Association of *IL-1B* gene polymorphism with IL-1β levels in gingival crevicular fluid (GCF) and bacterial profile in patients with periodontitis

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Abstract

Background

Inflammatory cytokines, such as interleukin-1β (IL-1β), play a crucial role in the pathogenesis of periodontal diseases. A high individual variation in the levels of IL-1β in gingival crevicular fluid (GCF) has been previously reported in patients with periodontitis that may affect the periodontal outcome. GCF IL-1β levels are possibly affected by genetic polymorphisms and/or by the presence of periodontal pathogens. Concomitant analysis of the impact of *IL-1B* gene polymorphisms and specific periodontal pathogens on IL-1β levels in GCF remains to be evaluated. The goal of this study was to investigate the role of an *IL-1B* single-nucleotide polymorphism at position +3954 [*IL-1B* (3954) SNP] and the presence of eleven (11) periodontal pathogens on GCF levels of IL-1β in patients with periodontitis.

Materials & Methods

In this cross-sectional study, clinical periodontal parameters and biological samples were collected from non-smoking patients with periodontitis in good general health (n=32). Clinical parameters were recorded at 6 sites/tooth and included: plaque accumulation, bleeding on

probing/ suppuration, pocket depth and attachment level. Third molars were excluded from analysis. DNA was extracted from buccal epithelial cells and was analyzed for the presence/absence of *IL-1B* (3954) gene polymorphism by polymerase chain reaction (PCR). GCF and subgingival plaque samples were collected from one healthy and one periodontitis site within the same periodontitis patient. GCF samples were used for the quantification of IL-1β levels with enzyme linked immunosorbent assay (ELISA), while plaque samples were analyzed by PCR for the detection of eleven periodontal pathogens. Statistical analysis was performed using SAS, Version 9.2 (SAS Institute, Cary, NC) and significance of difference was indicated at the level of 0.05 ($p<0.05$).

Results

The prevalence of *IL-1B* (3954) SNP was 40.63% in the study population. *IL-1B* (3954) SNP was significantly associated with higher GCF IL-1β levels in patients with periodontitis ($p<0.05$). *Porphyromonas gingivalis, Tanarella forsythia,* and *Fusobacterium nucleatum* were the only species that showed a higher frequency of detection in periodontitis patients with *IL-1B* (3954) SNP positive compared to patients with *IL-1B* (3954) SNP negative. No significant associations were found between the detection of any of the tested periodontal pathogens and the IL-1β levels in GCF.

Conclusions

Our results suggest that periodontitis patients with *IL-1B* (3954) SNP positive demonstrate higher GCF IL-1β levels and frequency of detection of *P. gingivalis, T. forsythia,* and *F. nucleatum* when compared to patients with *IL-1B* (3954) SNP negative. The increased levels of IL-1β in GCF in periodontitis patients seem to be associated more with the *IL-1B* genotype status rather than with the presence of several periodontal pathogens. Our data provide additional evidence and support previous studies showing that *IL-1B* (3954) SNP increases host susceptibility and risk to periodontitis.

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Association of *IL-1B* gene polymorphism with IL-1β levels in gingival crevicular fluid (GCF) and bacterial profile in patients with periodontitis

Introduction

Periodontitis

Periodontitis is an infectious - inflammatory disease, initiated primarily by specific bacteria, predominantly gram-negative anaerobes, which trigger chronic inflammatory and immune responses that are thought to determine the clinical outcome of the disease. Periodontal infection activates a cascade of host immune's mechanisms that ultimately lead to the destruction of the supporting tissues of the teeth. The primary clinical features of periodontitis include gingival inflammation, bleeding on probing, gingival edema, pocket formation, clinical attachment loss, recession of the free gingival margin, and alveolar bone loss. Periodontal disease progression can lead to root furcation exposure, increased tooth mobility, drifting and/or exfoliation of teeth.[1-5] Periodontal disease appears to have multiple etiologies, the most studied of which are microbial and immunological.

Bacterial challenge

The microbial aetiology of periodontal diseases has been already well established.[5,6] The dental bacterial biofilm is comprised by complex communities of organisms that colonize the tooth surface and lead to inflammation of periodontal tissues. The association of bacteria within mixed biofilms is not random, but there are specific associations among bacterial species. Specific bacterial complexes that establish on the tooth surface and proliferate at an early stage gradually lead to multiplication of bacteria from the "orange" and "red" complex species, that become numerically more dominant at late stages in plaque development.[6] Therefore, periodontitis has been referred as a "mixed bacterial infection", indicating that multiple microbial species contribute to the development of periodontal destruction.[7] Several studies have also shown that the proportions of periodontal pathogens are higher in periodontitis patients compared to healthy controls.[8,9] Additionally, the three bacterial species that have been designated the 'red-complex' periodontopathogens - *Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola*- and which are often associated with each other, showed a stronger association with disease.[10]

The long-standing paradigm is that, as periodontitis develops, the oral microbiota shifts from one consisting primarily of gram-positive aerobes to one consisting primarily of gram-negative anaerobes.[11] This shift gradually changes the symbiotic host–microbe relationship to a pathogenic one since the bacterial components and their virulence factors are involved in modulating inflammatory responses of the host.[6,12]

Host response

The major component of soft- and hard- tissue destruction associated with periodontitis is the result of activation of the host's immune-inflammatory response to the bacterial profile.[10] For disease to occur, bacteria must be virulent, exceed a quantitative critical threshold, conquer antagonistic microorganisms and overcome host response.[13] The host's immune system is activated in order to protect against local microbial attack and their damaging products from spreading or invading the gingival tissues. However, these ¨defensive¨ processes are also potentially harmful to the host by destroying surrounding cells and connective tissue structures.[14] Pro-inflammatory molecules and cytokine networks play an essential role in the pathophysiology of periodontal disease.[15] The strongest evidence for

cytokines functioning in networks exists for interleukin (IL)-1b, tumor necrosis factor-α (TNF-α), IL-6, Receptor activator of nuclear factor κappa-B ligand (RANKL) and matrix metalloproteinases (MMPs).[16] Interleukins (IL) play a central role in this pathogenic process. The pro-inflammatory cytokine IL-1, that exists in two forms IL-1α and IL-1β, has key regulatory roles in host immunity against periodontal pathogens. Increased levels of IL-1, mainly IL-1β, have been detected in gingival crevicular fluid (GCF) and gingival tissues of patients with periodontitis, showing a strong association with the severity of periodontal tissue destruction. [(17)] IL-1β has many functions, such as immunocyte activation, connective tissue catabolism stimulation, extracellular matrix regulation, other cytokine induction, adhesion molecules regulation, bone resorption stimulation, protease production and arachidonic acid metabolite synthesis. IL-1β has been particularly studied as a critical determinant of tissue destruction due to its pro-inflammatory and bone resorptive properties. Increased levels of IL-1β in gingival crevicular fluid have been associated with the severity of periodontal disease and periodontal tissue destruction.[17,18,19] In general, IL-1 proteins play a major role in chronic inflammation. Therefore, identification of genetic determinants, such as polymorphisms responsible for

overproduction of IL-1β, may be important in establishing a risk profile for a patient. [(15)]

Risk assessment

Periodontitis has been referred as a complex multifactorial disease.[14, 20] The extent and severity of periodontal lesions can be affected by numerous risk factors, such as genetic and familial background, systemic conditions, socioeconomic and environmental factors, smoking habits, and previous history of periodontitis.[1,3,4,21] Specific genes may determine the degree to which an individual's immune response is protective or destructive.[22] Periodontal disease has been associated with variations in multiple genes with each having a small overall contribution and relative risk for the disease process.[10,23] Common features of complex diseases (e.g. Alzheimer's disease, Crohn's disease, and cardiovascular diseases) are that those conditions present mostly with a relatively mild phenotype and are slowly progressive and chronic in nature.[20] Those characteristics are shared in conditions that are usually polygenic in origin, indicating that multiple genes play each one a limited role. Those genes are therefore considered to be disease-modifying genes.[24,25] For these disease modifying genes, the

Mendelian principles do not apply because both heterozygous and homozygous subjects for a given-disease modifying gene may not necessarily develop the disease. There are, however, other genetic risk factors, such as gene-gene interactions, and/or environmental risk factors, such as gene-environmental interactions, that need to be present simultaneously.[24] The pathophysiology of complex diseases is characterized by various biologic pathways that lead to similar clinical phenomena. Generally, genetic variance and environmental exposures are the key determinants to phenotypic differences between individuals.[13]

IL-1B gene polymorphism

Among the candidate genes for periodontitis, the *IL-1* gene polymorphisms have been broadly investigated for their relationship with the disease. There are three genes that regulate the production of IL-1: *interleukin-1A* (*IL-1A*), *interleukin-1B* (*IL-1B*), and *interleukin receptor antagonist* (*IL-1RN*). These genes are located on chromosome 2q14.[26] The specific form (allele) of each gene present in an individual can vary. Allele 2 polymorphism refers to a change in the sequence of nucleotides in the second most common form of a gene at a specific location.[15,27] Genes *IL-1A*

and *IL-1B* control the production of the pro-inflammatory cytokines IL-1α and IL-1β respectively. *Il-1RN* controls the synthesis of the antagonist protein IL-1ra that embeds IL-1α and IL-1β. Since these genes are known to exist in multiple forms, they are said to be polymorphic. The term genetic polymorphism refers to a change in the sequence of nucleotides comprising a gene.[15]

In 1997, an association was found between polymorphisms in the genes encoding for *IL-1A* (-889) and *IL-1B* (+3953) and an increased severity of periodontitis. It was shown that the combined presence of the allele 2 of the *IL-1A* gene at nucleotide position –889 (*IL-1A* –889T) and the allele 2 of *IL-1B* gene at nucleotide position +3953 (*IL-1B* +3953T) was associated with an increased risk for developing severe periodontitis in non-smoking Caucasian patients.[28] Many studies subsequently explored the role of *IL-1* gene polymorphisms in periodontitis with mixed results, mainly due to a substantial heterogeneity in the clinical phenotype and ethnicity across the reports.[29] No association was noted between *IL-1* polymorphisms and generalized aggressive periodontitis among Caucasians,[30] while in a different study on Indian population the polymorphism in the locus +3954 of *IL-1B* gene could be a risk factor for chronic periodontitis.[31] However, a

recent review and meta-analysis showed that *IL-1A* and *IL-1B* genetic variations are significant contributors to chronic periodontitis in white populations.[27] The same review and meta-analysis suggested that the allele frequencies, sample sizes, and number of studies made it impractical to include ethnicities other than Caucasian.[27]

Lately, the test has been modified to assess the presence of at least one copy of allele 2 at the *IL-1A*+4845 locus and at least one copy of allele 2 at the *IL-1B*+3954 locus. The *IL-1A*+4845 polymorphism has been determined to be more than 99% in linkage disequilibrium with the *IL-1A*–889 polymorphism (if one is present, the other usually is present), and it is being used because it is easier to identify. Additionally, the *IL-1B*+3953 polymorphism has been renumbered as *IL-1B*+3954.[15,29]

Single-nucleotide polymorphisms (SNPs), especially the T allele in the loci of the *IL-1B* gene at position 3954, have been associated with increased IL-1β production.[32,33] Several studies have indicated a role for such polymorphisms in the risk assessment for different inflammatory diseases due to the increased IL-1β production.[33-35] There have been though conflicting results, whether there is an association between the presence of

the T allele in the *IL-1B* gene at position 3954 and the risk for developing severe periodontitis.[36] A recent study demonstrated a significantly higher IL-1β expression in the diseased tissues of non-smoking patients in the CT and TT genotypes compared to the CC genotype.[36]

IL-1β GCF levels

Numerous studies have shown that patients with periodontitis exhibit higher levels of cytokines in GCF and periodontal tissues.[37-40] The relationship between genotype status and IL-1β production could manifest how the genotype status may impact periodontal health. Several investigators have evaluated the relationship between only allele 2 of the *IL-1B* polymorphism and the IL-1β production and provided conflicting data. No statistical significant difference has been reported between GCF levels of IL-1β from genotype-positive and genotype-negative patients, but allele 2 was associated with increased bleeding on probing (BOP) sites in chronic periodontitis patients.[41] On the other hand, another human study on an extensive panel of GCF mediators showed that only IL-1α and IL-1β levels were different, and more specifically were higher in initially diseased compared to initially healthy sites within the same patient, suggesting an

overall subject effect on cytokine profiles, which may be a reflection of periodontal status.[38] These inconclusive data on the association between *IL-1B* gene polymorphism and IL-1β in GCF need to be further clarified with studies including other major confounders of periodontal disease, such as the level of the bacterial profile, smoking history, and systemic diseases.[27]

A clear association between the subgingival microbial composition and the local cytokine milieu has been also reported.[39,42] Generalized aggressive periodontitis patients have statistically significantly higher GCF levels of IL-1β and higher proportions of orange and red complex species than periodontally healthy subjects.[39] Several studies have also indicated that genotype-positive patients manifest higher levels of pathogenic bacteria than genotype-negative individuals.[43-45] Another recent study of a small sample size reported that genetically positive patients had 3 times more bacteria from red complex and 2 times more bacteria from orange complex, as well as higher titers of *P. gingivalis* than patients with negative genotype, indicating a relationship between genotype and the environment of subgingival microflora.[46] It has been also shown that specific periodontal pathogens may also account for higher IL-1β levels in periodontal tissues,

since the red complex periodontal pathogens and *A. actinomycetemcomitans* characteristically induce IL-1β expression.[47,48] A recent study showed that *IL-1B* polymorphism at location (3954) and the red complex periodontal pathogens independently and additively modulated the levels of IL-1β in periodontal tissues as well as increase the scores of disease severity.[36] It has been previously speculated that over-expression of IL-1α and IL-1β in periodontal tissues in response to organisms in subgingival plaque may increase gingival inflammation, gingival crevicular fluid flow and induce an environment that favors further colonization and growth of subgingival species. These increases might then place the genotype positive subject at greater risk for periodontal disease progression.[45]

Reports of the role of *IL-1* genetic factors in periodontitis have been contradictory. Many studies have assessed the association between *IL-1* genetic variations and clinical measures (such as tooth loss or severity of periodontitis based on probing depths) or other outcomes associated with periodontitis, such as IL-1β levels in GCF. However, most of the studies include a small sample size and there is substantial heterogeneity in the phenotype and ethnicity across the reports. Polymorphisms in the *IL-1B* gene could predispose subjects to elevated IL-1β levels and may play a role as

risk factors, but there is insufficient evidence to conclude if *IL-1B* gene polymorphisms affect the susceptibility to periodontitis by ultimately modulating the levels of IL-1β in GCF.

Additionally, in above studies, there is still insufficient evidence regarding the concomitant role of periodontal pathogens and the levels of bacterial challenge, since several pathogens can induce increased expression of IL-1β in periodontal tissues. The exact role and contribution of *IL-1B* gene polymorphisms and periodontal pathogens on IL-1β levels in periodontal tissues has been evaluated separately and additional research is highly recommended.

There has not been a study, to the best author's knowledge, which has investigated concomitantly the relationship between *IL-1B* gene polymorphism, site specific IL-1β levels in GCF, and bacterial pathogens in patients with periodontitis. The influence of those parameters has been evaluated separately, but their combined contribution on a site to site basis in patients with periodontitis, as well as their possible association with the clinical parameters of disease extent and severity needs to be further evaluated. Comparing IL-1β GCF levels in association with the bacterial

levels in periodontitis and healthy sites among patients with and without *IL-1B* gene polymorphism, as well as in periodontitis and healthy sites within each patient, may clarify unanswered questions regarding the susceptibility to periodontitis and the biological plausibility for the *IL-1B* gene polymorphism.

Specific Aims and Hypothesis

The primary aim of the study is to compare the levels of IL-1β in GCF collected from healthy and periodontitis sites in *IL-1B* gene polymorphism positive and *IL-1B* gene polymorphism negative periodontitis patients in association with the bacterial profiles at the same periodontal sites.

The secondary aim will be to evaluate the prevalence of *IL-1B* gene polymorphisms among the ethnically diverse population of periodontitis patients treated at TUSDM.

Our hypothesis is that *IL-1B* gene polymorphism positive periodontitis patients have higher GCF IL-1β levels and higher levels of periodontal pathogens in both healthy and periodontitis sites than *IL-1B* gene polymorphism negative periodontitis patients.

Research Design and Methods

Material and Methods

Overall design and strategy

The present investigation was a clinical cross-sectional study, where gingival crevicular fluid (GCF), bacteria, and cells from the buccal mucosa were collected from patients with periodontitis (n=32). The GCF and the bacteria were collected from one healthy site and one periodontitis, one from each subject. The participants in the study were recruited from the Department of Periodontology and the Undergraduate Clinic at Tufts University School of Dental Medicine, Boston MA. The GCF samples were analysed for the presence and levels of IL-1β using enzyme-linked immunoassay (ELISA). The bacterial samples were analyzed for the detection and clinical threshold levels of 11 periodontal pathogens using polymerase chain reaction (PCR) amplification. The cells from buccal mucosa were analyzed for the presence of T allele in the loci of the *IL-1B* gene at position 3954.

Clinical parameters

Clinical parameters were evaluated by one examiner in all teeth, excluding third molars, and included the following: plaque index (PI) (0, 1); gingival redness (0,1); Bleeding on Probing (BoP) within 15 seconds (0, 1) (Mühlemann & Son 1971); Probing Depth (PD); Clinical Attachment Loss (CAL). Six sites were examined for each tooth: mesiobuccal, buccal, distobuccal, distolingual/distopalatal, lingual/palatal, and mesiolingual/palatal. PD and CAL were recorded to the nearest millimetre using a North Carolina periodontal probe (PCP-UNC 15; Hu-Friedy Manufacturing Company, Chicago, III). The same examiner also performed radiographic evaluation of any interproximal bone loss for each patient, based on radiographs that had been already exposed for each patient. In case, of absence of any radiographs, or radiographs exposed over 12 months before, new radiographs were exposed.

The above mentioned clinical and radiographic examination is part of the standard comprehensive periodontal examination of any patient who is screened and treated in the Department of Periodontology or the

Undergraduate Clinic at Tufts University School of Dental Medicine, Boston MA, for accurate diagnosis of periodontal disease.

Inclusion criteria

All patients had to be at least 18 years old and English speakers. All subjects were in good general health and had at least 20 teeth in their dentition, excluding 3rd molars.

Exclusion criteria

Patients were excluded from the study if they had any of the following exclusion criteria:

- Systemic disease that has an impact on periodontal status (e.g. diabetes mellitus, HIV/AIDS, rheumatoid arthritis)
- History of immunosuppressive chemotherapy or history of any disease known to severely compromise immune function (e.g. cytomegalovirus infection)

- Any acute intraoral infection (e.g. herpetic gingivostomatitis)
- Need for antibiotic premedication for routine dental procedures
- Use of antibiotics within the past 3 months of the study
- Chronic daily use of steroids or non-steroid anti-inflammatory therapy within the past 3 months of the study
- Pregnancy or lactation
- Inability or unwillingness to sign informed consent form.
- Current smokers
- Previous periodontal treatment (even prophylaxis) in the past 3 months prior to the examination

The actual protocol was submitted to Tufts Medical Center IRB for approval (#11000). The protocol was clearly explained to all patients, who were then asked to read and sign an appropriate Consent Form, before entering the study.

After patients' acceptance into the study and signing the informed consent, a detailed medical history and family history of periodontitis was obtained. Afterwards, a comprehensive periodontal evaluation was completed. The diagnosis of periodontitis was based on clinical parameters

and radiographic evidence of bone loss around the teeth. The appropriate kits were used afterwards for obtaining samples of cells from buccal mucosa, subgingival bacterial plaque, and GCF for *IL-1B* gene polymorphism, microbiological, and IL-1β levels analysis, respectively. All patients were advised to continue with their periodontal treatment that consisted of oral hygiene instructions and scaling/root planning within 2 weeks from being diagnosed with periodontitis.

Variables

The subjects were diagnosed with periodontitis according to the below described criteria. Then a healthy site and a periodontitis site were chosen for bacterial plaque and GCF sampling as described below:

Periodontitis Diagnosis:

Patients were diagnosed with periodontitis when they fulfilled the following criteria:

i. BoP $\geq$ 20 % of probing sites

ii. ≥ 6 teeth with at least one site each with PD ≥5 mm, CAL >3mm and BoP

iii. Radiographic indication of interproximal bone loss >2mm, measured from the cemento-enamel junction (CEJ) to the bone crest at sites with PD ≥5 mm and BoP

Healthy sites:

The healthy sites in periodontitis patients had to fulfill the following criteria:

i. Absence of BoP

ii. CAL ≤ 3mm

iii. PD ≤ 3mm

iv. Radiographic evidence of interproximal bone level at a distance of ≤2mm, measured from the CEJ to the bone crest at the periodontal ligament space

Periodontitis sites:

The periodontitis sites in periodontitis patients had to fulfill the following criteria:

i. BoP and/or Suppuration
ii. CAL > 3mm
iii. PD ≥ 5mm
iv. Radiographic indication of interproximal bone loss >2mm, measured from the CEJ to the bone crest

Determination of *IL-1B* gene polymorphism

Collection of cells from buccal mucosa:

A genetic test was used to identify participants who have specific variations in the *IL-1B* genes.[28] Cells from buccal mucosa were obtained from each individual for the *IL-1B* gene polymorphism assessment using a cytology brush. The cytology brush was used vigorously on both sites of buccal mucosa (internal surface of cheeks) for at least 30 seconds. The brush was then placed into a transfer tube. The samples were de-identified and shipped to a Clinical Laboratory Improvement Amendments-certified laboratory (OralDNA® Labs, MN) for DNA extraction and genotyping.

DNA Extraction:

Genomic DNA was extracted and tested for the *interleukin-1B* genetic variation located at position +3954. This genetic variation is tested by methods of the polymerase chain reaction (PCR), endonuclease digestion and resultant restriction fragment detection by automated microcapillary electrophoresis.

From the typical sample obtained with the cytology brush, DNA extraction was performed through a combination of mechanical disruption of the epithelial cell and ion-exchange column purification. The swab was placed in a tube containing 1mL sterile normal saline (0.9%) and then was agitated to dislodge the epithelial cells. Then, the swab was squeezed against the inside of the tube and above the saline level to remove as much liquid as possible and then it was returned to its original collection tube and stored in 4-8°C.

An amount of 1 mL of the processed sample with sterile saline was combined with 300 μL of zirconium beads and homogenized at 2500 rpm for 10 minutes (Thermo-Fisher). The resulting mixture was centrifuged to

sediment the zirconium beads, and 200 μL of the liquid fraction were purified using silica membrane technology (Qiacube HT DNA extractor; Qiagen, Germany).

Analysis of Interleukin-1B Polymorphisms:

The *interleukin-1B* 3954 (rs1143634) polymorphisms were detected using polymerase chain reaction followed by high-resolution melt analysis. For genotyping *interleukin-1B* (3954) Single Nucleotide Polymorphism (SNP), DNA fragments were amplified by the use of primer pairs 5'-TCGTGCACATAAGCCT-3' (forward) and 5'-CTCCACATTTCAGAACCT-3' (reverse) (59 bp). The primers are designed in-house specifically for high-resolution melt analysis. Smaller amplicons give better resolution for melt analysis and therefore the primers are put as close to the SNP as possible.

Amplification and detection were performed using a Qiagen RotorGene (Qiagen, Germany). Cycling was carried out for one cycle at 94°C for 2 minutes, followed by 50 cycles of 94°C for 5s; 54°C for 10s; 72°C for 5s. High-resolution melt analysis was performed with a ramp from 65°C to 80°C

with measurements every 0.05°C. C and T alleles were located, allowing, therefore, the determination of the CC, CT, and TT genotypes. The carriers of the polymorphic T allele (CT and TT genotypes) were combined into the same group that was called PST positive, while another group with the homozygous C allele (CC genotype) was named PST negative.

GCF collection

GCF was collected from the site with the deepest probing depth among the periodontitis sites, and from one healthy site.[40] In case of multiple sites with the same probing depth the most accessible one was chosen. After isolating the tooth with a cotton roll to prevent contamination with saliva, supragingival plaque was removed with curettes and wet gauges without touching the marginal gingiva. The crevicular site was then dried gently with an air syringe. GCF was collected with Periopaper strips (Periopaper, Interstate Drug Exchange, Amityville, NY, USA) which were inserted gently into the orifice of the sulcus/pocket, 1-2 mm subgingivally, in order to avoid any mechanical injury, and were left in place for 30 seconds. Strips visibly contaminated by saliva or blood were discarded. Following collection of GCF, the volume of the sample on the paper strips was

measured using a calibrated Periotron 8000 (Oraflow Inc, Plainview, NY, USA), calibrated by the investigators based on a protocol described before.[49] The readings from the Periotron 8000 were converted to an actual volume (µl) by reference to the standard (calibration) curve, by using the specific software that accompanies the device. After GCF collection, strips were placed immediately in de-identified labelled Eppendorf tubes. The samples were then stored at -80°C until subsequent cytokine analysis, which was performed at the laboratory of Dr. T.C. Theoharides, (Department of Integrative Physiology and Pathobiology, Tufts University School of Medicine, Boston, MA).

Calibration of Periotron 8000

The calibration of the Periotron 8000 was performed according to the protocol established by Chapple *et al.*[49] After the device was turned on and a minimum period of 60 minutes was allowed in order for the Periotron to warm up, the reading dial was set to zero (0). A series of sterile water aliquots were then applied to PerioCol papers (PerioCol paper, Oraflow Inc, Plainview, NY) according to the company's recommendations for better calibration readings. PerioCol papers can handle higher volume by

maintaining more fluid (up to 2 μl) and hereby raise the accuracy of the top part of the standard curve. The aliquots ranged in volume from 0.25μl to 1.25 μl (0.25, 0.50, 0.75, 1.00, 1.25 μl). Then, the PerioCol papers were placed between the sensors of the machine so that the entire sample portion of the collection strip covers the lower sensor of the Periotron device. The Periotron Scores on the digital readout were then recorded manually. Between each measurement, the sensors were wiped with a gauge to dry the electrodes. This procedure was repeated three (3) more times and the average Periotron score was recorded for each volume.

The Periotron scores recorded for each volume were transferred to the specific software that accompanies the device (Periotron Professional 3.0) and a calibration curve was generated. This calibration curve was used to calculate the GCF volume that was collected in the clinical samples.

Quantification of IL-1β levels in GCF

IL-1β levels in GCF were determined[39] in duplicate samples by an enzyme-linked immunosorbent assay (ELISA) (Human IL-1 beta/IL-1F2 DuoSet/ DY201, R&D systems, Minneapolis, MN). The IL-1β detection

range of the assay is 3.91 - 250 pg/ml. This assay was based on an antibody sandwich method, using microtiter plates, which were coated with the appropriate human cytokine.

The plate preparation included the following steps:

1. 100 μL/well of the capture antibody (diluted to the appropriate concentration in PBS) were transferred to the plates. The concentration of the capture antibody was calculated according to the total volume of 22,000μl that was necessary for 2 plates. The plates were then sealed and incubated overnight at room temperature for at least 16 hours.

2. Each well was aspirated and washed with Wash Buffer. An amount of 20 μl of the concentrated Wash Buffer was diluted in 480 μl of distilled water. The process was repeated for a minimum of 3 washes. Each well was forcefully filled with Wash Buffer (400 μL) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step was essential to good performance. After the last wash, any remaining Wash Buffer was removed by aspirating and by inverting the plate and blotting it against clean paper toweling.

3. An amount of 300 μL of recommended Blocking Buffer was added to each well. The plates were then incubated at room temperature for a minimum of 1 hour.

4. The aspiration/wash as in step 2 was repeated in order for the plates to be ready for the sample addition.

The samples were prepared following the below mentioned protocol:

Reagent Diluent 250 μL was added to each sample. Then each Eppendorf tube was Vortexed for 1 minute. The samples were then transferred to a shaker for 30 minutes in 4^0C. After shaking the tubes for 30 minutes, the Periopaper strip was removed and placed into a spin basket. The basket was then put again into the same tube and then all tubes were centrifuged in 15,000g for 8 minutes. The reason that the spin baskets were used was to purify more proteins of the strip into the Eppendorf tube.

For the assay procedure the following steps were followed:

1. An amount of 5ml Reagent Diluent was mixed with 45ml PBS. 300µl of this diluted concentrated Reagent Diluent was added to each well. The plates were then covered with an adhesive strip and incubated for 2 hours at room temperature.

2. The aspiration/wash as in step 2 of Plate Preparation was repeated.

3. An amount of 100 µL of the detection antibody was then added to each well. The plates were again covered with a new adhesive strip and incubated for 2 hours at room temperature.

4. The aspiration/wash as in step 2 of Plate Preparation was repeated.

5. An amount of 100 µL Streptavidin-HRP (R&D Systems, Catalog # DY998, dilute according to the directions on the vial label) was added to each well. The plates were again covered with a new adhesive strip and incubated for 20 minutes at room temperature. It was avoided to place the plates exposed to direct light.

6. The aspiration/wash as in step 2 of Plate Preparation was repeated.

7. Half amount of Color Reagent A and half amount of Color Reagent B were mixed to reach the total volume of 22.000µl.100 µL Substrate Solution (R&D Systems, Catalog # DY999) was added to each well. The plates were incubated for 20 – 30 minutes at room temperature. It was avoided to place the plates exposed to direct light.

8. An amount of 50 µL Stop Solution was added to each well. The plates were gently tapped to ensure thorough mixing.

9. The optical density (O.D.) of each well was determined within 30min using a microplate reader set to 450nm and 570nm (R&D Systems Catalog # DY999). The readings at 450nm were subtracted from the readings at 570nm in order to correct the optical imperfections in our plates. We then averaged the duplicate readings for each standard and sample, and subtracted the average zero O.D. The standard curve was created by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit.

Bacteria collection

Samples of subgingival plaque were collected[50] of the same sites after GCF collection, allowing for comparisons on a site-by-site basis.[49] Sterile paper points were placed for 20 seconds in each pocket (OralDNA® Labs, MN). Prior to sampling, the supra-gingival plaque was removed with a sterile curette and the site of sampling was dried with a sterile cotton roll. Using a pair of sterile forceps two paper points, one following the other, was inserted into the pre-defined sites, down to the base of the pocket. The samples from the single gingival pockets were then transferred in the transfer tube with de-identified labels and the sampling sites and maximum pocket depth were recorded on the order form. The samples were de-identified and shipped to a Clinical Laboratory Improvement Amendments-certified laboratory (OralDNA® Labs, MN) for further microbiological analysis.

Microbiological analysis

DNA extraction:

Bacterial DNA was extracted from the processed sample through a combination of mechanical disruption of the bacterial cell and ion-exchange column purification and tested for eleven bacterial species associated with periodontal disease. The paper strips were placed in 300 μL of zirconium beads and homogenized at 2500 rpm for 10 minutes (Thermo-Fisher). The resulting mixture was centrifuged to sediment the zirconium beads, and 200 μL of the liquid fraction was purified using silica membrane technology (Qiacube HT DNA extractor; Qiagen, Germany). 16SrRNA and species-specific primers were used to confirm the presence of bacterial DNA in each sample.

Analysis of periodontopathogenic bacteria:

The bacterial species that were analyzed were the following:

Aggregatibacter actinomycetemcomitans (A.a);

Red complex bacteria: *Porphyromonas gingivalis (P.g), Tannerella forsythia (T.f), Treponema denticola (T.d)*

Orange complex bacteria: *Eubacterium nodatum (E.n), Fusobacterium nucleatum (F.n), Prevotella intermedia (P.i), Campylobacter rectus (C.r) , Peptostreptococcus micros (P.m)*

Green complex bacteria: *Eikenella corrodens (E.c), Capnocytophaga species (C.s)*

The bacterial species were tested using asymmetric multiplexed polymerase chain reaction (PCR) with primers and molecular beacons designed to specific gene regions of each bacterial species. Amplification and detection were performed using a Qiagen RotorGene (Qiagen, Germany). Three PCR reactions each contain primers and beacons specific for three bacterial species and the fourth reaction contains primers and beacons for two species plus a set designed to amplify the human DNA sequence ApoB. Fluorescent emission resulting from molecular beacon hybridization is read at the end of the PCR reaction and compared to the fluorescence of known plasmid standards to provide a semi-quantitative analysis of patient sample concentration for each bacterium. (e.g. 10^3= 1000 bacteria copies per amplified reaction).

Sample size calculation

The primary aim of the study is to compare the levels of IL-1β in GCF obtained from periodontitis sites in periodontitis patients who possess, or do

not possess, *IL-1B* gene polymorphism. Based on data from previously published studies (Engebretson *et al.*) (37), we anticipate that the mean (SD) levels of IL-1β in the genotype positive group will be 160 (16) pg/μl, and the mean (SD) levels of IL-1β in the genotype negative group will be 137 (11) pg/μl. Assuming α=0.05, a sample of 30 subjects will provide 80% power to detect a difference between the two groups, if the prevalence of the genotype in this population is 20% (nQuery Advisor, 7.0).

Statistical Analysis

Analysis was performed using SAS, Version 9.2 (SAS Institute, Cary, NC).

Demographic data (age, gender, race) were analyzed and expressed in means, counts ± standard deviation (SD). The clinical variables (number of missing teeth, PD, CAL, BOP, PI) were analyzed using the same statistical software and expressed as frequencies and means ± SD.

Normality was assessed using the Kolmogorov-Smirnov test. If the assumptions of normality were found valid, the relationships were tested using the independent sample t-test. If the assumptions of normality were

not valid, the relationships were tested using the non-parametric Mann-Whitney U test. All p-values less than 0.05 were considered statistically significant.

An independent t-test was used to assess the statistically significant differences in age for the PST negative and PST positive subjects. A chi-square test was conducted to assess the differences in proportion of gender among PST status, while a fisher-exact test was conducted to assess the differences in proportion of race groups among PST status. A Fisher-exact test was used to determine the distribution of polymorphisms and alleles among the PST negative and positive groups at alpha 0.05 level.

Mann-Whitney U tests were used to assess the difference between groups for PD mean, CAL mean, BOP and PI that were not normally distributed.

The extent of the periodontal disease was assessed by calculating the number of teeth that were missing (tooth loss), the number of teeth affected with periodontitis, and the percentage of teeth affected with periodontitis in each patient as indicated in the inclusion criteria. Those values were given as

means ± SD and a Mann-Whitney U-test was conducted to determine any differences between the PST positive and PST negative group.

Clinical data, bacteria levels, IL-1β levels, and GCF volume were also analyzed for the specific sampled sites.

The frequency of detection of the periodontopathogenic bacteria was presented as counts and percentages. In terms of the analysis of the periodontopathogens in overall population, dependent test McNemar was conducted to compare this matched study design, since the healthy and periodontitis sites were in the same subject. Chi-square tests and Fisher Exact tests were conducted to compare frequencies of detection of bacteria between PST negative and PST positive in healthy and periodontitis sites. Chi-square tests and Fisher Exact tests were also conducted when comparing the differences of the clinical threshold levels of bacteria in healthy and periodontitis sites between PST positive and PST negative patients.

The volume of GCF that was collected from each site was determined by referring to the readings from the Periotron 8000, which were converted to an actual volume (μl) according to the standard (calibration) curve, by using

the specific software that accompanies the device (Periotron Professional 3.0). The amount (pg) and the concentration (pg/μl) of IL-1β in GCF, as well as the volume (μl) of GCF n healthy and periodontitis sites were expressed in means ± SD. The non-parametric test Wilcoxon Signed Rank Test (paired data) was used to determine the differences for the GCF variables between the healthy and periodontitis sites in overall population, while the non-parametric Mann-Whitney U test was used to assess the differences for the GCF variables (GCF volume, amount of IL-1β in GCF, concentration of IL-1β in GCF) between the PST positive and PST negative groups in the healthy and periodontitis sites. Significance was indicated at the 0.05 level ($p<0.05$).

Results

Table 1 summarizes the demographic data (age, gender, ethnic status) of all subjects who were included in this research project. Assumption for normality distribution for age was found valid (Fig. 1); thus an independent t-test was used to assess the differences in age (mean) between the PST negative and PST positive subjects. A chi-square test was conducted to access the differences in proportion of gender among PST status. A Fisher-exact test was conducted to access the differences in proportion of race groups among PST status. No significant differences were found in regards to age, gender and race distribution at alpha=0.05 level, indicating the lack of any confounding factors that would impede comparisons.

Table 1: Demographic data

Feature	PST +	PST-	Total	p-value
N subjects (count, %)	13 (40.6%)	19 (59.4%)	32 (100%)	
Age (mean, SD)	47.00 (12.51)	45.92 (16.12)	46.56 (14.56)	p=0.841[a]
Gender	4 females (30.8%) 9 males (69.2%)	9 females (47.4%) 10 males (52.6%)	13 females (40.6%) 19 males (59.4%)	p=0.348[b]
Ethnicity				
Caucasian	9 (69.2%)	8 (42.1%)	17 (53.1%)	
Black, African American	3 (23.1%)	4 (21.1%)	7 (21.9%)	p=0.112[c]
Asian	0 (0%)	6 (31.6%)	6 (18.8%)	
Hispanic, Latino, Other	1 (7.7%)	1 (5.3%)	2 (6.3%)	

Values are given as counts (percentages) except for the gender distribution where values are given as means (standard deviations)
[a] Determined by the independent t-test
[b] Determined by the chi-square test
[c] Determined by the fisher exact test
No statistical significant differences were found

Figure 1: Distribution of age in the population

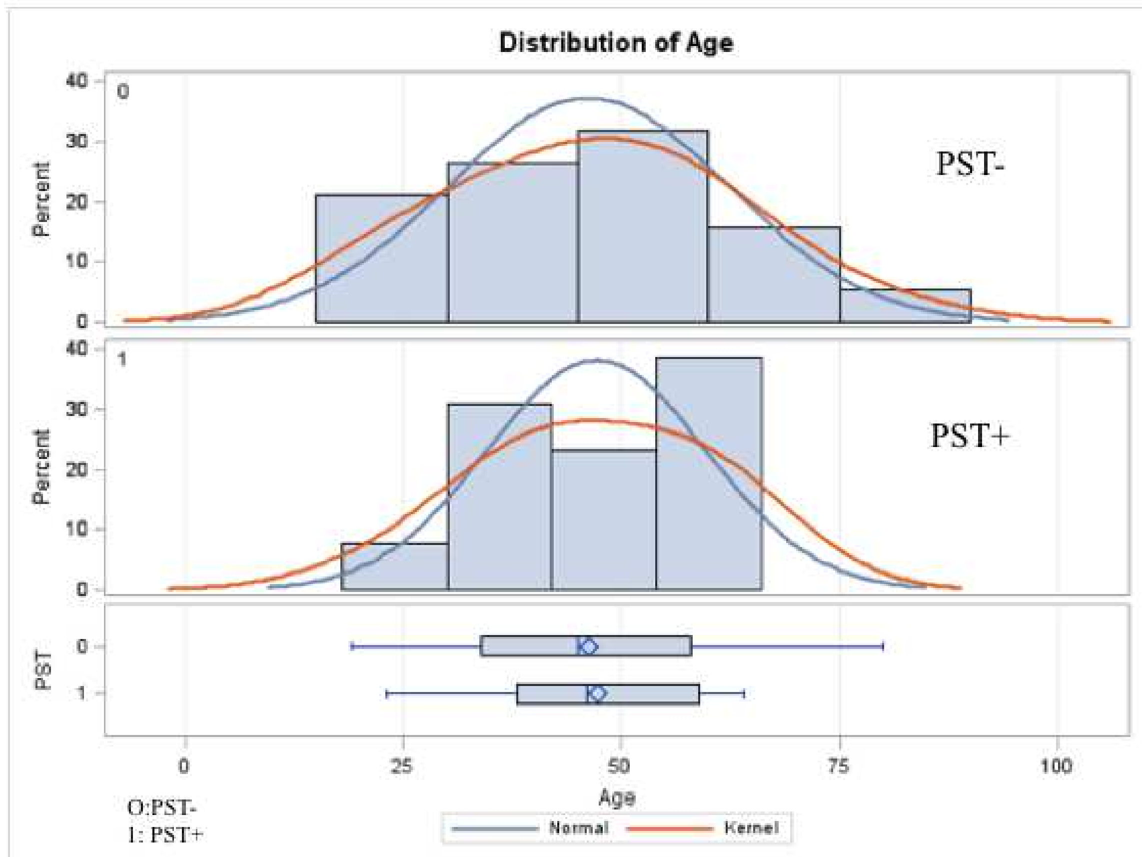


Table 2 summarizes the primer sequences and reaction properties for both *IL-1B* (3954) SNP and periodontal pathogens.

Table 2: Primer sequences and reaction properties

Reaction	Target	Forward Primer Sequence / Reverse Primer Sequence	Base Tm (°C)	Amplicon Length
	SNP IL-1B (+3954)	5’- TCGTGCACATAAGCCT- 3’		59 bp
		5’- CTCCACATTTCAGAACCT- 3’		
A	*Actinobacillus actinomycetemcomitans*	5' - GGCATCTACGGCGAAATC - 3'	50.3	219bp
		5' - AACTACGTCGCATTTGTTAC - 3'	47.7	
A	*Eubacterium nodatum*	5' - TGCAAGTTGAGCGAGAAA - 3'	45.8	187 bp
		5' - CTCAGGCCGGCTACTG - 3'	51.1	
A	*Porphyomonas gingivalis*	5' - CCGAAGCGAGCTTACATT - 3'	48	248 bp
		5' - GAGCCTGCGGATACCTAC - 3'	52.6	
B	*Prevotella intermedia*	5' - CAAGTATCCGAAGGCTGGTGAGGA - 3	59.1	122bp
		5' - TTATGCGTGGAATGTAACCATCG - 3'	53.5	
B	*Capnocytophaga spp. (gingavalis, ochracea, sputigenal)*	5' - TGGACAATGGTCGGAAGA - 3"	48	228 bp
		5' - TGACTTATTAGCCCGCCTAC - 3'	51.8	
B	*Peptostreptococcus micros*	5' - TAGCTTGTCCCGGTGCTTCAACAG - 3'	59.1	211 bp
		5' - GTGCTTTCAGACCCTGATGCAA - 3'	54.8	
C	*Campylobacter rectus*	5' - CGAGATCGTCTATCTAACGGCGA - 3'	57.1	238bp
		5' - TAGAGCACGGTTTGCGTCGTC - 3'	56.3	
C	*Tannerella forsythia*	5' - TTTATTGGTTGCGGATGG - 3'	45.8	82 bp
		5' - ACTGCTGCCTCCCGTAG - 3'	51.9	
C	*Eikenelia corrodens*	5' - CCCAAGCGAAGAAGCCGTAG - 3'	55.9	159bp
		5' - AGGATGTTGCGGGCGACT - 3'	52.6	
D	*Fusobacterium nucleatum/periodonticum*	5' - CATCCATAGGTTTTGTTGCATCTG - 3'	54	241 bp
		5' - GCTCTTGACGTAGCAGGAGCACCA - 3'	60.8	
D	Internal Control	5' - CCAGGTATATTCGAAAGTCCA - 3'	50.5	80 bp
		5' - CGAAAGGAAGTGTAATCACTA - 3'	48.5	
D	*Treponema denticola*	5' - TATACGGCAGCCTATCCT - 3'	48	112 bp
		5' - CCGAAACGCTTATCCAT - 3'	44.6	

Ta: Annealing temperature (54°C for all assays)
Tm: Melting temperature

Table 3 presents the frequencies of *IL-B* (3954) SNP in our population. The prevalence of the PST positive genotype (CT, TT) was 40.63% of the subjects in whole population. The heterozygous polymorphic genotype (CT) was more frequent among the PST positive subjects comparing to the homozygous polymorphic genotype (TT). In overall population the C allele was more prevalent comparing to the T allele.

Table 3: Frequencies of *IL-1B* (3954) SNP

Genotype or Allele	Total subjects (%)	Subjects with PST+
***IL-1B* genotype**		
CC	19 (59.38%)	0%
CT	8 (25%)	61.5%
TT	5 (15.63%)	38.5%
CT+TT	13 (40.63%)	
Allele		
C	46 (71.87%)	8 (30.7%)
T	18 (28.13%)	18 (69.3%)

Clinical data

The clinical characteristics of the study population are presented in Table 4. A Mann-Whitney U test was used to assess the difference between groups for PD (mean) and CAL (mean), as both parameters were not normally distributed (Fig. 2, 3). No significant difference was found for

either PD or CAL at alpha 0.05 level. Normality assumption was not valid for BOP and PI, therefore the non-parametric test Mann-Whitney was used to assess the difference of BOP and PI between the two groups. No significant difference was found for either BOP or PI at an alpha 0.05 level. Additionally, a Mann-Whitney U test was used to determine any differences between the PST groups in regards to number of teeth lost, number of teeth affected with periodontitis, and percentage of teeth affected with periodontitis, since normality assumption was not satisfied in those variables as well. The teeth affected with periodontitis were determined according to the inclusion criteria for our population, as described in the Materials and Methods section. Although PST positive patients showed an increased number of teeth affected with periodontitis compared to PST negative patients, there was no statistical significant difference between the two groups.

Table 4: Clinical characteristics

	Total	**PST Negative** (n=19)[a]	**PST Positive** (n=13)[a]	**Statistics p-value**
PD mean (SD)	3.24 (0.51)	3.23 (0.58)	3.25 (0.42)	p=0.43[b]
CAL mean (SD)	3.67 (0.57)	3.62 (0.64)	3.74 (0.46)	p=0.24[b]
BOP mean (%)	56.19 (13.77)	57.00 (13.25)	55.00 (14.97)	p=0.730[b]
PI mean (%)	80.31 (27.44)	86.01 (20.14)	71.98 (34.78)	p=0.241[b]
Tooth loss		2.11 ±1.89	1.69 ±2.31	p=0.436[b]
# Teeth Periodontitis		10.95 ±3.77	12.08 ±4.66	p=0.289[b]
% Teeth Periodontitis		0.42 ±0.13	0.46 ±0.17	p=0.748[b]

[a] Values are given as means ± standard deviations or as numbers (percentages)

[b] Determined by Mann-Whitney test (non-parametric test of the independent t-test was used since the normality assumption was invalid)
No statistical significant differences were found

Figure 2: The distribution of PD in the population.

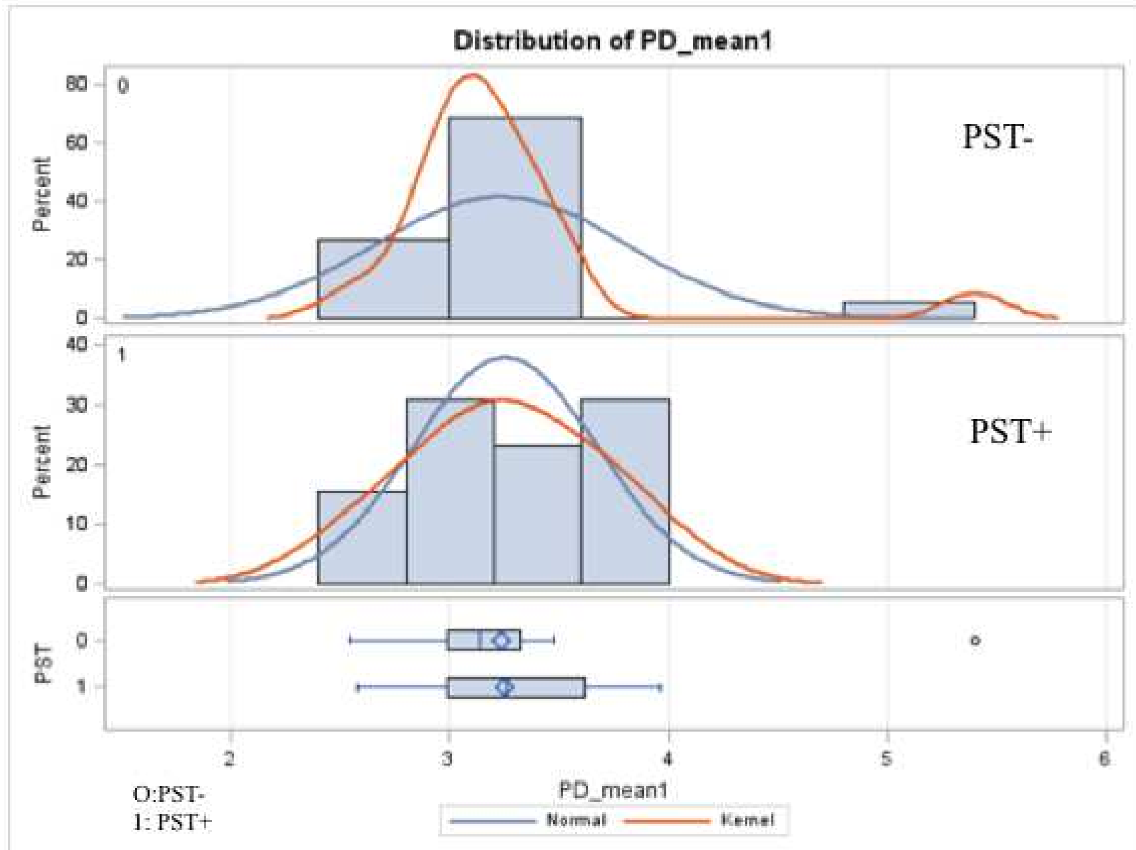
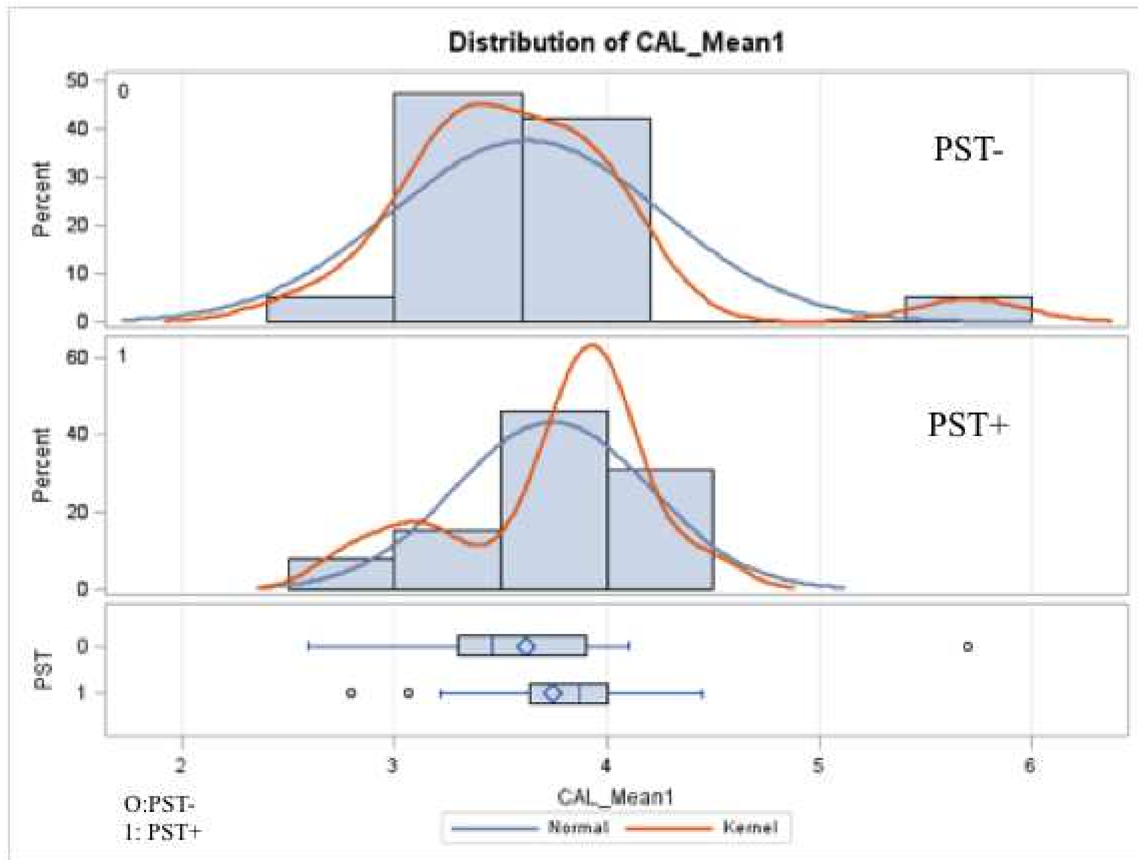


Figure 3: The distribution of CAL in the population.

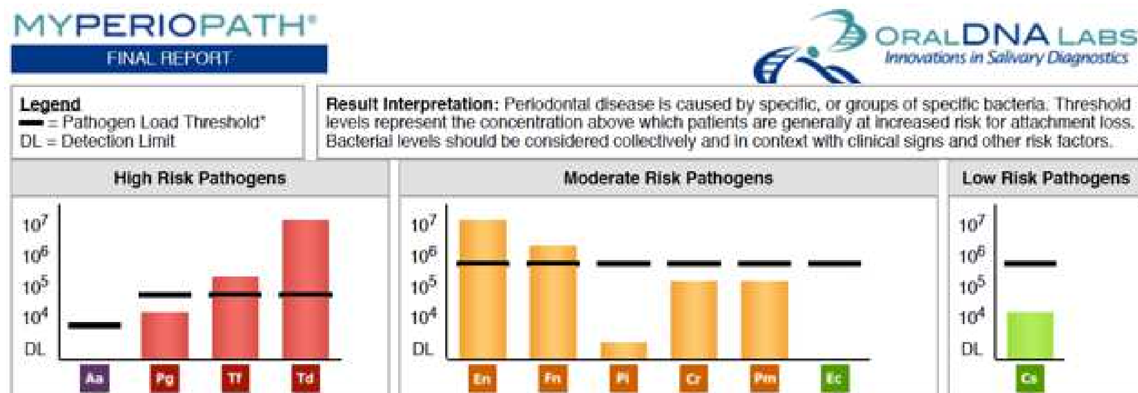


<u>Data for bacterial profile of subjects</u>

An example of a microbiological analysis report, as received by the company Oral DNA, for the periodontal pathogens detection and clinical threshold levels in the healthy and periodontitis site is presented in in Fig. 4. The detection limit for all eleven bacterial species was 10^3 (counts). The clinical threshold level for *A.a* was 10^4, for *P.g, T.f, T.d* (red complex bacteria) was 10^5, and for the orange complex bacteria (*E.n, F.n, P.i, C.r,*

P.m) and *E.c, C.s* (green complex bacteria) was 10^6. Any bacterial concentration above the clinical threshold level indicates that patients are generally at increased risk for attachment loss. In this particular example of Fig. 4, it is shown that *A.a* and *E.c* were not detected, *P.g, P.i, C.r, P.m, C.s* were in low concentration, while *T.f, T.d, E.n, F.n* were above the clinical threshold levels, thus in high concentration and therefore indicating a strong association with increased PD.

Figure 4: Example of Oral DNA report for periodontal pathogens detection.



A.a: Aggregatibacter actinomycetemcomitans

P.g: *Porphyromonas gingivalis*

T.f: Tannerella forsythia

T.d: Treponema denticola

E.n: Eubacterium nodatum

F.n: Fusobacterium nucleatum

P.i: Prevotella intermedia

C.r: Campylobacter rectus

P.m: Peptostreptococcus micros

E.c: *Eikenella corrodens*

C.s: Capnocytophaga species

Table 5 and Fig. 5 present the frequencies of the different types of bacteria in healthy and periodontitis sites in overall population. Due to the fact the healthy and periodontitis sites were in the same subject, dependent test McNemar was conducted to compare this matched study design. The count is given for each bacteria species along with the percentage out of 32 subjects. The final column states the p-value for the McNemar test given. All tests showed statistical significant differences ($p<0.05$). Obviously, higher frequencies of detection of bacteria were in periodontitis sites than in healthy sites.

Table 5: Frequency of detection of different types of bacteria in healthy and periodontitis sites in overall population.

	Healthy Sites	Periodontitis Sites	McNemar p-values
A.a	3 (9.38)	10 (31.25)	p=0.008*
C.r	7 (21.88)	26 (81.25)	p=<0.0001*
C.s	4 (12.50)	16 (50.00)	p=0.0027*
E.c	7 (21.88)	19 (59.38)	p=0.0013*
E.n	13 (40.63)	26 (81.25)	p=0.0003*
F.n	4 (12.50)	15 (46.88)	p=0.0023*
P.g	5 (15.63)	18 (56.25)	p=0.0003*
P.i	10 (31.25)	21 (65.63)	p=0.0009*
P.m	14 (43.75)	25 (78.13)	p=0.0045*
T.d	4 (12.50)	20 (62.50)	p=0.0002*
T.f	21 (65.63)	32 (100)	p=<0.0001*

Bacteria species are presented as counts and percentages
*Statistical significant difference ($p<0.05$)

Figure 5: Frequency of detection of different types of bacteria in healthy and periodontitis sites in overall population

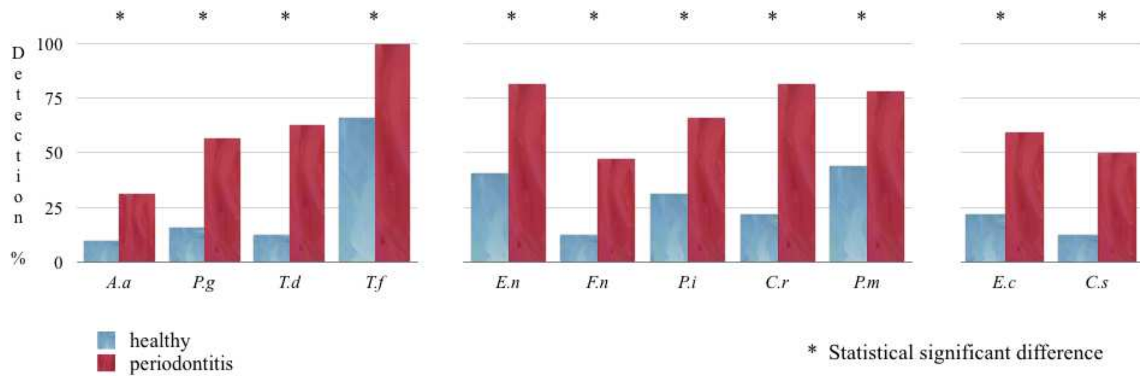


Table 6 and Fig. 6 show the frequency of detection of different types of bacteria in healthy sites between subjects with PST positive and PST negative. The counts for each bacteria species and percentages out of PST negative or PST positive are presented.

Chi-square tests were conducted to compare frequencies of *E.n* and *P.m* between PST negative and PST positive patients in healthy sites. Fisher Exact tests were conducted to compare frequencies of the remaining bacterial species (*A.a, C.r, C.s, E.c, F.n, P.g, P.i, T.d, T.f*) between PST negative and PST positive subjects in healthy sites. Only bacterial species

F.n and *T.f* were found to be significantly higher in healthy sites in PST positive compared to PST negative patients (p=0.02; p=0.01).

Table 6: Frequency of detection of different types of bacteria in healthy sites between subjects with PST positive and PST negative

Healthy sites

	PST negative n=19	PST positive n=13	Statistical test p-value
A.a	3 (15.79)	0 (0.00)	$p=0.25^{\alpha}$
C.r	4 (21.05)	3 (23.08)	$p=1.00^{\alpha}$
C.s	2 (10.53)	2 (15.38)	$p=1.00^{\alpha}$
E.c	3 (15.79)	4 (30.77)	$p=0.40^{\alpha}$
E.n	6 (31.58)	7 (53.85)	$p=0.21^{\beta}$
F.n	0 (0.00)	4 (30.77)	$p=0.02^{\alpha *}$
P.g	1 (5.26)	4 (30.77)	$p=0.13^{\alpha}$
P.i	6 (31.58)	4 (30.77)	$p=1.00^{\alpha}$
P.m	6 (31.58)	8 (61.54)	$p=0.09^{\beta}$
T.d	2 (10.53)	2 (15.38)	$p=1.00^{\alpha}$
T.f	9 (47.37)	12 (92.31)	$p=0.01^{\alpha *}$

Counts and percentages out of PST negative or PST positive are given.
Chi-square tests $^{\beta}$ and Fisher Exact tests $^{\alpha}$ were conducted to compare frequencies of bacteria between PST negative and PST positive patients in healthy sites.
*Statistical significant difference (p<0.05)

Figure 6: Frequency of detection of different types of bacteria in healthy sites between subjects with PST positive and PST negative

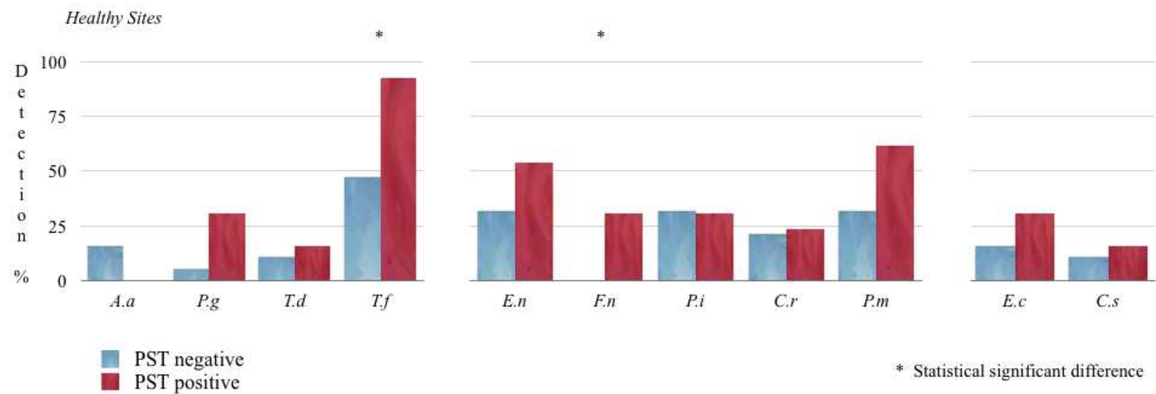


Table 7 and Fig. 7 present the frequency of detection of different types of bacteria in periodontitis sites between subjects with PST positive and PST negative. The counts for each bacteria species and percentages out of PST negative or PST positive are given. Chi-square tests were conducted to compare frequencies of *C.s, E.c, F.n, P.g,* and *T.f* between PST negative and PST positive subjects in periodontitis sites. Fisher Exact tests were conducted to compare frequencies of *A.a, C.r, E.n, P.i, P.m,* and *T.d* between PST negative and PST positive subjects in the same sites. Only bacteria species' *F.n* detection was found to be significantly higher in healthy sites in PST positive (69.23%) compared to PST negative (31.58%) patients (p=0.036). It is worth of mentioning that *P.g* was also detected in a higher number of periodontitis sites in PST positive (69.23%) compared to PTS negative (47.37%) patients, although the difference was not statistically significant (p=0.221).

Table 7: Frequency of detection of different types of bacteria in periodontitis sites between subjects with PST positive and PST negative.

Periodontitis sites

	PST negative n=19	PST positive n=13	Statistical test p-value
A.a	6 (31.58)	4 (30.77)	$p=1.00^{\alpha}$
C.r	15 (78.95)	11 (84.62)	$p=1.00^{\alpha}$
C.s	8 (42.11)	8 (61.54)	$p=0.28^{\beta}$
E.c	11 (57.89)	8 (61.54)	$p=0.84^{\beta}$
E.n	15 (78.95)	11 (84.62)	$p=1.00^{\alpha}$
F.n	6 (31.58)	9 (69.23)	$P=0.036^{\beta\,*}$
P.g	9 (47.37)	9 (69.23)	$p=0.221^{\beta}$
P.i	12 (63.16)	9 (69.23)	$p=1.00^{\alpha}$
P.m	15 (78.95)	10 (76.92)	$p=1.00^{\alpha}$
T.d	11 (57.89)	9 (69.23)	$p=0.713^{\alpha}$
T.f	19 (100)	13 (100)	$p=1.00^{\beta}$

Counts and percentages out of PST negative or PST positive are given.
Chi-square tests $^{\beta}$ and Fisher Exact tests $^{\alpha}$ were conducted to compare frequencies of bacteria between PST negative and PST positive patients in periodontitis sites.
*Statistical significant difference ($p<0.05$)

Figure 7: Frequency of detection of different types of bacteria in periodontitis sites between subjects with PST positive and PST negative.

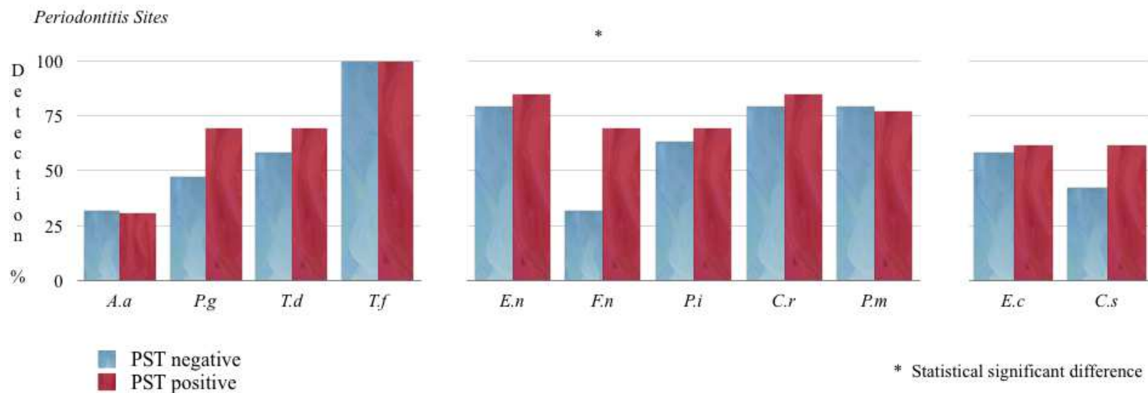


Table 8 presents the association of *IL-1B* gene polymorphism and the detection of *F.n, P.g, P.m, T.f.* Those four bacteria species showed some significance when tested in healthy and periodontitis sites between PST

positive and PST negative patients (Table 6, 7). Therefore, their association with the *B* genotype was tested. Fisher Exact test was only used for *T.f*, due to small cell counts, while regular chi-square test was used for the other three bacteria (*F.n, P.g, T.f*). All four bacteria showed a higher frequency of detection in both healthy and periodontitis sites between PST positive and PST negative patients ($p<0.05$), but difference of frequency of detection of *P.m* was not statistical significant ($p=0.261$).

Table 8: Association of *IL-1B* gene polymorphism and the detection of *F.n*, *P.g*, *P.m*, *T.f*.

	PST Negative	PST Positive	Statistical test p-value
F.n	6 (15.79)	13 (50.00)	p=0.003$^{\beta *}$
P.g	10(26.32)	13 (50.00)	p=0.053$^{\beta *}$
P.m	21(55.26)	18(69.23)	p=0.261$^{\beta}$
T.f	28 (73.38)	25 (96.15)	p=0.021$^{\alpha *}$

Counts and percentages out of PST negative or PST positive are given.
Chi-square tests $^{\beta}$ and Fisher Exact test $^{\alpha}$ were conducted to compare frequencies of bacteria between PST negative and PST positive patients overall (both healthy & periodontitis sites).
*Statistical significant difference ($p<0.05$)

Table 9 presents the clinical thresholds of different types of bacteria in healthy sites between subjects with PST positive and PST negative. The counts for each bacteria species and percentages out of PST negative or PST positive are presented. Fisher Exact tests were conducted to compare the clinical thresholds of all the bacteria species between PST negative and PST

positive patients in healthy sites. The clinical threshold of only the bacteria species *F.n* (p=0.020) and *T.f* (p=0.019) were found to be significantly different in healthy sites between PST positive and PST negative patients.

Table 9: Clinical thresholds of different types of bacteria in healthy sites between subjects with PST positive and PST negative.

Healthy sites

	PST negative n=19	**PST positive n=13**	**Statistical test p-value**
A.a **low**	3 (15.79)	0 (0.00)	p=0.253$^{\alpha}$
A.a **high**	0 (0.00)	0 (0.00)	
C.r **low**	4 (21.05)	3 (23.08)	p=1.00$^{\alpha}$
C.r **high**	0 (0.00)	0 (0.00)	
C.s **low**	2 (10.53)	2 (15.38)	p=1.00$^{\alpha}$
C.s **high**	0 (0.00)	0 (0.00)	
E.c **low**	3 (15.79)	3 (23.08)	p=0.666$^{\alpha}$
E.c **high**	0 (0.00)	0 (0.00)	
E.n **low**	5 (26.32)	6 (46.15)	p=0.446$^{\alpha}$
E.n **high**	1 (5.26)	1(7.69)	
F.n **low**	0 (0.00)	2 (15.38)	p=0.020$^{\alpha *}$
F.n **high**	0 (0.00)	2 (15.38)	
P.g **low**	1 (5.26)	3 (23.08)	p=0.124$^{\alpha}$
P.g **high**	0 (0.00)	1(7.69)	
P.i **low**	6 (31.58)	4 (30.77)	p=1.00$^{\alpha}$
P.i **high**	0 (0.00)	0 (0.00)	
P.m **low**	5 (26.32)	5 (38.46)	p=0.488$^{\alpha}$
P.m **high**	1 (5.26)	2 (15.38)	
T.d **low**	1 (5.26)	2 (15.38)	p=0.737$^{\alpha}$
T.d **high**	1 (5.26)	0 (0.00)	
T.f **low**	8 (42.11)	11 (84.62)	p=0.019$^{\alpha *}$
T.f **high**	1 (5.26)	1 (7.69)	

Counts and percentages out of PST negative or PST positive are given.
Fisher Exact tests $^{\alpha}$ were conducted to compare the clinical threshold of bacteria between PST negative and PST positive patients in healthy sites.
*Statistical significant difference (p<0.05)

Table 10 presents the clinical thresholds of different types of bacteria in periodontitis sites between subjects with PST positive and PST negative. The counts for each bacteria species and percentages out of PST negative or PST positive are presented. Chi-square tests were conducted to compare the clinical thresholds of *C.s,* and *T.f* between PST negative and PST positive in periodontitis sites. Fisher Exact tests were conducted to compare the clinical thresholds of the rest of the bacteria species (*A.a, C.r, E.c, E.n, F.n, P.g, P.i, P.m, T.d*) between PST negative and PST positive subjects in periodontitis sites. The clinical threshold of only the bacteria species *F.n* was found to be significant different in periodontitis sites between PST positive and PST negative patients (p=0.014).

Table 10: Clinical thresholds of different types of bacteria in periodontitis sites between subjects with PST positive and PST negative.

Periodontitis sites

	PST negative n=19	**PST positive n=13**	**Statistical test p-value**
***A.a* low**	4 (21.05)	3 (23.08)	p=0.697$^{\alpha}$
***A.a* high**	2 (10.53)	0 (0.00)	
***C.r* low**	12 (63.16)	5 (38.46)	p=0.183$^{\alpha}$
***C.r* high**	3 (15.79)	6 (46.15)	
***C.s* low**	8 (42.11)	8 (61.54)	p=0.280$^{\beta}$
***C.s* high**	0 (0.00)	0 (0.00)	
***E.c* low**	10 (52.63)	8 (61.54)	p=1.00$^{\alpha}$
***E.c* high**	1 (5.26)	0 (0.00)	
***E.n* low**	6 (31.58)	1 (7.69)	p=0.219$^{\alpha}$
***E.n* high**	9 (47.37)	10(76.92)	
***F.n* low**	2 (10.53)	1 (7.69)	p=0.014$^{\alpha *}$
***F.n* high**	4 (21.05)	9 (69.23)	
***P.g* low**	4 (21.05)	3 (23.08)	p=0.222$^{\alpha}$
***P.g* high**	5 (26.32)	7(53.85)	
***P.i* low**	11 (57.89)	7(53.85)	p=0.748$^{\alpha}$
***P.i* high**	1 (5.26)	2 (15.38)	
***P.m* low**	5 (26.32)	5 (38.46)	p=0.735$^{\alpha}$
***P.m* high**	10 (52.63)	5 (38.46)	
***T.d* low**	2 (10.53)	0 (0.00)	p=0.447$^{\alpha}$
***T.d* high**	9 (47.37)	9 (69.23)	
***T.f* low**	9 (47.37)	4 (30.77)	p=0.348$^{\beta}$
***T.f* high**	10 (52.63)	9 (69.23)	

Counts and percentages out of PST negative or PST positive are given.
Chi-square tests $^{\beta}$ and Fisher Exact tests $^{\alpha}$ were conducted to compare the clinical threshold of bacteria between PST negative and PST positive patients in periodontitis sites.
*Statistical significant difference ($p<0.05$)

Data for GCF results

Using both subjective tests (histograms and Q-Q plots) and objective tests (Kolmogorov test) for normality, the GCF variables were not normally distributed, and therefore non-parametric tests were used for the variables' comparisons.

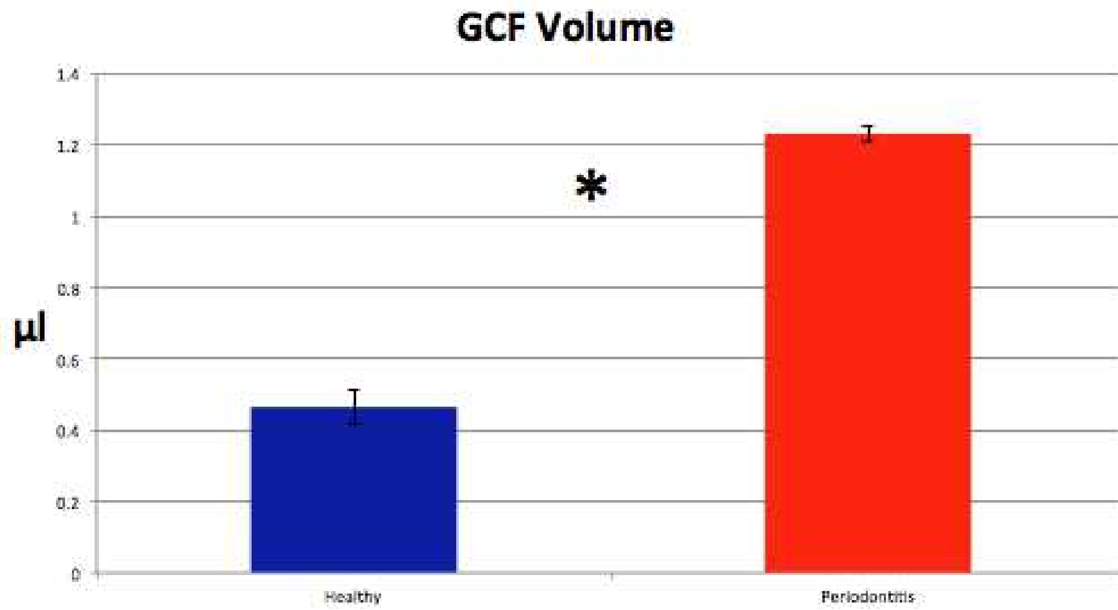
Table 11 presents the volume of GCF, the amount and concentration of IL-1β in GCF in healthy and periodontitis sites in overall population. Since those three GCF variables were not normally distributed, a non-parametric test Wilcoxon Signed Rank Test (paired data) was used to assess the difference between groups for GCF variables. It is important to mention that IL-1β was detected with ELISA as described before in 100% of samples of GCF from both healthy and periodontitis sites. Significant differences were found for the amount of IL-1β in GCF as well as the volume of GCF ($p<0.001$), while there was no significant difference in the concentration of IL-1β in GCF between healthy and periodontitis sites. The volume of GCF and the amount of IL-1β in GCF in healthy and periodontitis sites in overall population are also summarized in Fig. 8 and Fig. 9, respectively.

Table 11: Volume of GCF, amount and concentration of IL-1β in GCF in healthy and periodontitis sites in overall population.

	Healthy	Periodontitis	Statistics p-value
GCF Amount mean (SD)	19.75 (18.78)	81.69 (94.64)	<0.001*
GCF Concentration (SD)	67.42 (92.26)	67.49 (76.67)	0.715
GCF Volume (SD)	0.46 (0.27)	1.23 (0.11)	<0.001*

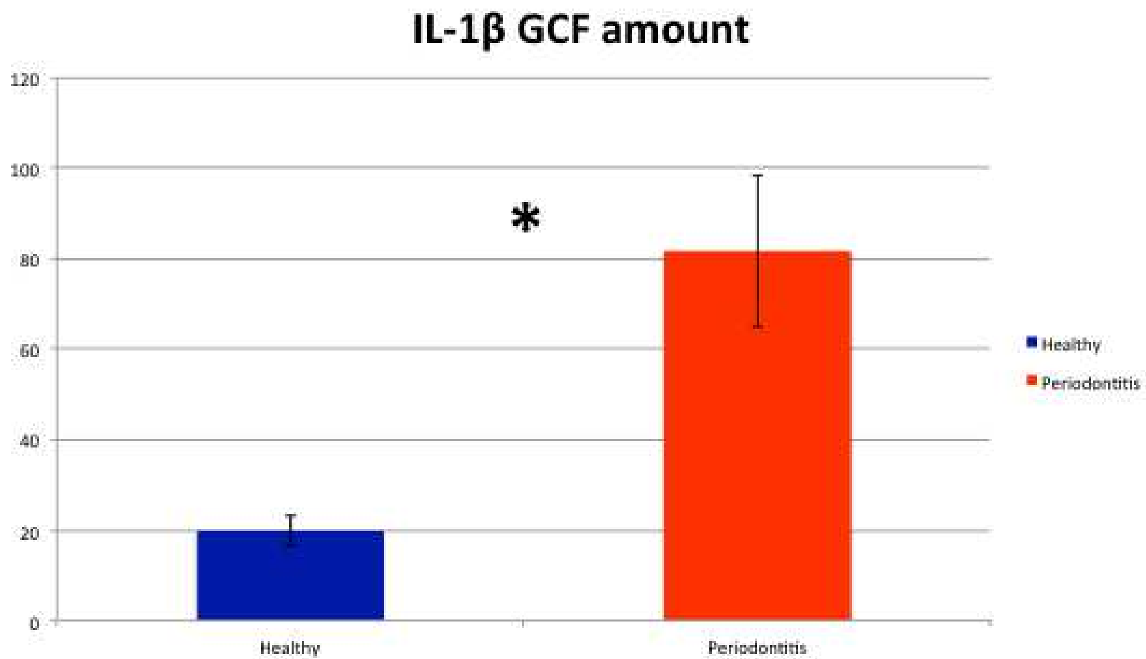
*Statistical significant difference ($p<0.05$)

Figure 8: Volume of GCF in healthy and periodontitis sites in overall population.



*Statistical significant difference ($p<0.05$)

Figure 9: Amount of IL-1β in GCF in healthy and periodontitis sites in overall population.



*Statistical significant difference ($p<0.05$)

Table 12 summarizes the volume of GCF, the amount and concentration of IL-1β in GCF in healthy sites between patients with PST positive and PST negative. Those three GCF variables were not normally distributed, thus the non-parametric test Mann-Whitney was used to assess the difference between groups for GCF variables. No significant differences were found.

Table 12: Volume of GCF, the amount and concentration of IL-1β in GCF in healthy sites between subjects with PST positive and PST negative.

	PST Negative	PST Positive	Statistics p-value
GCF Amount mean (SD)	18.15 (19.45)	22.10 (18.27)	0.565
GCF Concentration (SD)	52.77 (77.62)	88.83 (110.4)	0.250
GCF Volume (SD)	0.52 (0.27)	0.39 (0.26)	0.134

No statistical significant differences were found

Table 13 summarizes the volume of GCF, the amount and concentration of IL-1β in GCF in periodontitis sites between patients with PST positive and PST negative. Those three GCF variables were not normally distributed, thus the non-parametric test Mann-Whitney was used to assess the difference between groups for GCF variables. Statistical significant differences were found for the IL-1β amount (p=0.006) and concentration (p=0.005) in GCF.

The volume of GCF, the amount of IL-1β in GCF, and the concentration of IL-1β in GCF in healthy and periodontitis sites between the two groups are also summarized in Fig. 10, Fig. 11, and Fig. 12, respectively.

Table 13: Volume of GCF, the amount and concentration of IL-1β in GCF in periodontitis sites between subjects with PST positive and PST negative.

	PST Negative	PST Positive	Statistics p-value
GCF Amount mean (SD)	45.47 (33.60)	134.6 (127.8)	0.006*
GCF Concentration (SD)	36.37 (26.88)	113.0 (101.6)	0.005*
GCF Volume (SD)	1.25 (0)	1.20 (0.18)	0.252

*Statistical significant difference (p<0.05)

Figure 10: Volume of GCF in healthy and periodontitis sites in patients with PST positive and PST negative

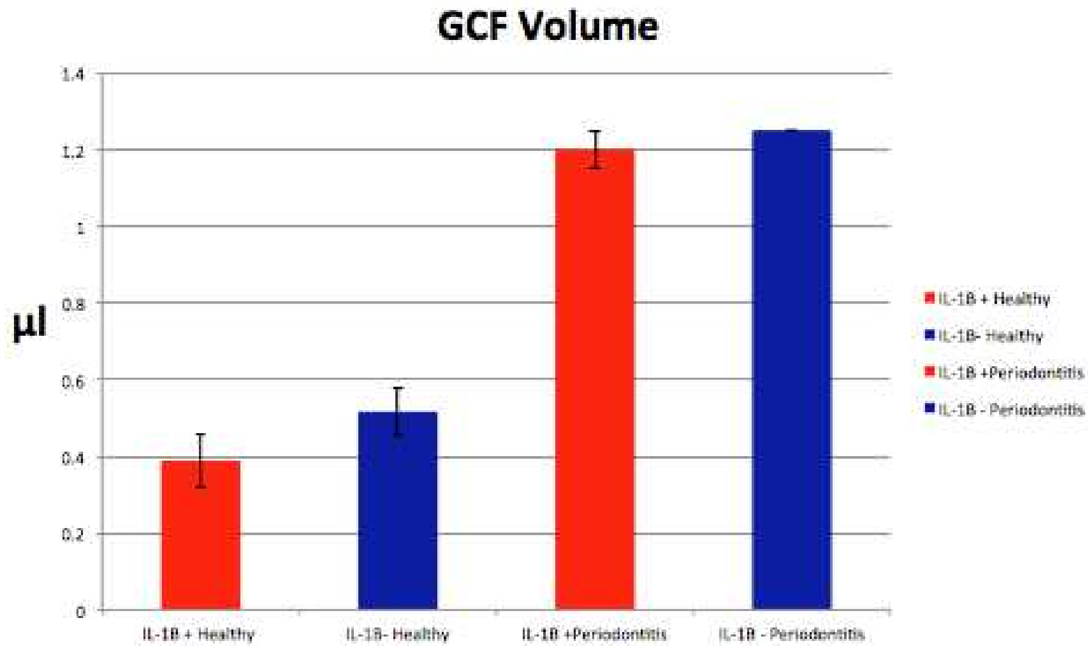
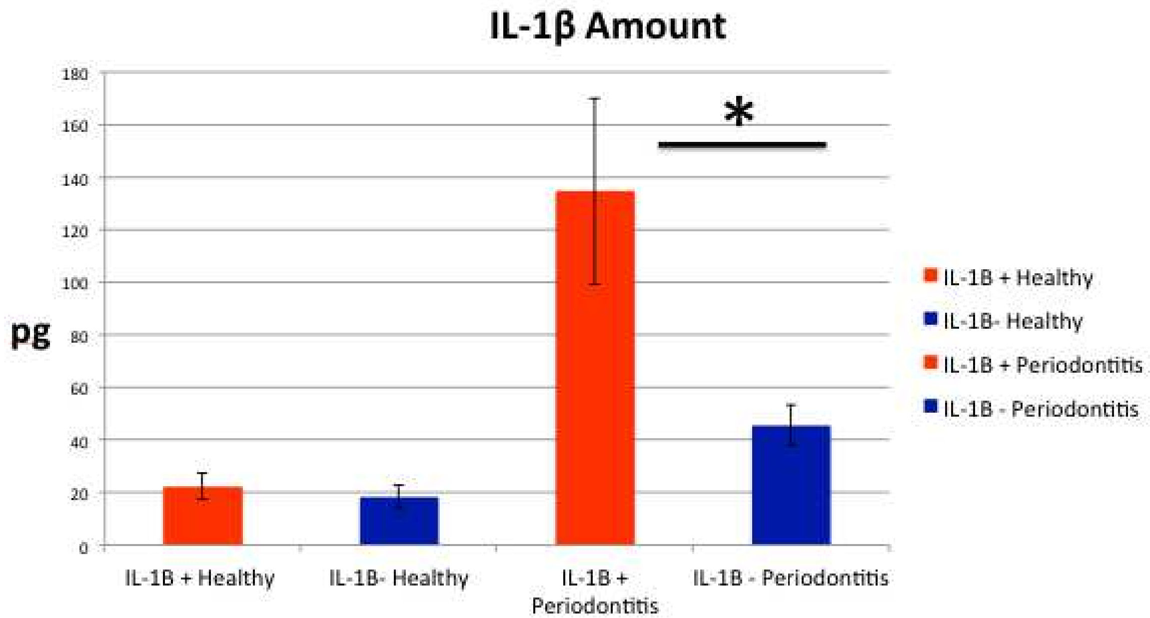
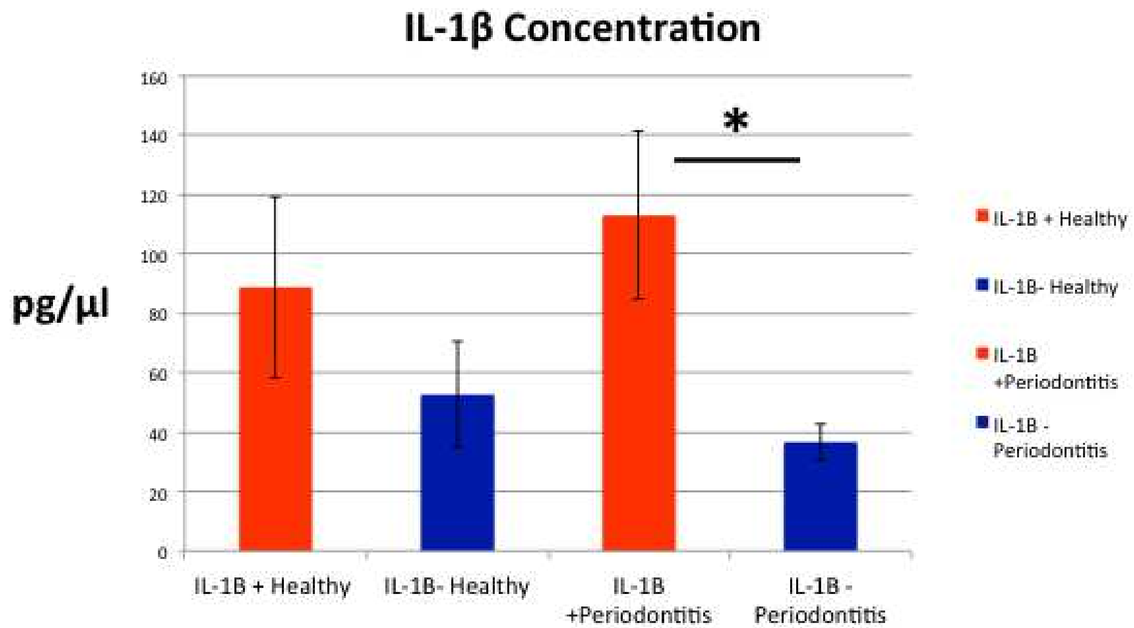


Figure 11: Amount of IL-1β in GCF in healthy and periodontitis sites between subjects with PST positive and PST negative.



*Statistical significant difference ($p<0.05$)

Figure 12: Presents the concentration of IL-1β in GCF in healthy and periodontitis sites between subjects with PST positive and PST negative.



*Statistical significant difference ($p<0.05$)

Table 14 summarizes the association between the GCF amount and GCF concentration of IL-1β in both healthy and periodontitis sites between patients with PST positive and PST negative. Regular chi-square test was used to compare those two variables. There is a statistical significant association between IL-1β amount and IL-1β concentration in GCF and *IL-1B* gene polymorphism. ($p<0.05$).

Table 14: Presents the association of IL-1β amount and IL-1β concentration in GCF and *IL-1B* gene polymorphism.

	PST negative	PST Positive	Statistics p-value
GCF Amount mean (SD)	31.81 (30.41)	78.36 (106.3)	0.045*
GCF Concentration (SD)	44.57 (57.89)	100.9 (104.5)	0.006*

*Statistical significant difference ($p<0.05$)

Tables 15 and 16 summarize the association between IL-1β GCF amount and IL-1β GCF concentration and the frequency of detection of *T.f*, *F.n*, and *P.g* in periodontitis sites, which were the most commonly detected periodontopathogenic bacteria that had significant differences between PST positive and PST negative subjects, as previously presented in Table 8. Mann-Whitney tests were used as none of the distributions for bacteria were normally distributed. The bacterial species *T.f* were all positive for the periodontitis sites, thus no analysis could be conducted. No statistical significant association was found for both IL-1β amount and IL-1β

concentration in GCF and the detection of *F.n*, and *P.g* in periodontitis sites ($p>0.05$).

Table 15: IL-1β amount and IL-1β concentration in GCF and their association with *F.n.* in periodontitis sites.

	F.n **Negative N=17**	***F.n*** **Positive N=15**	**Statistics p-value**
GCF Amount mean (SD)	51.94 (36.34)	115.4 (126.6)	0.113
GCF Concentration (SD)	41.55 (29.07)	96.89 (101.5)	0.097

No statistical significant differences were found

Table 16: IL-1β amount and IL-1β concentration in GCF and their association with *P.g.* in periodontitis sites.

	P.g **Negative N=14**	***P.g*** **Positive N=18**	**Statistics p-value**
GCF Amount mean (SD)	57.23 (55.44)	100.7 (114.5)	0.166
GCF Concentration (SD)	50.68 (50.10)	80.57 (91.58)	0.203

No statistical significant differences were found

Discussion

Periodontitis is an infectious disease, in which periodontal pathogens trigger chronic inflammatory immune responses that lead to destruction of tooth supporting tissues. Although the presence of microorganisms is the primary and crucial etiologic factor in periodontitis, host defense mechanisms within the periodontal tissues seem to be responsible for most of the tissue damage and for the progression and severity of periodontal diseases.[57]

The host immune response to bacterial challenge involves the secretion of pro-inflammatory cytokines, leading to extracellular matrix catabolism and bone resorption in periodontitis. IL-1β has been particularly studied as a critical determinant of tissue destruction due to its pro-inflammatory and bone resorptive properties.[16] The levels of the pro-inflammatory cytokine IL-1β are characteristically increased in diseased periodontal tissues and in gingival crevicular fluid around teeth affected with periodontitis and are thought to be a critical determinant of periodontitis outcome and the severity of periodontal disease.[17-19,51,52] In accordance, our data demonstrate significantly higher GCF volume and amount of IL-1β in GCF from periodontitis sites of our patients compared to their healthy sites.

Several risk factors, including genetic polymorphisms, smoking, diabetes mellitus and psychological stress, may modify the host response in periodontal tissues and affect the host's susceptibility to periodontitis. The *IL-1B* (3954) gene polymorphism has been also associated with increased IL-1β synthesis in different inflammatory conditions, including periodontal disease.[32, 33, 58]

In the present study we investigated the roles of *IL-1B* (3954) gene polymorphism and several bacterial pathogens in modulating the IL-1β levels in GCF in both healthy and periodontitis sites in patients with periodontitis. Our goal was to elucidate which parameter is more important (*IL-1B* gene polymorphism or bacterial pathogens) for patient's susceptibility to gingival inflammation and periodontal disease, due to different levels of IL-1β in GCF. The results of our study showed that *IL-1B* (3954) gene polymorphism is associated with higher IL-1β levels in GCF (amount and concentration) in healthy and periodontitis sites of patients with periodontitis. *IL-1B* (3954) gene polymorphism positive patients with periodontitis showed significantly higher IL-1β levels in GCF (amount and concentration) in periodontitis sites compared to *IL-1B* (3954) gene polymorphism negative ones. While IL-1B (3954) gene polymorphism

positive patients with periodontitis showed higher IL-1β levels in GCF (amount and concentration) in healthy sites compared to *IL-1B* (3954) gene polymorphism negative ones, no significant difference was observed.

The population of our study was diverse from different ethnic groups. The results revealed no significant differences in age, gender and ethnicity distribution between *IL-1B* (3954) gene polymorphism positive and *IL-1B* (3954) gene polymorphism negative patients. The prevalence of *IL-1B* (3954) gene polymorphism in our study population was 40.63% of the total number of participants, a prevalence that was consistent with ones from previously reported data.[36,41,54,59] The frequencies of C (71.87%) and T alleles (28.13%) in the total population (periodontitis patients) were found to be also similar to previously reported data.[36] It is important to mention that there were no significant differences in the clinical variables (PD, CAL, BOP, PI) between *IL-1B* (3954) gene polymorphism positive and *IL-1B* (3954) gene polymorphism negative patients. No significant differences in demographic and clinical variables between the two compared populations indicate that the two groups were random and comparisons on our primary outcomes (IL-1β GCF levels and bacterial profile) could be made, without any need for adjustment for the clinical variables, demographics or any other

possible confounding factors that could be controlled following strictly the inclusion/exclusion criteria of our study.

IL-1B gene polymorphisms may modulate the host response to the bacterial challenge and influence host's susceptibility to periodontitis. Inconclusive data on the role of *IL-1B* gene polymorphism and the synthesis of IL-1β in periodontal tissues indicate the need for additional research.[33,52-56] Some of those studies were *in vitro* and they were mainly indicating the positive impact of *IL-1B* gene polymorphism on synthesis and production of IL-1β by either peripheral or oral polymorphonuclear leukocytes (PMN's). Limited data from clinical studies and inconsistent research design criteria exist in this topic. For instance, Engebretson *et al.* showed that the *IL-1* genotype influences the amount and concentration of IL-1β in GCF and that, patients with *IL-1* genotype positive are at increased risk for periodontitis.[37] However, it is worth of highlighting that in their study IL-1β levels were higher in the GCF of only shallow sites (<4 mm) in periodontitis patients that possessed the *IL-1B* (3954) gene polymorphism, while in deeper sites (≥4mm) there were no differences in IL-1β levels in GCF among *IL-1B* (3954) gene polymorphism positive and negative patients. In addition, although the mean concentration of IL-1β in tissue collected from *IL-1B*

genotype positive patients was 3.6 times higher than what was observed for the *IL-1B* genotype negative patients at baseline, no statistical significant differences were observed in mRNA expression of *IL-1B* in periodontal tissues.[37] However, they evaluated the effect of *IL-1B* genotype (composite genotype as previously described by Kornman *et al.*) on the expression levels of IL-1β in periodontal tissues and GCF, while in our study we evaluated the sole effect of *IL-1B* gene polymorphism on IL-1β levels in GCF.

Our findings on the positive effect of *IL-1B* gene polymorphism on IL-1β GCF levels are in accordance with data published by Fereirra *et al.,* who reported significantly higher IL-1β mRNA expression in diseased periodontal tissues of chronic periodontitis patients with *IL-1B* (3954) polymorphism.[36] On the other hand, Yücel *et al.* found no significant differences for IL-1β levels in GCF regarding *IL-1B* (3954) genotype in their studied population.[41] However, it is important to mention that although Yücel *et al.* used a similar kit for ELISA analysis to the one that we used in our study, they compared IL-1β levels from GCF samples that were collected from 6 maxillary sites per patient and were studied as pooled

sample, while in our study we analyzed and compared IL-1β levels from GCF samples from one periodontal site (site-specific analysis).

At the same time, the influence of *IL-1B* genotype on bacterial profile in periodontitis patients has been also investigated.[45,46] It has been indicated that composite genotype positive patients manifested higher levels of "red" and "orange" complex bacterial species than genotype negative patients.[45,46] Those studies, that evaluated the composite genotype instead, used also different techniques for bacterial detection, and the collected samples were pooled, which would not allow for a site – to – site comparison. It is important to mention that in those previous investigations the bacterial detection limit has been higher (10^5 counts),[45,46] while using the Oral DNA bacteria detection kit (MyPerioPath®) the bacterial detection limit was as low as 10^3 counts, which allowed to detect more periodontopathogens. Despite this fact, our data demonstrated no associations between the bacteria detection levels and the *IL-1B* (3954) gene polymorphism, except for *Tanarella forsythia* and *Fusobacterium nucleatum*, which showed a higher tendency to be detected in periodontitis patients with *IL-1B* (3954) gene polymorphism positive compared to those with *IL-1B* (3954) gene polymorphism negative. Furthermore, the present study did not demonstrate

any associations between the bacteria clinical threshold levels and the *IL-1B* (3954) gene polymorphism status. Supporting our findings, Fereirra *et al.* found that the different *IL-1B* (3954) genotypes were not associated with statistical differences in the frequency of detection of red complex (*P. gingivalis, T. forsythia,* and *T. denticola)* periodontal pathogens in periodontal pockets in chronic periodontitis patients.

While bacteria are considered the primary etiologic agents for periodontal disease to occur, several periodontal pathogens and their virulence factors may also contribute to increased levels of several cytokines, including IL-1β, in GCF, that could modify further the severity of periodontal destruction.[39,45-48] Generalized aggressive periodontitis patients had statistically significantly higher GCF levels of IL-1β and IL-8 and higher proportions of orange and red complex species than periodontally healthy subjects, indicating distinct associations of different microbial complexes with GCF biomarkers.[39] In our study, no significant associations between the detection frequencies of periodontal pathogens, and especially *Tanarella forsythia* and *Fusobacterium nucleatum*, which were found statistically higher in *IL-1B* (3954) gene polymorphism positive patients- and the IL-1β levels in GCF. Our data differ from Fereirra *et al.* who previously published

that in chronic periodontitis patients, *P. gingivalis, T. forsythia,* and *T. denticola* detection was associated with significantly higher IL-1β mRNA expression of periodontal tissue biopsies. However, these differences could be attributed to different research design between two studies, but also due to the fact that probing depths were different among groups that were compared, and Fereirra *et al.* speculated that the increased IL-1β levels could be a consequence of deeper probing depths of tested sites and not of the presence of specific bacteria. However, in our study, no significant differences in clinical variables of the tested sites were observed in compared groups and there was no need for adjustment for any of the clinical variables.

The concomitant influence of periodontal pathogens and genetic polymorphisms on levels of IL-1β in GCF, has been mainly investigated separately though, and consequently, their exact individual and/or combined contributions to the determination of IL-1β levels remain unidentified. Only one study performed such comparisons, but with only four periodontal pathogens.[36] Furthermore, no investigation has been performed yet, to the author's knowledge, on a site-specific basis. Therefore, we collected samples from two sites (healthy, periodontitis site) of the same patient,

diagnosed with periodontitis. Previous data report results from pooled samples, giving an overall estimation of the bacterial profile and IL-1β levels, without taking into consideration the factor "subject effect and their susceptibility to disease". Fereirra *et al.* investigated the concomitant association of only four periodontopathogens (*P.g, T.f, T.d, A.a)* on *IL-1B* genotype and IL-1β levels in periodontal tissues in a chronic periodontitis group and a control (healthy) group. They found that the detection of only one red complex bacterial species does not result in increased levels of IL-1β, while the presence of two or three red complex species (*P.g, T.f, T.d)* was found to be associated with increased IL-1β mRNA levels in diseased tissues. Additionally, they demonstrated that in the absence of *P.g, T.f, T.d* (analyzed individually or as a complex), the presence of the T allele (IL-1B genotype positive) was associated with higher IL-1β levels.[36] Similarly, in the presence of the red complex bacteria, the T allele was also associated with higher IL-1β levels, the simultaneous occurrence of these periodontopathogens and the polymorphic T allele being associated with the highest IL-1β levels. The presence of *A.a* was not found to modify the levels of IL-1β in the different genotype groups. Similarly, no associations between the bacteria detection levels and the *IL-1B* (3954) SNP, except for *Tanarella forsythia* and *Fusobacterium nucleatum*, which showed a higher tendency to

be detected in periodontitis patients with *IL-1B* (3954) SNP positive compared to those with *IL-1B* (3954) SNP negative. The absence of any association between the detection of several periodontal pathogens and the IL-1β levels in GCF leads us to the conclusion that the increased expression of IL- 1β in GCF in diseased sites was mainly due to the positive *IL-1B* gene polymorphism, and not due to different detection frequencies of periodontal pathogens.

Limitations

There were no statistical significant associations between the variables of IL-1 β in GCF and the *IL-1B* genotype in the healthy sites of our population, but the amount and concentration of IL-1β in GCF was higher in the patients with *IL-1B* PST positive. This may be due to our small sample size. Our study did not show any difference on the clinical variables and severity of periodontitis between the *IL-1B* gene polymorphism positive and *IL-1B* gene polymorphism negative groups. There were also no statistical significant differences in the bacteria detection between the examined groups, apart from 2 bacterial species. Both outcomes could be also attributed to the small sample size, but also since the primary outcome of our study was the effect

of *IL-1B* gene polymorphism on IL-1β GCF levels, the sample size was calculated according to this variable. For possible statistical differences in clinical variables and bacteria detection levels, a different and larger sample size may be necessary to evaluate this.

There were no statistical significant differences in the IL-1β concentration in GCF between healthy and diseased sites. The reason was that the volume of GCF in the diseased sites that was collected with Periopapers usually exceeded the volume range that is given when the Periotron values are calibrated using the software. This resulted to have the volume of 1.25 μl in almost all periodontitis sites (the maximum volume that can be obtained using the software calibration). Therefore, although the IL-1β amount in GCF was statistically significant higher in the periodontitis sites, when dividing these values with the obtained volume using the software for Periotron in order to obtain the respective concentration, no statistical significant differences were found.

This study demonstrated that *IL-1B* (3954) genetic polymorphisms are significant contributors to periodontitis. It was shown that increased expression of IL- 1β in GCF in periodontitis sites was mainly due to the

positive *IL-1B* gene polymorphism, and not due to different detection frequencies of periodontal pathogens. This finding supports the already established hypothesis that, while bacteria are undoubtedly the principal cause of the initial inflammatory lesion leading to periodontal disease, it is the host response, not the type of bacteria, which dictates the disease progression and clinical manifestation. The inflammation in the gingival tissues leads to micro-environmental changes within the sulcus/pocket, which in turn favor the growth of gram-negative bacteria. This modifies the state of dynamic equilibrium or host-microbial homeostasis, which is usually seen in healthy periodontal sites. Taking that into consideration, it is crucial to realize the important role of the host to the disruption of the homeostasis and the periodontal disease outcome. Understanding more the role of gene polymorphisms and their impact on host susceptibility to periodontitis could allow us to develop more effective diagnostic chair-side techniques, as well as preventive, and therapeutic strategies for the clinical management of periodontal disease.

Conclusions

i. Our study demonstrated that the *IL-1B* (3954) SNP is associated with higher IL-1β levels in GCF (amount and concentration) in healthy and periodontitis sites of patients with periodontitis, supporting previous studies that supported the role of *IL-1B* (3954) SNP in increasing host susceptibility to periodontitis.

ii. The prevalence of the *IL-1B* (3954) SNP was 40.63% of the subjects in whole population, confirming previous data of prevalence from other published studies.

iii. *Porphyromonas gingivalis, Tanarella forsythia,* and *Fusobacterium nucleatum* were the only species that showed a higher frequency of detection in periodontitis patients with *IL-1B* (3954) SNP positive compared to patients with *IL-1B* (3954) SNP negative.

iv. The results of the present study could not reveal any associations between the detection frequencies of periodontal pathogens, and especially *Tanarella forsythia* and *Fusobacterium nucleatum*, and the IL-1β levels in GCF.

v. The present study did not demonstrate any associations between the bacteria clinical threshold levels and the *IL-1B* (3954) SNP.

vi. Within our study's limitations, the absence of any association between the detection of several periodontal pathogens and the IL-1β levels in GCF leads us to the conclusion that the increased expression of IL- 1β in GCF in periodontitis sites was mainly due to the positive *IL-1B* gene polymorphism, and not due to different detection frequencies of periodontal pathogens.

vii. Further studies are needed to improve our knowledge on how *IL-1B* gene polymorphisms may affect the outcome (IL-1β levels in GCF/bacterial profile) of periodontal therapy in patients with different *IL-1B* gene polymorphism status.

viii. Understanding deeper the role of gene polymorphisms and their impact on host susceptibility to periodontitis may lead to more effective diagnostic, preventive, and therapeutic strategies for the clinical management of periodontal disease.

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