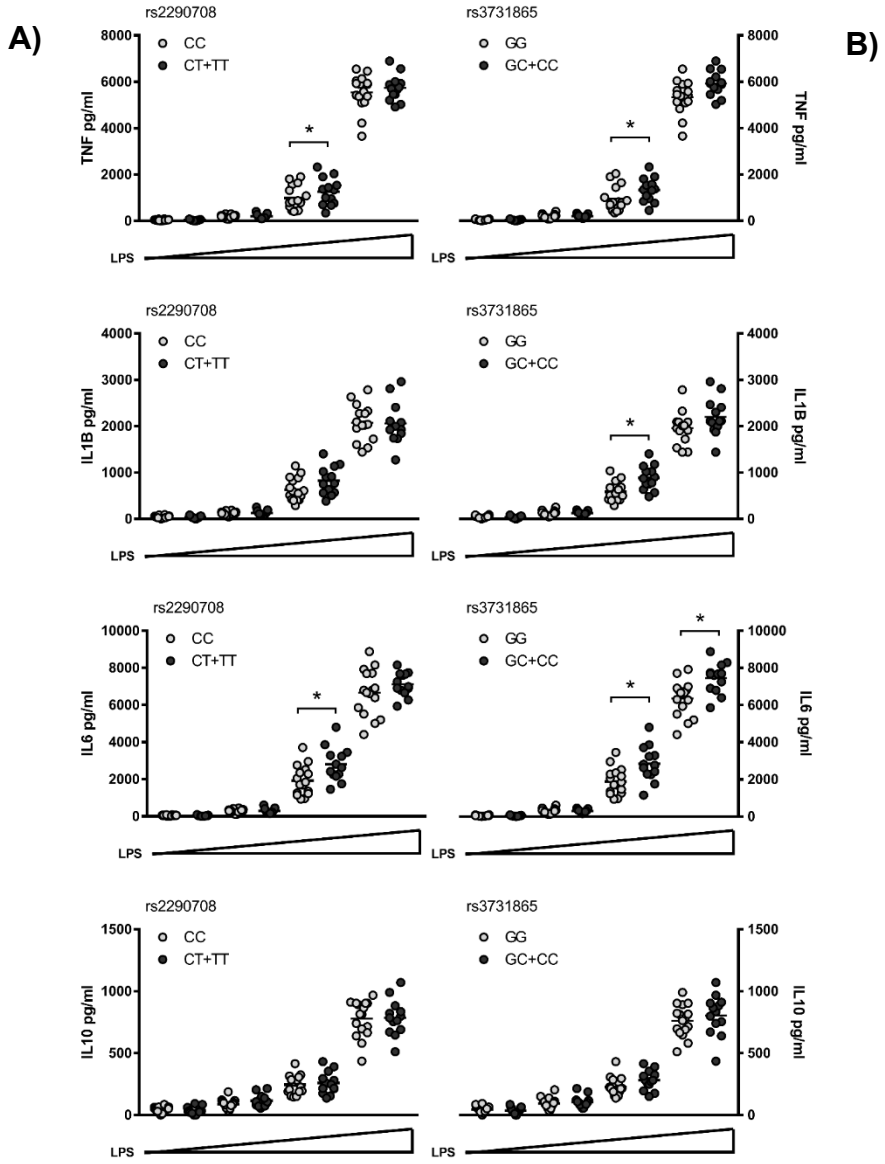


Figure 4: *Slc11a1* rs2290708(A) and rs3731865(B) genotypes influence in inflammatory cytokine production by human monocytes *in vitro*. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood from systemically and periodontally healthy subjects [N=29; presenting *Slc11a1* rs2290708 CC (N=16) or CT+TT (N=13), or *Slc11a1* rs3731865 GG (N=15) or GC+CC (N=14) genotypes] by density gradient centrifugation. CD14+ monocytes were purified from fresh PBMCs with anti-CD14 magnetic beads, and cultured at 5 x 10^5 cells/mL in RPMI medium supplemented with 10% FBS, and subjected to *P. gingivalis* LPS stimulation (0, 1, 10 or 100 ng/mL; defined to achieve a low, intermediate and intense degree of stimuli). After 12h, the cells supernatants were colleted TNF, IL1B, IL6 and IL-10 ELISA analysis. *$p<0.05$, t test; CC vs CT+TT or GG vs GC+CC within the same stimulation protocol.

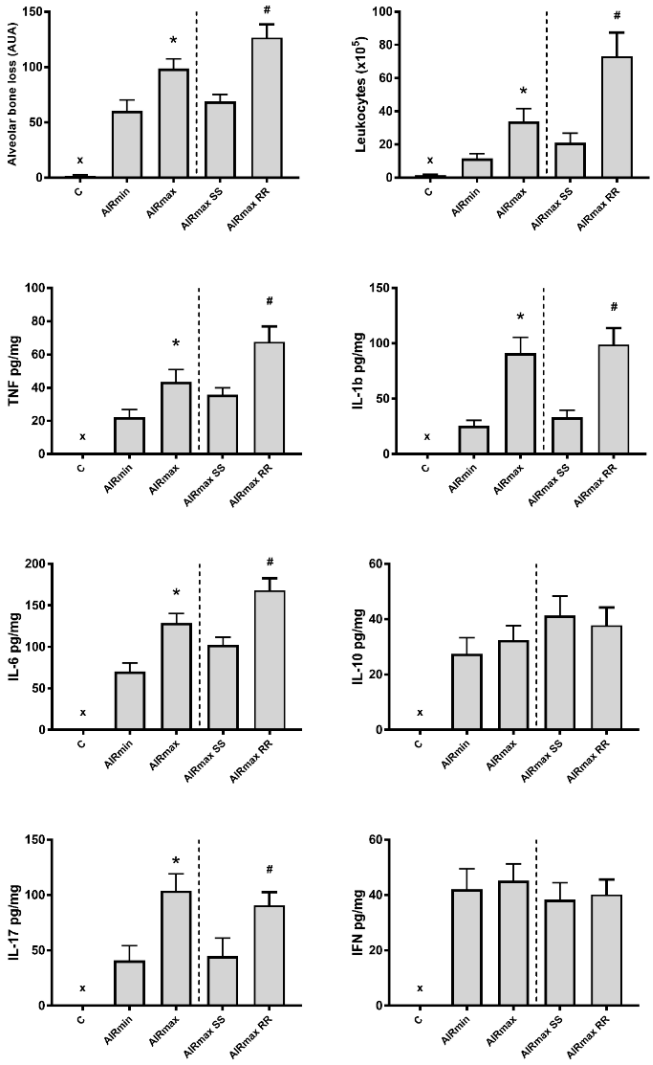


Figure 5: Impact of murine *Slc11a1* hipo/hiper-reactive variants in the outcome of experimental periodontitis. AIRmin (characterized by the predominance of 'S' hipo-responsive *Slc11a1* allele), AIRmax (characterized by the predominance of 'R' hiper-responsive *Slc11a1* allele), AIRmaxRR and AIRmaxSS (strains presenting the AIRmax background but homozygous for R and S *Slc11a1* alleles) mice were submitted to an experimental periodontitis protocol via *A. actinomycetemcomitans* JP2 inoculation. Samples from experimental and control groups were collected for histomorphometric and molecular analysis, and evaluated for (A) extent of alveolar bone loss (the increase in CEJ–ABC area in comparison with control mice); (B) quantification of inflammatory leukocytes in periodontal tissues; and (C-H) the concentrations of cytokines in periodontal extracts, determined by ELISA. The analysis of all readouts demonstrated a significant difference (represented by x) when controls and lesions were compared ($p<0.05$; Kruskal-Wallis); * represent statistically significant differences ($P<0.05$; Mann-Whitney test) between AIRmin and AIRmax strains; # represent statistically significant differences ($P<0.05$; Mann-Whitney test) between AIRmaxSS and AIRmaxRR strains.

TABLES

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

	Healthy (N=476)	Chronic Periodontitis (N=444)	Chronic Gingivitis (n=207)
N and gender distribution*	**244 f / 232 m**	**230 f / 214 m**	**106 f / 101 m**
Age	46.52 ± 6.21	46.83 ± 7.51	48.62 ± 6.71
Clinical parameters	***value ± SD***	***value ± SD***	***value ± SD***
Probing depth	2.23 ± 0.67	4.31 ± 0.76	2.69 ± 0.51
Clinical Attachment Loss	0.61 ± 0.24	3.95 ± 0.67	0.68 ± 0.28
% BOP	4.93 ± 2.89	63.68 ± 8.69	62.22 ± 11.36
Plaque index	33.66 ± 8.19	53.06 ± 9.78	53.75 ± 10.32
16S DNA ($x10^9$)	0.10 ± 0.24	15.58 ± 35.44	14.86 ± 34.32

Table 2. Frequencies of *Slc11a1* SNP (rs2290708) in healthy individuals and patients with chronic periodontitis and chronic gingivitis

*Slc11a*1 SNP (rs2290708)	Healthy (H) (N = 476)	Chronic Periodontitis (CP) (N = 444)	Chronic Gengivitis (CG) (N=207)	H vs CP	H vs CG	CG vs CP
Genotype*	n (%)	n (%)	n (%)			
CC	249 (52.31%)	253 (56.98%)	103 (49.75%)			
CT	218 (45.80%)	164 (36.93%)	102 (49.25%)	P=0.0276 OR=0.7404 CI: 0.569 to 0.969	P=0.4624 OR=1.131 CI: 0.818 to 1.564	P=0.0135 OR=0.654 CI: 0.465 to 0.919
TT	9 (1.89%)	27 (6.08%)	2 (1.00%)	P=0.0043 OR=2.953 CI: 1.399 to 6.443	P=0.4248 OR= 0.5372 CI: 0.114 to 2.092	P=0.0104 OR=5.496 CI: 1.491 to 23.78
CT + TT	227 (47.69%)	191 (43.02%)	104 (50.24%)	P=0.1551 OR=0.8281 CI: 0.6409 to 1.078	P=0.5396 OR=1.108 CI: 0.804 to 1.525	P=0.0847 OR= 0.7477 CI: 0.538 to 1.038
Allele*						
C	716 (75.21%)	670 (75.45%)	308 (74.40%)			
T	236 (24.79%)	218 (24.55%)	106 (25.60%)	P=0.8739 OR=0.983 CI: 7.957 to 1.213	P=0.7496 OR= 1.044 CI: 0.801 to 1.365	P=0.6820 OR= 0.945 CI: 0.724 to 1.238

* chi-square test, OR: odds ratio; Cl: confidence interval

Table 3. Frequencies of *Slc11a1* SNP (rs3731865) in healthy individuals and patients with chronic periodontitis and chronic gingivitis

*Slc11a*1 SNP (rs3731865)	Healthy (H) (N = 476)	Chronic Periodontitis (CP) (N = 444)	Chronic Gengivitis (CG) (N=207)	H vs CP	H vs CG	CG vs CP
Genotype*	n (%)	n (%)	n (%)			
GG	244 (51.26%)	237 (53.38%)	104 (50.24%)			
GC	209 (43.91%)	160 (36.04%)	96 (46.37%)	P=0.0868 OR= 0.788 CI: 0.600 to 1.032	P=0.660 OR=1.078 CI: 0.771 to 1.503	P=0.0729 OR=0.731 CI: 0.522 to 1.024
CC	23 (4.83%)	47 (10.58%)	7 (3.38%)	P= 0.0052 OR= 2.104 CI: 1.253 to 3.532	P= 0.449 OR= 0.914 CI: 0.280 to 1.669	P=0.0077 OR=2.946 CI:1.282 to 6.958
GC + CC	232 (48.74%)	207 (46.62%)	103 (50.24%)	P= 0.5204 OR= 0.918 CI: 0.706 to 1.193	P= 0.8066 OR=1.042 CI: 0.756 to 1.434	P=0.4555 OR=0.881 CI: 0.635 to 1.223
Allele*						
G	697 (73.21%)	634 (71.40%)	304 (73.42%)			
C	255 (26.79%)	254 (28.60%)	110 (26.57%)	P= 0.3837 OR=1.095 CI: 0.892 to 1.347	P=0.9340 OR=0.989 CI: 0.380 to 1.283	P= 0.4464 OR= 1.107 CI: 0.851 to 1.439

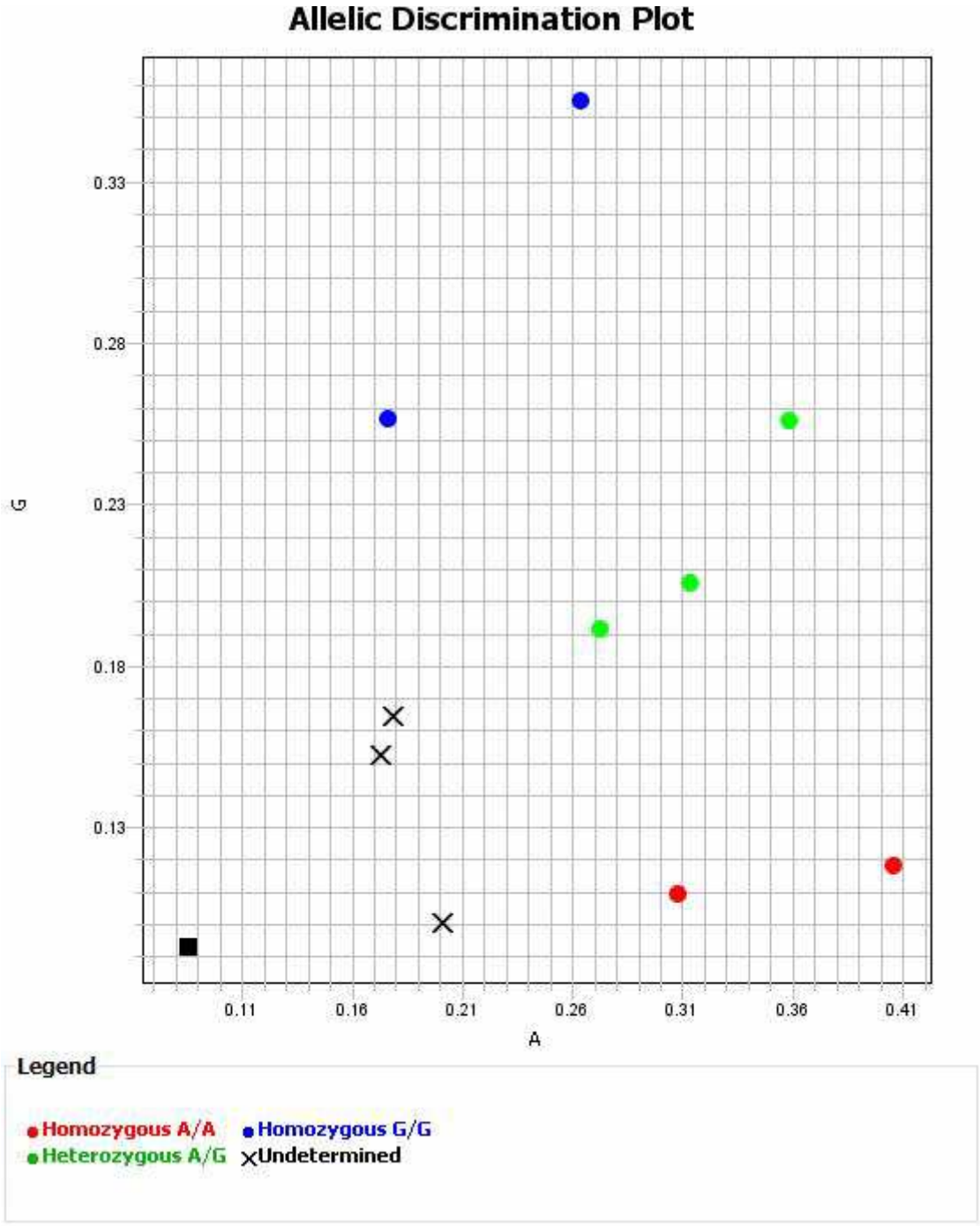
* chi-square test, OR: odds ratio; Cl: confidence interval

SUPPLEMENTARY MATERIAL

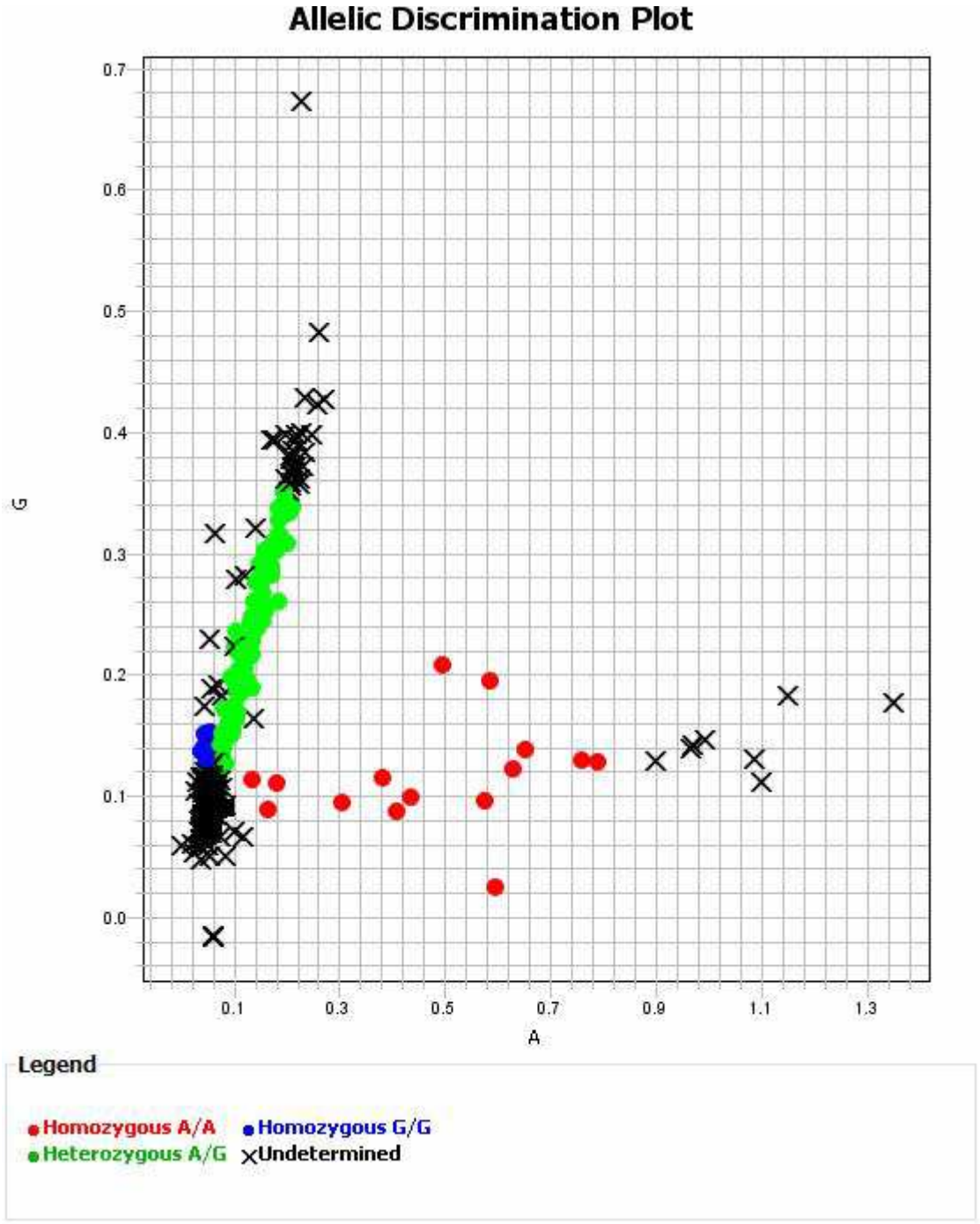
Supplementary Table 1.

Bacterial species assayed by DNA-DNA hybridization checkerboard

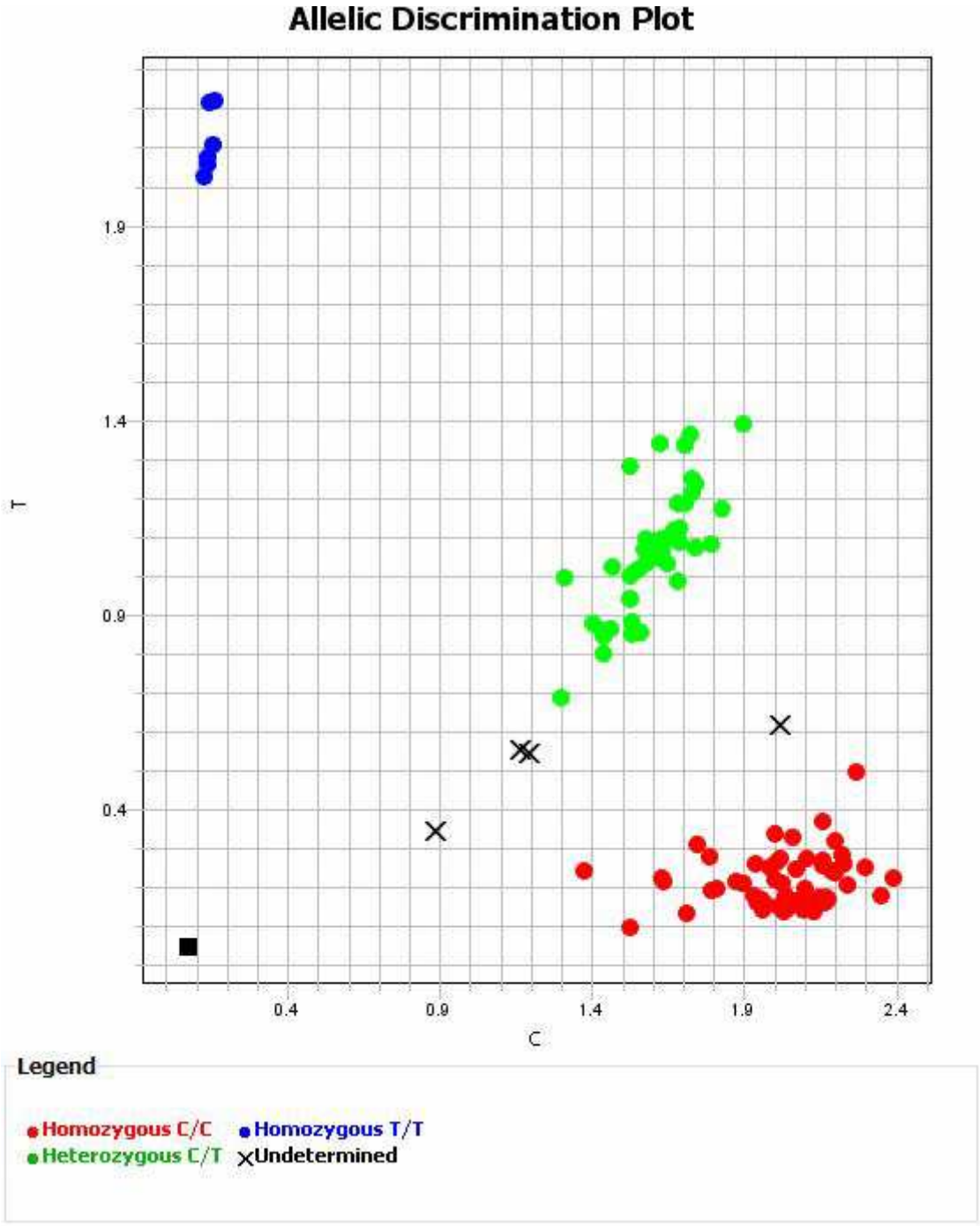
A.gerencseriae	*S.sanguinis*	*F.nucleatum.ssp.nucleatum*	*T.denticola*
A.israelii	*Aa*	*F.nucleatum.ssp.polymorphum*	*E.saburreum*
A.naeslundii	*C.gingivalis*	*F.nucleatum.ssp.vincentii*	*G.morbillorum*
A.oris	*C.ochracea*	*F.periodonticum*	*L.buccalis*
A.odontolyticus	*C.sputigena*	*P.micra*	*P.acnes*
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*



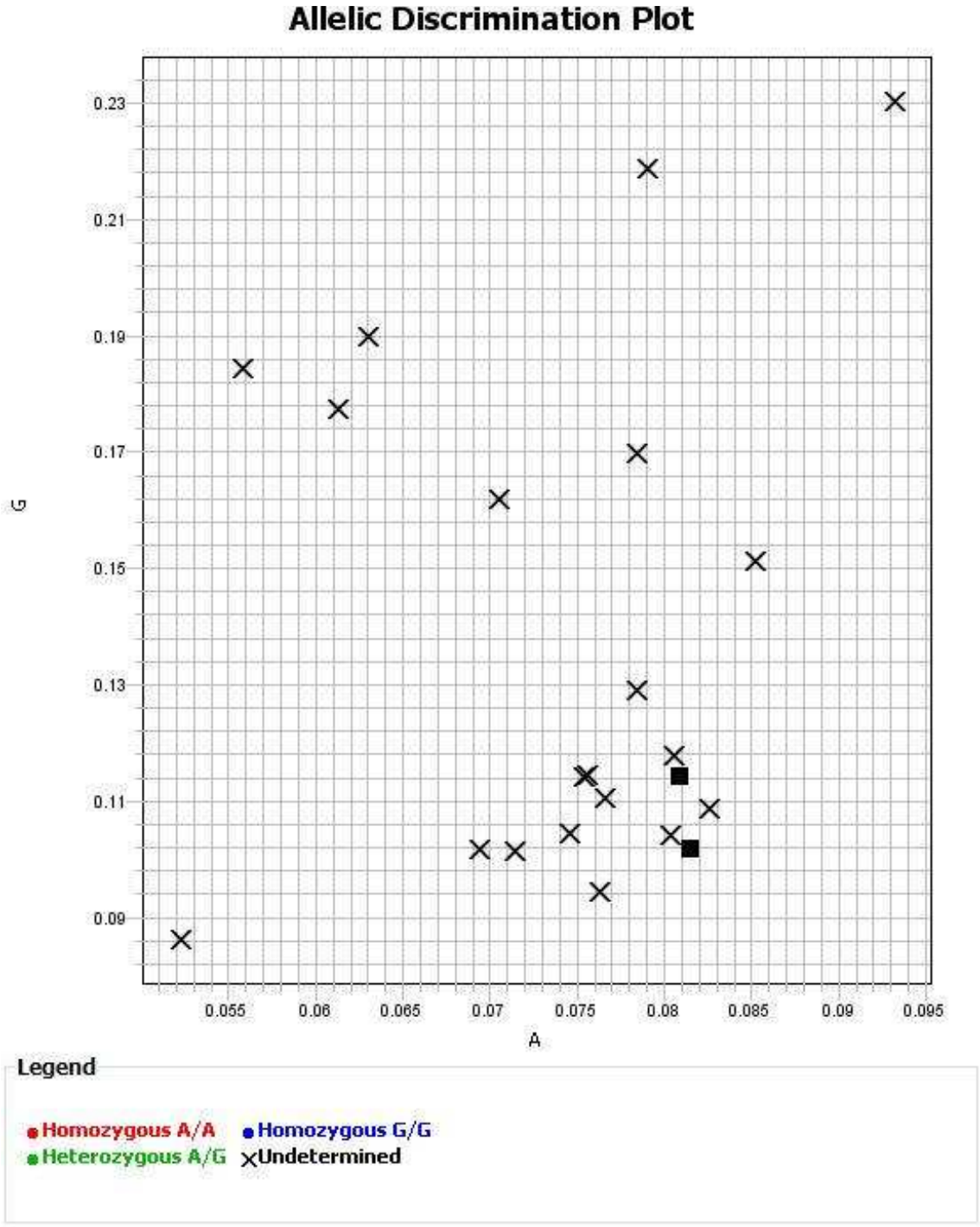
Supplementary Figure 1: Allelic discrimination plot of rs17228995. Not reviewed due to technical issues.



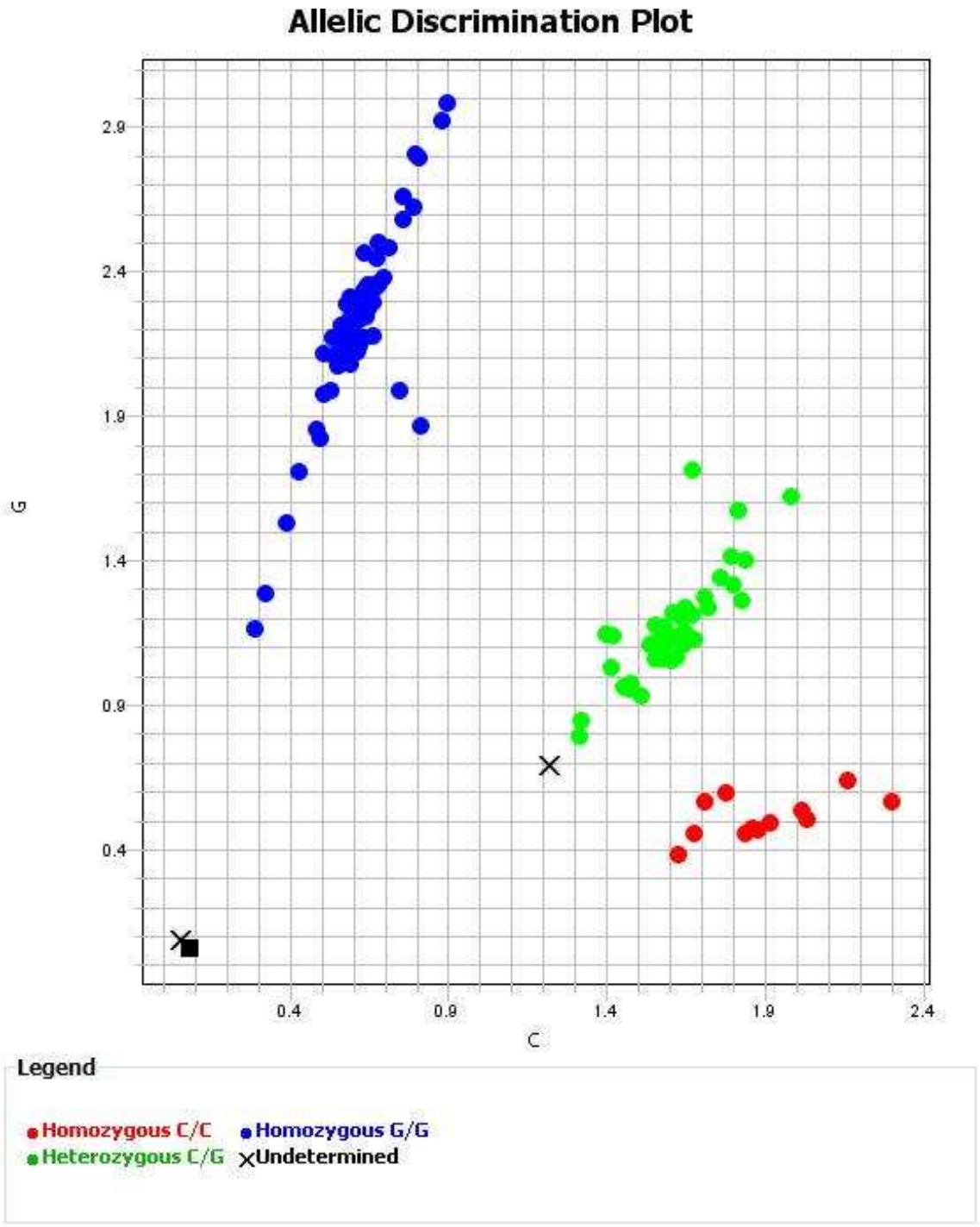
Supplementary Figure 2: Allelic discrimination plot of rs17235409. Not reviewed due to technical issues.



Supplementary Figure 3: Allelic discrimination plot of rs2290708.



Supplementary Figure 4: Allelic discrimination plot of rs2695343. Not reviewed due to technical issues.



Supplementary Figure 5: Allelic discrimination plot of rs3731865.

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3 DISCUSSION

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Periodontal diseases are chronic and multifactorial involving in this pathogenesis microbial, inflammatory and immunological factors, that together can be modulated by local, environmental, genetic and epigenetic factors (MARTINEZ and HOLT, 1999; LALLA *et al.*, 2003). Periodontal disease initiation and propagation are triggered by the dysbiosis of the commensal oral microbiota, which then interacts with the immune defenses of the host, leading to inflammation and subsequently to the development of disease symptoms (KINANE *et al.*, 2017). In this context, understanding host mechanisms in the face of the microbial challenge are essential for the understanding and treatment of periodontal disease.

One of the factors that interfere in the response of the host that has been much studied are the genetic polymorphisms. Among the genes with potential to modulate periodontitis outcome, the predominance of *Slc11a1* hipo/hiper-responsive variants has been demonstrated to influence the severity of experimental periodontitis in mice. In humans, a recent study suggests that one SNP in *Slc11a1* could account for the risk to periodontitis development. Our results, showed that 639+22C/T (rs2290708) is related the development of chronic periodontal disease, since the CT and TT genotypes presented a statistically significant difference. The same relation was found for the same CT and TT genotypes when comparing the groups with chronic gingivitis and chronic periodontitis. This study comprises the first description of the potential association of such variant with periodontal disease. A previous study described the lack of association between rs2290708 with leishmania in Pakistani individuals (SOPHIE *et al.*, 2017). However, we must consider that the pathogenesis of leishmanial significantly differ for periodontitis pathogenesis, and also that Brazilian and Pakistani individuals present distinct genetic backgrounds.

Our results also demonstrate that rs3731865 was also associated with periodontitis. Indeed, allele C and the CT and TT genotypes are related to the presence of periodontal diseases, since its frequency is higher when comparing healthy groups and chronic gingivitis with that of periodontal diseases. Importantly, the frequency of rs3731865 was similar to that previously reported in the Brazilian population (BROCHADO *et al.*, 2016).

Also, polymorphism rs17235409 quoted initially to be studied is associated with a protective factor against periodontal diseases in the Iranian population. And as mentioned above, due to technical problems this polymorphism was not analyzed (KADKHOZADEH *et al*.,2016). Additionally, rs3731865 was associated with the risk to other infectious conditions, such as susceptibility in tuberculosis in Turkish patients (ATES *et al.*, 2009), and in Brazilian populations was associated with the risk of leprosy (BROCHADO *et al.*, 2016).

While the case-control data from suggests the involvement of *Slc11a1* SNPs in periodontitis susceptibility/resistance, additional experimental approaches were conducted to support such potential association from the functional and mechanistic viewpoints. When analyzing the expression of cytokines taking into account the genotypes of the chronic periodontitis group of both polymorphisms, we noticed an increase in the expression of the cytokines of the innate immune response, TNF-α, IL-1β and IL-6 (Fig. 1), associated the presence of the T allele (rs2290708) and the allele C (rs3731865). To date, no previous studies have been investigated the possible association between *Slc11a1* and host response parameters *in vivo*, being the immunomodulatory effects of *Slc11a1* studied basically in experimental models (BAULER *et al.*, 2017; CORREA *et al.*, 2017). In human cells, the expression of *Slcl11a1* have been associated with enhancement of pro-inflammatory responses, promotes efficient resolution of infection, but is associated with autoimmunity and inflammation, such as type 1 diabetes (O'BRIEN *et al.*, 2008).

In periodontitis context, and it is already known that direct effect on the pathogenesis of periodontal diseases, TNF-α up-regulates the production of other classic pro-inflammatory innate cytokines, such IL-1β and IL-6 (OKADA *et al.*, 1997; GRAVES *et al.*, 2008; GARLET, 2010). And both IL-1β and IL-6 also have been characteristically associated with inflammatory cell migration and osteoclast genesis processes (GRAVES *et al.*, 2008; FONSECA *et al.*, 2009). This was also confirmed by our results, since the levels of RANKL expression and the RANKL/OPG ratio (Fig. 2), since in both polymorphisms these markers are with increased expression associated with polymorphic alleles. An earlier study, showed the blockage of RANKL by OPG leads to a reduction in alveolar bone loss throughout experimental periodontal disease in mice(JIN *et al.*, 2007), and analysis of experimental data supports results from human studies, since RANKL/OPG balance was associated with alveolar bone loss rate and experimental disease progression (GARLET *et al.*, 2006).Certainly, the absence of an exaggerated inflammatory response decreases bone loss in individuals with periodontal disease.

While a significant association between *Slc11a1* SNPs and host response parameters was observed in periodontal lesions, we must consider the complexity of periodontitis pathogenesis, where multiple factors can modulate host response and periodontitis outcome.

In this scenario, we observed that *Slc11a1* SNPs were not associated with variations in the patterns of periodontal infection. No difference was observed in the pattern of microbiological colonization of sites with chronic periodontitis with respect to SNPs rs2290708 and 3731865 (Fig.3). However, the presence of these microbial agents induces the microbial challenge, because they present lipopolysaccharide (LPS), bacterial DNA, diacyl lipopeptides and peptidoglycan, where there is poor oral hygiene favors the development of periodontal disease (MAHANONDA and PICHYANGKUL, 2007). But, strong evidence supporting a direct connection between the host's genetic profile, specifically rs2521634, rs10010758, rs666702 and rs10043775 polymorphisms and the occurrence of chronic periodontitis associated bacteria(CAVALLA *et al.*, 2018). It shows that there is an association between genetic polymorphisms and the presence of certain microorganisms.

In order to gain further insight into the potential modulation of host response ant periodontal environment, we next performed an *in vitro* analysis of macrophages derived from donors with distinct *Slc11a1* genotypes. Macrophages are considered key cells in periodontitis pathogenesis, in the view of its properties, which can range from pro-inflammatory M1 cells to pro-reparative M2 phenotype. Furthermore, the expression of *Slc11a1* have been associated with macrophage response *in vitro* increase after infection with osteopathogens (RYE *et al.*, 2013). *In vitro* analysis confirms the hyper-reactive response of the polymorphic alleles T (rs2290708) and C (rs3731865), because in both the presence of these alleles induced a greater production of TNF-α, IL-1β, IL-6 and IL-10 (Fig. 4). Although IL-6, has been produced by macrophage with increased LPS stimulation in the C (rs3731865) allele. This can be explained because high IL-6 levels are frequently observed in patients with chronic diseases, in addition to there is evidences that IL-6 is capable of mediating both proinflammatory effects. Thus, the increase in the LPS stimulus causes increases of IL-6 to occur as the balance between the proinflammatory and anti-inflammatory effects of IL-6 may influence the development of chronic inflammation and diseases (CRONSTEIN, 2007).

Finally, in order to test the impact of *Slc11a1* hipo/hiper-responsive variants in periodontitis outcome in a cause-and-effect manner, experimental periodontitis was induced in AIRmin (characterized by the predominance of 'S' hipo-responsive *Slc11a1* allele), AIRmax

(characterized by the predominance of 'R' hiper-responsive *Slc11a1* allele), AIRmaxRR and AIRmaxSS (strains presenting the AIRmax background but homozygous for R and S *Slc11a1* alleles). Ultimately, we observed that hyperactivity variants of *Slc11a1*, characterized in the AIRmin and AIRmax murine lines, are associated with increased alveolar bone leukocyte influx and increased production of TNF-α, IL-1β, IL-6 and IL-17 (Fig.5). Accordingly, a previous study demonstrated thatgenetic bases that result the differential phenotypes between the AIRmax and AIRmin strain, demonstrated that the gene *Slc11a1* ("solute carrier family 11a member 1") it is one of the genes responsible for the deferential response between the strains. *Slc11a1* alleles are named alleles R or S once they demonstrate resistance (R) or susceptibility (S) of determined infections/diseases (ARAUJO *et al.*, 1998; RIBEIRO *et al.*, 2003). Also, our data demonstrate that the presence of homozygous R and S *Slc11a1* genotypes in AIRmax background was also associated with a significant modulation of experimental periodontitis severity in mice.

4 CONCLUSION

4 CONCLUSION

Thus, it is possible to conclude that polymorphisms in the *Slc11a1* gene show to be functional, being associated to the increase of inflammatory responsiveness, and that consequently influence the risk to the development of periodontitis.

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ANNEX

UNIVERSIDADE GUARULHOS - UNG

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: INFLUÊNCIA DO MOMENTO DA ADMINISTRAÇÃO DE METRONIDAZOL E AMOXICILINA NO TRATAMENTO DE INDIVÍDUOS COM PERIODONTITE CRÔNICA E DEFINIÇÃO DE POSSÍVEIS PERFIS - CLÍNICOS, MICROBIOLÓGICOS, IMUNOLÓGICOS E GENÉTICOS - COM DIFERENTES RESPOSTAS AO TRATAMENTO.

Pesquisador: Magda Feres Figueiredo

Área Temática:

Versão: 2

CAAE: 32465714.4.1001.5506

Instituição Proponente: Universidade Guarulhos - UNG

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 746.355

Data da Relatoria: 12/08/2014

Apresentação do Projeto:

Projeto está bem redigido, claro e bem fundamentado.

Objetivo da Pesquisa:

O objetivo está claro e pretende avaliar o melhor momento de administração de MTZ+AMX sistêmicos adjuntos à RAR no tratamento da periodontite crônica generalizada: a)na fase ativa da terapia periodontal,ou b) após a fase de cicatrização e reparo da terapia mecânica

Avaliação dos Riscos e Benefícios:

Estão descritos adequadamente.

Comentários e Considerações sobre a Pesquisa:

A casuística e o método adotados estão adequados para responder aos objetivos.

Considerações sobre os Termos de apresentação obrigatória:

O pesquisador apresentou todos os termos obrigatórios.

Recomendações:

Nada a declarar.

Endereço: Praça Tereza Cristina, 229
Bairro: Centro **CEP:** 07.023-070
UF: SP **Município:** GUARULHOS
Telefone: (11)2464-1779 **Fax:** (11)2464-1187 **E-mail:** comite.etica@ung.br

UNIVERSIDADE GUARULHOS - UNG

Continuação do Parecer: 746.355

Conclusões ou Pendências e Lista de Inadequações:

Foram adequados o TCLE e também os riscos.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Esta aprovação é válida pelo período previsto no cronograma postado. Enviar relatório final até 30/11/2020, via Plataforma Brasil.

GUARULHOS, 11 de Agosto de 2014

Assinado por:
Regina de Oliveira Moraes Arruda
(Coordenador)

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