COMPARISON OF SALIVARY AND SERUM LEVELS OF TNF ALPHA AND IL-4 IN CHRONIC PERIODONTITIS AND PERIODONTAL HEALTH

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BRANCH II

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CERTIFICATE

This is to certify that this dissertation titled "**COMPARISON OF SALIVARY AND SERUM LEVELS OF TNF ALPHA AND IL-4 IN CHRONIC PERIODONTITIS AND PERIODONTAL HEALTH**" is a bonafide record of work done by **Dr. Teenu Abraham** under my guidance during the study period of 2010-2013.

This dissertation is submitted to **THE TAMIL NADU Dr. MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY**. It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVIATIONS

BS	-	Bleeding Score
CAL	-	Clinical Attachment Level
CRP	-	C Reactive Protein
ELISA	-	Enzyme Linked Immuno Sorbent Assay
GCF	-	Gingival Crevicular Fluid
ICAM	-	InterCellular Adhesion Molecule
IFN	-	Interferon
Ig	-	Immunoglobulin
IL	-	Interleukin
LPS	-	Lipopolyssacharide
NK	-	Natural Killer
Pg	-	Porphyromonas gingivalis
PAMPs	-	Pathogen Associated Molecular Patterns
PPD	-	Periodontal Probing Depth
PI	-	Plaque index
NFκB	-	Nuclear Factor Kappa b
RANKL	-	Receptor Activator of Nuclear Factor – κ B Ligand
STAT	-	Signal Transduction and Activation of Transcription
Treg	-	Regulatory T Cell
TCR	-	T Cell Receptor

Th	-	Helper T Cell
TLR	-	Toll Like Receptor
TNF	-	Tumor Necrosis Factor
VCAM	-	Vascular Cell Adhesion Molecule
WBC	-	White Blood Cell

ABSTRACT

BACKGROUND:

Peridontitis is a chronic inflammatory disease that results in the destruction of the tooth supporting apparatus. It is believed to be mediated by the exaggerated host response that results from a disharmony between the pro inflammatory cytokine and anti inflammatory cytokine released during the inflammatory process. Recent advancements have enabled to detect the levels of proteins in oralfluids that may act as a bio marker for periodontal disease. This study was therefore conducted to assess the levels of salivary cytokines in patients with advanced periodontitis and healthy subjects.

AIM:

To analysis the levels of pro inflammatory cytokine TNF alpha and anti inflammatory cytokine IL-4 in saliva and serum and correlate it with the level of systemic cytokine hs-CRP in saliva and serum of chronic periodontitis patients and healthy subjects.

MATERIALS AND METHODS:

30 patients were included in the study. The study group was segregated as health group and chronic periodontitis group. Serum and saliva were collected from both the groups and the levels of TNF alpha and IL-4 was detected using ELISA. hs-CRP levels were detected using immunoturbidity

method. Statistical analysis was done using independent sample student T test and Pearson correlation test.

RESULTS:

The levels of salivary TNF alpha in disease showed a statistical significance ($P=0.035$, $P<0.05$). There was a significant correlation between the salivary levels of hs-CRP and TNF alpha in serum ($p<0.001$). However the levels of both cytokine in saliva and serum were not significant.

CONCLUSION:

Chronic periodontitis may be mediated by the Th2 response and TNF alpha may be used as a bio marker for periodontal diseases as its level showed a significant increase in periodontitis. The nonspecific serum marker for systemic inflammation hs-CRP may be well correlated to the pro inflammatory cytokine TNF alpha than the anti-inflammatory marker IL-4.

[key words: TNF alpha, IL-4, pro inflammatory cytokine, chronic periodontitis, anti-inflammatory cytokine, saliva]

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INTRODUCTION

Periodontitis is a chronic inflammatory disorder that may eventually result in breakdown of the tooth supporting apparatus. It has also been suggested that it may also play a role as modifying factor of the systemic health of patients.[112]

Microbial antigens present within the periodontal tissue initiate an inflammatory response in the host. The tissue destruction and disease progression that occurs in periodontal diseases is related to a prolonged but unprotected host response which may be mediated by genetic, environmental and systemic factors. This host response leads to a prolonged release of inflammatory mediators like kinins, eicasonoids, and cytokines.[84] Pro inflammatory cytokines are linked to extensive tissue destruction while anti-inflammatory cytokines counteract and attenuate disease progression. The imbalance between the pro inflammatory and anti-inflammatory cytokines may be an important determinant of periodontal disease progression.

Tumor necrosis factor-alpha, a pro inflammatory cytokines is released from T cells, macrophages, cells of the junctional epithelium, connective tissue fibroblasts and polymorphonuclear leukocytes as a response of innate immunity.[114] Prolonged production of TNF alpha results in excess bone resorption as it enhances osteoclastogenisis by up regulating RANK-RANKL coupling mechanism and by increasing the production of other pro inflammatory cytokines like IL-1,6,8 etc.[82]

IL-4 is generally considered as an anti-inflammatory cytokine as it suppresses the inflammatory function by inhibiting the transcription of pro inflammatory cytokine, suppressing the polarization of TH-1 cells and enhancing further production of other anti-inflammatory cytokines like IL-10.[3] They also help to reduce the ongoing tissue degeneration process by inhibiting the production of RANKL & MMP's and at the same time up regulating the expression of TIMP &OPG.[42]

These inflammatory cytokines play an important role in the regulation of host response into Th1 or Th2 immune response in periodontal diseases. The balance between Th1 and Th2 cytokines regulate the immune response to infection. The overall effect of the Th1 cytokines is to enhance cell-mediated responses, while the effect of Th2 cytokine IL-4 is to suppress cell-mediated responses and enhance the resistance associated with humoral immunity.[67]

There is a need for newer diagnostic tests that would help in early recognition of the microbial challenge, assessment of current disease activity, prediction of sites that are vulnerable for future breakdown and prediction of the periodontal therapy administered. Various researchers into this field are currently investigating the use of different oral fluids like saliva and GCF for diseases assessment.[62]

GCF may be regarded as a good diagnostic medium due to its proximity to the disease site but its difficulty in accurate volume determination, high technical demand and increased time consumption for

multiple sampling of individual tooth reduces its efficacy as a diagnostic medium.

Saliva is an alternative diagnostic medium that acts as a noninvasive diagnostic aid to measure biomarkers released during disease initiation and progression.[49] The localized nature and its proximity to periodontal lesions in the oral cavity, ready availability & ease in collection have increased the utilization of saliva as a natural biological fluid for measurement of microbial and protein biomarkers of the diseases process.[65] Periodontal disease has been reported to influence the course of systemic diseases through the release of cytokines into circulation.[58] Analysis of a systemic bio marker like hs-CRP in saliva would help in detection or assessment of any ongoing systemic inflammation in various chronic inflammatory conditions like diabetes mellitus, artherosclerosis . Hence this study aimed at a correlation between the levels of TNF alpha, IL-4 and hs-CRP in serum and saliva in both periodontitis patients and periodontaly healthy subjects.

AIMS AND OBJECTIVES

The aims of the present study are:

1. To evaluate the salivary level of the pro inflammatory cytokine TNF alpha and the anti-inflammatory cytokine IL-4 in healthy subjects and in patients with chronic periodontitis.
2. To evaluate the circulatory levels of TNF alpha and IL-4 in periodontal health and disease.
3. To correlate salivary and serum levels TNF alpha and IL-4 in both periodontal health and disease.
4. To correlate levels of the salivary and serum cytokines (TNF alpha & IL-4) and levels of hs-CRP in periodontal diseases.

REVIEW OF LITRATURE

PATHOGENISIS OF PERIODONTITIS

The progression of gingivitis to periodontitis is a slow process that occurs as loss of attachment over a long period of time and also this result in rapid attachment loss that occurs in episodic burst in short time.[103] It is now evident that periodontal diseases in humans has heterogeneous etiologies stemming from the development of biofilm in the subgingival environment ,social and behavioral factors and genetic or epigenetic traits, all of which are modulated and controlled by the underlying immune and inflammatory responses of the host. As a result of biofilm maturation, the pathogenic species developing in the periodontal pockets release an assay of virulence factors, antigens, or by products that evade the host defense mechanism, causing damage to cells and tissue via dysregulated inflammatory interactions, which typically consists of neutrophil, monocytes/macrophages, dentritic cells, T-cells and predominantly immunoglobulin producing B-cells.[85] The infected tissue/cells are overwhelmed by persistent pathogens accompanied with a lasting chronic inflammation where the potent pro inflammatory mediators and cytokines prevail. Inflammation is a characteristic feature that depicts the trigger of the defense mechanism against an invading pathogen in the body which is accompanied by a release of many cytokines.

Mahanonda R et al suggested that a critical aspect of the host response is the detection of bacteria by Toll-like receptors (TLRs).[61] Activation of the innate immune response by the binding of various bacterial components (i.e., diacyllipopeptides, peptidoglycan, LPS, flagellin, and bacterial DNA) to TLRs results in the production of cytokines and chemokines. Upon activation of TLRs, an intracellular signaling cascade is stimulated that leads to the activation of transcription factors (e.g., nuclear factor-kappa B, activator protein 1 (AP-1) and p38) and the production of various cytokines, many of which directly or indirectly stimulate osteoclast formation.

Evidence that cytokines played a critical role was shown in a non-human primate model. In this report, inhibition of interleukin (IL)-1 and tumor necrosis factor (TNF) reduced the progression of periodontal bone loss and loss of attachment, which was attributed to the recruitment of inflammatory cells (notably monocytes and lymphocytes) toward the bone.[40]

Cytokines are low molecular protein involved in initiation and effector stages of immunity and inflammation, in which they regulate the amplitude and duration of the response. They are the cell regulators that have a major influence on the production and activation of different effector cells which decides the type of immune response that occurs on exposure to a pathogen which is in turn is vital in determining resistance or susceptibility to diseases. Cytokines interact with specific cell surface receptors which are usually

expressed in relatively low numbers. Many cytokines are pleiotrophic, having multiple activation on different target cells and or overlapping cell regulatory actions. But despite this overlap cytokine function may be identical6. Cytokines interact in a network firstly by inducing each other, secondly, trans modulating cell surface receptors and thirdly, by a synergistic, additive or antagonistic interaction on cell function.[9]

PRO AND ANTI INFLAMMATORY CYTOKINES

Kelso et al suggested that a successful immune response to an infectious agent depends on activation of appropriate effector functions.[51] As periodontitis is a chronic inflammatory disease, it is certainly reasonable that such a condition establishes a local cytokine environment that influences the immune response. 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. Production of appropriate cytokine is essential for the development of protective immunity. If inappropriate cytokines is elicited destructive or progressive disease can result.

The extent of periodontal tissue destruction is mainly determined by the balance maintained between the pro- and anti- inflammatory cytokines and the regulation of their receptor and signaling pathways. Pro inflammatory cytokines mediate the tissue damage, which leads to loss of function and clinical disease. The persistent activation of immune responses leads to

increased synthesis and secretion of these pro inflammatory cytokines with concomitant effects on function and turnover of periodontal cells.

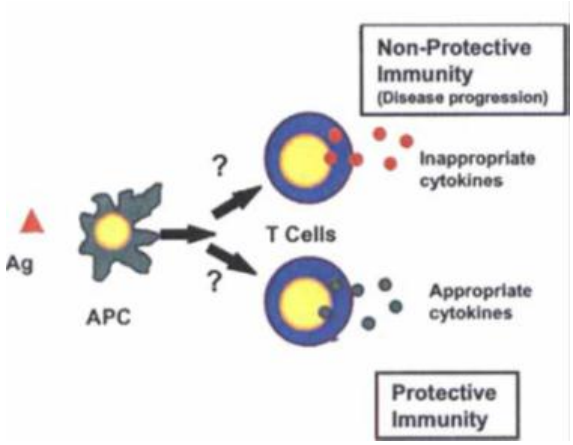


Fig 1: release of protective and non-protective cytokine

Snyderman R et al, suggested that periodontitis has high levels of proinflammatory cytokines, including IL-1b and tumor necrosis factor-alpha (TNF-alpha) and low levels of cytokines which suppress the immunoinflammatory response such as IL-10 and IL-4.[102] These cytokines are associated with the active stages of periodontitis.

Kawai and Akaira et al found that these cytokines (TNF alpha and IL-1β) are synthesized by the activation of transcription factor NF-ƙB which is activated by the recognition of LPS by a macromolecular complex involving CD14, MD-2 and TLR-4.[50]

Tumour necrosis factor-a (TNF-a) is one among the pro-inflammatory cytokines that induces bone resorption and leads to up regulated production of prostaglandin E2 (PGE2) and MMP secretion.

Graves et al showed that TNF-α plays a central role in inflammatory reaction, alveolar bone resorption, and the loss of connective tissue attachment. He studied that IL-1β and TNF-alpha induce up regulation of adhesion molecules on leucocytes and endothelial cells, they stimulate the production of chemokines (which recruit circulating leucocytes to sites of inflammation) and they induce expression of other inflammatory mediators that potentiate inflammatory responses, such as the prostaglandins and MMPs.[35]

Cytokines up regulate the production of inflammatory mediators in the periodontium (e.g. prostaglandins, MMPs, cytokines, chemokines) leading to tissue destruction. Multiple feedback loops develop; for example, cytokines induce the secretion of prostaglandins, and increased prostaglandin concentrations result in increased cytokine secretion.[37]

Nakao et al demonstrated that IL-1β and TNF-a synergistically increase PGE2 and TNF-α was shown to upregulate PGE2 and COX production in HGFs via the JNK and NF-kB signaling pathways.[73]

Palmqvist studied that there was a dose-dependent stimulation of IL-6 and LIF mRNA and protein by IL-1b and TNF-alpha and dose-dependent stimulation of IL-11 mRNA and protein by IL-1b.[83]

In another study, **Noguchi et al** found that TNF-alpha, IL-1b and PGF2a all stimulated IL-6 production in cultured HGFs and PGF2a synergistically increased IL-6 production stimulated by TNF-alpha and IL-1b.[78] **Yucel-Lindberg** also found that TNF-alpha also induced IL-1a and IL-1b production in HGFs and this was synergistically enhanced by the presence of bradykinin.[119]

Kent et al studied that IL-1b and TNF-alpha act synergistically in stimulating IL-6 secretion by HGFs and this combination of cytokines was shown to be many hundreds of times more potent in stimulating IL-6 production than LPS.[52]

Kobayashi and Okada et al found that IL-1b and TNF-a induced IL-1a secretion in HGFs and this production was differentially modulated by T-cell derived cytokines including IFN-g and IL-4.[53]

Ohta et al studied that Chemokine expression is stimulated by cytokines; for example, IL-1b and TNF-alpha increase the production of RANTES/CCL5 in HGFs and IFN-ϒ, TNF-α and IL-4 cooperatively regulate CXCR3- agonistic chemokines in oral keratinocytes and fibroblasts.[81]

Liu et al studied enhanced accumulation of PMNLs has been reported in the gingival tissues of patients with periodontitis, and has been associated with up regulated IL-8, ICAM-1, IL-1b and TNF-alpha expression.[57]

Delima et al showed that inhibition of IL-1 using human soluble IL-1 receptor type I (IL-1R) significantly reduced inflammation, connective tissue attachment loss, and bone resorption induced by periodontal pathogens.[21]

In response to P. gingivalis oral gavage, **Baker PJ et al** studied that mice with genetically deleted IL-6 had decreased bone loss compared to wild-type mice, suggesting that the production of IL-6, which is pro inflammatory, contributed to bone resorption.[8]

Garlet et al have demonstrated that similar cytokines considered harmful in the context of tissue destruction may play important roles in the control of periodontal infection.[31]

Dinarello et al found that TNF-α plays a critical role in both innate and adaptive immune responses, up-regulating antigen presentation and the bactericidal activity of phagocytes.[22]

Clements et al proposed that Tumor necrosis factor alpha is produced mainly by macrophages in response to agents such as lipopolysaccharide. The decrease in TNF-a seemed to reduce the host response, thereby leading to higher levels of bacteria; however, because the host response was less, there

was a reduced expression of the cytokines that stimulate bone resorption, which resulted in net bone loss.[18]

Hosokawa et al studied that IL-4, IL-13 and IL-10 inhibited CXCL10 production by IFN-g- or TNF alpha stimulated cells.[40]

Agnello et al showed that IL-4 is also supposed to attenuate periodontitis progression, in contrast to its putative destructive role, previously discussed. Similar to IL-10, IL-4 presents marked suppressive and anti-inflammatory properties mediated by its capacity to inhibit the transcription of pro inflammatory cytokines and IFN-γ, then suppressing the polarization of Th1 cells.[2]

Jarnicki and **Fallon et al** found that IL-4 induces the production of cytokines with similar or complementary suppressive properties, such as IL-10.[44]

Ihn et al 2002 studied that IL-4 is also able to inhibit the production of MMPs and RANKL and concomitantly induces the up-regulation of its respective inhibitors TIMPs and OPG reinforcing its potential protective role in PD pathogenesis.[41]

Immune Regulation in Periodontal Pathogenesis

The importance of cytokines in the pathogenesis of periodontal disease is apparent at a number of levels. Not only do they act as initiators and regulators of innate and adaptive immunity but they also mediate the tissue

damage, which leads to loss of function and clinical disease. Many of the non-immune cell types of the periodontium (e.g. keratinocytes and fibroblasts) synthesize cytokines in response to bacteria and other cytokines, and cytokines also influence turnover of extracellular matrix components and the fibres of the periodontal ligament (PDL).[57] Cytokines also have a central role in osteoclast activation. Cytokines drive the tissue destruction that results in the clinical manifestations of periodontitis through myriad overlapping effects on cells and mediators in the periodontium. The complex interactions between cytokines and immune responses make it difficult to distinguish and compartmentalize different aspects of the role of cytokines in driving tissue destruction.

Numerous immune histochemical studies conducted have proposed the paradigm that periodontitis is a B cell lesion and the immunoregulatory role of T cell in periodontitis have been proposed by **Seymoer et al.**[100]

Romagnani et al,[96] has characterized 3 subsets of T-helper cells based on their cytokine profile. Typical secretory products of Th1 cells are IL-2.IL-12, TNFα and INFϒ and those of Th2 cells are IL-4, IL-5, IL-6,IL-10 and Th3 are known to secret TGFβ.

Mosman et al[69] studied that Thl cytokines are involved in cell-mediated inflammatory reactions. They increase the ability of macrophages to lull intracellular and extracellular pathogens and also mediate delayed type hypersensitivity reactions.

Reinhardt RA et al[94] suggested that Th2 cytokines are found in association with strong antibody and allergic responses. These cells stimulate mast cells, eosinophils and immunoglobulin E (IgE) antibodies and are elevated in allergic diseases and helminth infections

Ebersole & Taubman et al found that Cytokine profiles of cells extracted from 6 patients were consistent with Thl cells in that they were IL-2 and IFN-ϒ positive but negative for IL-5.[23] This study speculated that Thl cells may be destructive in periodontal disease via the production of IFN-ϒ by potential stimulation of macrophage secretion of IL-1 with subsequent bone resorption. However, the presence of messenger RNA for IFN-.I, IL-6 and IL-13 but not for IL-2, IL-4 or IL-5 in CD4+ T cells extracted from periodontal disease lesions indicates that Tho cells may be involved in periodontal disease.

Takeichi et al, showed that IFN-ϒ and IL-1-p messenger RNA was expressed by some gingival cells on extraction, indicating Type 1 cells and upon stimulation, IL-6 transcripts were also expressed but no IL-2 or IL-2 receptor messenger RNA could be detected. These studies indicate that periodontal lesions are predominated by Th-1 cell types.[107]

However other set of studies conducted by **Yamazhk**i found that an increased percentage of IL-4-positive cells proportional with an increasing ratio of B cells to T cells.[116] IL-4 was the prominent cytokine in periodontally affected tissues compared with IL-2, IFN-ϒ and IL-6. The demonstration of concentrations of IgG4 many times higher in sites of active periodontitis than

in serum as well as significantly elevated concentrations compared with stable lesions also suggests a role for IL-4 and Th2 responses in periodontitis lesions. Analysis of IL-2: IL-4 ratios revealed significantly lower ratios for cells derived from periodontitis tissues compared with cells from gingivitis tissues. Taken together, these data seem to support the hypothesis that Thl cells are associated with the stable lesion, whereas a Th2 response may lead to nonprotective antibodies and disease progression. A Th2 response that results in protective antibodies may result in elimination of organisms. (**Gemmell E, et al**)[33]

Karatzas et al showed that T-cell lines and clones specific for P. gingivalis have been reported to resemble Tho cells, although one CD4-positive clone did produce IL-4 and IL-5 messenger RNA, suggesting a Th2 profile.[47]

Another study showed that P gingivalis-reactive T-cell lines derived from the peripheral blood of a P. gingivalis infected adult periodontitis and a gingivitis subject and from the gingival tissues of the adult periodontitis patient, contained both the Th2 cytokine, IL-4 and the Thl cytokines IFN-) I and IL-2, although there was a higher percentage of IL-4-positive T cells in the adult periodontitis-derived lines.[32]

These data may therefore indicate that the full range of cytokine-producing T cells (Tho, Thl and Th2) are all found in both gingivitis and

periodontitis lesions, or may in fact offer supporting evidence for the concept that such distinction of T cells does not in reality exist.

Effect of cytokines on vascular endothelium

Cytokines have increased effect on endothelial cells. Circulating cytokines interact with specific receptors on various cell types and activate JAK-STAT, NFKB & smad signaling pathway leading to an inflammatory response involving cell adhesion, permeability & apoptosis. Cytokine induced activation of these pathways in endothelial cells modifies the production/activity of vasodilatory mediators like nitric oxide, prostacyclin, endothelium derived hyperpolarizing factor & bradykinin etc.

When endothelial cells undergo inflammatory activation, an increase in expression of adhesion molecules such as selectins, vascular cell adhesion molecule -1 (VCAM-1) & intercellular adhesion molecule -1 (ICAM-1) promotes the adherence of the inflammatory cells monocytes, neutrophils, lymphocytes and macrophages and recruitment of additional cytokines growth factors and MMPs. The increased expression of these cell adhesion molecules are brought out by the release of cytokines like IL-1beta, IL-6, 8, TNF alpha etc during the inflammatory process.

Cytokines could also induce vascular cell growth and migration.it was shown that TNF–α and IL-6 induce VGEF expression in cultured A431 human endothelial carcinoma and skeletal myoblast cell lines.

Tumor necrosis factor a (TNF-a) and IL-1 mediate adhesion molecule expression on endothelial cells and hence play a role in the migration of polymorphonuclear neutrophils (PMNs), lymphocytes and macrophages (MQ) into the periodontal tissue.

Dinarello CA et al showed that TNF-α acts in the cell migration process at multiple levels, inducing the up-regulation of adhesion molecules and the production of chemokines, which are chemotactic cytokines involved in cell migration to infected and inflamed sites.[22]

Springer et al suggested that Many of the endothelial surface cell adhesion molecules, including Eselectin, VCAM- l and ICAM- l, are increased by inflammatory cytokines, such as TNF-a, IL-I.[104]

Bevilacqua MI et al suggested that both tumor necrosis factor αand IL-1 have been shown to act on endothelial cells to increase the attachment of polymorphonuclearneutrophils and monocytes and thus help to recruit these cells into sites of inflammation.[12] TNFα can initiate apoptosis. It can stimulate apoptosis in many cell types by the recruitment of the DED containing protein caspase-8 to the receptor complex following association of TRADD & FADD to TNRF-1. This receptor recruitment results in autocatalytic activation of caspase-8.it then initiates a hierarchical series of caspase activation steps culminating in the activation of effector caspase like caspase-3. Cytokines also promote adhesion of immune cells to EC and cause an increase in vascular permeability.

M F Lademarco et al proposed that when TNF-alpha and IL-4 were combined, there was a synergistic increase in VCAM-1 expression and a dramatic prolongation of the appearance of VCAM-1 on the cell surface.[59] This synergy results from a combination of transcriptional activation by TNF-alpha and the stabilization of resulting transcripts by IL-4. IL-4 allows sub threshold concentrations of TNF-alpha (concentrations that would not normally activate expression of adhesion molecules on the endothelium) to selectively increase VCAM-1 expression and to prolong its appearance on the surface of endothelial cells.59Interaction between vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells and alpha 4 integrins on leukocytes is thought to mediate the selective recruitment of eosinophils and lymphocytes that occurs in allergic diseases. IL-4 is associated with allergic conditions and it has been shown to selectively increase expression of VCAM-1 on endothelial cells in vivo, suggesting that it could be responsible for VCAM-1 expression in allergic disease. Using a combination of immunofluorescence, flow cytometry and Northern analysis, we compared the effect of TNF-alpha and IL-4 on VCAM-1 expression.

Toi, M et al suggested that IL-4 has emerged as a potent mediator of endothelial cell function. It acts as an endothelial cell growth factor, specifically increases vascular cell adhesion molecule (VCAM)-1.[115]

M H Thornhill suggest that whereas IL-1 and TNF- alpha alone are unselective in terms of leukocyte adhesion to EC, the combination of TNF (or

LT) with IL-4 or IFN-gamma may be of key importance in determining the recruitment of a lymphocyte-predominant infiltrate in immune mediated inflammation, and in initiating the transition from acute to chronic inflammation.[60]

Effect of cytokines on bone cells

Graves DT et al suggested that inflammation and bone loss are hallmarks of periodontal disease.[37] Accumulated evidence demonstrates that PD involves bacterially derived factors and antigens that stimulate a local inflammatory reaction and activation of the innate immune system. Proinflammatorymolecules and cytokine networks play essential roles in this process. Interleukin-1 and tumor necrosis factor-alpha seem to be primary molecules that, in turn, influence cells in the lesion. Eventually, a cascade of events leads to osteoclastogenesis and subsequent bone loss via the receptor activator of nuclear factor-kappa B (RANK)–RANK ligand (RANKL)–osteoprotegerin (OPG) axis. The initial response to bacterial infection is a local inflammatory reaction that activates the innate immune system. Amplification of this initial localized response results in the release of an array of cytokines and other mediators and propagation of inflammation through the gingival tissues.

The failure to encapsulate this ‘‘inflammatory front’’ within gingival tissue results in expansion of the response adjacent to alveolar bone. Whether bone loss will occur in response to an inflammatory reaction is now known to

depend on two critical factors. First, the concentration of inflammatory mediators present in gingival tissue must be sufficient to activate pathways leading to bone resorption.

Lerner UH et al said that the inflammatory mediators must penetrate gingival tissue to reach within a critical distance to alveolar bone.[56] Achieving critical concentrations of inflammatory mediators that lead to bone resorption depends on the expression of proinflammatory cytokines, such as interleukin (IL)-1, -6, -11, and -17, tumor necrosis factor-alpha (TNF-a), leukemia inhibitory factor and oncostatin M. This is the opposite of the expression of anti-inflammatory cytokines and other mediators, such as IL-4, -10, -12, -13, and -18, as well as interferon-beta (IFN-b) and -gamma (IFN-g), which serve to inhibit bone resorption.

Assuma R et al demonstrated that in an animal model, Porphyromonasgingivalis (Pg)-soaked silk ligatures applied to posterior mandibular teeth to induce experimental periodontitis showed significant inflammatory cell recruitment and osteoclast formation surrounding bone in the control primates.[6] Thus, infection with Pg in these animals was associated with expansion of the inflammatory front to alveolar bone. In contrast, antagonists to cytokines TNF-alpha and IL-1 reduced the appearance of inflammatory cells in this region and the formation of bone resorbing osteoclasts. Injection of these antagonists reduced recruitment of inflammatory cells by 80%, osteoclast formation by 67% and bone loss by 60% compared to

control sites (P <0.01). These findings suggested that inhibition of the inflammatory mediators can prevent the inflammatory front from reaching alveolar bone, and it was associated with a reduction in bone loss in this animal model.

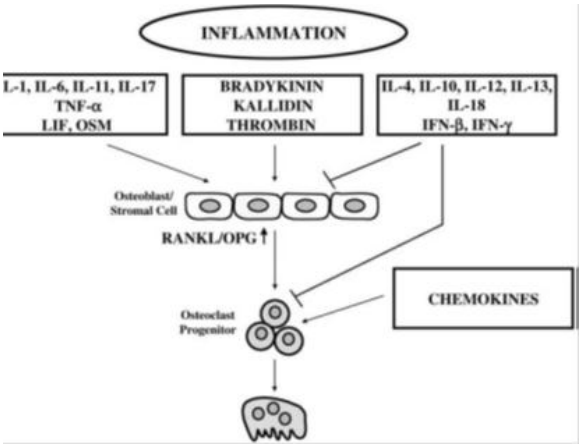


Fig 2-Stimulation and inhibition of osteoclast formation and bone resorption involves the interplay between a number of inflammatory cytokines and other mediators acting through RANKL binding to RANK on osteoclast progenitor cells. LIF = leukemia inhibitory factor; OSM = oncostatin M. Reprinted with permission from the International and American Associations for Dental Research.

Nakashima T et al proposed that During an inflammatory response, proinflammatorcytokines, such as IL-1b, -6, -11 and -17 and TNF-a, can induce osteoclastogenesis by increasing he expression of RANKL while decreasing OPG production in osteoblasts/stromal cells.[74] Whereas anti-

inflammatory mediators, such as IL-13 and IFN-g, may lower RANKL expression and/or increase OPG expression to inhibit osteoclastogenesis.

Mundy et al studied that Tumor necrosis factor molecules stimulate bone resorption by inducing the proliferation and differentiation of osteoclast progenitors and activating formed osteoclasts indirectly.[70] It is now well accepted that a large consortia of cytokines, cell-signaling molecules and matrix metalloproteinases are dysregulated and intimately involved in the pathogenesis of periodontitis. The task ahead of us is to identify the various roles of these important biological mediators of inflammation and how to control them. A principal feature of inflammatory-mediated bone loss in periodontitis is enhanced osteoclast activity without a corresponding increase in bone formation. Osteoclasts are multinucleated cells that are derived from the monocyte/macrophage lineage and are considered to be the principal cell responsible for bone resorption (**Boyle WJ et al).**[15]

Pettit AR et al said that multinucleated osteoclasts have been shown to resorb alveolar bone in both animal and human studies of periodontitis.[91] The formation of osteoclasts is driven by cytokines present in the inflamed periodontal tissues, and understanding this process is central in the development of strategies to control this process.

EFFECT OF CYTOKINES ON SOFT TISSUE

Reynolds JJ et al found that the effects of cytokines and cell-signaling molecules on normal and pathological cellular process are important and it is proposed that their roles in the pathophysiology of extracellular matrix destruction result from their excessive production, dysregulation or inadequate inhibition of these cytokines.[95]

Le J et al suggested that tumor necrosis factor is also a multipotential cytokine having a wide variety of biological effects and has been suggested to have similar effects as IL- 1.[55]

Clemens M J et al found that tumor necrosis factor alpha is produced mainly by macrophages in response to agents such as lipopolysaccharide. IL-1 and tumor necrosis factor a are key mediators of chronic inflammatory diseases and have the potential to initiate tissue destruction and bone loss in periodontal disease IL-1 has been shown to stimulate fibroblasts in culture to produce collagenase.[18]

Meikle MC, et al[64] found that tumor necrosis factor also mediates tissue destruction by stimulating collagenase and degradation of type 1 collagen by fibroblasts leading to connective tissue destruction. Cytokines drive the secretion of inflammatory mediators and destructive enzymes

Noguchi et al studied in 2007 that Cytokines upregulate the production of inflammatory mediators in the periodontium (e.g. prostaglandins, MMPs,

cytokines, chemokines) leading to tissue destruction. Multiple feedback loops develop; for example, cytokines induce the secretion of prostaglandins and increased prostaglandin concentrations result in increased cytokine secretion (Noguchi et al. 2007).[78]

Morton &Dongari-Bagtzoglou[68] in 2001 found that IL-1b and TNF-a induce COX-2 in oral epithelial cells and IL-1b upregulates COX-2 expression in HGFs.

Yucel-Lindberg et al in 1999 found that IL-1b and TNF-a synergistically increase PGE2 production in HGFs and TNF-a was shown to upregulate PGE2 and COX production in HGFs via the JNK and NF-kB signaling pathways.[118]

Agarwal et al in 1995, identified that cytokines induce the secretion of other cytokines. IL-1b induces the expression of IL-6, IL-8 and TNF-a in HGFs, and also acts in an autocrine manner to induce further IL-1b expression.

Palmqvist et al in 2008 conducted a study of cytokine expression in HGFs obtained from non-inflamed gingiva where there was a dose-dependent stimulation of IL-6 and LIF mRNA and protein by IL-1b and TNF-a and dose-dependent stimulation of IL-11 mRNA and protein by IL-1b. In another study, TNF-a, IL-1b and PGF2a all stimulated IL-6 production in cultured HGFs and PGF2a synergistically increased IL-6 production stimulated by TNF-a and IL-

1b.[83] TNF-alpha also induced IL-1a and IL-1b production in HGFs, and this was synergistically enhanced by the presence of bradykinin. It was observed that the up regulation of IL-6 production by HGFs that is induced by IL-1b is mediated by the p38 MAPK and NFkB signalling pathways. IL-1b and TNF-a act synergistically in stimulating IL-6 secretion by HGFs and this combination of cytokines was shown to be many hundreds of times more potent in stimulating IL-6 production than LPS.

Cytokine effect on epithelium

Bacteria by up regulate cytokine secretion and expressing adhesion molecules. Keratinocytes when challenged with bacterial infection, express a large variety of cytokines like IL-1α, IL-1β, IL-8 and TNF-α etc. Physiologically keratinocytes have high turnover rate.

Cytokine effect on gingival fibroblast

The gingival fibroblast and periodontal ligament cells are involved in release of various cytokines which not only function of inflammatory cells but also remodeling of gingiva, periodontal ligament and alveolar bone. An additional illustration of the important role of gingival fibroblasts in PD pathogenesis is its long-lasting ability to produce inflammatory cytokines after LPS challenge.

Uehara & Takada[1] said that during inflammation, resident GF are triggered by cytokines released by macrophages to enhance their synthesis of

cytokines. Thus, IL-1β and TNF-α stimulate the expression and release of IL-6 and IL-11 in human gingival fibroblast. HGFs are responsive to LPS and constitutively express mRNA for a variety of TLRs and NLRs, stimulation of which leads to production of pro-inflammatory cytokines such as IL-6, IL-8. HGFs challenged with P. gingivalis and E. coli LPS secrete IL-6 and IL-8 with no evidence of LPS tolerance, indicating that HGFs can sustain the inflammatory response in the periodontium.

In addition to LPS, HGFs also respond to outermembrane protein and polysaccharide of P. gingivalis by producing inflammatory cytokines. It was also found that primary HGFs and PDL fibroblasts respond to P. gingivalis by increasing gene expression for IL-1b, IL-6, IL-8, TNF-a and regulated on activation normal T-cell expressed and secreted (RANTES), with heterogeneity in responsiveness between fibroblasts from different donors and this may be important in determining susceptibility to periodontitis.

Elevated levels of cytokines can also locally amplify responses to LPS. For example, CD141 HGFs those were primed with IFN-gamma increased production of IL-8 in response to LPS through augmentation of the CD14–TLR system.

Nakajima[72] reported increased serum levels of IL-1b, TNF, OC, soluble intercellular adhesion molecule (sICAM), IL-6, MMP-9 have been reported in experimental studies in animal models as well as in clinical studies in humans mediated by metalloproteinases.

Biomarkers in periodontal diagnosis

Traditional clinical assessment of periodontal diseases such as pocket probing depth, Clinical attachment level, bleeding on probing etc., though provide information on past periodontal progression ,are error prone methods which makes it difficult for the clinicians to determine the current and future clinical progression of the diseases

A systematic review **by Buduneli N**[16] suggested that host-derived diagnostics are at an early stage of development. Analysis of the cytokine levels in serum, saliva, GCF etc are considered to be a good diagnostic marker as they help in assessing the progression of the disease and helps in a better evaluation of therapeutic prognosis.

Cytokines in gingival tissues and cell culture supernatants

Yin L, et al found that the relative amount of IL-1 beta mRNA expression of gingival samples from patients with adult periodontitis was much higher than that from the normal control. The relative amount of TNF-alpha mRNA expression, gingival samples from AP patients was also much higher than that from the normal control.[121]

K. Fujihashi et al conducted a study on gingival mononuclear cells and showed that GMC from localized inflammatory tissues in severe stages of AP possess a distinct cytokine profile represented by high levels of IL-5 and

IL-6 mRNA expression and protein synthesis, whereas IL-2 and IL-4 were not detected.[45]

Tervahartiala et al found that Aggressive periodontitis gingival tissue has an elevated expression of TNF-alpha and especially its p55 receptor, suggesting that TNF-alpha may contribute to tissue degradation in periodontitis.[109]

To elucidate the mechanisms of tissue breakdown in periodontitis, **Kamagata Y et al[46]** examined cytokine production by human periodontitis gingival tissue. Twelve periodontitis patients were included in this study. Control subjects with healthy periodontium consisted of nine individuals. Gingival samples were biopsied from inflamed or healthy gingival tissues. Biopsy specimens were dissected into fragments 3 mm in diameter and plated onto 24 well culture plates with RPMI 1640 medium. IL-1 activity was measured by a growth inhibition assay using melanoma cell line A 375. An enzyme-linked immunosorbent assay (ELISA) was used for measuring levels of human IL-1 alpha, IL-1 beta. TNF alpha activity was measured by a growth inhibition assay using cell line LM2D6. IL-1 activity was detected in significantly (p less than 0.001) higher levels in culture supernatants from gingival tissues in periodontitis (48.0 +/- 23.3 units/ml) than in control tissues (2.3 +/- 0.6 units/ml), however, levels of IL-1 activity were not associated with periodontal pocket depth or extent of alveolar bone resorption in periodontitis. Cytokines in serum.

Hence, it has been proposed by **Beck** and **Offenbacher et al**[11] that patients with periodontitis may have elevated circulating levels of some inflammatory markers. Monocytes, macrophages and other cells (including fibroblasts and endothelial cells) respond to the dental plaque microorganisms, membrane associated vesicles, lipopolysaccharides (LPS) and other soluble and particular fractions by secreting a number of chemokines and inflammatory cytokines, especially tumor necrosis factor (TNF)-α, prostaglandin (PGE2), interleukins (IL-1β and IL-6). These inflammatory cytokines and prostaglandin have been associated with the presence of various bacterial infections including periodontitis.

Górska et al assessed the relationship between clinical parameters and concentrations of the key cytokines (IL-1β, TNF-α, IL-2, IFN-γ, IL-4, IL-10), important in the initiation and progression of periodontal disease, within inflamed gingival tissues and serum samples from patients with severe chronic periodontitis. The concentrations of IL-1β, TNF-α, IL-2, IFN-γ were, on average, significantly higher in serum samples and gingival tissue biopsies from periodontitis patients than in healthy controls. However, serum samples from both groups showed high individual variability of cytokine profiles, and no association between cytokine concentrations and clinical parameters of periodontitis was found. On the contrary, IL-4 and IL-10 levels in both kinds of samples obtained from patients and controls were generally low or even undetectable and remained, on average, on the same level. These results

indicate that high variability of cytokine concentrations and low frequency of their detection in serum samples from periodontitis patients make these determinations useless for the detection of disease presence and/or its severity.[34]

Cytokine levels in GCF

Hence researches were conducted in the direction where oral fluids were analyzed as diagnostic medium for biomarker evaluation. Gingival crevice fluid (GCF) is a complex mixture of substances derived from serum, leukocytes and structural cells of the periodontium and oral bacteria. These substances possess a great potential for serving as indicators of periodontal disease and healing after therapy. The host-derived substances in GCF include antibodies, cytokines, enzymes and tissue degradation products. The antibodies in gingival crevicular fluid are comprised of both locally and systemically synthesized molecules and they reflect periodontal colonization by particular microbial species. They may also be crucial in eliciting destructive inflammatory reactions in the periodontium.

Beck et al[10] studied that Low GCF levels of monocytic products such as interleukin-1 or tumor necrosis factor are detected in gingivitis which indicated a low activation level of cells associated with chronic inflammation.

Figueredo et al[30] found that levels of GCF interleukin-1 were increased in samples from periodontitis patients, regardless of the severity of disease at the sample site.

Catherine Giannopoulou et al[17] found that in periodontally diseased subjects the total amounts of IL-1β, IL-6 and IL-8 were significantly elevated as compared to healthy subjects, whereas IL-4 showed an inverse relationship to periodontal status and higher amounts were found in the healthy group. The amounts of all four cytokines were positively correlated with probing depths. IL-4, IL-6 and IL-8 were significantly correlated to smoking while stress was associated with IL-1β, IL-6 and IL-8 levels. Hence they concluded that crevicular IL-1β, IL-6 and IL-8 reflect the activity of periodontal destruction, whereas IL-4 shows an inverse correlation to it. The enhanced production of inflammatory cytokines in the presence of smoking and stress may have clinical consequences.

Saliva as diagnostic medium

Recent reviews have reported presence of several mediators of chronic inflammation and tissue destruction in whole saliva of periodontitis patients.[48]

S. Chiappin et al[98] said that Salivary glands have rich vasculature from which saliva is filtered and processed. Salivary components may originate entirely from the salivary glands or may be derived from the blood by passive diffusion or active transport. In cases where components in saliva

are derived from the blood, levels of biochemical and immunological components measured in saliva may reflect blood levels. Substitution of saliva samples for blood in analysis of biomarkers is of considerable interest because collection of saliva is less invasive and does not have any of the risks associated with collection of blood. While there is some information about single biomarkers in saliva (such as cortisol), the correspondence to blood levels varies widely by biomarker and to date there has been little published regarding how well blood levels of specific cytokines or other biomarkers are represented in saliva, or how saliva collection technique affects recovery of specific bio-markers.

Whole saliva represents pooled samples with contribution from all periodontal sites and thus helps to provide an overall assessment of diseases status.[65] Saliva can be collected with or without stimulation. Stimulated saliva is collected by masticatory action (i.e., from a subject chewing on paraffin) or by gustatory stimulation (i.e., application of citric acid on the subject's tongue; Mandel, 1993).[63] Stimulation obviously affects the quantity of saliva; however, the concentrations of some constituents and the pH of the fluid are also affected. Unstimulated saliva is collected without exogenous gustatory, masticatory, or mechanical stimulation. Unstimulated whole saliva often correlates to systemic clinical conditions more accurately than stimulated saliva, since materials use to stimulate flow may change salivary composition

Navaszesh et al[75] suggested that the best two ways to collect whole saliva are the draining method, in which saliva is allowed to drip off the lower lip, and the spitting method, in which the subject expectorates saliva into a test tube.

Gursory et al[38] in the year 2009 conducted a study to analysis the levels of IL-1,1L-6, & TNF alpha in saliva of 84 chronic periodontitis patients and 81 controls. There was no statistical difference in the level of IL-6 & TNF alpha.it was therefore stated that IL-1 alone can differentiate periodontitis.

kaufman E et al[49] suggested that Saliva is of additional advantage as it acts as a noninvasive diagnosis fluid to measure biomarkers released during disease initiation and progression.

Miller et al[65] suggested that The localized nature and proximity of periodontal lessions to the oral cavity, ready availability & ease in collection rewards saliva with additional credits for saliva to be used as a natural biological fluid for measurement of microbial and protein biomarkers of the disease process. Furthermore, **Frodge et al** evaluated salivary concentrations of TNF-a, RANKL, and ICTP in 35 subjects with moderate to severe chronic periodontitis in comparison with 39 healthy controls. The authors reported that salivary TNF alpha levels were significantly elevated in chronic periodontitis patients suggesting the utility of this biomarker in a panel of salivary parameters that could facilitate the screening, diagnosis and management of periodontal disease.

Analysis of saliva for Systemic inflammatory response in periodontal disease

Chronic periodontal infections are associated with systemic changes to blood and blood forming organs. Periodontal disease is capable of predisposing to vascular disease given the abundance of gram negative species involved, easily detectable levels of pro inflammatory cytokines, dense immune cell infiltrate involved, association of peripheral fibrinogen and white cell count, extent and chronicity of disease.

Periodontitis can elicit a systemic inflammatory response by activating the hepatic acute phase response. This occurs presumable as a consequence of systemic appearance of transient and recurrent bacteremia of oral origin, which has been a long recognized characteristic of periodontal infections.

Offenbacher S et al[80] hypothesized that gram negative anaerobe pathogens from periodontium trigger release of biologically active mediators such as PGE2, and TNFα in circulation causing premature labor.

Cross sectional evidence indicates that periodontitis elicits a mild elevation in markers of acute phase response including C reactive protein, haptoglobin, alpha 1 antitrypsin, and fibrinogen. The liver in response to systemic challenge of organisms secretes acute phase proteins. This acute phase response is triggered by blood borne oral LPS and oral bacteria which elicit release of cytokines IL-6, TNF α. Markers of acute phase response

associated with periodontitis and cardiovascular risk include C reactive protein, increase in WBC count and increase in α1 anti-trypsin, haptoglobin and fibrinogen and decrease in albumin.

Noack B et al[77] determined that CRP plasma levels are increased in periodontitis. Also, there are elevated levels of CRP associated with infection with subgingival organisms often associated with periodontal disease, including Porphromonasgingivalis, PrevotellaIntermedia, Campylobacter rectus, Bacteriodesforsythus.

Havemose-Poulsen A et al[35] reported an increase in the proinflammatory cytokines including IL-1α, IL-1β, IL-RA, IL-6, IL-10, TNF α levels in systemic circulation following periodontal disease

Floriano PN et al[28] stated that CRP remains a non-specific inflammatory response factor that increases in many conditions including periodontal diseases.

However, whole saliva is most frequently studied when salivary analysis is used for the evaluation of systemic disorders. Whole saliva (mixed saliva) is a mixture of oral fluids and includes secretions from both the major and minor salivary glands, in addition to several constituents of non-salivary origin, such as gingival crevicular fluid (GCF), expectorated bronchial and nasal secretions, serum and blood derivatives from oral wounds, bacteria and

bacterial products, viruses and fungi, desquamated epithelial cells, other cellular components, and food debris.

Floriano et al[29] reported that a group of salivary biomarkers can complement findings of an electrocardiogram (ECG) following an acute myocardial infarction. These markers include CRP, myoglobin and myeloperoxidase, in combination with an ECG, showed a highly significant correlation with myocardial infarct patients as compared to healthy controls. The clinical value of salivary proteomic biomarkers in periodontal disease diagnosis is under experimental development and is based on profile changes in molecules involved in inflammation, collagen degradation and bone loss.

Tatjana Todorovic et al[114] in 2006 examined the activity of CK, LDH, AST, ALT, GGT, ALP and ACP in saliva from patients with periodontal disease before and after periodontal treatment and in saliva from healthy patients .Patients with periodontal disease were under conventional periodontal treatment. There was statistically significant increase of activity of CK, LDH, AST, ALT, GGT, ALP, ACP in saliva from patients with periodontal disease in relation to control group and also a positive correlation between the activity of examined salivary enzymes and value of the gingival index. After conventional periodontal therapy the activity of all salivary enzymes was significantly decreased. He concluded that activity of these enzymes in saliva, as biochemical markers for periodontal tissue damage, may

be useful in diagnosis, prognosis and evaluation of therapy effects in periodontal disease.

Balwant Rai et al[93] estimated salivary tumor necrosis factor alpha levels in periodontitis and healthy normal. Significantly higher levels Salivary tumor necrosis factor alpha of was observed in periodontitis patients as compared to controls ($p<0.001$). He concluded that Saliva provides an ideal medium for the detection of proinflammatory markers of the oral cavity. Salivary TNF-alpha analysis may be a useful diagnostic tool and a potential prognostic marker in periodontal disease.

Patricia Yen Bee Ng 2007[90] evaluated the association between radiographic evidence of alveolar bone loss and the concentration of host-derived bone resorptive factors (interleukin-1 beta, tumor necrosis factor-alpha, interleukin-6, prostaglandin-E2) and markers of bone turnover [pyridinolinecrosslinkedcarboxyterminaltelopeptide of type I collagen (ICTP), osteocalcin, osteonectin] in stimulated cells.

Saliva has been proposed as a noninvasive diagnostic fluid that could be used in the diagnosis of oral and systemic diseases. The levels of salivary biomarkers, such as cytokines, could potentially be used as a surrogate to distinguish periodontally healthy individuals from subjects with periodontitis.

Drawbacks of salivary analysis

Various authors like **Zhang et al** suggested that In spite of this progress, some of the biomarkers identified are not disease specific.[120] Therefore, **R. P. Teles** investigated the levels of 10 different cytokines in saliva between a group of periodontally healthy individuals and a group of subjects with periodontitis. Correlations between the concentrations of these 10 cytokines and clinical parameters of periodontal disease were also examined. They found that mean salivary levels of granulocyte–macrophage colony-stimulating factor, interleukin-1β, interleukin-2, interleukin-4, interleukin-5, interleukin-6, interleukin-8, interleukin-10, interferon-γ and tumor necrosis factor-α could not discriminate between periodontal health and disease.[108]

Future trends in salivary bio markers.

However, Diagnostic methods used in clinical practice today lack the ability to detect the onset of inflammation and to identify those patients who are susceptibity to future disease progression. oral fluid based point of care (POC) diagnostics are commonly used in medicine and, more recently, is being adapted for the potential " chair side " determination of oral diseases. The latest clinical applications use new "lab-on-a-chip" (LOC) technologies as rapid POC diagnostic test for systemic infectious diseases and periodontal disease. The development of rapid Point of Care chair side diagnostics has the

potential for the early detection of periodontal infection and progression to identify the incipient disease.

Amy et al[4] developed a portable microfluidic device for detection of potential biomarkers of periodontal disease in saliva.[4] The device performs rapid microfluidic chip-based immunoassays (<3–10 min) with low sample volume requirements (10 μL) and appreciable sensitivity (nM–pM). This method facilitates hands-free saliva analysis by integrating sample pretreatment (filtering, enrichment, mixing) with electrophoretic immunoassays to quickly measure analyte concentrations in minimally pretreated saliva samples. The microfluidic chip has been integrated with miniaturized electronics, optical elements, such as diode lasers, fluid-handling components and data acquisition software to develop a portable, self-contained device. The device and methods are being tested by detecting potential biomarkers in saliva samples from patients diagnosed with periodontal disease. The microchip-based analysis can readily be extended to detection of biomarkers of other diseases, both oral and systemic, in saliva and other oral fluids.

MATERIALS AND METHODS

30 patients who attended the outpatient Department of periodontology, Ragas Dental College and Hospitals, Chennai were enrolled in the study. Patients were divided into two groups based on their periodontal health status as group A and B. Informed consent was obtained from all the patients. The patients were informed that this research work was in no way directly related to the therapy or cure of the disease. The study was undertaken following approval from the institutional review board.

Selection criteria

Group A: Periodontaly healthy group included 15 subjects of age between 25- 50 exhibiting no signs of periodontal disease, determined by clinical attachment loss <2mm, absence of bleeding on probing and PPD <3mm.

Group B: Periodontitis group included 15pateints of age between 25-55 with more than 6 sites exhibiting PPD ≥ 5 mm, CAL ≥ 3mm and radiographic evidence of bone loss.

Exclusion criteria included

- Patients with history of periodontal therapy or antibiotic therapy in the past 6 months

- Patients with history of systemic diseases that may affect the periodontal status
- Pregnancy and Lactation
- Smokers
- Evidence of any other active oral infections eg: pulpal pathology.

Clinical evaluation:-

Clinical evaluation was done using mouth mirror and William's periodontal probe. The probing depth, clinical attachment loss, bleeding on probing and plaque index was evaluated.

Serum collection:

3 ml of Peripheral blood drawn from patients using venepuncture from the antecubital fossa to be used for ELISA analysis.

Peripheral blood was drawn prior to onset of Phase I periodontal therapy in periodontitis patients. All patients underwent a complete physical examination and hematological investigation in Ragas General Hospital to rule out any systemic diseases.

Saliva collection:-

Salivary collection is done according to the technique by Navazesh et al 2008.[75] The patients were advised to refrain from intake of any food or beverage (water exempted) for one hour before sampling. The subjects were

advised to rinse his or her mouth several times with distilled water and then to relax for five minutes. The patient is asked to lean the head forward over the container with the mouth slightly open and allow the saliva to drain into the container with the eyes open. The time lasted for saliva collection is five minutes, the saliva is collected in a sterile disposable plastic container and the samples then centrifuged at 2600rpm and were stored at -80° C and used for further analysis.

Armamentarium

Vaccutainers

Test Tubes

Centrifuge Tubes

Laboratory Centrifuge

Refridgerator

autoclavable containers for saliva collection

Ice pack (for transfer)

Microtitre plate reader fitted with appropriate filters (450nm required with optional 620nm reference filter)

Microplate washer or wash bottle 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips 50-300ml multi-channel micropipette with disposable tips

Multichannel micropipette reagent reservoirs

Distilled water

Vortex mixer

Miscellaneous laboratory plastic and/or glass

Principle of the method

The Diaclone ELISA kit used in the study is a solid phase sandwich ELISA for the in-vitro qualitative and quantitative determination of proteins in oral or biological fluid. A capture Antibody highly specific for the protein to be detected has been coated to the wells of the microtitre strip plate provided during manufacture. Binding of protein samples and known standards to the capture antibodies and subsequent binding of the biotinylated a secondary antibody to the analyte is completed during the incubation period. Any excess unbound analyte and secondary antibody is removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation. Excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The

intensity of the produced coloured complex is directly proportional to the concentration of the protein present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of protein in the sample tested.

Ellisa kit contents:-

TNF alpha kit included 96 well microtitre strip plate, Plastic plate covers, Standard (800pg/ml), Standard Diluent (Human serum), Control, Biotinylated anti-TNF alpha, Biotinylated Antibody diluent, Streptavidin-HRP, HRP Diluent, Wash Buffer, TMB Substrate, Stop reagent.

IL-4 kit content included 96 well microtitre strip plate, Plastic plate covers, Standard (35pg/ml), Standard Diluent (Human serum), Control, Biotinylated anti-IL-4, Biotinylated Antibody diluent, Streptavidin-HRP, HRP Diluent, Wash Buffer, TMB Substrate, Stop reagent.

ASSAY preparation for TNF alpha

The number of microwell strips required to test the desired number of samples and appropriate number of wells needed for running zeros and standards were determined and the wash buffer and standard diluent buffer for TNF alpha were prepared according to the instruction given by the manufacturer.

Preparation of the standard for TNF alpha-

Standard vials must be reconstituted with the volume of standard diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 800pg/ml of TNF alpha and it is mixed gently by repeated aspiration /ejection. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 800 to 25pg/ml. A fresh standard curve is produced for each new assay. Immediately after reconstitution 200ml of the reconstituted standard is added to well's A1 and A2, which provides the highest concentration standard at 800pg/ml. 100ml of appropriate standard diluent is added to the remaining standard wells B1 and B2 to F1 and F2. 100ml from wells A1 and A2 is transfered to B1 and B2. The well contents are mixed by repeated aspirations and ejections taking care not to scratch the inner surface of the wells. 1:1 dilution wascontinued using 100ml from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 800pg/ml to 25pg/ml. 100ml from the final wells of the standard curve (F1 and F2) are discarded.

Preparation of controls for TNF alpha

Freeze-dried control vials are reconstituted with the most appropriate Standard Diluent to the samples. The supplied Controls are also reconstituted with the volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, gives a solution at the concentration as was stated on the vial.

Biotinylated anti-TNF alpha is prepared by dilutingbiotinylated anti-TNF alpha with biotinylated antibody diluent. 5 µl of steptavidin is mixed with 0.5 ml of HRP diluent immediately before use. Further the HRP solution to volumes appropriate for the required number of wells is diluted in a clean glass vial.

ELISSA test for TNF alpha was carried as mentioned below.

ASSAY STEP		DETAILS
1.	Addition	Standard curve was prepared as mentioned above
2.	Addition	100ml of each, Sample, Standard, Control and zero (appropriate standard diluent)was added in duplicate to appropriate number of wells
3.	Addition	50ml of diluted biotinylated anti-TNFalpha was added to all wells
4.	Incubation	the plate is covered with a plastic plate cover and incubated at room temperature (18 to 25°C) for 3 hour(s)
5.	Wash	Plate was washed as as follows: a) The liquid from each well is aspirated b) 0.3 ml of 1x washing solution was dispensed into each well c) The contents of each well were aspirated again. d) step b and c were repeated another two times
6.	Addition	100µl of Streptavidin-HRP solution were added into all wells
7.	Incubation	The plate was again coveedr and incubate at room temperature (18 to 25°C) for 30 min
8.	Wash	Wash step5 was repeated again.
9.	Addition	100µl of ready-to-use TMB Substrate Solution was added into all wells
10.	Incubation	The plates were covered with aluminum cover and then incubated in the dark for 12-15 minutes at room temperature. .
11.	Addition	100µl of H2SO4:Stop Reagent was added into all wells

H_2SO_4: Stop Reagent is added in the end of the incubation and the readings are measured by an spectrophometer using 450nm as the primary wave length and 620nm as the reference wave length.

ASSAY preparation for IL-4

The number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards were determined and the wash buffer and standard diluent buffer for IL-4 were prepared according to the instruction by the manufacturer.

Preparation of the standard for IL-4

Standard vials were reconstituted with the volume of standard diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 35pg/ml of IL-4 and it is mixed gently by repeated aspiration /ejection. Serial dilutions of the standard were made directly in the assay plate to provide the concentration range from 35 to 1pg/ml. A fresh standard curve is produced for each new assay. Immediately after reconstitution 200µl of the reconstituted standard is added to well's A1 and A2, which provides the highest concentration standard at 35pg/ml. 100µl of appropriate standard diluent is added to the remaining standard wells B1 and B2 to F1 and F2. 100µl from wells A1 and A2 to B1 and B2 is transferred. The well contents are mixed by repeated aspirations and ejections taking care not to scratch the inner surface of the wells. 1:1 dilution was continued using 100µl from wells

B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 35pg/ml to 1pg/ml. 100µl from the final wells of the standard curve (F1 and F2) were discarded.

Preparation of controls for IL-4

Freeze-dried control vials were reconstituted with the most appropriate Standard Diluent to the samples. The supplied Controls are also reconstituted with the volume of Standard Diluent indicated on the vial. Reconstitution of the freeze-dried material with the indicated volume, gave a solution at the concentration as was stated on the vial.

Biotinylated anti-IL-4 was prepared by diluting biotinylated anti- IL-4 with biotinylated antibody diluent. 5 µl of streptavidin was mixed with 0.5 ml of HRP diluent immediately before use. Further the HRP solution to volumes appropriate for the required no: of wells was diluted in a clean glass vial.

Method for IL-4 assay

ASSAY STEPS

1	Addition	Standard curve is prepared as mentioned above
2	Addition	100µl of each sample and zero is added into duplicate to appropriate number of wells
3	Incubation	The plates are covered with plastic cover and incubated at room temperature (18 to 25°c) for 2 hours
4	Wash	Plate was washed as as follows: a) The liquid from each well is aspirated b) 0.3 ml of 1x washing solution was dispensed into each well c) The contents of each well were aspirated again. d) step b and c were repeated another two times
5	Addition	50µl of diluted biotinylated anti-IL-4 was added to all wells
6	Incubation	The plates are covered with plastic cover and incubated at room temperature (18 to 25°c) for 1 hour.
7	Wash	Step5 was repeated.
8	Addition	100µl of streptavidin –HRP solution is added into all wells.
9	Incubation	The plates are covered with plastic cover and incubated at room temperature (18 to 25°c) for 30min.
10	Wash	Wash step 5 is repeated again
11	Addition	100µl of ready-to-use TMB Substrate Solution was added into all wells
12	Incubation	The plates were covered with aluminum cover and then incubated in the dark for 12-15 minutes at room temperature
13	Addition	100µl of H2SO4:Stop Reagent was added into all wells

H_2SO_4: Stop Reagent is added in the end of the incubation and the readings are measured by a spectrophometer using 450nm as the primary wave length and 620nm as the reference wave length.

Measurement of High Sensitivity C - reactive protein (hs-CRP):-

Measurement of hs-CRP was performed using a immunoturbidimetric assay performed on a dirui cs-400 auto analyser. The assay range was 1.45-6.80 mg/l.

Statistical analysis:

The circulatory and salivary levels of TNF alpha and IL-4 in periodontal health and disease was compared by calculating the mean and standard deviation for each group. Sample student T test was used for statistical analysis and P value was calculated. Correlation coefficient of serum, saliva levels of TNF alpha, IL-4 and salivary hs-CRP in periodontal health and disease was analyzed using Pearson's correlation.

RAGAS DENTAL COLLEGE AND HOSPITALS, CHENNAI.

DEPARTMENT OF PERIODONTICS

PROFORMA

NAME: AGE: SEX: DATE:

ADDRESS: OCCUPATION:

OP.NO:

CHIEF COMPLAINT:

PAST DENTAL HISTORY:

MEDICAL HISTORY:

PERSONAL HABITS:

INTRAORAL EXAMINATION:

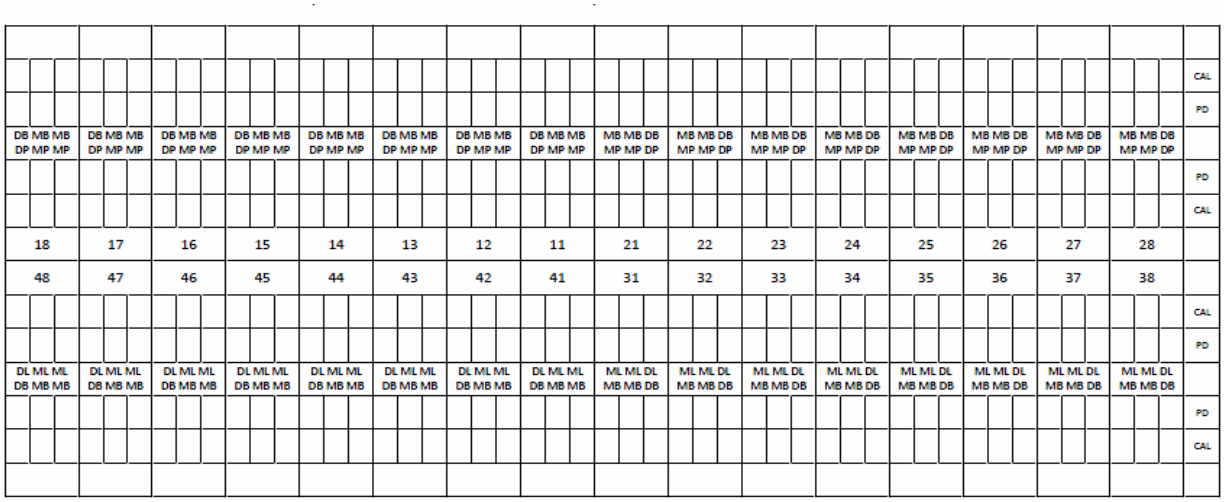
A.HARD TISSUE EXAMINATION:

B.SOFT TISSUE EXAMINTAION:

- GINGIVAL FINDINGS

- DENUDED ROOTS (MILLERS CLASSIFICATION)

Measurement of Probing Depth and Clinical Attachment Loss



PROVISIONAL DIAGNOSIS:

TREATMENT PLAN:

CONSENT FORM

I,_____s|o,w|o,d|o_____ _____ aged about _____ years Hindu/Christian/Muslim/ _____ residing at _____do hereby solemnly and state as follows.

I am the deponent herein; as such I am aware of the facts stated here under.

I state that I came to Ragas Dental College Hospital, Chennai for my treatment for ……

I was examined by Dr. _____ and I was requested to do the following tests.

1.

2.

3.

I was also informed and explained about the pros and cons of the treatment / test in the _____ (language) known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequences of the dissertation/ thesis /study and I undertake to cooperate with the doctor for the study.

I also assure that I shall come for each and every sitting without fail.

I also authorize the doctor to proceed with further treatment or any other/suitable/alternative method for the study.

I have given voluntary consent to undergo treatment without any individual pressure.

I am also aware that I am free to withdraw the consent at any time during the study in writing.

Signature of the Patient/Attendant

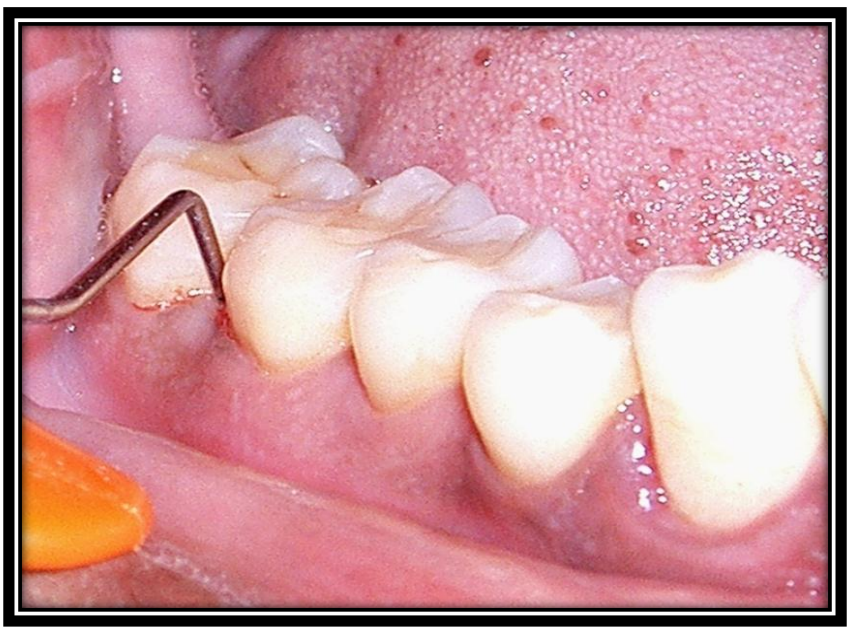
The patient was explained the procedure by me and he has understood the same and with full consent signed in (English/ Tamil/ Hindi/ Telugu/ _____) before me.

Signature of the Doctor

PATIENT GROUPS

GROUP A HEALTHY GINGIVA

GROUP B PERIODONTITIS



ARMAMENTARIUM

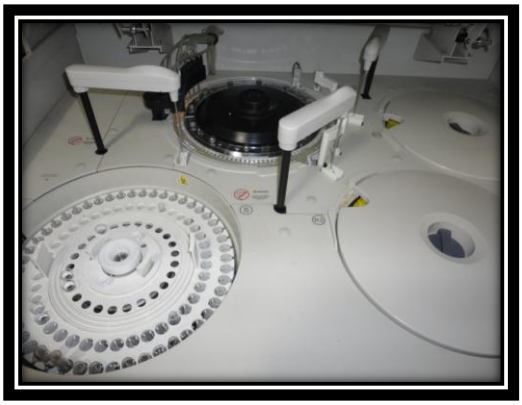
TNF ALPHA KIT CONTENT

IL-4 KIT CONTENT

ELISA AUTOMATED WASHE

ELISA READER BIO RAD

AUTO ANALYSER FOR HS CRP



BLOOD SAMPLE

VACUTEINERS

STERILE CONTAINER FOR SALIVA COLLECTION

RESULTS

The present study was done to assess the circulatory and salivary cytokine levels of TNF alpha and IL-4 in patients with periodontal health and disease and correlate them with a non-specific systemic inflammatory marker, hs-CRP.

30 peripheral blood samples and saliva samples were collected from the 2 groups - Group A and Group B (15 periodontal health and 15 periodontal disease). Cytokine levels in serum and saliva were assessed using sandwich ELISA technique. Absorbance was measured at 450nm as primary wavelength and 650 as reference wave length in terms of pg/ml, and the results were obtained.

Evaluation of serum and salivary levels of TNF alpha in periodontal health and disease

The mean salivary TNF alpha level in periodontal disease was 209.36±20.63pg/ml whereas the mean salivary TNF alpha level in periodontal health was 136.08±25.01 pg/ml. There was a significant increase in the periodontal disease when compared to health at $P=0.035$, $P< 0.05$

The mean serum TNF alpha level in periodontal disease was 77.82±14.79 pg/ml and the mean serum TNF alpha level in periodontal health was 76.14±7.45 pg/ml. There was no significant difference between

periodontal disease when compared to health at P=0.920, P>0.05 [refer table no:1 and graph no:1]

Correlation between salivary and circulatory TNF alpha levels in periodontal disease

There was no significant correlation between the levels of TNF alpha in saliva and circulation at P=0.175(P>0.01) (refer table no:3)

Evaluation of salivary and serum levels of IL-4 in periodontal health and disease

The mean salivary IL-4 level in periodontal disease was 0.82±0.26 pg/ml whereas the mean salivary IL-4 level in periodontal health was 1.18±0.29 pg/ml. Though IL-4 level was decreased in disease, the difference was not statistically significant when compared to health at P=0.22, P>0.05

The mean serum IL-4 level in periodontal disease was 1.12±0.21 pg/ml and the mean serum IL-4 level in periodontal health was 1.38±0.13pg/ml which was not statistically significant at P=0.325, P>0.05 [refer table no:2 and Ghraph no:2]

Correlation between salivary and circulatory IL-4 levels in diseases

There was no significant correlation between the levels of IL-4 in saliva and serum at P=0.548(P>0.01)

Evaluation of serum and salivary hs-CRP in Periodontal Health and Disease

The mean salivary hs-CRP level in periodontal disease was 1.024±0.19 pg/ml and the mean salivary hs-CRP levels in periodontal health was 0.83±0.18 pg/ml, which was not statistically significant at P= 0.471, P>0.05.

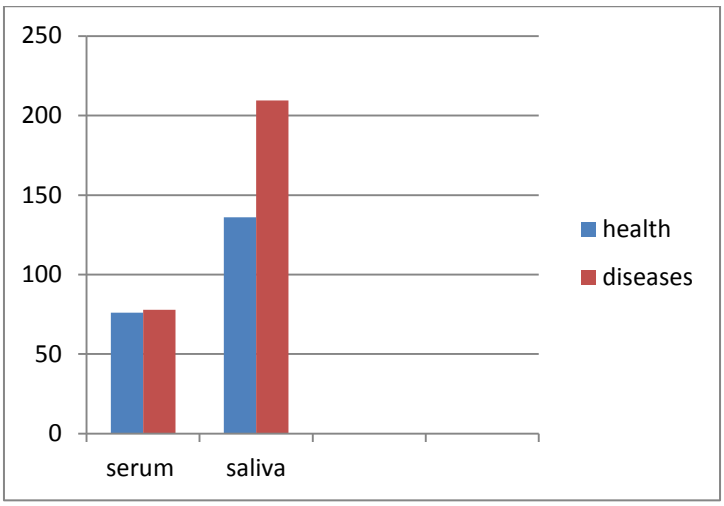
The mean serum hs-CRP level in periodontal disease was 2.05±0.28 pg/ml and the mean serum hs-CRP levels in periodontal health was 1.42±0.21 pg/ml, which was not statistically significant at P= 0.103, P>0.05.[refer table no03]

Correlation of hs-crp to the serum and salivary levels of cytokines (TNF-α and IL-4)

No significant correlation was found between hs-crp and the circulatory and salivary levels of the cytokines, except for salivary hs-CRP and serum levels of TNF alpha, where a statistically significant correlation was found at P=0.727(P>0.01). [refer table no:3]

GRAPH 1

COMPARISON OF SERUM AND SALIVARY LEVELS OF TNF ALPHA IN HEALTH AND DISEASES.



GRAPH 2

COMPARISON OF SERUM AND SALIVARY LEVELS OF IL-4 IN HEALTH AND DISEASES

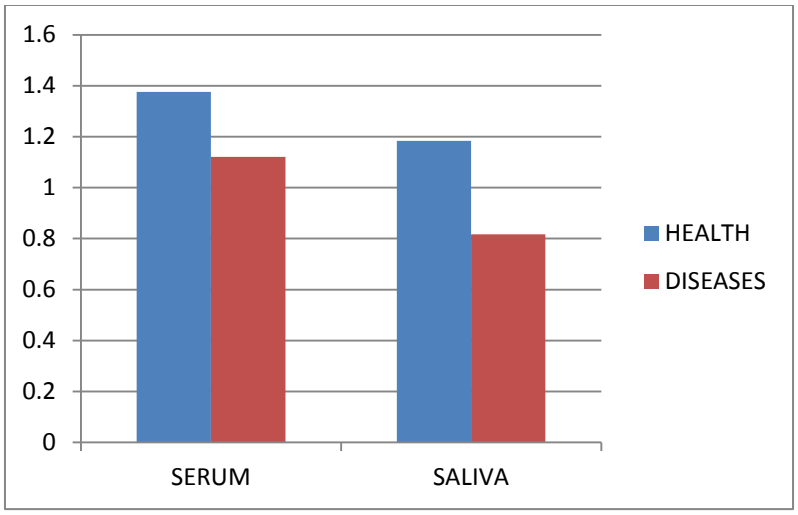


TABLE 1

COMPARISON OF SERUM AND SALIVARY LEVELS OF TNF ALPHA IN HEALTH AND DISEASES

	Mean +std error mean in health	Mean +std error mean in diseases	P value
SERUM	76.1392 ± 7.4467	77.8283 ±14.7860	P=0.920,p>0.05
SALIVA	136.0757±20.6350	209.3643±25.0162	0.035p<0.05

TABLE 2

COMPARISON OF SERUM AND SALIVARY LEVELS OF IL-4 IN HEALTH AND DISEASES

	Mean +std error mean in health	Mean +std error mean in diseases	P value
SERUM	1.3760±0.1355	1.1210±0.2122	P=0.325, p>0.05
SALIVA	1.1830±-0.2916	0.8162±0.2608	P=0.22, p>0.05

TABLE 3

CORRELATIONS

		TNF A	IL-4 A	TNF B	IL-4 B	HSCRP B
TNF A	Pearson Correlation	1	.081	.357	.071	.727(**)
	Sig. (2-tailed)	.	.775	.175	.800	.001
	N	16	15	16	15	16
IL-4 A	Pearson Correlation	.081	1	-.187	-.162	-.040
	Sig. (2-tailed)	.775	.	.488	.548	.883
	N	15	16	16	16	16
TNF B	Pearson Correlation	.357	-.187	1	.327	.414
	Sig. (2-tailed)	.175	.488	.	.216	.099
	N	16	16	17	16	17
IL-4 B	Pearson Correlation	.071	-.162	.327	1	-.079
	Sig. (2-tailed)	.800	.548	.216	.	.770
	N	15	16	16	16	16
HSCRP B	Pearson Correlation	.727(**)	-.040	.414	-.079	1
	Sig. (2-tailed)	.001	.883	.099	.770	.
	N	16	16	17	16	17

DISCUSSION

Periodontal diseases is reported to be a chronic inflammatory condition that may, if left untreated, eventually result in tooth loss.[85] This low grade inflammation has also been associated with systemic effects which modulate the course of other diseases such as atherosclerosis, diabetes mellitus etc.[76]

An "inappropriate" cytokine milieu into periodontal tissues is thought to be one of the pathophysiological pathways that lead to destruction of periodontal tissues.[51] This is also thought to be an important biological mechanism through which periodontal diseases mediates its systemic effects.

Inappropriate cytokine release is generally believed to be a result of an imbalance between destructive pro inflammatory and protective anti-inflammatory cytokines. This study was hence undertaken to evaluate the imbalance between pro inflammatory cytokine TNF alpha and anti-inflammatory cytokine IL-4 in both saliva and circulation and correlate this with the inflammatory marker, hs-CRP in chronic periodontitis patients and periodontaly healthy subjects.

The use of saliva as a diagnostic medium has attracted much attention over the decades. The lack of site specificity, greater inter individual variability and its second hand state (constituents being primarily from GCF) were thought to be its disadvantage as a marker.

In recent years there has been an interest in research on salivary diagnosis with identification of the salivary proteome and transcriptome using advanced techniques such as NMR, MALDI-TOF. It has also been

demonstrated that the serum proteome panel that consists of over 2690 different proteins are almost completely present in saliva.[71] This has lead to the development of lab on chip applications wherein saliva is used as a medium for detection of several chronic oral and systemic inflammatory diseases like atherosclerosis, diabetes mellitus.[105] The ease of collection, less demand on trained personnel and infrastructure are advantages of saliva over GCF. However it must be recognized that for detection of periodontal diseases most salivary constituents are obtained from GCF.[25]

The present study was cross sectional in nature where the salivary cytokine levels were assessed in both chronic periodontitis patients and healthy controls. The disease group included patients with severe chronic periodontitis, the sub classification of mild and moderate were avoided. These subdivisions were excluded to avoid confounders in the cytokine profile arising from the clinical staging of periodontal disease. Saliva samples were all collected between 11A.M and 1 PM as circadian variations are reported to affect salivary protein content.[101] All samples collected were centrifuged at 2600rpm for 10 minutes and the supernatants were collected to avoid contamination. The samples were stored at -80°c to prevent any loss of antigen as per well established protocol.[99]

Enzyme-linked immunosorbent assay (ELISA) is used in both experimental and diagnostic techniques. It is a highly sensitive assay that can detect proteins at the picomolar to nanomolar range (10-12 to 10-9 moles per liter)as per the studies reported by Ruo-Pan Huang et al.[97] This technique was

used in our study as high sensitivity to detect proteins can be achieved with less laboratory demands, thereby reducing cost. Techniques such as Real Time PCR, NMR etc provide greater ability to detect minute quantities of protein but they are expensive and extensive, placing them beyond the reach of the socio economically challenged sections of our population.

The results of this study suggested that there was a marginal decrease in IL-4 levels in periodontal disease when compared to health but the difference was not statistically significant.

IL-4 is known to be an anti-inflammatory cytokine that exerts its effects on several inflammatory events.IL-4 is primarily a Th2 cytokine that is particularly involved in differentiation of Bcells and Th2 cells. IL-4 is known to suppress the Th1 cytokine levels by, firstly, exerting a direct antagonistic effect on IFN which is a signature cytokine for Th1 cell,[97] by down regulating the master transcription factor T-bet of Th1 cells[106] and thirdly by affecting the epigenetic mechanism that may influence the DNA methylation involved in T cell differentiatio.[113] A reduction in IL4 levels is therefore thought to be associated with increased Th1 activity, macrophagic conversion to osteoclasts and consequently disease progression.[54] Therefore, down regulation of anti-inflammatory cytokine - IL4 in the periodontitis samples is in accordance with previous literature.[116]

These results were comparable to the study conducted by Teles et al where a statistically insignificant difference in the salivary IL-4 levels were reported between chronic periodontitis patients and healthy subjects.[108]

Similarly, Ivaniushko TP et al reported a decrease in the levels of IL-4 while there was an increase in THF alpha in saliva samples of patients with chronic periodontitis.[43]

There was a statistically significant increase in the salivary levels of TNF alpha in the periodontal disease samples when compared to the health samples in our study.

TNF alpha is one of the key cytokines that plays an important role in the pathogenesis of periodontal diseases. It is produced mainly by macrophages in response to agents such as lipopolysaccharide.[18] The release of TNF alpha during an inflammatory process mediates increased expression of cell adhesion molecules like VCAM-1, ICAM-1 on the vascular endothelial cells that will lead to increased infiltration of leukocytes into the periodontal tissues. It promotes adhesion of immune cells to endothelial cells and causes an increase in vascular permeability.[57,59] It also acts as a chemotactic cytokine which helps in increased cell migration into the inflamed area.[22] TNFα can initiate apoptosis by stimulating many cell types by the recruitment of the DED containing protein caspase-8 to the receptor complex following association of TRADD & FADD to TNRF-1. This receptor recruitment results in autocatalytic activation of caspase-8. It then initiates a series of caspase activation steps culminating in the activation of effector caspase like caspase-3. TNF alpha up regulates the expression of RANKL and thereby leads to increased osteoclastogenisis and contributes to the increased bone loss seen in periodontal diseases.[74] Tumor necrosis factor is responsible for the connective

tissue destruction as it mediates the release of collagenase and degradation of type 1 collagen by fibroblasts.[64] Considering the key role this cytokine plays in mediating the disease process, the significant increase observed in periodontitis samples was along expected lines.

Our results were in accordance with Frodge et al, who found an increase in the levels of salivary TNF alpha in chronic periodontitis patients compared to health.[30]

The results of our study indicate that there was no significant difference in the circulating TNF alpha and IL 4 levels between periodontal health and disease. This would indicate that there is not much release of TNF alpha or IL4 released into the circulation in periodontal disease. Although most literature would seem to suggest otherwise, a previous study by Nakajima T et al had reported a significantly lower level of TNF alpha in serum of periodontitis patients compared to control.[72] Our results would seem to follow this study more closely as against those of Anthony M. Iacopino[5] who demonstrated that periodontitis-induced bacteremia/endotoxemia causes elevations of serum pro inflammatory cytokine tumor necrosis factor-alpha (TNF-α).

There was no significant correlation between saliva and circulatory interleukin levels in our study. Whole saliva is a mixture of oral fluids and includes secretions from both the major and minor salivary glands, several constituents of non-salivary origin, such as gingival crevicular fluid, expectorated bronchial and nasal secretions, serum and blood derivatives from

oral wounds, bacteria and bacterial products, viruses and fungi, desquamated epithelial cells, other cellular components, and food debris.[63] Therefore, it is entirely possible that the salivary interleukin level may well correlate better with its levels in GCF than in serum. This may explain the reason why there was no correlation between the salivary and serum cytokine levels in our study.

Our results are in agreement with those of Fiorini T, has reported that the GCF levels of TNF alpha did not well correlate with that of serum.[27] Although this study was performed on GCF samples, the results may be extrapolated to our study because GCF may the main source of salivary constituents in periodontal disease, as previously indicated.

CRP is considered as a systemic biomarker of inflammation and plays a direct role in the pathogenesis of inflammation. Pasceri et al found that CRP directly induces the expression of adhesion molecules by endothelial cells.[89] Presence of acute phase protein in immunological response of periodontitis is thought to confirm the involvement of a systemic inflammatory response.[58] This acute-phase reactants have pro-inflammatory properties wherein they help to neutralize pathogens in the periodontal environment and stimulate repair and regeneration of tissues. CRP has been focused in many studies because its elevated levels constitute a risk factor for cardiovascular disease (CVD) and for chronic inflammatory conditions like diabetes mellitus.[13] Several reports have demonstrated elevated serum CRP levels in patients with periodontitis compared with healthy controls.[92] Pan HB and coworkers

reported that the circulatory levels of hs-CRP significantly correlated with severity of periodontitis.[87] Craig RG determined the effect of destructive periodontal disease status, severity and progression on components of the acute-phase response and found that destructive periodontal disease and disease progression are consistent with increase in serum components of hsCRP.[19]

Most of the reported literature is related to serum hs-CRP levels, few studies have focused on its salivary levels. In the present study the circulatory hs-CRP levels were not investigated as we have previously reported on the lack of correlation between periodontal disease and circulating hs-CRP levels (Dissertation submitted to Dr. M.G.R. UNIVERSITY). In contradiction to the previously mentioned reports, the results of our study showed no correlation in salivary hs-CRP levels between periodontal health and disease. These results may be explained on the basis that most blood constituents unlike GCF, undergo a filtration process in the oral mucosa before they reach saliva. Consequently, there is a weaker expression in saliva when compared to blood, leading to no statiscally significant difference between health and disease. The salivary hs-CRP levels in our study were much lower when compared to the serum levels we obtained in a previous study in patients with periodontal disease.

On the other hand, our study was more in accordance with Yakob et al where they investigated circulatory high-sensitivity C-reactive protein (hs-CRP) in relation to symptomatic subjects with and without long-term

periodontitis and found the circulatory levels of hs-CRP was not significantly associated with periodontitis.[115] Offenbecker et al also reported no significant reduction in the hs-CRP levels in of chronic periodontitis patients after 6 months of periodontal treatment.[79] Another study by Nakajima T and coworkers reported that the concentration of hs-CRP is not affected by periodontal treatment although there was an increased concentration of hs-CRP in periodontal infections.[72]

We tried to correlate both the pro and anti inflammatory cytokine levels with hs-CRP. The results of our study indicate that there was no correlation between the salivary cytokine and hs-CRP. However, there was a significant correlation between the TNF alpha level in serum and hs-CRP level in saliva.

This was similar to the study done by Bahceci M, on diabetic patients where they demonstrated an association between TNF alpha and hs-CRP levels in circulation.[7] Parks HS and coworkers also demonstrated an association of serum TNF alpha and hs-CRP in obese patients with increased visceral adiposity.[88]

The implications of these results are not immediately apparent but it may be suggested that the nonspecific inflammatory marker salivary hs-CRP may correlate with circulating pro inflammatory cytokines, indicating that it has value as a surrogate marker in systemic inflammatory disorders. Further studies are needed to confirm this hypothesis.

Taking together, the results of this study indicate that salivary markers need to be interpreted with a lot of caution if they are used to detect systemic diseases as they may not accurately reflect serum levels. On the other hand, it shows some promise as a marker of systemic inflammation. However, salivary cytokines may be used to detect periodontal disease with reasonable accuracy as there was a statistically significant difference obtained in the TNF alpha levels.

It must be clearly understood that periodontal diseases involve interaction of various cytokines that take part in the inflammatory process. Hence analysis of a few cytokines would not depict the progression of inflammatory process in the periodontal tissues. Therefore an array of other cytokines that are released into the periodontal environment should be analyzed to map the pathogenesis that leads to periodontal diseases. Thus a panel of salivary cytokines may provide accurate prediction of the periodontal disease activity and may be used for diagnostic and prognostic purposes.

The cross sectional study design may minimize the strength of evidence as the limited samples size may not represent the entire population from where they are drawn, may provide differing results if another time frame is chosen and is prone for Neyman bias.[14] Hence further studies are required to asses multiple cytokine profile in periodontal diseases to predict the progression of diseases and therapeutic outcome of the periodontal intervention. Scientific data to establish a benchmark for the diagnostic value

of saliva in comparison with that of other biomedia will be necessary to assess the disease discriminatory value of saliva.[20]

Within the limitations of the study, these results demonstrate both the value of salivary diagnostics in periodontal disease and its limitations in reflecting systemic cytokine levels.

SUMMARY AND CONCLUSION

The aim of the present study was to estimate the circulatory and salivary levels of inflammatory cytokines TNF alpha and IL-4 between periodontal health and disease and to assess whether they correlate with each other and with the systemic inflammatory marker, hs-CRP. 30 patients who attended the outpatient Department of periodontology, Ragas Dental College and Hospitals, Chennai were enrolled in the study. Patients were divided in to two groups based on their periodontal health status- 15 periodontaly healthy patients, 15 Periodontitis patients.

Peripheral blood and saliva was collected prior to phase I periodontal therapy from these patients and processed for ELISA analysis. Processing for ELISA involved the preparation of serum from peripheral blood.

Statistical analysis was done using independent sample student T test and pearson correlation test.

From the results of this study, we may conclude that

1) The significantly increased levels of pro inflammatory cytokine TNF alpha in chronic periodontitis patients indicates that it may be used as a marker for chronic periodontitis.
2) Salivary and circulating levels of cytokines TNF alpha, IL4 do not correlate with each other.

3) Salivary levels of hs-CRP may not prove to be an ideal marker of ongoing inflammation associated with periodontal diseases; it may instead reflect serum pro inflammatory cytokine levels.

Further long term studies with an array of cytokines conducted on a greater sample size are required to confirm these results.

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