´´Levels of interferon-γ (IFN-γ), interleukin-4 (IL-4), interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP) in gingival crevicular fluid from patients with chronic periodontitis´´

Evangelos Papathanasiou, DDS

Thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

Tufts University School of Dental Medicine

Masters Committee Members

Terrence Griffin, DMD

Chairman/Director of Postdoctoral Periodontology,

Tufts University School of Dental Medicine

Theoharis C Theoharides, MS, PhD, MD, FAAAAI

Professor of Pharmacology, Internal Medicine and Biochemistry; Director, Molecular Immunopharmacology and Drug Discovery Laboratory,

Department of Molecular Physiology and Pharmacology,

Tufts University School of Medicine

Flavia Teles, D.D.S., M.S, D.M.Sc

Instructor in Oral Medicine, Infection and Immunity,

Harvard School of Dental Medicine
Assistant Member of the Staff, Department of Periodontology
The Forsyth Institute

Emilio Arguello, D.D.S., M.S

Clinical Director, Postgraduate Periodontology

Harvard University School of Dental Medicine

Matthew Finkelman, Ph.D,

Assistant Professor, Department of Research Administration

Tufts University School of Dental Medicine

Acknowledgement

To my father Sotirios, my mother Kyriaki, and my brother Anastasios, for their inexhaustible source of encouragement and constant support in my efforts to pursue all endeavors I have dreamed of.

To my mentor Dr Terrence Griffin for offering me the unique opportunity to complete my residency in Periodontology and to obtain a Master of Science degree at Tufts University School of Dental Medicine. Over these past years, Dr. Griffin has been exceptionally giving in his time and efforts to help me achieve professional excellence.

To my mentor Dr Theoharis Theoharides, in appreciation to all the trust and support towards me. His thirst for life-long learning and commitment to innovation and education always remind me how much further I have to go.

To my mentor Dr Flavia Teles, for her constant support, guidance and encouragement. Special thanks to Dr Teles, who has committed a lot of her valuable time to teach me and to constantly motivate me to broaden my knowledge and advance my skills.

To my teacher, Dr Matthew Finkelman, for making biostatistics very interesting to me and highlighting the *p*-value of setting, reaching and exceeding your goals.

To my mentor, Dr Emilio Arguello, for his fundamental contribution on establishing a solid foundation on this research project and continuously encouraging our ambitious collaboration between the department of Periodontology of TUSDM, the department of Periodontology of Forsyth Institute and the Department of Molecular Physiology and Pharmacology at Tufts University School of Medicine.

To all my friends for surrounding me with their constant love and support.

"If we knew what we were doing,
it would not be called research, would it?"

Albert Einstein

ABSTRACT

Background

The tissue destruction in periodontal diseases appears to result from a complex interplay between the pathogenic bacteria and the host's immune and inflammatory responses.

Numerous cytokines that play key roles in the pathophysiology of periodontitis can be identified and measured in gingival crevicular fluid (GCF). In the last 20 years, the Th1/Th2 paradigm has represented a useful framework for the investigation of the pathogenesis of periodontal diseases. Many studies have supported the hypothesis that the balance of the cytokines regulated by the Th1 or Th2 response would determine if an inflamed gingival lesion would remain stable or would progress.

Our goals are to provide additional information on the profile of Th1/Th2 responses in periodontal disease, by comparing the levels of interferon-γ (IFN-γ) and interleukin-4 (IL-4) in GCF, and to investigate the presence of novel cytokines, interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP), in periodontal tissues.

Materials & Methods

In this cross-sectional study, clinical data and GCF samples were collected from sites with different clinical characteristics (healthy, gingivitis and periodontitis sites) from periodontally healthy (n= 14), plaque-induced gingivitis (n=17) and chronic periodontitis (n=11) subjects.

GCF samples were analyzed for the presence and levels of IFN-γ, IL-4, IL-33, and TSLP using a multiplex bead immunoassay (Luminex).

Results

Inflamed sites in both plaque-induced gingivitis and chronic periodontitis patients showed statistically significantly higher volume of GCF compared to non-inflamed sites in all categories of patients.

IFN-γ could be detected in about 50% of the GCF samples, especially in inflamed sites in patients with plaque-induced gingivitis and chronic periodontitis.

A very low number of the GCF samples showed detectable levels for IL-4 and TSPL (4% and 3% of samples, respectively), while IL-33 was below the curve of detection for all samples collected.

Conclusions

The present study did not demonstrate major differences in the frequency of detection or amounts of IFN-γ, IL-4, IL-33 and TSLP in GCF from patients with chronic periodontitis compared to patients with gingivitis and periodontally healthy subjects.

Further studies are needed to clarify the relevance of IL-33 and TSLP in the pathogenesis of periodontitis.

Table of Contents

List of Tables and Figures

Tables

Figures

INTRODUCTION

Periodontal disease

The most common periodontal diseases (PD), namely plaque-induced gingivitis and chronic periodontitis, are infectious in nature and elicit an inflammatory response, clinically characterized by local redness, edema and bleeding on probing of periodontal tissues upon accumulation of bacterial plaque. If left untreated, destruction of the periodontal attachment apparatus may occur, which can lead to tooth mobility and tooth loss. [1]

Dental bacterial biofilms have been shown to be the primary aetiological factor of periodontal diseases. [2, 3] It has been shown recently that the gingival crevice and the periodontal pocket may harbor several hundred different bacterial taxa. [4] Studies that investigated the periodontal microbiota have demonstrated that they are comprised by many different species. [5-9] Therefore, periodontal disease has often been referred as a ¨mixed bacterial infection¨. [10] Most of those taxa seem to be host-compatible species and only a subset of that consortium may contribute directly to the pathogenesis of periodontal disease. [5]

Interestingly, the presence of pathogens *per se* is required, but not sufficient for disease initiation. [11] Classical experiments have demonstrated that inadequate oral hygiene and subsequent accumulation of bacterial plaque triggers host inflammatory responses in associated gingival tissues, [12] which play a major role in the pathological alterations and ultimately destruction of periodontal structures.

In the mid-1960s, animal and human studies demonstrated the critical role of bacteria in the initiation of gingivitis and periodontitis, leading to the ¨bacterial¨ concept of pathogenesis, i.e., bacteria cause periodontal disease. [12, 13] This model that proposed bacterial plaque as the primary causative factor of periodontal destruction predominated until the mid-1970s, and changed the approaches for prevention and treatment of periodontal diseases. [14] Further studies broadened the knowledge on the concept of bacterial plaque and led to the observations that, on a ¨patient-level¨ analysis, not all individuals were equally susceptible to periodontal disease. Around late 1970s, a major shift in periodontal research occurred, from pocket elimination therapy *per se* to an investigation of cells, mediators and enzymes, involving also the host response in the progression of periodontitis. [15]

Further microbiological studies showed that the pathogenesis of periodontal disease is mediated by an overgrowth of specific Gram-negative anaerobic bacteria in the gingival sulcus. [16, 17] *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*, collectively known as the *red complex* [5] were identified as major players in the initiation and progression of periodontitis. [3] In fact, in 1996, *P.gingivalis* and *T. forsythia* were classified as periodontal pathogens by the American Academy of Periodontology, due to the extensive body of evidence available. [18] However, the major component of soft- and hard- tissue destruction associated with periodontal disease is the result of activation of the host's immune-inflammatory response to the bacterial challenge. [19] Over the past two decades, there has been intense interest to investigate the molecular mechanisms of the host response underlying the periodontal tissue destruction and identify several host factors as possible diagnostic and therapeutic targets for the management of the periodontal inflammation.

Host response

The tissue destruction in periodontal disease appears to result from a complex interplay between the pathogenic bacteria and the host's immune and inflammatory responses. 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. [10] The accumulation of bacterial plaque in the gingival crevice triggers the host's immune responses, which are divided into *innate* and *adaptive* responses.

Innate immune response

The *innate immune system* involves non-specific responses, that require no prior exposure to the stimulus, aiming to provide immediate protection against infection. Early reactions to the bacterial challenge include secretion of vasoactive substances, like histamine and vascular endothelial growth factor (VEGF), mainly produced by mast cells in the periodontium, [20] which increase the vascular permeability in gingival tissues. Vasodilation enhances the recruitment of phagocytic cells, like polymorphonuclear cells (PMNs), monocytes and macrophages. They possess a number of general, non-specific antimicrobial peptides, proteins and enzymes against many different pathogens. In response to a microbial challenge, the periodontal tissues are infiltrated by many phagocytic cells, which amplify a cascade of cellular and biochemical events in the periodontal tissues by secreting different inflammatory mediators, like cytokines, chemokines and prostaglandins. [21]

One of the most critical pathways in the initiation of the immune response is the recognition of the microbe-associated molecular patterns (MAMPs) by the pattern recognition

receptors (PRRs) of the host cells. Toll-like receptors (TLRs) have been identified as a family of mammalian homologs of Drosophila Tollis, [22] expressed predominantly in cells which mediate the first-line defense, such as neutrophils, monocytes/macrophages, dendritic cells and epithelial cells. [10] The most studied pathway is the recognition of lipopolysaccharides (LPS), derived from Gram-negative bacteria, by a macromolecular complex, involving CD-14, MD-2 and TLR-4. After TLR activation, an intracellular signaling cascade is stimulated, leading to the translocation of the transcription factor NF-kB and consequent synthesis of pro-inflammatory mediators, including tumor necrosis factor-α (TNF-α) and interelukin-1β (IL-1β), [22, 23] leukocyte migration, and osteoclastogenesis. [24]

The pro-inflammatory mediators produced by the first-line defense cells stimulate the expression of cell adhesion molecules, like endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ILAM-1). The expression of these molecules enhance the ‘‘diapedesis’’ and establish a fast flow of PMNs through the junctional epithelium and into the gingival sulcus, [25] making them the predominant leukocyte within the gingival crevice/pocket both in health and disease. Once in the gingival sulcus, the PMNs either phagocytose the bacterial cells present in the area, accomplishing their effector function or die by apoptosis. In both cases, they release their lysosomal contents into the gingival sulcus. These contents, which include enzymes such as elastases and collagenases, can contribute to the local destruction of connective tissues. [26] In addition, cells from the innate immune response, such as neutrophils, monocytes and macrophages may produce cytokines that can compromise periodontal tissue integrity and interfere with the coupling mechanism of bone formation. [27] In fact, previous studies have shown that pro-inflammatory cytokines inhibit osteogenic

differentiation, [28] and activation of TLRs in osteoblasts induces the production of osteoclastogenic cytokines. [29]

Antigen-presenting cells (APCs)

Antigen-presenting cells (APCs) play a crucial role in the initiation and maintenance of inflammation in periodontal diseases. Langerhans cells, macrophages and dendritic cells are professional APCs and contribute to antigen recognition and early response mechanisms in host defense. [10, 30] The release of pro-inflammatory mediators induces the Langerhans cells, which are dendritic cells located in the epithelia, to migrate to regional lymph nodes, [31] and initiate antigen-specific T-cell proliferation. [32] The presentation of the processed antigen involves interactions with specific receptors on the T-cells: T-cell receptors (TCRs), which recognize the antigen in association with the major histocompatibility complex (MHC I or MHC II) molecules on the surface of APCs. [30]

Bacteria are necessary for both initiation and progression of periodontitis. They play a major role in the disease by constantly shaping the T-cell response through differential TLR-mediated activation of APCs and secreted cytokines. The innate and adaptive host response, in turn, determines the fate of the infection, and consequently whether inflammation will progress to disease or be controlled. [33]

Adaptive immune response

The *adaptive immune response*, which is a second line of defense, aims to improve the host's ability to recognize and respond against the pathogen. The adaptive response utilizes strategies of recognition, memory, and binding to support the immune systems in the elimination of the causative factors. [10] Whereas innate immunity comprises macrophages,

dendritic cells, neutrophils, monocytes/macrophages and epithelial cells that identify and respond temporarily to MAMPs; the adaptive immune response relies on T- and B-cell recognition of specific antigen structures, resulting in immune responses which are highly specific and sustained, synchronized by the communication through signals, the cytokines, between specific groups of cells. [23] Antibodies are produced by plasma cells and aim to protect the host by enhancing the phagocytosis of the antigens by the PMNs and the macrophages, a process known as opsonization. However, the antibodies' actions might be protective or further destructive, pending on their ability to eliminate the causative organisms. [34]

Cytokines

Cytokines are soluble proteins that bind to specific receptors on target cells and initiate intra-cellular signaling cascades resulting in phenotypic changes in the cell via altered gene regulation. They are effective at low concentrations, are produced transiently in the tissues and primarily act locally. This is documented by the fact that cytokine-producing cells are often physically located immediately adjacent to the responding cells. It is becoming increasingly clear, however, that cytokines do not function in isolation, but rather in complex networks involving both pro- and anti-inflammatory effects. [23]

Numerous cytokines play key roles in the pathophysiology of periodontitis. The strongest evidence for cytokines functioning in networks exists for interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), IL-6, Receptor activator of nuclear factor κappa-B ligand (RANKL) and matrix metalloproteinases (MMPs). [35] Regarding the control of cytokine levels, it has been recognized that under normal homeostasis, there is a degree of balance between the pro- and anti-inflammatory activity, with specific T-cell cytokines, receptor antagonists, such as

interleukin-1 receptor antagonist (IL-1Ra), inhibitory soluble forms of IL-1 and TNF-α receptors, and the tissue inhibitors of metalloproteinases (TIMPs), all having some form of protective effect. The balance between pro- and anti-inflammatory cytokines and regulation of their receptors and signaling pathways will determine the extent of periodontal tissue destruction. [23]

Immunoregulation

The link between the innate and adaptive immunity, as previously mentioned, is created by the APCs. Mature dendritic cells express co-stimulatory molecules and produce distinct patterns of cytokines that will determine the subsequent polarization and activation of antigen-specific lymphocytes. [36] The nature of the adaptive immune response is dependent on a complex interplay between various immunological networks. [26] The immune response polarization involves the selective migration of CD4 T-helper subsets and the subsequent production of characteristic cytokines at the areas of response. [37] The adaptive immune response is under the control of those T-helper subsets, in that they will either stimulate the cell-mediated immunity or activate the differentiation of B-cells and plasma cells and antibody production.

Th1/Th2 concept

In the last 20 years, the Th1/Th2 paradigm has represented a useful framework for the investigation of the pathogenesis of periodontal diseases. As a general rule, immune responses mediated by T-cells polarized into a Th1 phenotype are characteristically cellular and pro-inflammatory, while Th2 cells are associated with humoral immunity and anti-inflammatory properties. [37] However, recent studies describing two new well-defined CD4 T-cell subsets, the

Th17 cells, which may amplify the pro-inflammatory responses, and the regulatory T-cells (Tregs), which suppress immune reactions, [38] raised new questions on the potential of the Th1/Th2 concept to answer accurately the pathogenesis of periodontal diseases. The development and the regulation of an immune response depends to a large extent on the local cytokines milieu, which can determine whether the response will be a protective or a destructive one. [34]

CD4 T-cells were initially subdivided into two subsets, named Th1 and Th2 cells, on the basis of their pattern of cytokine production. [37, 39] Th1 cells, by mainly secreting IFN-γ and IL-2, promote cell-mediated immunity, by activating macrophages, natural killers cells (NK) and cytotoxic CD8 T-cells. On the other hand, Th2 cells release IL-4, IL-5, IL-10, IL-13 and regulate humoral (antibody-mediated) immunity. [40]

IL-12 and IL-18 have been also associated with periodontal tissue destruction. [41] A strong innate immune response results in large amounts of IL-12 and IL-18 production by macrophages and neutrophils. IL-18 will then enhance the differentiation of naive T-cells into Th1 and the secretion of IFN-γ, which will further improve the phagocytic activity of both neutrophils and macrophages and will lead to the control of the infection. [42] However, if the innate response is poor, low levels of IL-12 will be secreted and a poor Th-1 response might not be able to eliminate the infection. [34] At that point, IL-18 can stimulate the production of IL-4 and IL-13 by NK cells, mast cells and basophils, encouraging further a Th2 response, B-cell activation and antibody secretion. [42]

In early studies, histopathology demonstrated that the periodontitis lesions (advanced lesion) are dominated by B-cells and plasma cells, while the gingivitis lesions (established

lesion) are mainly characterized by the infiltrate of T-cells. [43] More specifically, two types of established lesion appear to exist: one that remains stable without any progression, and a second that becomes more active and progresses into the advanced destructive lesion. Recent studies have shown that the established lesions that progress are those that exhibit an increased infiltrate of B-cells and plasma cells (53%), compared to reduced existence of plasma cells (31%) in the lesion that remains stable. [44] These findings led to the concept that the establishment of periodontitis involves a switch from a gingival lesion infiltrated by T-cells to one that is dominated by a large number of B-cells and plasma cells. [26] Many studies supported the hypothesis that the balance of the cytokines regulated by the Th1 or Th2 response would determine if an inflamed gingival lesion would remain stable or would progress. [34]

Gingival Crevicular Fluid (GCF) and its potential diagnostic value

At present, periodontal diagnosis and treatment plan is based on an accurate and comprehensive clinical periodontal examination. This assessment includes probing depths, clinical attachment levels, plaque index, gingival index, bleeding on probing, suppuration, furcation involvement, mobility and radiographic findings. The collection of all these clinical data represents the clinician's attempt to define the periodontal diagnosis and prognosis at a patient level, as well as at a tooth and site levels. However, these clinical parameters are not sufficiently sensitive and specific to identify disease activity in individual sites or to predict future attachment loss. [45] Therefore, there is a need for the development of diagnostic tools that could screen the susceptible subjects in the general population, differentiate the active from inactive inflamed sites and predict future tissue destruction. [46] Oral fluids, like saliva or gingival crevicular fluid (GCF), have gained great interest on their possible diagnostic value in periodontal disease. They contain a large number of proteins and peptides derived from

inflamed host tissues. The analysis of their contents can reflect the disease status of individual sites and thus identify potential biomarkers of periodontitis. [47]

GCF can be easily collected from the gingival sulcus surrounding the teeth. GCF exists as a serum transudate or an inflammatory exudate, originating from the gingival plexus of blood vessels in the connective tissue, close to the epithelium lining of the dentogingival space. [46, 48] GCF is mainly composed by serum and contains tissue breakdown products, inflammatory mediators, antibodies, inflammatory cells, bacteria, epithelial cells and keratinocytes. [49] The potential diagnostic value of GCF has been recognized since early 1970s, when different host enzymes and inflammatory mediators were identified in GCF. [50] The amount of GCF increases with the severity of inflammation and is directly associated with the increased vascular permeability and ulceration of the pocket epithelium at inflamed sites. [51] However, no studies have indicated that increased volume of GCF is related to the risk for periodontal tissue destruction. [52]

Since GCF can reflect tissue and serum concentrations of inflammatory mediators very precisely, [49] 90 different components have been evaluated as diagnostic biomarkers for the identification of periodontal disease. [53] These markers can be divided into three major groups: host-derived enzymes and their inhibitors, inflammatory mediators and host-response modifiers, and tissue breakdown products. [49] The predominance of host enzymes and inflammatory mediators in the GCF clearly emphasizes the fundamental role of the host response in the pathogenesis of periodontal diseases. Since periodontal disease is a non-continuous process, characterized by cyclic periods of exacerbation and remission, [54] it is critical to define stable and progressing inflamed sites for the management of periodontal disease. Based on the concept that the stable lesion is characterized by a Th1 response and a

progressive lesion by a Th2 response, [34] many studies have attempted to elucidate the Th1/Th2 cytokine profile in GCF and to correlate it with disease activity. [34, 55]

A number of studies have attempted to identify disease-associated cytokine profiles, both in saliva, [56] and GCF. [57-59] Ultimately, such cytokine signatures would foster the understanding of the mechanisms involved in the pathogenesis of periodontitis as well as provide a model for early detection of periodontal tissue breakdown. In that scenario, the use of GCF offers a number of advantages in comparison with saliva, including the fact that samples are collected from individual sites. This is important because periodontal diseases are site-specific, hence, a GCF sample from a given site is truly representative of the disease status associated with that site.

In the present research project, cytokine levels were determined by multiplexed bead immunoassay technology rather than enzyme-linked immunosorbant assay (ELISA) technique. Although ELISA is relatively specific and sensitive, it precludes the systematic examination of multiple mediators in a small sample of biological material, such as GCF. [60] The multiplex bead technology utilizing flow cytometry was preferred in our study, since it enables the simultaneous quantitative assessment of several analytes in very small samples (less than 1μl). [61] This significant advantage has made the multiplexed sandwich immunoassay based technology the technique of choice in the most recent studies on inflammatory mediators. [59, 62, 63] Many inflammatory mediators that are involved in periodontal tissue destruction, including interleukins and metalloproteinases, can be identified and measured in GCF, [53, 62, 63] like IL-1β, IL-6, IL-12, IL-18, MMP-8. [41, 63, 64] In addition, many Th1/Th2 cytokines can also be identified in GCF, like IFN-γ, IL-2, IL-4 and IL-10. [55]

Interferon-gamma (IFN-γ)

IFN-γ is the signature cytokine of Th-1 type responses, playing a critical role in the innate cell-mediated immunity. [24, 65] IFN-γ is one of the key cytokines that stimulates the phagocytic activity of the host cells, amplifying the production of pro-inflammatory mediators and chemokines against the periodontal pathogens. [24, 37, 65] IFN-γ production is mainly controlled by the cytokine milieu of the inflamed area, and especially by the presence of IL-12 and IL-18, produced by the APCs. [42, 65] The recognition of the pathogens by macrophages and other APCs initially induces the release of different pro-inflammatory mediators, like IL-12, and chemokines, such as macrophage-inflammatory protein -1α (MIP-1α), that stimulate the recruitment of NK cells. [66]

IFN-γ, initially released by NK cells, [67] promotes the cell-mediated immunity by influencing naive CD4+ cell differentiation towards a Th1 phenotype. [24, 65]Moreover, IFN-γ upregulates the major histocompatibility complex (MHC I or MHC II) molecules on the surface of APCs, [68] which contribute in the increased production of IFN-γ. [65] IFN-γ enhances the ‘‘diapedesis’’ of more innate immune cells in the inflamed area by increasing the expression of adhesion molecules and chemokines. [65, 68] IFN-γ is a remarkable cytokine that acts as a link between the innate immune and the T-cell mediated response, by orchestrating the leukocyte attraction and enhancing the phagocytic activity of macrophages. [24, 65]

Many studies have shown that IFN-γ is detected in high levels in inflamed periodontal tissues, highlighting the major role that this molecule plays in the development of periodontal inflammation. [24, 69] Based on the Th1/Th2 concept of pathogenesis of periodontal diseases, the role of IFN-γ in progressing lesions and periodontal destruction has been controversial. It has

been proposed that a strong innate immune response, mainly characterized by high levels of IL-12, will lead to an effective Th1-mediated response, regulated by increased levels of IFN-γ, resulting in a stable periodontal lesion. [34, 70] In vitro studies support also this hypothesis, since they have shown that IFN-γ strongly suppresses osteoclastogenesis, by interfering with the RANKL-RANK signaling pathway and providing a negative link between T-cell activation and bone resorption. [71]

On the other hand, more recent *in vivo* studies seem to support that IFN-γ promotes bone resorption by stimulating antigen-dependent T cell activation and T cell secretion of the osteoclastogenic factors RANKL and TNF-alpha. [72] Moreover, a positive co-expression relationship and interactions between IFN-γ and RANKL-mediated osteoclastogenesis have been clearly demonstrated in a mouse periodontitis model. [73] In addition, studies have also shown that the concentration of IFN-γ was higher in serum samples and gingival tissue biopsies from periodontitis patients than from gingivitis patients and healthy controls. [69, 74] Furthermore, the total amount of IFN-γ in the GCF of active sites in patients exhibiting progression of periodontitis was significantly higher than in inactive sites. [75]

Interleukin-4 (IL-4)

IL-4 is the key cytokine for the growth and proliferation of B-cells, promoting the humoral immunity against the pathogens. [76] IL-4 is one of the major cytokines produced by Th2 cells, which activates B-cells and antibody production, but also suppresses the cell-mediated immunity. [34] Except for Th2 cells, IL-4 is also secreted by macrophages, monocytes, mast cells, basophils, fibroblasts and endothelial cells. [77] IL-4 plays an important role in the regulation of the immune response in inflammatory diseases, especially due to the marked anti-inflammatory

properties that it demonstrates. [34] IL-4 has been shown to have opposite effects to IFN-γ, since IL-4 suppresses the abilities of LPS-stimulated monocytes/macrophages to secrete prostaglandin E_2 (PGE_2) and to produce IL-1 and TNF-α. [78] In addition, IL-4 is able to inhibit the production of MMPs and RANKL in the periodontium, [79] suggesting a protective role against periodontal inflammation.

Cytokines drive tissue destruction through their impact on lymphocytes and subsequent T-cell subsets differentiation. The role of IL-4 in periodontal destruction has been debated in the literature. Based on the concept of Th1/Th2 cells, the dominance of B cells/plasma cells in the progressive periodontal lesions reflects the role of Th2 cells in periodontal inflammation. If the innate immune response against the periodontal pathogen is poor, low levels of IL-12 are produced. [34] In the absence of IL-12, IL-18 induces the production of IL-4 and other Th2 cytokines, which will further activate B-cells and the production of antibodies, contributing to progress of periodontal disease. [42] However, many studies indicate a protective role of IL-4 against the periodontal pathogens. Localized absence of IL-4 was proposed to be associated with progression of periodontal inflammation and it was assumed that absence of IL-4 might trigger the periodontal destruction. [80] Further studies confirmed that hypothesis, since they showed that there is an inverse relationship between the levels of IL-4 in GCF and periodontal disease activity. [81] Moreover, the concentration of IL-4 has been shown to decrease from periodontal health to disease, suggesting that this Th2 cytokine is more associated with the remission of periodontal disease. Since several studies demonstrated higher levels of IFN-γ in GCF than IL-4 in areas of periodontal destruction, it was recommended that the ratio of IL-4/ IFN-γ could be a useful indicator of periodontal disease activity. [55, 82]

Interleukin-33 (IL-33)

IL-33 is a newly described member of the IL-1 family, first reported in 2005, and initially described as a cytokine with a major role in creating pathological changes in mucosal organs. [83] IL-33 was identified as the ligand for the then "orphan" receptor ST2, [83] which was first reported in 1989 as a member of the IL-1 receptor (IL-1R) family, that belongs to the Toll-like receptor (TLR) superfamily. [84] Since 1998, when ST2 receptor was reported that it is expressed on the cell surface of Th2 cells and not of Th1 cells, [85] the interest of investigating the role of ST2 in immunology was further encouraged.

IL-33 is widely expressed in several tissues and cell types including smooth muscle cells, epithelial cells, fibroblasts, keratinocytes, dendritic cells and activated macrophages. [86] IL-33 seems to act more as an immunoregulatory than a pro-inflammatory cytokine, [87] since it enhances the secretion of IL-4, -5, -13 by Th2 cells. [83] Moreover, by acting on cells of the innate immune response, like mast cells and basophils, IL-33 plays a major role in the trafficking of T-cells in an inflamed site. [88] IL-33 can exacerbate the Th2 response by stimulating DCs to produce IL-5, IL-6 and IL-13 by $CD4^+$ T cells, [89] suggesting a new pathway between DCs and Th2 cells activation.

Binding of IL-33 to ST2 receptor activates a cascade of signaling events leading to the activation of NF-kappaB and several kinases (MAPKs). Through this mechanism, IL-33 has been found to stimulate the production of pro-inflammatory mediators from mast cells, like TNF-a, IL-1β, IL-6, IL-8 and VEGF, suggesting a central role for IL-33 and mast cells in the inflammatory processes. [90, 91] On the other hand, depending on the cytokine milieu, IL-33 has been reported to induce, in combination with IL-12, an increase in IFN-γ production in NK

cells *in vivo*. [92] Moreover, observations in mice showed that IL-33 is a crucial amplifier of innate, rather than acquired, immune responses. [93] This finding of a possible dual role for IL-33 adds to the complexity of the regulation of lymphoid cells in the inflamed site.

Thymic stromal lymphopoietin (TSLP)

Thymic stromal lymphopoietin (TSLP) has also been shown to be highly involved in the pathogenesis of inflammatory diseases, like asthma, atopic dermatitis and allergic rhinitis. [94] TSLP is an IL-7-like cytokine molecule that was first cloned in 2001. [95] While TSLP was initially identified and characterized as a novel cytokine produced by thymic stromal cells (Friend SL,1994), [96] it is highly expressed by epithelial cells, skin keratinocytes, mast cells, stromal cells, smooth muscle cells, and lung fibroblasts. [97] Exposure to viral, bacterial or parasitic pathogens and stimulation of Toll-like receptors (TLRs) can induce the release of TSLP from epithelial cells. [98]

TSLP was shown to activate myeloid dendritic cells (mDCs) by upregulating MHC class I and II molecules. [97] By acting on mDCs, TSLP promotes the differentiation of naive $CD4^+$ T cells into Th2 lymphocytes and the production of IL-4, IL-5, IL-6, IL-13 and TNF-α. [94, 99] TSLP may also stimulate an innate response by triggering DCs, mast cells and NK cells to secrete Th2 cytokines, chemokines and pro-inflammatory mediators. [94] One recent study demonstrated that mast cells can express and produce TSLP through caspase-1 and NF-kB signal pathway, regulating further the immune response against the pathogens. [100] TSLP might also enhance the effect of IL-33 on mast cells to amplify Th2 responses in inflamed sites. [101] Since TSLP seems to represent a cytokine-link in the epithelial - DC interface and the

differentiation of T-cells, it might play a pivotal role in regulation of the inflammatory response.

SPECIFIC AIMS & HYPOTHESIS

Purpose

The goals of the present study are to investigate:

i. To examine the profile of Th1/Th2 responses in periodontal disease, by comparing the levels of signature cytokines of Th1 or Th2 cells in GCF (IFN-γ, IL-4, IL-33, TSLP);

ii. To assess the presence of IL-33 and TSLP in periodontal tissues, due to their presence in other diseases that present strong inflammatory components (asthma, rheumatoid arthritis) and secretion by cells that are prominent in the periodontium (epithelial cells, keratinocytes, dendritic cells, fibroblasts);

iii. To evaluate the potential diagnostic properties of each mediator (IFN-γ, IL-4, IL-33, TSLP) in differentiating health and periodontal disease and distinguishing levels of periodontal inflammation.

Specific Aims

- Evaluate the frequency of detection of IFN-γ, IL-4, IL-33 and TSLP in gingival crevicular fluid (GCF) and measure their levels in inflamed deep sites, inflamed shallow sites and healthy shallow sites in patients with chronic periodontitis, and compare these findings with those obtained from inflamed shallow sites and healthy shallow sites in

patients with plaque-induced gingivitis and healthy shallow sites in periodontally healthy subjects.

Hypothesis

- The frequency of detection and the levels (means) of total amount and concentration of IFN-γ, IL-4, IL-33 and TSLP in GCF from patients with chronic periodontitis would be different from that of patients with plaque-induced gingivitis and periodontally healthy subjects.

RESEARCH DESIGN AND METHODS

Materials & Methods

Overall design and strategy

The present investigation was a cross-sectional pilot study, in which gingival crevicular fluid (GCF) samples were collected from sites with different clinical characteristics (healthy, gingivitis and periodontitis sites, as presented above) from periodontally healthy (n=14), plaque-induced gingivitis (n=17) and chronic periodontitis (n=11) subjects. 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 analyzed for the presence and levels of IFN-γ, IL-4, IL-33, and TSLP using a multiplex bead immunoassay (Luminex). Differences in the presence and levels of the selected analytes were sought among the different clinical groups.

Clinical measurements

Clinical parameters were evaluated by one calibrated examiner in all teeth, excluding third molars, and included the following: Plaque Accumulation (0,1), Gingival Redness (0,1), Suppuration (0,1), Bleeding on Probing (BoP), Probing Depth (PD), Clinical Attachment Loss (CAL). Six sites were examined for each tooth: mesiobuccal, buccal, distobuccal, distolingual/distopalatal, lingual/palatal, and mesiolingual/mesiopalatal surface (up to 168 sites/subject). PD and CAL were recorded to the nearest millimeter using a North Carolina periodontal probe. Moreover, the examiner performed radiographic evaluation of any interproximal bone loss for each patient, based on radiographs that had been already obtained for each patient. All the above clinical measurements and radiographic examination are part of the standard examination of any patient who is screened in the Department of Periodontology and the Undergraduate Clinic at Tufts University School of Dental Medicine, Boston MA.

Inclusion criteria

In order to be included in the study, subjects needed to meet the following criteria:

In general, study participants had to be at least 21 years old and present at least 20 natural teeth in their dentition.

For each of the 3 clinical groups defined in this study, the following criteria were employed:

a) <u>Chronic Periodontitis:</u>

i. BoP $\geq$ 20 % of surfaces

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

iii. At least one site with PD and CAL ≤3 mm and BoP

iv. At least one site with PD and CAL ≤3 mm and absence of BoP

v. Radiographic indication of interproximal bone loss at sites with PD ≥5 mm and BoP

b) Plaque-induced Gingivitis:

i. BoP ≥ 20 % of surfaces

ii. No sites with PD and CAL >3 mm

iii. Radiographic absence of interproximal bone loss

c) Healthy Subjects:

i. BoP < 20 % of surfaces

ii. No sites with PD and CAL > 3 mm

iii. Radiographic absence of interproximal bone loss

Exclusion criteria

The following patients were excluded from the study:

a. Patients that suffer from any systemic disease with an impact on periodontal status (e.g. diabetes mellitus, HIV/AIDS, rheumatoid arthritis).

b. Patients that require antibiotic prophylaxis for routine dental procedures.

c. Patients who had received antibiotics, steroids or non-steroid anti-inflammatory therapy in the 3-month period prior to the study.

d. Patients who had received any periodontal treatment (even prophylaxis) 3 months before entering the study.

e. Pregnant or nursing women.

f. Patients who currently smoke.

g. Patients who are undergoing immunosuppressive therapy.

The study protocol was submitted to the Tufts Medical Center IRB for approval (#9516/07-09-2010) and all patients were asked to read and sign an appropriate consent form, before entering the study. The protocol was clearly explained to all study participants, mentioning that, within 2 weeks of the detection of any periodontal disease, all patients would be provided with periodontal treatment, that consisted of oral hygiene instructions for all of them and prophylaxis or scaling/root planning, according to the patient's disease status.

Variables

Subjects in each one of the clinical categories described above, namely periodontally healthy, plaque-induced gingivitis and periodontitis harbored up to three different types of sites: healthy, gingivitis and periodontitis. Therefore, the following independent variables were compared among the groups:

a) <u>Chronic Periodontitis:</u>

i. Periodontitis-periodontitis (Inflamed Deep sites) (PP) (PD ≥5 mm, CAL >3 mm and BoP)

ii. Periodontitis-gingivitis (Inflamed Shallow sites) (PG) (PD & CAL ≤ 3 mm and BoP)

iii. Periodontitis-healthy (Healthy Shallow sites) (PH) (PD & CAL ≤ 3 mm and absence of BoP)

b) Plaque-induced Gingivitis:

i. Gingivitis-gingivitis (Inflamed Shallow sites) (GG) (PD & CAL ≤ 3 mm and BoP)

ii. Gingivitis-healthy (Healthy Shallow sites) (GH) (PD & CAL ≤ 3 mm and absence of BoP)

c) Healthy Subjects:

Healthy (Healthy Shallow sites) (HH) (PD & CAL ≤ 3 mm and absence of BoP)

Collection of GCF

GCF was collected from one mesiobuccal site of a posterior tooth (molar or premolar), in order to match to the extent possible the location and tooth type in each one of the above categories. In case a mesiobuccal site was not available, a disto-buccal site was selected. Among the inflamed deep sites, the one with the deepest probing depth (PD) was chosen, while among the inflamed shallow and healthy shallow sites, there was random selection from any mesiobuccal site of a posterior tooth (molar or premolar).

After isolating the tooth with a cotton roll to prevent contamination with saliva, supragingival plaque was removed with curettes and wet gauzes without touching the marginal gingiva. The crevicular site was then dried gently with an air syringe. GCF was collected with Periopaper strips (PerioPaper GCF collection strips, Oraflow Inc, Plainview, NY), that 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 s. 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). After GCF collection, strips were placed immediately in Eppendorf tubes, which contained 120 μl of Phosphate Buffered Saline (PBS) with 0.05% Tween 20. The samples then were stored at -80°, until they were shipped to Millipore Corporation, (Millipore Corporation, MO, USA) for subsequent cytokine analysis with Luminex.

The readings from the Periotron 8000 were converted to an actual volume (μl) according to the standard (calibration) curve, by using the specific software that accompanies the device (Periotron MLCONVRT software).

Calibration of Periotron 8000

The calibration of the Periotron 8000 was performed according to the protocol established by Chapple et al. [102] In brief, after the device was switched on and a minimum waiting period of 60 min was allowed in order for the Periotron 8000 to warm up, the reading dial was set to zero (0). Then, a series of sterile water aliquots were applied to the Periopaper strips (PerioPaper GCF collection strips, Oraflow Inc, Plainview, NY). The aliquots ranged in volume from 0.25 μl to 1.25 μl (0.25, 0.50, 0.75, 1.00, 1.25 μl). Then, the paper strips were placed between the jaws of the machine and the values displayed on the reading dial of the device were recorded manually. Between each measurement, the jaws were wiped with a gauge to dry the electrodes. The Periotron values recorded for each volume were transferred to the specific software that accompanies the device (Periotron MLCONVRT software) and a

calibration curve was generated. This calibration curve was used to calculate the GCF volume in the clinical samples. The calibration of Periotron was performed every month.

Quantification of Cytokines Using Luminex

Cytokine levels were determined by multiplexed sandwich immunoassay based on flowmetric Luminex™ xMAP technology (Millipore Corporation, MO, USA). Four cytokines , IFN-γ, IL-4, IL-33 and TSLP were measured. The assay was performed in a 96-well filter plate. First, the filter plate was pre-wetted with washing buffer and then this solution would be aspirated from the wells using a vacuum manifold. Microsphere beads coated with the monoclonal antibody against each of the four analytes were added to the wells. Standards were pipetted in each well in concentrations ranging from 0.6 to 10,000 pg/ml for IFN-γ and IL-4; ranging from 4.9 to 20,000 pg/ml for IL-33 and ranging from 2.4 to 10,000 for TSLP. Each Eppendorf tube contained 120 µl of Phosphate Buffered Saline (PBS) with 0.05% Tween 20 plus the volume of GCF that was collected from each individual site. From the Eppendorf tube, 12.5 µl was pipetted and diluted with 12.5 µl of 1:500 dilution of the protease inhibitor (1:2). Samples were pipetted in each well in duplicates. Then, the plate containing the standards and samples were incubated for 2 hours at 4°C with the beads. The wells were washed using a vacuum manifold (Millipore Corporation) and a mixture of biotin-conjugated secondary antibodies was added. After incubation for 1 hour, beads were washed followed by an incubation of 30 minutes with streptavidin conjugated to the fluorescent protein, R-phycoerythrin (streptavidin-RPE). After washing to remove the unbound streptavidin-RPE, the beads (minimum of 100 per cytokine) were analyzed in the Luminex 100 instrument (MiraiBio, Alameda, CA). The device monitors the spectral properties of the beads to distinguish the

different analytes, while simultaneously measuring the amount of fluorescence associated with R-phycoerythrin, reported as median fluorescence intensity. Raw data (median fluorescence intensity, MFI) were analyzed using MASTER PLEX QT v 2.0 software and converted into concentration, expressed as pg/ml, by using the standard curve (MiraiBio, Alameda, CA). Samples below the detection limit of the assay were recorded as zero in our data.

Sample size & Power

A sample size calculation was conducted to estimate the number of subjects needed in each clinical group using the nQuery (Version 7.0) software program. Assuming that the squared multiple correlation would be greater than or equal to 0.2621 ($R^2 \geq 0.2621$), a total of 42 subjects is adequate to attain a significance level α of 0.05 and a power of 80%.

Statistical analysis

Initially, demographic data (age, sex) for each category of the patients (healthy, gingivitis, periodontitis) were analyzed using statistical software SPSS Inc (PASW statistics 18) and expressed in means ± standard deviation (SD). Moreover, clinical data were collected from 6 sites of each tooth, excluding third molars. The clinical variables (number of missing teeth, PD, CAL, BOP, PI, gingival redness, suppuration) were analyzed using also statistical software SPSS for each category of patients and expressed as frequencies and means ± SD.

One-way ANOVA and Tukey tests for post-hoc comparisons were used to determine statistically significant differences in age. The chi-square test and Fisher's exact tests for post-hoc comparisons were used to determine statistically significant differences in categorical variables where the independence was not violated (sex), while generalized estimating

equations (GEE) was used for the categorical variables where the independence was violated (PI, BOP, redness). The Kruskal-Wallis and the Mann-Whitney U-tests for post-hoc comparisons were used in continuous variables (number of missing teeth, PD, AL) that were not normally distributed. Probability values >0.016 (the new probability value α is calculated by dividing the significance level α of 0.05 over the number of tests that were performed, so $\alpha=\alpha/3=0.05/3=0.016$) were considered not significant, since there should be a Bonferroni correction, in order to counteract the problem of multiple comparisons.

Clinical data (PD, BOP, PI, gingival redness, suppuration) were also analyzed for the specific sites that we collected the GCF and were also expressed as frequencies and means ± SD.

In addition, the volume of GCF that was collected from each site (inflamed deep, inflamed shallow and healthy shallow) from each category (healthy, gingivitis, periodontitis) 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. The volume of GCF was expressed in means ± SD for each group and the significance of difference was determined by using a mixed effects model with SAS version 9.2. The mixed effects model also produces follow-up (post-hoc) tests, in order to define which groups are the ones which show statistically significant differences in terms of the volume of GCF that is collected.

Regarding the levels of the inflammatory mediators (IFN-γ, IL-4, IL-33 and TSLP) that were assessed, the data were analyzed and expressed in frequencies, means ± SD for the amount (pg) and concentration (pg/μl) for each inflammatory mediator. For the samples that the

level of the inflammatory mediator was below the level of detection, in our data analysis it was recorded with the value of "0". [59] The significance of difference for frequency of detection was determined with generalized estimating equations (GEE) by using SAS version 9.2. The significance of difference for the amount and concentration was determined with the Mann-Whitney U-test for sites between different categories of patients, and with the Wilcoxon signed-rank test for sites within the same category of patients. Probability values >0.003 (the new probability value α is calculated by dividing the significance level α of 0.05 over the number of tests that were performed, so $\alpha=\alpha/15=0.05/15=0.003$) were considered not significant, since there should be a Bonferroni correction, in order to counteract the problem of multiple comparisons.

We also tested if there was a statistically significant association between any of the clinical variables (PD, BOP, PI, Redness) and the detection of IFN-γ in the samples of GCF that were collected by using generalized estimating equations (GEE), since independence was violated. P-values >0.05 were considered not significant.

RESULTS

Table 1 presents the mean demographic and clinical parameters for the three groups. One-way ANOVA and Tukey tests for post-hoc comparisons were used to determine statistically significant differences in age. The chi-square test and Fisher's exact tests for post-hoc comparisons were used to determine statistically significant differences in categorical variables where the independence was not violated (sex), while generalized estimating equations (GEE) was used for the categorical variables where the independence was violated

(PI, BOP, Redness). The Kruskal-Wallis and the Mann-Whitney U-tests were used in continuous variables (number of missing teeth, PD, AL). In all comparisons, the omnibus test (ANOVA, chi-square test and the Kruskal-Wallis test) showed statistically significant differences ($p<0.05$); therefore, we had to use post-hoc tests (Tukey tests, Fisher's exact tests and the Mann-Whitney U-tests) where the probability values <0.0167 were considered significant, as there was a Bonferroni correction due to multiple comparisons.

Chronic periodontitis subjects were significantly older than healthy and gingivitis individuals ($p<0.001$). There was a higher percentage of males in the periodontitis group in comparison with the healthy group.

Regarding the clinical characteristics of the study population, periodontitis patients presented a greater mean number of missing teeth in comparison with the healthy group ($p<0.05$). In addition, probing depth and attachment levels mean values were greater in the periodontitis group ($p<0.001$). Periodontitis subjects also exhibited, in average, more plaque and suppuration than the other clinical groups. Regarding BOP and redness, periodontitis and gingivitis individuals presented significantly higher values in comparison with healthy individuals ($p<0.001$), but no statistically significant differences were observed when periodontitis and gingivitis subjects were compared.

The differences in age and % of males could act as confounders in our analyses; however, due to the lack of significant differences in the levels of GCF cytokines between groups of sites of GCF collection, analysis of covariance adjusting for these variables was not performed.

Table 1: Demographic and clinical parameters for Periodontally Healthy, Plaque-induced Gingivitis and Chronic Periodontitis Subjects.

Mean (± SD)	Healthy (n=14)	Gingivitis (n=17)	Periodontitis (n=11)	Healthy vs Gingivitis	Healthy vs Periodontitis	Gingivitis vs Periodontitis
Age	26.3±2.6	30.6±7.0	49.2±14.9	NS (p=0.382)	* (p<0.001)	* (p<0.001)
% males	21.4	47.1	72.7	NS (p=0.258)	NS (p=0.017)	NS (p=0.253)
Number of missing teeth	0.64±1.22	0.94±1.60	2.73±2.61	NS (p=0.785)	* 0.014	NS (p=0.034)
Pocket Depth (PD) (mm)	2.65±0.62	2.62±0.65	2.97±1.66	NS (p=0.187)	* (p<0.001)	* (p<0.001)
Attachment Level (AL) (mm)	2.62±0.67	2.59±0.69	2.99±1.71	NS (p=0.230)	* (p<0.001)	* (p<0.001)
PI	12.2	47.9	79.2	* (p<0.001)	* (p<0.001)	* (p<0.001)
BOP	11.8	47.5	46.6	* (p<0.001)	* (p<0.001)	NS (p=0.356)
Redness	11.7	46.2	46.2	* (p<0.001)	* (p<0.001)	NS (p=0.219)
Suppuration	0.0	0.0	1.0	#	#	#

*: statistically significant, p<0.0167; NS: not statistically significant, p>0.0167

#: No comparisons could be made

Data for groups of sites of GCF collection

In order to test if the periodontal sites where GCF was collected met the classification criteria that were described in the Materials & Methods section, we summarize in table 2 all the clinical variables for the GCF collected sites, classified into 6 different groups (Healthy, Gingivitis healthy-Gingivitis gingivitis, Periodontitis healthy-Periodontitis gingivitis-Periodontitis periodontitis). Moreover, these clinical data are also presented in bar graphs (Figures 1-3).

Table 2: Clinical parameters for all groups of sites of GCF collection (Healthy, Gingivitis healthy-Gingivitis gingivitis, Periodontitis healthy-Periodontitis gingivitis-Periodontitis periodontitis)

	Healthy	Gingivitis	Gingivitis	Periodontitis	Periodontitis	Periodontitis
	n=14	n=17	n=17	n=11	n=11	n=11
	H	GH	GG	PH	PG	PP
Pocket Depth (PD) (mm)	2.86±0.36	2.94±.24	2.94±.24	2.91±0.30	3.00±0.0	6.00±0.63
% of sites with (prevalence):						
PI	7.1	17.6	100.0	9.1	100.0	100.0
Redness	0.0	0.0	100.0	0.0	100.0	100.0
BOP	0.0	0.0	100.0	0.0	100.0	100.0
Suppuration	0.0	0.0	0.0	0.0	0.0	0.0

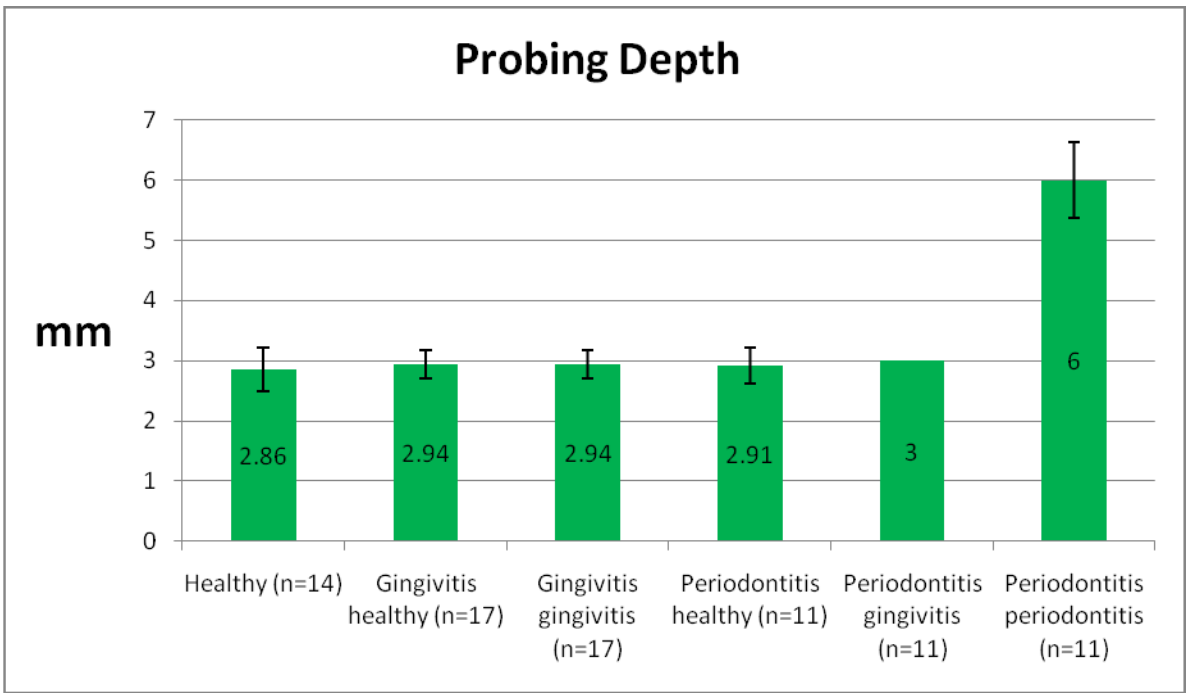

Figure 1: Bar chart of the mean levels of probing depth in each group of GCF collection. Brackets indicate standard deviations.

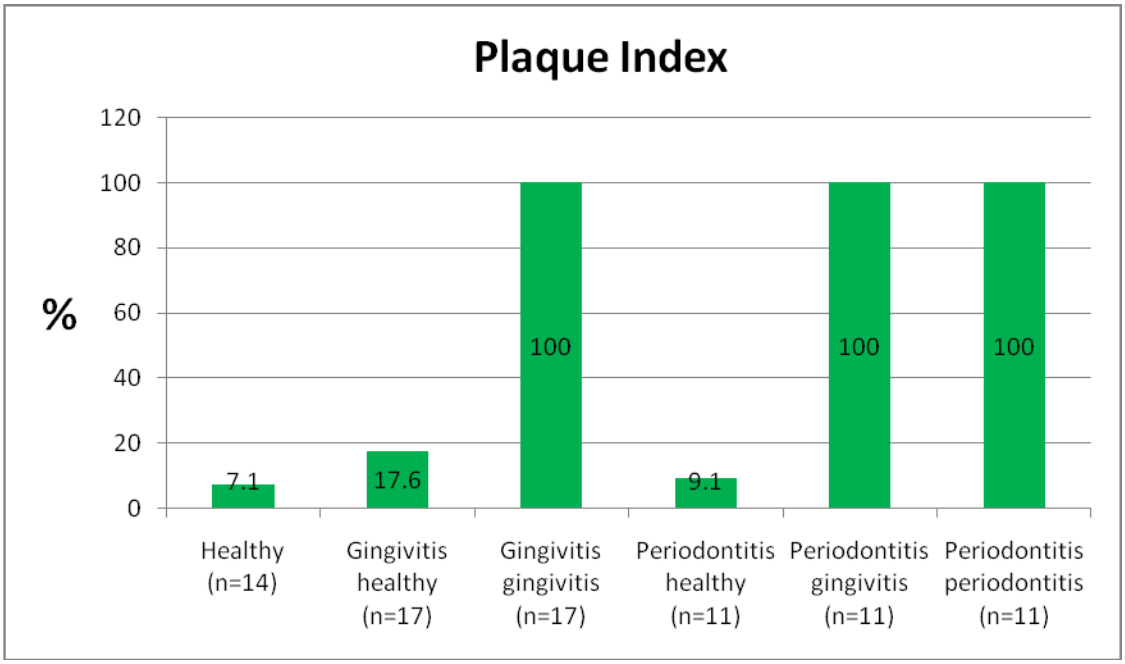

Figure 2: Bar chart of the % plaque index in each group of GCF collection.

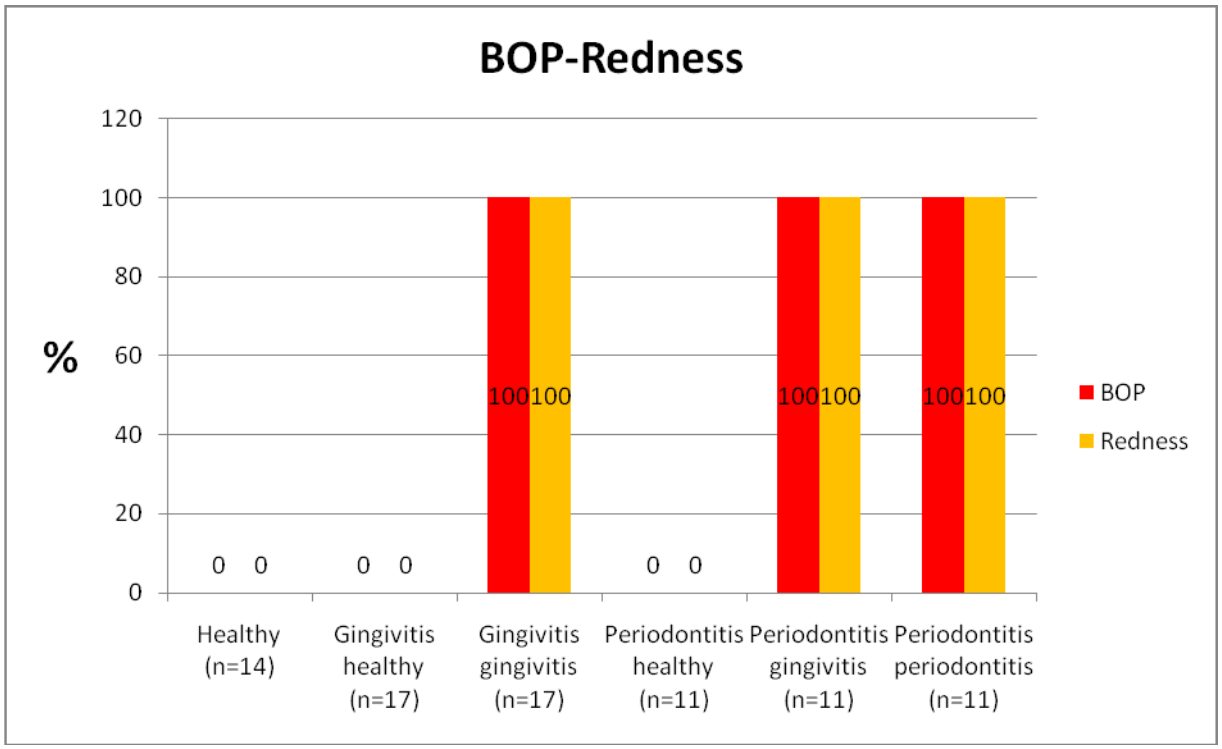

Figure 3: Bar chart of the % bleeding on probing (BOP) and redness in each group of periodontal sites of GCF collection.

Data for GCF volume for all groups of GCF collection

Table 3 summarizes the mean levels and the standard deviations of the volume of Gingival Crevicular Fluid (GCF) (μl) that was collected in each group of periodontal sites (Healthy, Gingivitis healthy-Gingivitis gingivitis, Periodontitis healthy-Periodontitis gingivitis-Periodontitis periodontitis). GCF volume data are also presented below in a bar graph and a box plot (Figures 4 and 5).

A statistically significant association between the group of the site of GCF collection and the volume of GCF that is collected was observed, since the p-value that was determined by

using a mixed-effects model with SAS version 9.2 was p<0.0001. Moreover, inflamed sites in both Plaque-induced Gingivitis and Chronic Periodontitis patients showed statistically significantly higher volume of GCF compared to non-inflamed sites in all categories, determined by follow-up (post-hoc) tests that were produced by the mixed-effects model. Furthermore, GCF volume was statistically significantly higher in the group Periodontitis-periodontitis, compared to all other groups.

Table 3: GCF volume mean levels (±SD) for all groups of GCF collection

	Healthy	**Gingivitis**	**Gingivitis**	**Periodontitis**	**Periodontitis**	**Periodontitis**
	n=14	n=17	n=17	n=11	n=11	n=11
	H	**GH**	**GG**	**PH**	**PG**	**PP**
GCF Volume	0.20±0.09	0.18±0.08	0.46±0.19	0.19±0.06	0.41±0.17	0.84±0.26
Healthy	-	NS (p=0.819)	* (p<0.0001)	NS (p=0.953)	* (p=0.014)	* (p<0.0001)
Gingivitis healthy	-	-	* (p<0.0001)	NS (p=0.879)	* (p=0.0005)	* (p=0.0005)
Gingivitis gingivitis	-	-	-	* (p=0.0001)	NS (p=0.481)	* (p<0.0001)
Periodontitis healthy	-	-	-	-	* (p=0.0012)	* (p<0.0001)
Periodontitis gingivitis	-	-	-	-	-	* (p<0.0001)

*: statistically significant, p<0.003; NS: not statistically significant, p>0.003

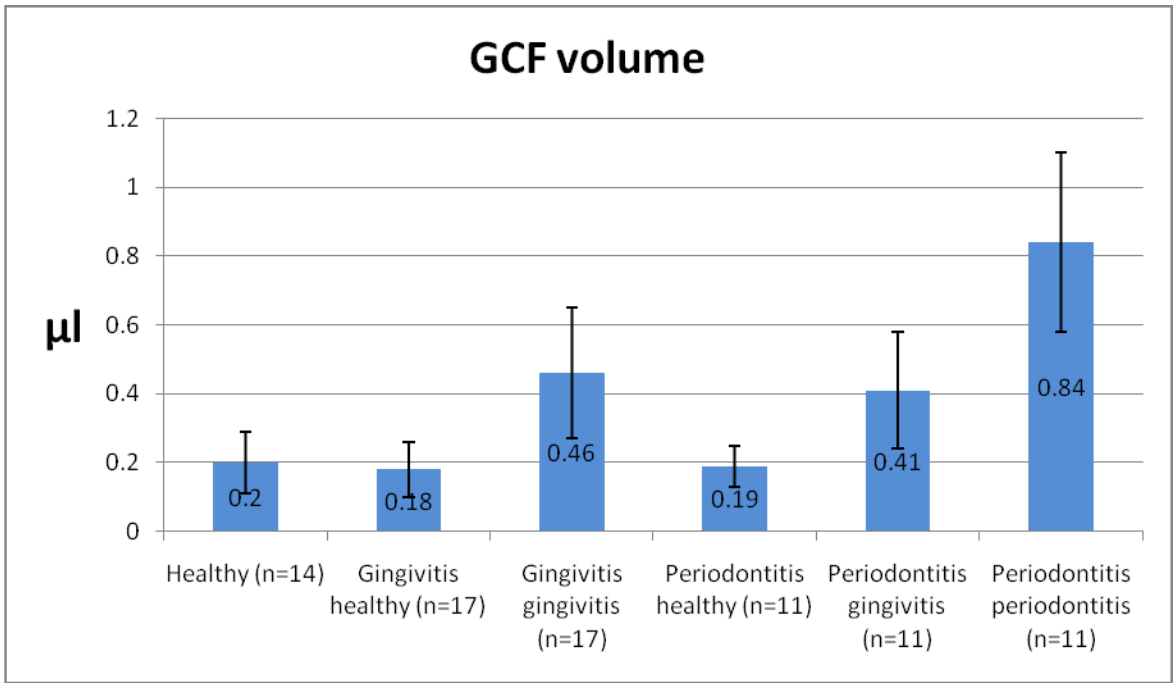

Figure 4: Bar chart of the volume of GCF (µl) in each group.

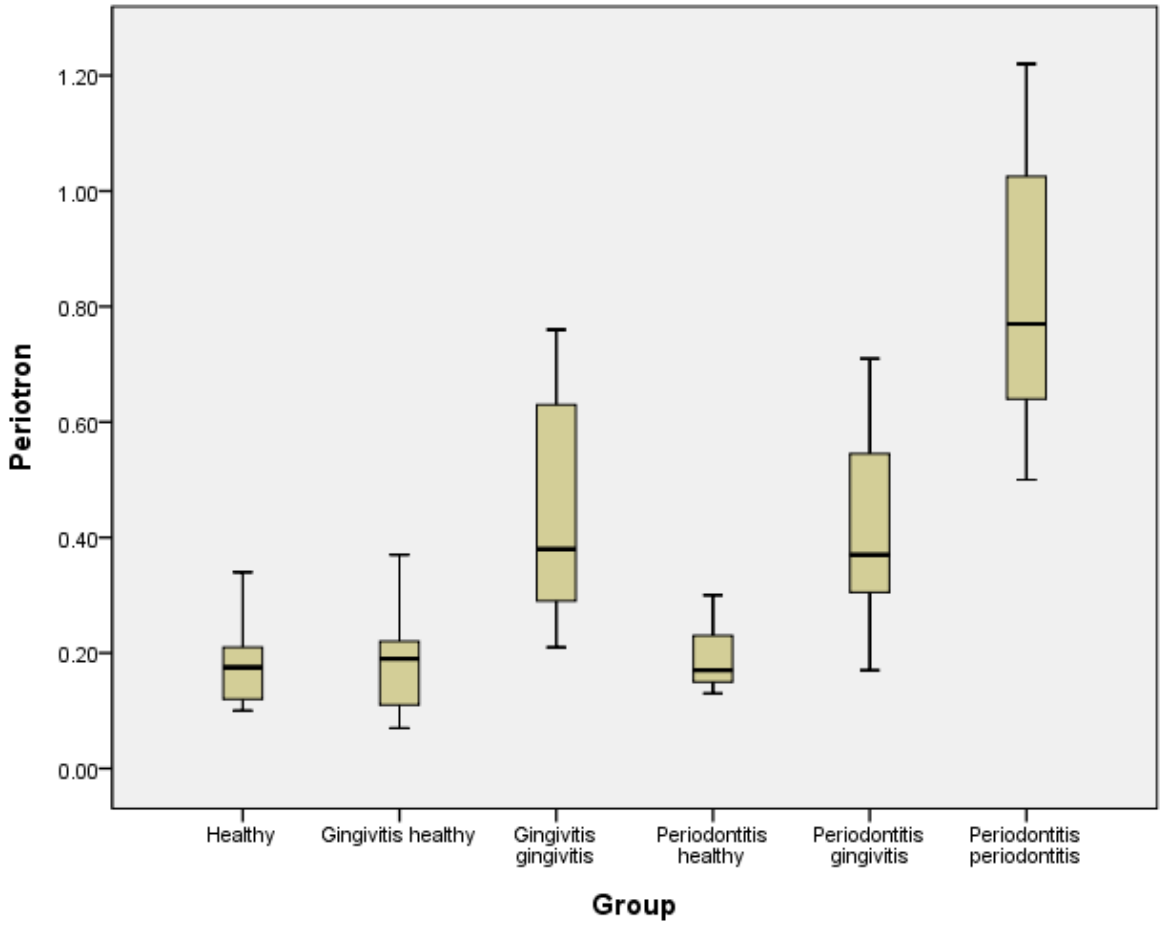

Figure 5: Box plot of volume of GCF (µl) in each group. Follow-up (post-hoc) tests produced by the mixed-effects model showed statistically significantly higher volume of GCF in inflamed sites in both Plaque-induced Gingivitis and Chronic Periodontitis patients compared to non-inflamed sites in all categories. Moreover, GCF volume was statistically significantly higher in the group Periodontitis-periodontitis, compared to all other groups.

Frequency of detection of IFN-γ, IL-4, IL-33 & TSLP in GCF

Table 4 summarizes the frequencies of detection of the inflammatory mediators in GCF that were analyzed (IFN-γ, IL-4, IL-33and TSLP) by using the Luminex technology. It must, however, be noted that IFN-γ was detected in about 50% of all samples collected. A very low number of the samples showed detectable levels for IL-4 and TSPL, while IL-33 was below the detection limit of the assay for all samples. Data are presented also below in bar graphs (Figures 6-8). The significance of difference for frequency of detection was determined with generalized estimating equations (GEE) by using SAS version 9.2. No statistically significant association was found between the group of site of GCF collection and the frequency of detection of IFN-γ (p=0.35).

Table 4: Frequency detection of IFN-γ, IL-4, IL-33 & TSLP for all groups of sample collection

	Healthy	Gingivitis	Gingivitis	Periodontitis	Periodontitis	Periodontitis
	n=14	n=17	n=17	n=11	n=11	n=11
	H	GH	GG	PH	PG	PP
% Sites positive for						
IFN-γ	50.0	35.3	64.7	36.4	45.5	63.6
IL4	0.0	5.9	5.9	9.1	0.0	0.0
IL33	0.0	0.0	0.0	0.0	0.0	0.0
TSLP	0.0	0.0	5.9	0.0	0.0	9.1

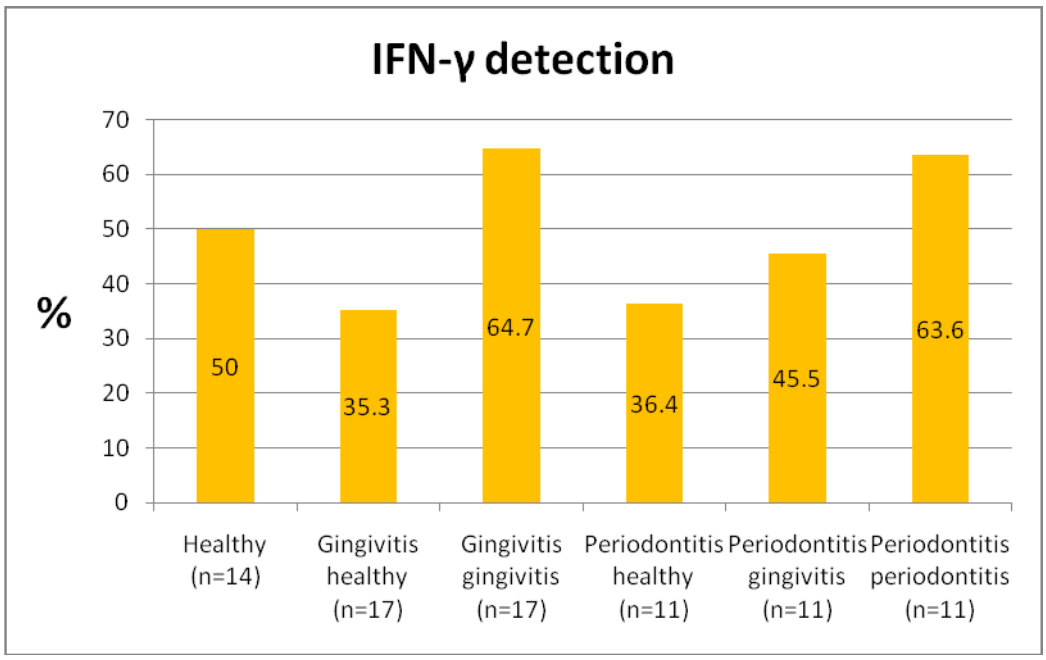

Figure 6: Bar chart of % IFN-γ detection. About 50 % of the samples were above the curve of detection for IFN-γ. No statistically significant association between the group of site of GCF collection and the frequency of detection of IFN-γ (p=0.35).

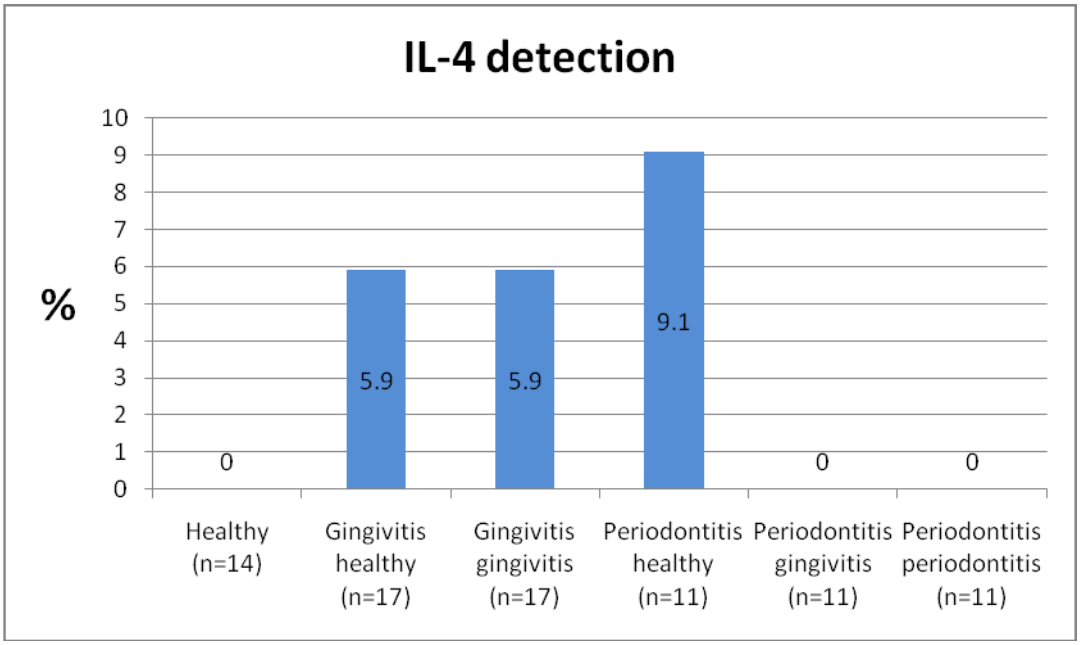

Figure 7: Bar chart of % IL-4 detection. A very low number of samples showed detectable levels of IL-4 (3/81).

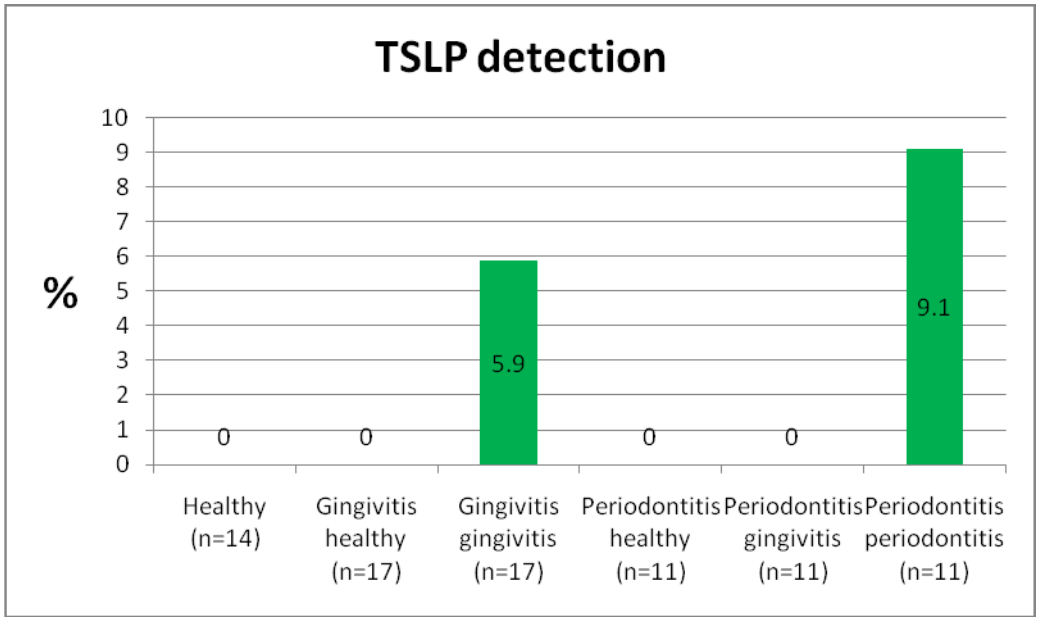

Figure 8: Bar chart of % TSLP detection. A very low number of samples showed detectable levels of TSLP (2/81).

Amount and concentration of IFN-γ in GCF

Table 5 summarizes the mean levels±SD of the amount of IFN-γ (pg/site) and table 6 the concentration of IFN-γ (pg/μl) in GCF samples that was collected from all groups (Healthy, Gingivitis healthy-Gingivitis gingivitis, Periodontitis healthy-Periodontitis gingivitis-Periodontitis periodontitis). IFN-γ amount and concentration data are also presented below in bar graphs and box plots (Figures 9-12).

The significance of differences in the amount and the concentration of IFN-γ in GCF samples was determined with the Mann-Whitney U-test for sites between different categories of patients, and with the Wilcoxon signed-rank test for sites within the same category of patients. No statistically significant differences were found either in IFN-γ amount or in IFN-γ concentration between any of the groups of periodontal sites, where GCF was collected. (p-values >0.003 were considered not significant, because of the Bonferroni correction). In addition, for the samples that the level of the inflammatory mediator was below the level of detection, in our data analysis it was recorded with the value of "0". [59] The concentration (pg/μl) of IFN-γ in GCF was calculated by dividing the amount of IFN-γ over the volume of GCF in each sample of GCF that was collected (IFN-γ amount (pg)/GCF volume (μl)

Table 5: Mean levels (±SD) of amount of IFN-γ for all groups of sample collection

	Healthy	Gingivitis	Gingivitis	Periodontitis	Periodontitis	Periodontitis
	n=14	n=17	n=17	n=11	n=11	n=11
	H	**GH**	**GG**	**PH**	**PG**	**PP**
Mean Amounts (pg/site)						
IFN-γ	0.19±0.20	0.14±0.21	0.27±0.19	0.13±0.19	0.17±0.20	0.31±0.26
Healthy	-	NS (p=0.544)	NS (p=0.262)	NS (p=0.344)	NS (p=0.727)	NS (p=0.166)
Gingivitis healthy	-	-	NS (p=0.050) #	NS (p=0.926)	NS (p=0.746)	NS (p=0.111)
Gingivitis gingivitis	-	-	-	NS (p=0.047) #	NS (p=0.161)	NS (p=0.378)
Periodontitis healthy	-	-	-	-	NS (p=0.610)	NS (p=0.035) #
Periodontitis gingivitis	-	-	-	-	-	NS (p=0.069)

NS: not statistically significant, p>0.003

#: Although the level of significance was initially determined as α=0.05, a Bonferroni correction created a new level of significance α=0.003 (α=α/15=0.05/15=0.003).

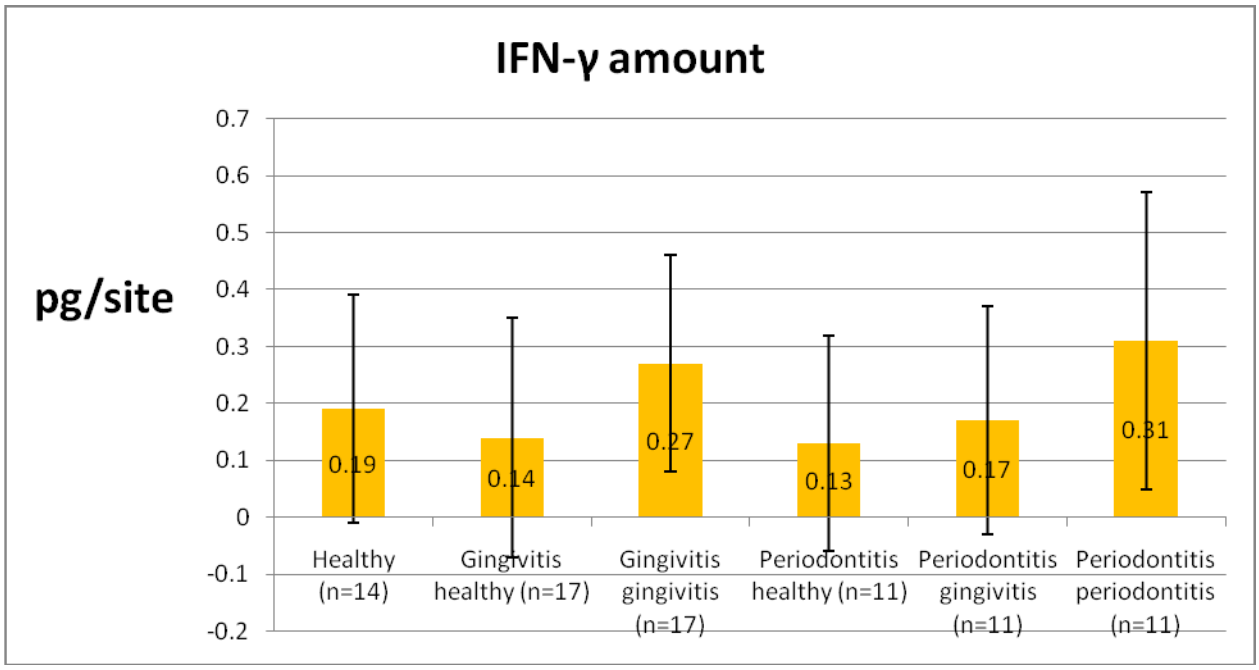

Figure 9: Bar chart of the mean levels of IFN-γ amount (pg/site) in each group of GCF collection. Brackets indicate standard deviations.

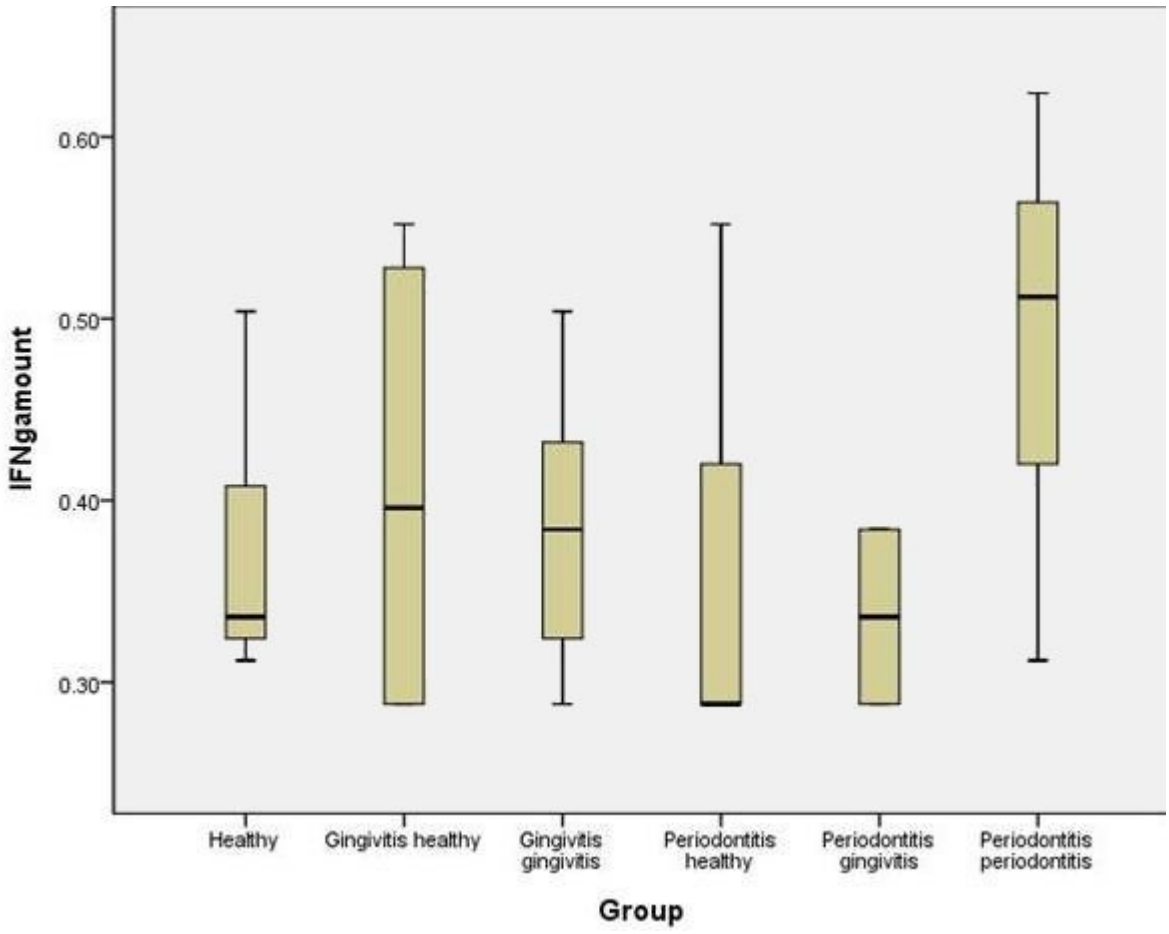

Figure 10: Box plot of IFN-γ amount (pg/site) in each group of GCF collection.

No statistically significant differences in IFN-γ amount were found between any of the groups of periodontal sites, determined with the Mann-Whitney U-test for sites between different categories of patients, and with the Wilcoxon signed-rank test for sites within the same category of patients ($p<0.003$).

Table 6: Mean levels (±SD) of concentration of IFN-γ for all groups of sample collection

	Healthy	Gingivitis	Gingivitis	Periodontitis	Periodontitis	Periodontitis
	n=14	n=17	n=17	n=11	n=11	n=11
	H	**GH**	**GG**	**PH**	**PG**	**PP**
Mean Concentration (pg/µl)						
IFN-γ	1.06±1.44	0.59±0.86	0.70±0.58	0.54±0.86	0.40±0.50	0.38±0.34
Healthy	-	NS (p=0.399)	NS (p=0.799)	NS (p=0.403)	NS (p=0.317)	NS (p=0.467)
Gingivitis healthy	-	-	NS (p=0.507)	NS (p=0.817)	NS (p=0.890)	NS (p=0.817)
Gingivitis gingivitis	-	-	-	NS (p=0.264)	NS (p=0.208)	NS (p=0.161)
Periodontitis healthy	-	-	-	-	NS (p=0.866)	NS (p=0.889)
Periodontitis gingivitis	-	-	-	-	-	NS (p=0.08)

NS: not statistically significant, p>0.003

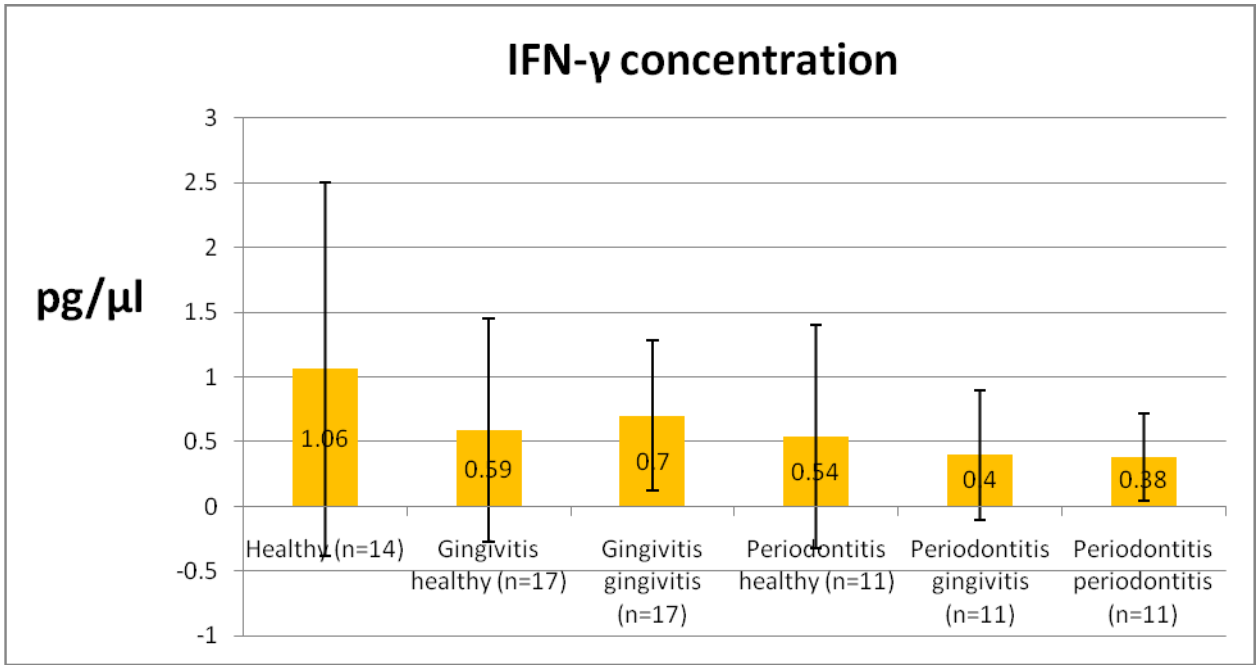

Figure 11: Bar chart of the mean levels of IFN-γ concentration (pg/µl) in each group of GCF collection. Brackets indicate standard deviations.

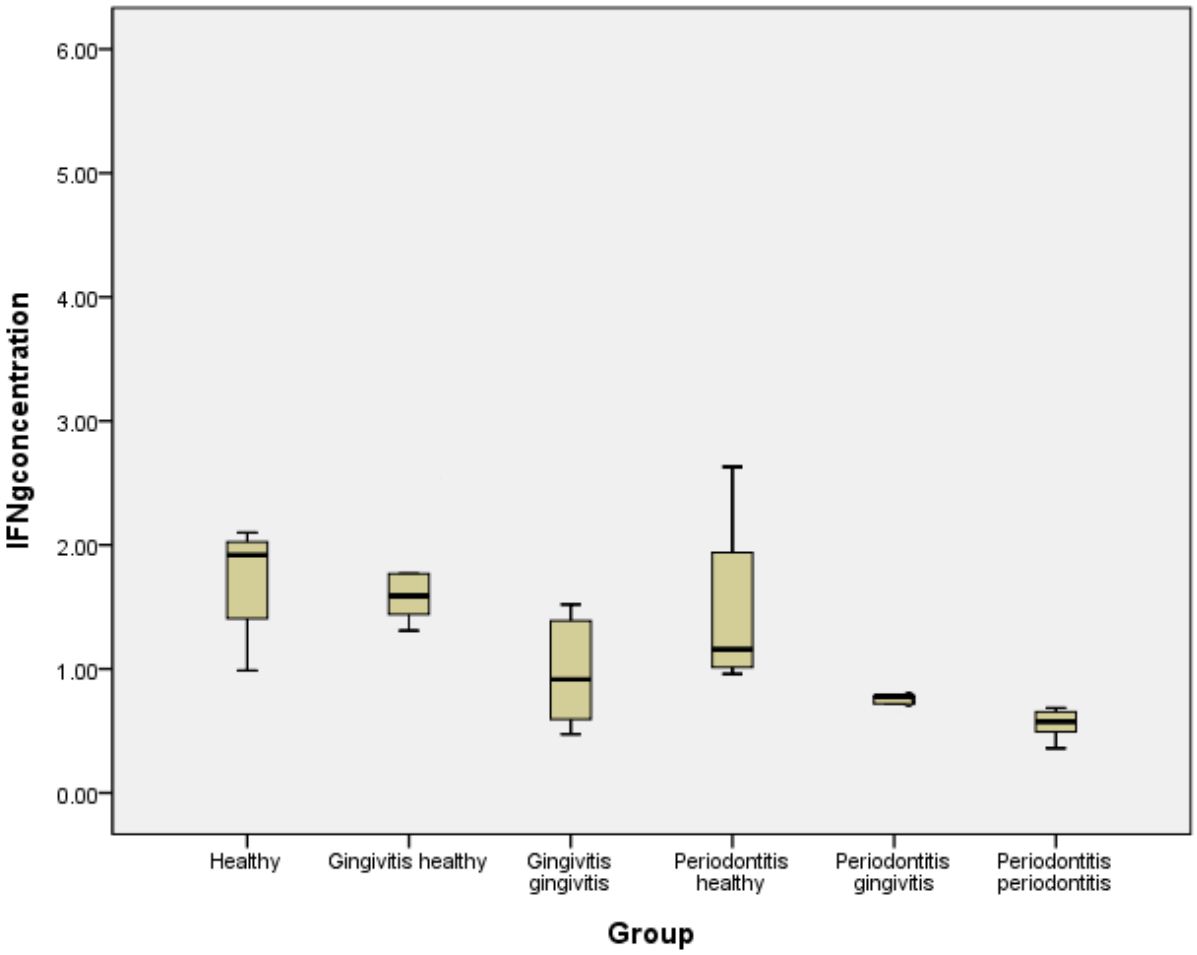

Figure 12: Box plot of IFN-γ concentration (pg/µl) in each group of GCF collection.

No statistically significant differences in IFN-γ concentration were found between any of the groups of periodontal sites, determined with the Mann-Whitney U-test for sites between

different categories of patients, and with the Wilcoxon signed-rank test for sites within the same category of patients ($p<0.003$).

Association between clinical variables and detection of IFN-γ

We also tested if there was a statistically significant association between any of the clinical variables (PD, BOP, PI, Redness) and the detection of IFN-γ in GCF samples collected by using generalized estimating equations (GEE), since independence was violated. No statistical significant association was observed. p-values >0.05 were considered not significant. Data are presented in bar graphs (Figures13-16).

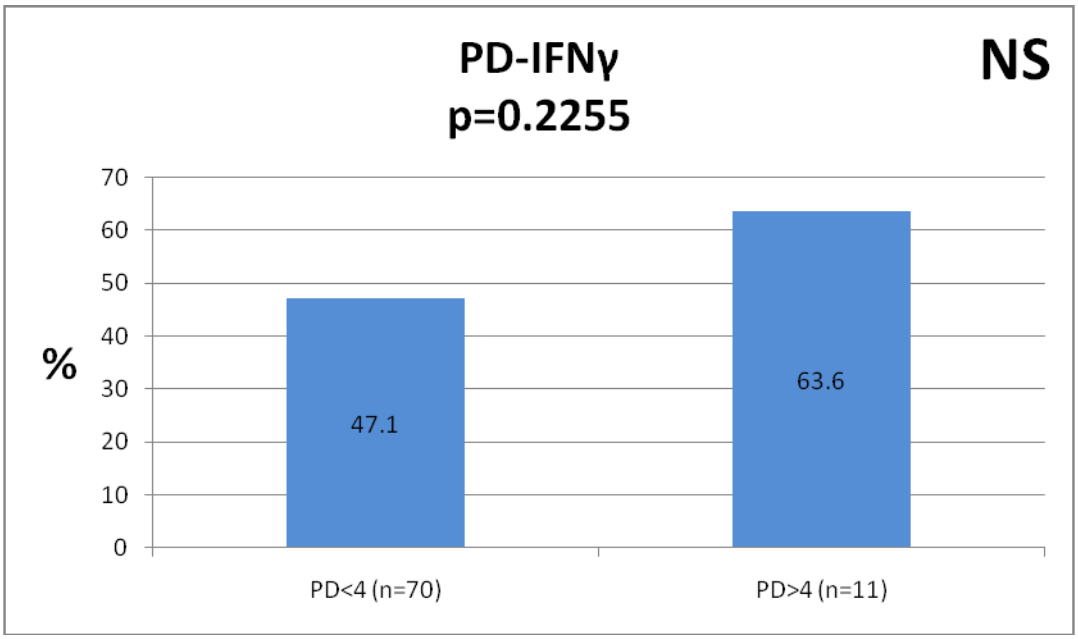

Figure 13: Bar chart of the association of probing depth (PD) and IFN-γ detection in the GCF samples. No statistically significant association was found between the periodontal sites that had PD>4 mm and IFN-γ detection.

NS: No statistically significant

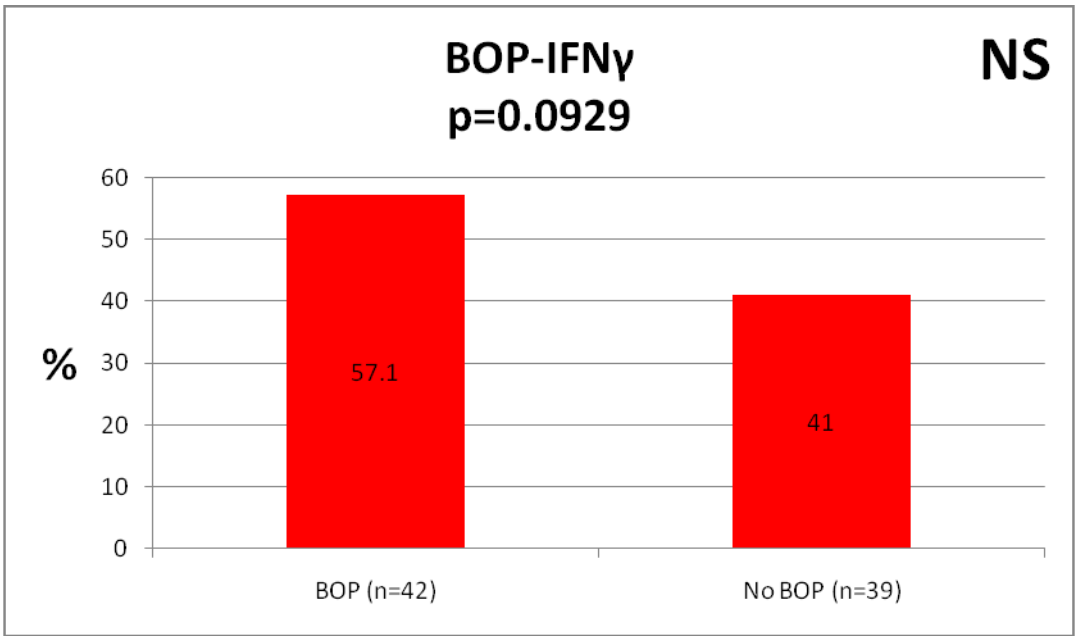

Figure 14: Bar chart of the association of bleeding on probing (BOP) and IFN-γ detection in the GCF samples. No statistically significant association was found between the periodontal sites that showed BOP and IFN-γ detection.

NS: No statistically significant

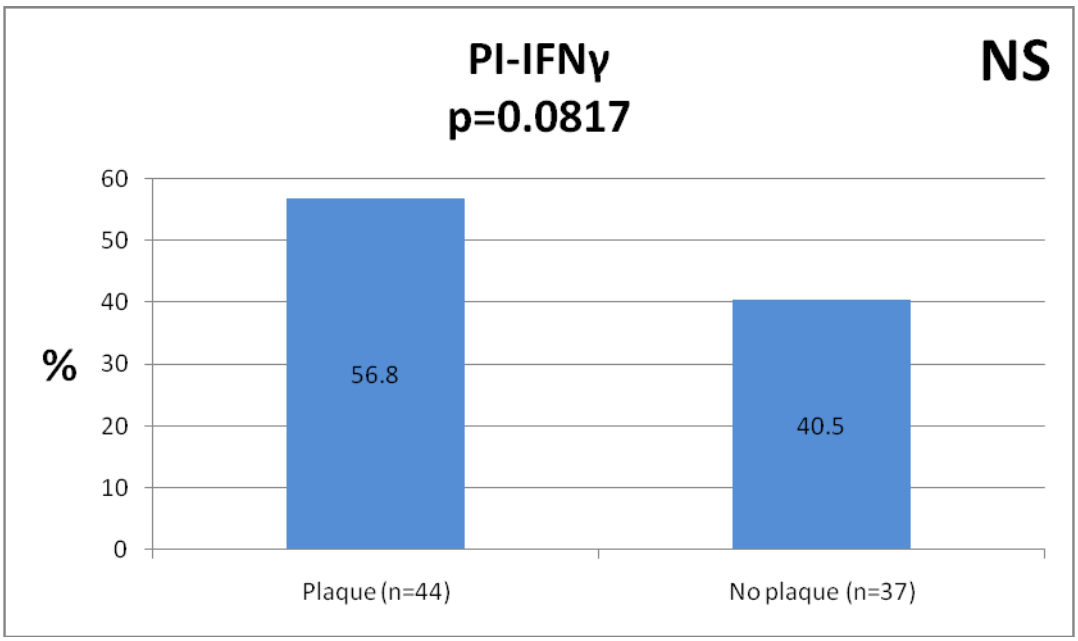

Figure 15: Bar chart of the association of plaque index (PI) and IFN-γ detection in the GCF samples. No statistically significant association was found between the periodontal sites that showed presence of plaque and IFN-γ detection.

NS: No statistically significant

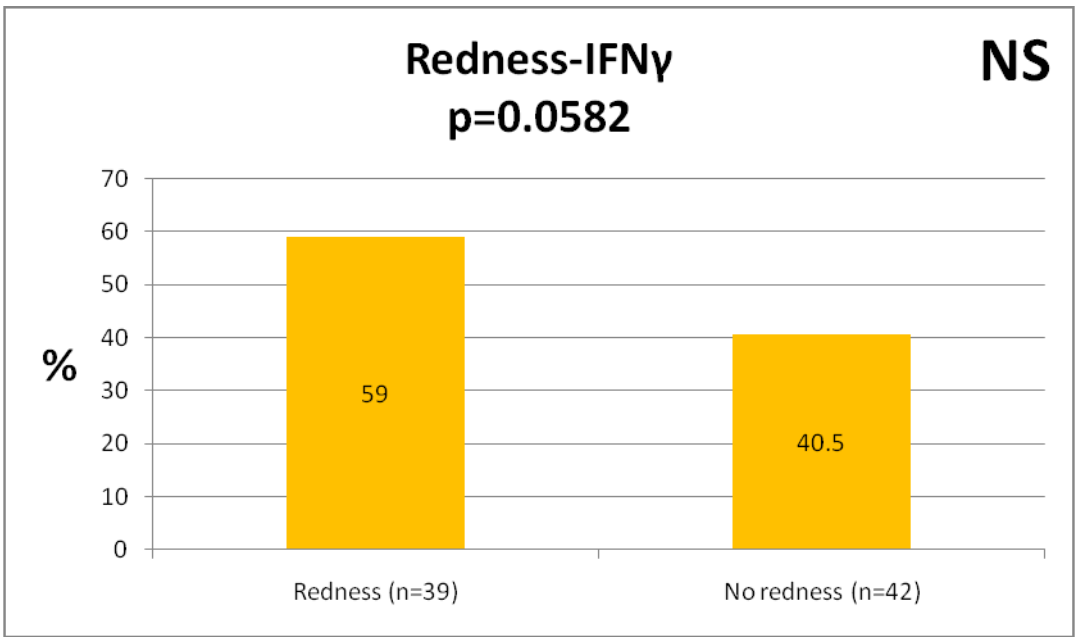

Figure 16: Bar chart of the association of redness and IFN-γ detection in the GCF samples. No statistically significant association was found between the periodontal sites that showed redness and IFN-γ detection.

NS: No statistically significant

DISCUSSION

The goal of the present study was to evaluate the frequency of detection of IFN-γ, IL-4, IL-33 and TSLP in gingival crevicular fluid (GCF) and measure their levels in inflamed deep sites, inflamed shallow sites and healthy shallow sites in patients with chronic periodontitis. This research project also aimed at comparing these findings with those obtained from inflamed shallow sites and healthy shallow sites in patients with plaque-induced gingivitis and healthy shallow sites in periodontally healthy subjects. The hypothesis to be tested was that the

frequency of detection and the levels (means) of total amount and concentration of IFN-γ, IL-4, IL-33 and TSLP in GCF samples from patients with chronic periodontitis would be different from that of patients with plaque-induced gingivitis and periodontally healthy subjects.

Most of the studies that investigated the detection and the role of inflammatory mediators in periodontal inflammation compared the levels of cytokines in GCF between different categories of subject health, like between periodontally healthy subjects, patients with plaque-induced gingivitis or patients with chronic periodontitis. [75, 103] In the present study, the proper selection of chronic periodontitis, plaque-induced gingivitis and periodontally healthy is demonstrated by demographic and clinical characteristics of the study population presented in table 1. For instance, periodontitis patients presented a greater mean number of missing teeth in comparison with the healthy group ($p<0.016$). In addition, pocket depth and clinical attachment level mean values were greater in the periodontitis group ($p<0.001$). Periodontitis subjects also exhibited, in average, more plaque and suppuration than the other clinical groups. Regarding BOP and redness, periodontitis and gingivitis individuals presented statistically significantly higher values in comparison with healthy individuals ($p<0.001$), but no statistically significant differences were observed when periodontitis and gingivitis subjects were compared.

In the present study, we also aimed at exploring any possible diagnostic value of those inflammatory mediators in periodontal disease. Therefore, we assessed and compared the levels of IFN-γ, IL-4, IL-33 and TSLP in GCF not only in patients belonging to different categories of periodontal disease, but also between different categories of sites distinguished by clinical variables (i.e., sites with different levels of disease) in the same patient (for instance, healthy, gingivitis and periodontitis sites in a periodontitis patient). In order to achieve that, healthy, gingivitis and periodontitis sites from patients in the different clinical categories were selected.

The proper selection of sites for GCF sample collection is demonstrated in table 2 and figures 1-3. Analysis of the clinical variables of the sites selected for GCF collection showed that they met the classification criteria that were initially defined. As shown in table 2, all "Periodontitis" (inflamed deep) sites had PD≥5 mm (mean PD±SD=6.00±0.63), CAL >3 mm and BoP (100%), all "Gingivitis" (inflamed shallow) sites had PD & CAL ≤ 3 mm and BoP (100%) and all "Healthy" (healthy shallow) sites had PD & CAL ≤ 3 mm and absence of BoP (0%).

In the current project, a statistically significant association between the clinical status of the site of GCF collection and the volume of GCF collected was observed ($p<0.0001$) (Table 3, Fig 4-5). In addition, inflamed sites in both Plaque-induced Gingivitis and Chronic Periodontitis patients showed statistically significantly higher volume of GCF compared to non-inflamed sites in all categories of health. These findings confirm results from previous studies, where it was demonstrated that the amount of GCF increases with the severity of inflammation and is directly associated with the increased vascular permeability and ulceration of the pocket epithelium at inflamed sites. [51, 59, 62] Quantitative assessments of GCF has no practical clinical application in the management of patients, since no studies have indicated that increased volume of GCF is related to the risk for periodontal tissue destruction. [52] However, in our study and other research studies, estimation of GCF volume can be used as an objective measurement of gingival inflammation that can supplement assessments based on more subjective clinical indices of inflammation (like PI, BOP, Redness). [49]

Despite the proper selection of patients and sites, as well as collection of GCF samples, the results of this cross-sectional study did not demonstrate any statistically significant difference in the frequency of detection, amount or concentration of IFN-γ, IL-4, IL-33 and TSLP in GCF from patients with chronic periodontitis compared to patients with plaque-

induced gingivitis and periodontally healthy subjects, in part due to low frequency of detection of most of the selected analytes in GCF.

IFN-γ was the most frequently detected inflammatory mediator in the present study. It could be detected in about 50% of the clinical samples. No statistically significant association was found between the clinical status of the site of GCF collection and the frequency of detection of IFN-γ (p=0.35). It is noteworthy that samples in which the level of IFN-γ was below the level of detection, in our data analysis it was recorded with the value of "0". [59] Therefore, no statistically significant differences were found either in the IFN-γ amount (pg/site) or in the IFN-γ concentration (pg/μl) between any of the groups of periodontal sites, where GCF was collected. However, comparing the mean amounts of IFN-γ between Periodontitis healthy and Periodontitis periodontitis sites, the results approach a statistically significant difference (p=0.035). The Bonferroni correction applied during the data analysis of the present study decreased the level of significance from α=0.05 to α=0.003 in order to counteract the multiple comparisons (tables 5-6, fig 9-12). In case a less conservative approach had been employed as an alternative to the Bonferroni correction, significantly higher values of IFN-γ would also have been identified for gingivitis sites in gingivitis patients when compared with healthy sites in the same patients, gingivitis sites in gingivitis patients when compared with healthy sites in periodontitis patients and deep inflamed sites when compared to healthy shallow in periodontitis patients (table 5).

Dutzan et al demonstrated that the total amount of IFN-γ in the GCF of active sites in patients presenting signs of progression of periodontitis was significantly higher than in inactive sites, where disease activity was defined by the ‘‘tolerance method’’, [104] suggesting a destructive role of IFN-γ and Th1 response in the periodontium. [75] The design of their study was

very similar to that of the present study, except that in the Dutzan et al study GCF samples were analyzed with ELISA. In addition, the authors did not present any data about regarding the frequency of detection of IFN-γ in GCF samples. Furthermore, the investigators did not mention what value was recorded when IFN-γ was below the level of detection. Recently, Thunell et al using a multiplexed bead immunoassay, the same technique employed in the present study, showed that IFN-γ levels in GCF samples were higher in diseased sites when compared to healthy sites in patients with generalized severe chronic periodontitis. [63] However, in their statistical analysis, the authors mentioned that they did not adjust the level of significance for multiple comparisons, since they claimed that this would have resulted in very conservative tests, hiding clinically meaningful differences.

Figueredo et al compared the GCF levels of IFN-γ in systemically healthy patients, patients with Crohn's disease and ulcerative colitis, all diagnosed with chronic untreated periodontitis. [105] IFN-γ was not detected in any samples of any category of patients, despite the fact that all samples were pooled together in one Eppendorf tube and analyzed with multiplex bead technology.

Our results regarding the frequency of detection of IFN-γ in GCF samples seem to be in accord with the findings reported by Rescala et al. The authors showed that this analyte could be detected in 57% of the samples from patients with chronic and aggressive periodontitis. [59] Although Rescala et al used also a multiplexed bead immunoassay for determining the cytokine levels in GCF samples, the levels were measured in pooled sites from each site category of each patient, while we analyzed each GCF sample from each site of each patient individually.

Based on our results, it can be reported that IFN-γ has been detected more frequently and in higher amounts in inflamed sites in patients with Plaque-Induced Gingivitis and Chronic

Periodontitis compared to non-inflamed sites in all categories of patients. This finding could be somewhat anticipated, since several studies have suggested that IFN-γ plays a critical role in periodontal inflammation, enhancing the phagocytic activity of the host cells and amplifying the innate immune host response to the periodontal pathogens. [24, 65] However, our data cannot provide any additional information on the debated role of IFN-γ in the stability or progression of periodontal lesions, since there was a lack of statistically significant difference in the levels of IFN- γ in GCF in sites of different health status and in patients with different levels of periodontal disease.

In the present study, we also assessed the associations between the clinical variables (PI, BOP, Redness) with the detection of IFN-γ in order to evaluate a potential diagnostic value of the detection of IFN-γ in GCF. There was no statistically significant association between the detection of IFN-γ in GCF and any of the clinical variables that were recorded during the periodontal examination (PI, BOP, Redness).

In this study, levels of IL-4 were below the detection limit of the assay in the vast majority of the GCF samples, since approximately only 4% (n=3) of the total samples (n=81) of GCF showed detectable levels. Hence, our data cannot support results of previous studies that indicated a protective role of IL-4 against the periodontal pathogens. Pradeep et al suggested that IL-4 is associated with the remission of periodontal disease, since they reported that IL-4 levels decreased progressively in GCF from healthy to periodontitis. [106] The authors mentioned that their study was the first one to compare the levels of IL-4 in GCF in periodontal health and disease. There are a number of factors in their study that might have favored the detection of IL-4 in GCF. The authors highlighted that in healthy subjects, GCF was pooled from multiple sites without any sign of inflammation in order to ensure an adequate volume of biological

material that could be analyzed. Moreover, they employed a commercially available, sensitive ELISA kit to detect the smallest amount of IL-4 in the samples (sensitivity of 0.5 pg/ml), while the sensitivity of the kit of multiplex bead technology that was used in the present study was much lower (6.4 pg/ml). Finally, in the Pradeep et al study, GCF samples were collected with microcapillary pipettes to avoid non-specific attachment of the analyte which was observed happening with filter papers. [107]

The inconsistency in the detection of IL-4 in GCF suggests that high variability of its levels among different subjects. This finding has already been reported for IL-4 in serum samples. [69] In addition, it is possible that even though IL-4 might be relevant to the pathogenesis of periodontal diseases, it might not be present in GCF samples. It is noteworthy that, even though GCF is a useful and convenient sample to represent the ongoing processes associated with the periodontal tissues, it is not a perfect snapshot of the periodontal status. Only mediators that are secreted in sufficient amounts that manage to cross the junctional epithelium and remain intact within the crevicular milieu might be detected. It is possible that IL-4 might be secreted and consumed within the periodontal tissues and might never reach the gingival crevicular area in measurable amounts. Or else, it might transverse the junctional epithelium but may be degraded once it reaches the GCF.

Levels of IL-4 have also been investigated in periodontally healthy subjects and patients with chronic periodontitis employing samples other than GCF, such as gingival biopsies and serum samples, with a great diversity in the results that were presented. Gorska et al showed that detectable levels of IL-4 were found in about 50% of the serum samples; however no statistically significant differences between periodontally healthy and patients with severe chronic periodontitis (mean PD: 5.09 ±1.02) were observed. [69] Moreover, Gorska et al reported

that IL-4 was detected in 88% of healthy tissue supernatants versus 35% of samples from periodontitis patients. Duarte et al compared periodontally healthy subjects, aggressive and chronic periodontitis patients for the serum levels of IL-4. The authors observed that serum samples present undetectable or low levels of IL-4 and no statistically significant difference could be observed among the different groups. [108] Finally, Bastos et al showed very low means of amount of IL-4 (about 0.2 pg/site) in GCF in both aggressive periodontitis and periodontally healthy subjects, and again no statistically significant differences in the levels of IL-4 between the two groups could be detected. [103]

Recently, in the 7th European Workshop on Periodontology, Kinane et al encouraged the exploration of novel cytokines in the pathophysiology of periodontitis, including IL-33. [35] According to our knowledge, this is the first study that investigates the existence and the role that IL-33 and TSLP might play in periodontal inflammation. Our results showed that TSLP was detected only in 2 of the samples of GCF that we collected (2/81), while IL-33 was below the curve of detection for all samples. Although both TSLP and IL-33 have been reported to be produced by cells similar to the ones that they exist in periodontium (epithelial cells, fibroblasts, dendritic cells, macrophages) and to play a significant role in inflammation in mucosal organs, no conclusion can be drawn regarding the role that IL-33 and TSLP play in periodontal inflammation based on the present data. There are a number of reasons that might explain the lack of detection of both IL-33 and TSLP. First, it is possible that these mediators might be secreted and consumed within the periodontal tissues and might never reach the gingival crevicular area in measurable amounts. Second, it is also possible that they might transverse the junctional epithelium but may be degraded once they reach the GCF, where a number of proteases are present. In either case, the analysis of their mRNA from gingival

biopsies rather than their actual levels in GCF may provide additional information regarding their presence within periodontal tissues. Therefore, IL-33 and TSLP could be assessed by reverse transcription polymerase chain reaction (RT-PCR) of m-RNA extracted from complete gingival biopsies, in order to evaluate whether the cells of the inflamed periodontium express the information to produce IL-33 and TSLP. It is also possible that these analytes are present in the periodontium, although in levels lower that the detection limit of the assay used in the present study. In that case, a more sensitive ELISA kit could be used only to analyse GCF samples for IL-33 and TSLP separately, in order to counteract the restriction of the small volume of biologic material that is collected from the gingival sulcus and increase the chances of determining their levels in GCF. Finally, the hypothesis that these cytokines might not be involved with periodontal diseases cannot be discarded.

The comparison of the results from different studies that aimed at analyzing biomarkers in GCF pose a number of challenges. Different studies employ diverse sample collection protocols and definitions of diseases and they use different platforms for analysis and different approaches to report results. For example, few studies report on the frequency of detection of analytes, and choose to report only the mean amounts or concentrations. This strategy can be misleading in that a few "positive" samples may be averaged with many "zero" samples. As a result, a theoretical number of limited value might be reported. In addition, often times it is not reported which value was recorded in cases that samples exhibited levels of the inflammatory mediator below the detection limit of the assay. Collectively, these differences in the available manuscripts compromise our ability to draw conclusions about the definite role of different cytokines in the pathogenesis of periodontal disease.

One of the main limitations of the present study was its size. It was a small exploratory study, comprising a total of 81 GCF samples from 42 patients that represented 3 clinical groups. Moreover, different clinical groups of patients were selected and multiples sites from the same patient were included for the comparison of the levels of IFN-γ, IL-4, IL-33 and TSLP in GCF. As a result, a Bonferroni correction was applied, in order to adjust for multiple comparisons. This correction decreased the level of significance from $\alpha=0.05$ to $\alpha=0.003$, creating very stringent criteria for the determination of statistically significant differences.

Another limitation of the present study was the lack of assessment of the local bacterial insult, the etiological factor of periodontal diseases and the trigger for the host-immune responses. Studies that have evaluated simultaneously distinct patterns of GCF cytokine expression and different subgingival biofilm profiles [62] have gained interest recently, because they cover both ends of host-microbial interactions involved in the pathogenesis of periodontal diseases. Hence, they are highly recommended in order to identify several host factors as possible therapeutic targets for the management of the periodontal inflammation.

CONCLUSIONS

i. The present study could not demonstrate any statistically significant difference in the frequency of detection, amount or concentration of IFN-γ, IL-4, IL-33 and TSLP in gingival crevicular fluid from patients with chronic periodontitis compared to patients with plaque-induced gingivitis and periodontally healthy subjects;

ii. The results cannot provide additional information for the profile of Th1/Th2 cells in periodontal disease, by comparing the levels of signature cytokines of Th1 or Th2 cells (IFN-γ, IL-4, IL-33, TSLP);

iii. The present data could not support the presence of IL-33 and TSLP in periodontal tissues, due to lack of detection of IL-33 and TSLP in GCF samples;

iv. The results of the present study could not reveal any diagnostic properties of any mediator (IFN-γ, IL-4, IL-33, TSLP) in differentiating health and periodontal disease and distinguishing levels of periodontal inflammation, due to lack of any statistically significant association between their levels in GCF and the clinical signs of inflammation.

REFERENCES

1. Tonetti MS, Claffey N, European Workshop in Periodontology group C. Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. group C consensus report of the 5th european workshop in periodontology. *J Clin Periodontol*. 2005;32 Suppl 6:210-213.

2. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000*. 1994;5:78-111.

3. Socransky SS, Haffajee AD. Periodontal microbial ecology. *Periodontol 2000*. 2005;38:135-187.

4. Paster BJ, Boches SK, Galvin JL, et al. Bacterial diversity in human subgingival plaque. *J Bacteriol*. 2001;183(12):3770-3783.

5. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL,Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol*. 1998;25(2):134-144.

6. Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. *J Clin Periodontol*. 2000;27(10):722-732.

7. Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. *J Clin Periodontol*. 2000;27(9):648-657.

8. Cugini MA, Haffajee AD, Smith C, Kent RL,Jr, Socransky SS. The effect of scaling and root planing on the clinical and microbiological parameters of periodontal diseases: 12-month results. *J Clin Periodontol*. 2000;27(1):30-36.

9. Madianos PN, Bobetsis YA, Kinane DF. Generation of inflammatory stimuli: How bacteria set up inflammatory responses in the gingiva. *J Clin Periodontol*. 2005;32 Suppl 6:57-71.

10. Lindhe J, Lang NP, Karring T. *Clinical Periodontology and Implant Dentistry.* 5th ed. Oxford ; Ames, Iowa: Blackwell Munksgaard; 2008.

11. Graves D. Cytokines that promote periodontal tissue destruction. . 2008;79(8 Suppl):1585-1591.

12. Loe H, Theilade E, Jensen SB. Experimental gingivitis in man. *J Periodontol*. 1965;36:177-187.

13. Lindhe J, Hamp S, Loe H. Experimental periodontitis in the beagle dog. *J Periodontal Res*. 1973;8(1):1-10.

14. Kornman KS. Mapping the pathogenesis of periodontitis: A new look. *J Periodontol*. 2008;79(8 Suppl):1560-1568.

15. Williams RC. Understanding and managing periodontal diseases: A notable past, a promising future. *J Periodontol*. 2008;79(8 Suppl):1552-1559.

16. Tanner AC, Kent R,Jr, Kanasi E, et al. Clinical characteristics and microbiota of progressing slight chronic periodontitis in adults. *J Clin Periodontol*. 2007;34(11):917-930.

17. Loesche WJ. Microbiology of dental decay and periodontal disease. In: Baron S, ed. *Medical Microbiology.* 4th ed. Galveston (TX): The University of Texas Medical Branch at Galveston; 1996.

18. Periodontal diseases of children and adolescents. *J Periodontol*. 1996;67(1):57-62.

19. Salvi GE, Lang NP. Host response modulation in the management of periodontal diseases. *J Clin Periodontol*. 2005;32 Suppl 6:108-129.

20. Holgate ST. The role of mast cells and basophils in inflammation. *Clin Exp Allergy*. 2000;30 Suppl 1:28-32.

21. Van Dyke TE, Kornman KS. Inflammation and factors that may regulate inflammatory response. *J Periodontol*. 2008;79(8 Suppl):1503-1507.

22. Kopp EB, Medzhitov R. The toll-receptor family and control of innate immunity. *Curr Opin Immunol*. 1999;11(1):13-18.

23. Preshaw PM, Taylor JJ. How has research into cytokine interactions and their role in driving immune responses impacted our understanding of periodontitis? *J Clin Periodontol*. 2011;38 Suppl 11:60-84.

24. Garlet GP. Destructive and protective roles of cytokines in periodontitis: A re-appraisal from host defense and tissue destruction viewpoints. *J Dent Res*. 2010;89(12):1349-1363.

25. Moughal NA, Adonogianaki E, Thornhill MH, Kinane DF. Endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in gingival tissue during health and experimentally-induced gingivitis. *J Periodontal Res*. 1992;27(6):623-630.

26. Ohlrich EJ, Cullinan MP, Seymour GJ. The immunopathogenesis of periodontal disease. *Aust Dent J*. 2009;54 Suppl 1:S2-10.

27. Behl Y, Siqueira M, Ortiz J, et al. Activation of the acquired immune response reduces coupled bone formation in response to a periodontal pathogen. *J Immunol*. 2008;181(12):8711-8718.

28. Kwan Tat S, Padrines M, Theoleyre S, Heymann D, Fortun Y. IL-6, RANKL, TNF-alpha/IL-1: Interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev*. 2004;15(1):49-60.

29. Bar-Shavit Z. Taking a toll on the bones: Regulation of bone metabolism by innate immune regulators. *Autoimmunity*. 2008;41(3):195-203.

30. Berglundh T, Donati M. Aspects of adaptive host response in periodontitis. *J Clin Periodontol*. 2005;32 Suppl 6:87-107.

31. Cumberbatch M, Griffiths CE, Tucker SC, Dearman RJ, Kimber I. Tumour necrosis factor-alpha induces langerhans cell migration in humans. *Br J Dermatol*. 1999;141(2):192-200.

32. Barrett AW, Cruchley AT, Williams DM. Oral mucosal langerhans' cells. *Crit Rev Oral Biol Med*. 1996;7(1):36-58.

33. Gaffen SL, Hajishengallis G. A new inflammatory cytokine on the block: Re-thinking periodontal disease and the Th1/Th2 paradigm in the context of Th17 cells and IL-17. *J Dent Res*. 2008;87(9):817-828.

34. Gemmell E, Seymour GJ. Immunoregulatory control of Th1/Th2 cytokine profiles in periodontal disease. *Periodontol 2000*. 2004;35:21-41.

35. Kinane DF, Preshaw PM, Loos BG, Working Group 2 of Seventh European Workshop on Periodontology. Host-response: Understanding the cellular and molecular mechanisms of host-microbial interactions--consensus of the seventh european workshop on periodontology. *J Clin Periodontol*. 2011;38 Suppl 11:44-48.

36. Cutler CW, Jotwani R. Antigen-presentation and the role of dendritic cells in periodontitis. *Periodontol 2000*. 2004;35:135-157.

37. Murphy KM, Reiner SL. The lineage decisions of helper T cells. *Nat Rev Immunol*. 2002;2(12):933-944.

38. Appay V, van Lier RA, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: Consensus and issues. *Cytometry A*. 2008;73(11):975-983.

39. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*. 1986;136(7):2348-2357.

40. Mosmann TR, Coffman RL. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*. 1989;7:145-173.

41. Orozco A, Gemmell E, Bickel M, Seymour GJ. Interleukin-1beta, interleukin-12 and interleukin-18 levels in gingival fluid and serum of patients with gingivitis and periodontitis. *Oral Microbiol Immunol*. 2006;21(4):256-260.

42. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev*. 2001;12(1):53-72.

43. Page RC, Schroeder HE. Pathogenesis of inflammatory periodontal disease. A summary of current work. *Lab Invest*. 1976;34(3):235-249.

44. Liljenberg B, Lindhe J, Berglundh T, Dahlen G, Jonsson R. Some microbiological, histopathological and immunohistochemical characteristics of progressive periodontal disease. *J Clin Periodontol*. 1994;21(10):720-727.

45. Halazonetis TD, Haffajee AD, Socransky SS. Relationship of clinical parameters to attachment loss in subsets of subjects with destructive periodontal diseases. *J Clin Periodontol*. 1989;16(9):563-568.

46. Buduneli N, Kinane DF. Host-derived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. *J Clin Periodontol*. 2011;38 Suppl 11:85-105.

47. Ozmeric N. Advances in periodontal disease markers. *Clin Chim Acta*. 2004;343(1-2):1-16.

48. Giannobile WV, Beikler T, Kinney JS, Ramseier CA, Morelli T, Wong DT. Saliva as a diagnostic tool for periodontal disease: Current state and future directions. *Periodontol 2000*. 2009;50:52-64.

49. Armitage GC. Analysis of gingival crevice fluid and risk of progression of periodontitis. *Periodontol 2000*. 2004;34:109-119.

50. Goodson JM, Dewhirst FE, Brunetti A. Prostaglandin E2 levels and human periodontal disease. *Prostaglandins*. 1974;6(1):81-85.

51. Ozkavaf A, Aras H, Huri CB, et al. Relationship between the quantity of gingival crevicular fluid and clinical periodontal status. *J Oral Sci*. 2000;42(4):231-238.

52. Lamster IB, Ahlo JK. Analysis of gingival crevicular fluid as applied to the diagnosis of oral and systemic diseases. *Ann N Y Acad Sci*. 2007;1098:216-229.

53. Loos BG, Tjoa S. Host-derived diagnostic markers for periodontitis: Do they exist in gingival crevice fluid? *Periodontol 2000*. 2005;39:53-72.

54. Goodson JM, Tanner AC, Haffajee AD, Sornberger GC, Socransky SS. Patterns of progression and regression of advanced destructive periodontal disease. *J Clin Periodontol*. 1982;9(6):472-481.

55. Tsai CC, Ku CH, Ho YP, Ho KY, Wu YM, Hung CC. Changes in gingival crevicular fluid interleukin-4 and interferon-gamma in patients with chronic periodontitis before and after periodontal initial therapy. *Kaohsiung J Med Sci*. 2007;23(1):1-7.

56. Ramseier CA, Kinney JS, Herr AE, et al. Identification of pathogen and host-response markers correlated with periodontal disease. *J Periodontol*. 2009;80(3):436-446.

57. Offenbacher S, Barros SP, Paquette DW, et al. Gingival transcriptome patterns during induction and resolution of experimental gingivitis in humans. *J Periodontol*. 2009;80(12):1963-1982.

58. Teles RP, Likhari V, Socransky SS, Haffajee AD. Salivary cytokine levels in subjects with chronic periodontitis and in periodontally healthy individuals: A cross-sectional study. *J Periodontal Res*. 2009;44(3):411-417.

59. Rescala B, Rosalem W,Jr, Teles RP, et al. Immunologic and microbiologic profiles of chronic and aggressive periodontitis subjects. *J Periodontol*. 2010;81(9):1308-1316.

60. Rakmanee T, Olsen I, Griffiths GS, Donos N. Development and validation of a multiplex bead assay for measuring growth mediators in wound fluid. *Analyst*. 2010;135(1):182-188.

61. Cook EB, Stahl JL, Lowe L, et al. Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: Allergics vs. non-allergics. *J Immunol Methods*. 2001;254(1-2):109-118.

62. Teles RP, Gursky LC, Faveri M, et al. Relationships between subgingival microbiota and GCF biomarkers in generalized aggressive periodontitis. *J Clin Periodontol*. 2010;37(4):313-323.

63. Thunell DH, Tymkiw KD, Johnson GK, et al. A multiplex immunoassay demonstrates reductions in gingival crevicular fluid cytokines following initial periodontal therapy. *J Periodontal Res*. 2010;45(1):148-152.

64. Hernandez M, Gamonal J, Tervahartiala T, et al. Associations between matrix metalloproteinase-8 and -14 and myeloperoxidase in gingival crevicular fluid from subjects with progressive chronic periodontitis: A longitudinal study. *J Periodontol*. 2010;81(11):1644-1652.

65. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: An overview of signals, mechanisms and functions. *J Leukoc Biol*. 2004;75(2):163-189.

66. Salazar-Mather TP, Hamilton TA, Biron CA. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. *J Clin Invest*. 2000;105(7):985-993.

67. Kikuchi T, Hahn CL, Tanaka S, Barbour SE, Schenkein HA, Tew JG. Dendritic cells stimulated with actinobacillus actinomycetemcomitans elicit rapid gamma interferon responses by natural killer cells. *Infect Immun*. 2004;72(9):5089-5096.

68. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annu Rev Immunol*. 1997;15:749-795.

69. Gorska R, Gregorek H, Kowalski J, Laskus-Perendyk A, Syczewska M, Madalinski K. Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. *J Clin Periodontol*. 2003;30(12):1046-1052.

70. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 is a unique cytokine that stimulates both Th1 and Th2 responses depending on its cytokine milieu. *Cytokine Growth Factor Rev*. 2001;12(1):53-72.

71. Takayanagi H, Ogasawara K, Hida S, et al. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature*. 2000;408(6812):600-605.

72. Gao Y, Grassi F, Ryan MR, et al. IFN-gamma stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation. *J Clin Invest*. 2007;117(1):122-132.

73. Teng YT, Mahamed D, Singh B. Gamma interferon positively modulates actinobacillus actinomycetemcomitans-specific RANKL+ CD4+ th-cell-mediated alveolar bone destruction in vivo. *Infect Immun*. 2005;73(6):3453-3461.

74. Honda T, Domon H, Okui T, Kajita K, Amanuma R, Yamazaki K. Balance of inflammatory response in stable gingivitis and progressive periodontitis lesions. *Clin Exp Immunol*. 2006;144(1):35-40.

75. Dutzan N, Vernal R, Hernandez M, et al. Levels of interferon-gamma and transcription factor T-bet in progressive periodontal lesions in patients with chronic periodontitis. *J Periodontol*. 2009;80(2):290-296.

76. Paul WE, Ohara J. B-cell stimulatory factor-1/interleukin 4. *Annu Rev Immunol*. 1987;5:429-459.

77. Fujihashi K, Kono Y, Beagley KW, et al. Cytokines and periodontal disease: Immunopathological role of interleukins for B cell responses in chronic inflamed gingival tissues. *J Periodontol*. 1993;64(5 Suppl):400-406.

78. Hart PH, Vitti GF, Burgess DR, Whitty GA, Piccoli DS, Hamilton JA. Potential antiinflammatory effects of interleukin 4: Suppression of human monocyte tumor necrosis factor alpha, interleukin 1, and prostaglandin E2. *Proc Natl Acad Sci U S A*. 1989;86(10):3803-3807.

79. Saidenberg-Kermanac'h N, Bessis N, Lemeiter D, de Vernejoul MC, Boissier MC, Cohen-Solal M. Interleukin-4 cellular gene therapy and osteoprotegerin decrease inflammation-associated bone resorption in collagen-induced arthritis. *J Clin Immunol*. 2004;24(4):370-378.

80. Shapira L, van Dyke TE, Hart TC. A localized absence of interleukin-4 triggers periodontal disease activity: A novel hypothesis. *Med Hypotheses*. 1992;39(4):319-322.

81. Giannopoulou C, Cappuyns I, Mombelli A. Effect of smoking on gingival crevicular fluid cytokine profile during experimental gingivitis. *J Clin Periodontol*. 2003;30(11):996-1002.

82. Salvi GE, Brown CE, Fujihashi K, et al. Inflammatory mediators of the terminal dentition in adult and early onset periodontitis. *J Periodontal Res*. 1998;33(4):212-225.

83. Schmitz J, Owyang A, Oldham E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity*. 2005;23(5):479-490.

84. Tominaga S. A putative protein of a growth specific cDNA from BALB/c-3T3 cells is highly similar to the extracellular portion of mouse interleukin 1 receptor. *FEBS Lett*. 1989;258(2):301-304.

85. Xu D, Chan WL, Leung BP, et al. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp Med*. 1998;187(5):787-794.

86. D'Acquisto F, Maione F, Pederzoli-Ribeil M. From IL-15 to IL-33: The never-ending list of new players in inflammation. is it time to forget the humble aspirin and move ahead? *Biochem Pharmacol*. 2010;79(4):525-534.

87. Castellani ML, Kempuraj D, Salini V, et al. The latest interleukin: IL-33 the novel IL-1-family member is a potent mast cell activator. *J Biol Regul Homeost Agents*. 2009;23(1):11-14.

88. Komai-Koma M, Xu D, Li Y, McKenzie AN, McInnes IB, Liew FY. IL-33 is a chemoattractant for human Th2 cells. *Eur J Immunol*. 2007;37(10):2779-2786.

89. Rank MA, Kobayashi T, Kozaki H, Bartemes KR, Squillace DL, Kita H. IL-33-activated dendritic cells induce an atypical TH2-type response. *J Allergy Clin Immunol*. 2009;123(5):1047-1054.

90. Theoharides TC, Zhang B, Kempuraj D, et al. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. *Proc Natl Acad Sci U S A*. 2010;107(9):4448-4453.

91. Moulin D, Donze O, Talabot-Ayer D, Mezin F, Palmer G, Gabay C. Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. *Cytokine*. 2007;40(3):216-225.

92. Bourgeois E, Van LP, Samson M, et al. The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. *Eur J Immunol*. 2009;39(4):1046-1055.

93. Oboki K, Ohno T, Kajiwara N, et al. IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci U S A*. 2010;107(43):18581-18586.

94. Allakhverdi Z, Comeau MR, Jessup HK, et al. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. *J Exp Med*. 2007;204(2):253-258.

95. Quentmeier H, Drexler HG, Fleckenstein D, et al. Cloning of human thymic stromal lymphopoietin (TSLP) and signaling mechanisms leading to proliferation. *Leukemia*. 2001;15(8):1286-1292.

96. Friend SL, Hosier S, Nelson A, Foxworthe D, Williams DE, Farr A. A thymic stromal cell line supports in vitro development of surface IgM+ B cells and produces a novel growth factor affecting B and T lineage cells. *Exp Hematol*. 1994;22(3):321-328.

97. Soumelis V, Reche PA, Kanzler H, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol*. 2002;3(7):673-680.

98. Kato A, Favoreto S,Jr, Avila PC, Schleimer RP. TLR3- and Th2 cytokine-dependent production of thymic stromal lymphopoietin in human airway epithelial cells. *J Immunol*. 2007;179(2):1080-1087.

99. Ito T, Wang YH, Duramad O, et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med*. 2005;202(9):1213-1223.

100. Moon PD, Kim HM. Thymic stromal lymphopoietin is expressed and produced by caspase-1/NF-kappaB pathway in mast cells. *Cytokine*. 2011;54(3):239-243.

101. Pecaric-Petkovic T, Didichenko SA, Kaempfer S, Spiegl N, Dahinden CA. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood*. 2009;113(7):1526-1534.

102. Chapple IL, Landini G, Griffiths GS, Patel NC, Ward RS. Calibration of the periotron 8000 and 6000 by polynomial regression. *J Periodontal Res*. 1999;34(2):79-86.

103. Bastos MF, Lima JA, Vieira PM, Mestnik MJ, Faveri M, Duarte PM. TNF-alpha and IL-4 levels in generalized aggressive periodontitis subjects. *Oral Dis*. 2009;15(1):82-87.

104. Haffajee AD, Socransky SS, Goodson JM. Comparison of different data analyses for detecting changes in attachment level. *J Clin Periodontol*. 1983;10(3):298-310.

105. Figueredo CM, Brito F, Barros FC, et al. Expression of cytokines in the gingival crevicular fluid and serum from patients with inflammatory bowel disease and untreated chronic periodontitis. *J Periodontal Res*. 2011;46(1):141-146.

106. Pradeep AR, Roopa Y, Swati PP. Interleukin-4, a T-helper 2 cell cytokine, is associated with the remission of periodontal disease. *J Periodontal Res*. 2008;43(6):712-716.

107. Griffiths GS. Formation, collection and significance of gingival crevice fluid. *Periodontol 2000*. 2003;31:32-42.

108. Duarte PM, da Rocha M, Sampaio E, et al. Serum levels of cytokines in subjects with generalized chronic and aggressive periodontitis before and after non-surgical periodontal therapy: A pilot study. *J Periodontol*. 2010;81(7):1056-1063.