EVALUATION OF STRESS, SERUM AND SALIVARY CORTISOL AND INTERLEUKIN-1β LEVELS IN SMOKERS AND NON-SMOKERS WITH CHRONIC PERIODONTITIS

Dissertation submitted to

Maharashtra University of Health Sciences, Nashik

in the Partial Fulfillment of Regulations

for the award of the Degree of

MDS

IN

PERIODONTICS

BRANCH II

2018

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EVALUATION OF STRESS, SERUM AND SALIVARY CORTISOL AND INTERLEUKIN-1β LEVELS IN SMOKERS AND NON-SMOKERS WITH CHRONIC PERIODONTITIS

By

DR. PRANJALI BAWANKAR

Under the Guidance of

DR. ABHAY KOLTE

Through

VSPM DENTAL COLLEGE AND RESEARCH CENTRE
HINGNA, NAGPUR

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Has been prepared by

DR. PRANJALI BAWANKAR

under my direct supervision and guidance in partial fulfillment of the regulations for the award of the

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IN

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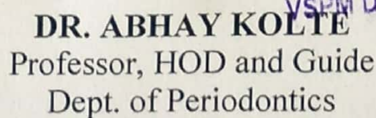

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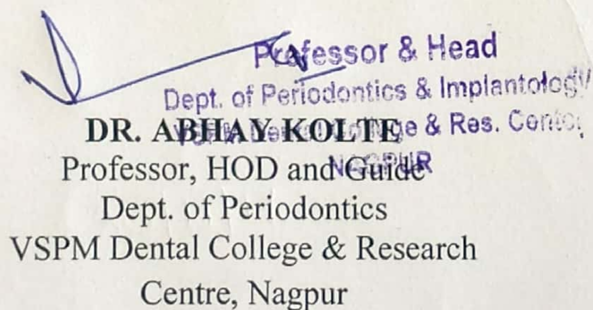

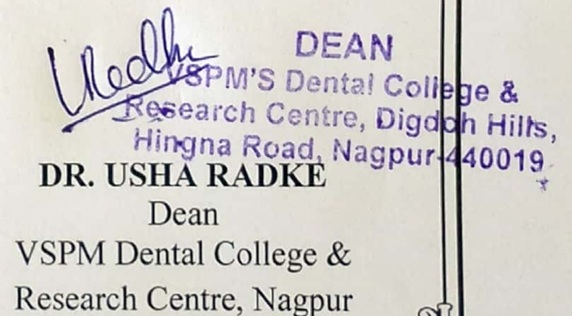

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I hereby declare that the dissertation entitled

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PERIODONTICS

BRANCH II

2018

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LIST OF ABBREVIATIONS

Sr. No.	Short Form	Full Form
1	CP	Chronic periodontitis
2	CS	Chronic stress
3	HPA	Hypothalamus-pituitary-adrenal axis
4	IFN-γ	Interferon-gamma
5	IL-1α	Interleukin-1 alpha
6	IL-1β	Interleukin-1 beta
7	IL	Interleukin
8	GCF	Gingival crevicular fluid
9	TNF-α	Tumor necrosis factor-alpha
10	PGE2	Prostaglandin E2
11	LPS	Lipopolysaccharides
12	IgG	Immunoglobulin
13	Bf	Bacteroidesforsythus
14	Pg	Porphyromonasgingivalis
15	Aa	Actinobacillusactinomycetemcomitans
16	AP	Aggressive periodontitis
17	PDI	Periodntal disease index
18	PI	Plaque index
19	GI	Gingival index
20	PPD	Probing pocket depth
21	CAL	Clinical attachment level
22	BOP	Probing pocket depth
23	OHI-S	Oral hygiene index –simplified
24	ELISA	Enzyme-linked immunosorbent assay
25	OPG	Osteoprotegrin
26	MMP	Matrix metalloproteinase
27	CRP	C-reactive protein
28	GM-CSF	Granulocyte –macrophage colony stimulating factor
29	MIPs	Macrophage inflammatory proteins

Sr. No.	Short Form	Full Form
30	TOS	Total oxidant status
31	TAS	Total antioxidant status
32	LF	Lactoferrin
33	α-1-AT	Alpha 1 antitrypsin
34	α-2-MG	Alpha 2 macroglobulin
35	SRP	Scaling and root planning
36	GCF	Gingival crevicular fluid
37	SDS	Self rating depression scale
38	EOP	Early onset periodontitis
39	SIg A	Secretory immunoglobulin A
40	RANTES	Regulated on activation ,normal T cell expressed and secreted
41	PMN	Polymorphonuclear neutrophils

Introduction

The etiopathogenesis of periodontal disease indicates that periodontitis is a multifactorial disease caused by periopathogens in which host and environmental factors play an important role. Micro-organisms play a crucial role as primary etiological agents but alone seem to be insufficient to explain occurrence or progression of the disease. The onset and progression of periodontal disease is influenced by various systemic diseases, environmental factors and psychologic stress that have the potential to alter periodontal tissues and host immune response. This results in more severe destruction of collagen fibres and other matrix constituents of periodontal ligament and alveolar bone around the teeth resulting in formation of periodontal pockets, loss of attachment apparatus and ultimately leading to tooth loss.

Numerous epidemiological studies have reported risk factors associated with periodontitis. In particular, cross-sectional studies have revealed a clear-cut

correlation between the progressive course of a periodontal disease and the psychosocial stress status of a patient.[1] Stress is defined as a nonspecific reaction of the body to any request for adjustment or adaptation, performed in a stereotyped manner on the base of identical biochemical changes.[2,3]

Stress has been known for more than 40 years to be an important predisposing factor in the development of necrotising periodontal diseases.[4] Psychosocial stress probably interacts with lifestyle factors, such as smoking, in the initiation of periodontal disease.[5]

Marcenes & Sheiham in 1992 [6] have found associations between work stress and periodontal status. Stressful life events have been related to degree of periodontal disease (**Green et al. 1986**)[7]. **Belting & Gupta (1961)**[8] in a study reported that subjects with psychiatric illness had more periodontal disease than subjects with non-psychiatric disorders.

Impact of Stress on Periodontium

A bidirectional relationship has been observed amongst stress and inflammation. These processes involve genetic, neural, endocrine and immune interactions. Both animal and human studies have proved that stress affects the immune system in multiple ways. Stress increases neuro-endocrine hormones, such as glucocorticoids and catecholamines. Through the activation of these hormones, stress has detrimental effects on immune functions, including reduction of lymphocyte populations, lymphocyte proliferation, natural killer cell activity and antibody production and the re-activation of latent viral infections.[9] The limbic–hypothalamic–pituitary–adrenal axis and the sympathetic nervous system are the major neural pathways activated by

physical (i.e. pathogens or toxins) and psychological (i.e. major life events, abuse, or work or relationship-related factors) stressors.[10,11]

Chronic stress provokes a state of chronic inflammation through the activation of macrophages, dendritic cells, microglia, adipocytes and endothelium, which secrete cytokines. Other effects include altered cell trafficking, natural killer cell cytotoxicity changes and alterations in the T-helper 1/T-helper 2 balance, all of which could contribute to the potential for poor immune responsiveness to microorganisms and vaccines, susceptibility to infections, re-activation of latent viruses and delays in wound healing.[12] In stress, there may be activation of the sympathoadrenal system and the limbic–hypothalamic–pituitary–adrenal axis, both of which may be involved in the production of the chronically inflamed state.[13]

Genco et al. (1999) [14] found a higher risk of more severe periodontal attachment loss (odds ratio = 2.24) and alveolar bone loss (odds ratio = 1.91) among individuals with financial strain and an inadequate coping style, when compared with those with low levels of financial strain within the same coping group, after adjustment for age, gender and cigarette smoking.

Chronic stress and depression have been hypothesized to reduce immune responsiveness, resulting in more pathogenic infection and concomitant periodontal tissue destruction. Evidence also indicates that chronic stress and depression can mediate risk and progression of periodontitis through changes in health-related behaviours, such as oral hygiene, smoking and diet.[15,16] Although stress can adversely impact multiple health-related behaviours, including oral hygiene,[17] there is strong evidence that stress plays a contributory role in the pathophysiology of periodontitis.

Stress, Cortisol and Chronic Periodontitis

Psychosocial stress or emotional disturbances produce a transient reduction in salivary flow and changes in the salivary components. Saliva in turn, relates to plaque formation, calculus deposition, and antibacterial and proteolytic activities, all of which may progress to periodontal disease.

Cortisol acts as an anti-inflammatory and immunosuppressive hormone by inhibiting the formation of T lymphocytes and suppressing the function of natural killer cells (NK) or macrophages.[18] In addition to these effects, it induces an increase in blood glucose concentration and influences fat metabolism.[19] Recently, salivary-free cortisol was suggested to offer advantages over serum cortisol.[20] In serum, cortisol is to 90-95% bound to proteins, about 60% to transcortin and 30% to albumin. Transcortin binds cortisol with high affinity but low capacity, whereas albumin has a low affinity but an almost infinite capacity for cortisol.[21] In saliva, cortisol appears mainly in free form. Its concentration is approximately two thirds of unbound cortisol in serum and correlates well with this serum fraction. About 15% of salivary cortisol is bound to transcortin.[21]

Cortisol shows circadian variations. **Akerstedt T and Levi L (1978)** [22] reported plasma cortisol levels to be greatest in the early morning and decrease to their lowest level in the late evening sometimes with a small secondary peak after amid-day meal. There is growing evidence that the cortisol change over the first hour after waking represents a distinct psychobiological phenomenon that is under different control mechanisms from cortisol over the remainder of the day.

Role of Interleukin-1β in chronic periodontitis

Cytokines are small polypeptides with a wide range of inflammatory, metabolic and immunomodulatory properties. They are manufactured by macrophage, lymphocytes, monocyte, dendritic cells, lymphocytes, neutrophils, endothelial cells and fibroblasts. Cytokines are the means of communication between immune and non-immune cells.[23] Inflammatory mediators are important to the pathogenesis of periodontal diseases and may be used as diagnostic markers. Interleukin (IL)-1 is present in two active forms, IL-1α andIL-1β. Both are potent pro-inflammatory molecules and are the main components of osteoclast activating factor. Interleukin (IL)-1 is produced by macrophages and marrow stromal cells. It stimulates bone resorption and participates in pathological conditions with bone loss.[24] Interleukin-1β (IL-1 β) which is a vital pro-inflammatory cytokine play a major role in inflammation and bone resorption, therefore it becomes an important parameter in periodontal research. IL-1β is a cell immune response mediator released as an outcome of bacterial components for example, lipopolysaccharides interacting with toll-like receptors. This cytokine increases the neutrophils recruitment and the expression of adhesion molecules as well as causing vascular modification. When produced constantly, it can result in destruction of periodontal tissue. [25]

The biological activity of IL-1β is extremely diverse, with the focus on the activation of acute-phase proteins, prostaglandins, other cytokines, the induction of collagen and collagenase synthesis, and calcium resorption in the bone. [26] In addition, a stimulating effect on the proliferation of lymphocytes, an inhibited interferon (IFN) synthesis and an elevated production of lymphokines from T cells and of immunoglobulins from

plasma cells were reported in immunocompetent cells.[27] These lead to pro-inflammatory activity which regulates and coordinates the course of the immune response but may also give rise to destructive effects in the event of over-reactions.

The release of IL-1β is triggered by numerous substances, such as lymphokines other cytokines, prostaglandin E2 (PGE2), complement factors and bacteria or lipopolysaccharides (LPS).[28-30] In particular, the bacteria- stimulated local release of IL-1βis of special interest in inflammatory periodontal disease, as the infections involved are bacterially induced. Some in vitro studies reported that secretion of IL-1βcould be stimulated by activating mononuclear phagocytes with LPS. [31] Also, the released IL-1βmay stimulate the gingival fibroblasts to liberate substantial amounts of PGE2 and collagenolytic enzymes. In addition, a demineralising effect on the alveolar bone was reported. [32]

Smoking and Periodontium

Cigarette smoking is considered to be one of the most important environmental risk factors that is closely related not only with the risk but also the prognosis of periodontitis. Smoking is the second strongest modifiable risk factor for periodontal disease after the microbial dental plaque.[33] Smokers are more likely to harbour a higher prevalence of potential periodontal pathogens, and significantly higher clinical attachment loss and bone loss have been observed in smokers.[34] Smoking impairs many aspects of acquired and innate immunity. Studies on the mechanism of how smoking modifies the host response and eventually results in the progression of periodontal tissue destruction have suggested that smoking alters vascular function, neutrophil/monocyte activities, adhesion molecule expression, antibody production

and cytokine and inflammatory mediator release,[35] implying that phagocytes could be the key cells through which the effect of smoking is mediated.

Previous experimental studies have found that cigarette craving is increased after acute stress exposure in smokers.[36,37] On the other hand, chronic exposure to nicotine is thought to increase subjective stress levels and exacerbate a depressed mood by inducing changes in neurotransmitter systems and neural pathways implicated in mood regulation.[38] Bidirectional communication between the HPA axis and immune system plays a key role in the response to chronic and repeated stressors. Cortisol, which is an effector of the HPA axis, increases after nicotine administration and decreases in response to acute tobacco abstinence.[39] In the context of chronic stress, salivary cortisol levels are higher in depressed individuals, compared to non-depressed individuals.[40] The effect of smoking-associated chronic stress on salivary cortisol production is unclear. Proinflammatory cytokines, including interleukin (IL)-1β, interleukin (IL)-6, and tumor necrosis factor (TNF)-α, which increase during early inflammation, are believed to activate the HPA axis.[41]

In light of the above facts, the present study is designed to further investigate the association of salivary and serum cortisol and Interleukin-1β in smokers and non-smokers with chronic periodontitis and its role in the pathogenesis of chronic periodontitis.

Aim and Objectives

The present study aimed to evaluate the association of stress, serum and salivary cortisol and interleukin-1β levels in smokers and non-smokers with chronic periodontitis (CP).

Also glued to this aim were the following objectives.

Objectives

1. To evaluate and compare the stress levels in smokers and non-smokers with CP.
2. To evaluate and correlate the serum and salivary cortisol levels in smokers and non-smokers with CP
3. To evaluate and correlate the serum and salivary interleukin-1β levels in smokers and non-smokers with CP.
4. To correlate the stress with cortisol and IL-1 β levels in smokers and non-smokers with CP.

Review of Literature

Chronic periodontitis being the most ubiquitous disease is characterized by the destruction of connective tissue and alveolar bone support following an inflammatory host response secondary to infection by periodontal bacteria. It is also known to have environmental influences as associated risks, one of which being psychosocial stress. Numerous studies till date have proved the direct association between psychosocial stress and chronic periodontal disease. In chronic stress, stress hormones e.g., cortisol have been reported to be elevated in both saliva as well as blood serum. Plenty of literature is available demonstrating elevated cortisol levels to be a risk factor in chronic periodontitis patients. Also pro-inflammatory cytokines especially IL-1β is found to be constantly associated with the inflammatory disease like chronic periodontitis. To understand the association between stress, cortisol and interleukin 1β it is important to explore related literature.

For the sake of better understanding, the review of literature has been divided into three parts-

A. Review of studies on association of stress, cortisol levels and chronic periodontitis

B. Review of studies on association of IL-1β and chronic periodontitis.

C. Review of studies on association of smoking and chronic periodontitis

A) STUDIES PROVING ASSOCIATION OF STRESS, CORTISOL AND PERIODONTAL DISEASES

Moss ME et al. (1996)[42] explored the association between social factors and adult periodontitis by comparing self-reported information for daily strains and symptoms of depression in 71 cases and 77 controls. They examined serum antibody, for three periodontal pathogens (Bacteroides forsythus [IgG Bf], Porphyromonas gingivalis [IgG Pg], Actinobacillus actinomycetemcomitans [IgG Aa]), and assessed interaction between antibody levels and a Depression score derived from the Brief Symptom Inventory. IgG Pg and IgG Aa were both found to be strongly associated with case status. IgG Bf was associated with periodontal disease but only among individuals who had higher scores for depression. Smoking status was associated with case status. They assessed these findings prospectively by examining factors associated with more extensive disease among the 71 case subjects after 1 year of follow-up. They found baseline smoking status and IgG Bf among individuals scored high on depression at baseline to be associated with more extensive disease. This exploratory analysis has served to identify specific lines of inquiry concerning psychosocial measures as important environmental factors in adult periodontitis.

Genco RJ et al. (1999)[14] in a cross sectional study of 1426 subjects evaluated association between stress, distress and coping behaviour with periodontal disease. They were asked to complete a set of 5 psychosocial questionnaire which measured psychological traits and attitudes including discrete life events and their impact; chronic stress or daily strains; distress; coping styles and strategies; and hassles and uplifts. Clinical assessment of supragingival plaque, gingival bleeding, subgingival calculus, probing depth, clinical attachment level (CAL) and radiographic alveolar crestal height (ACH) was performed, and 8 putative bacterial pathogens from the subgingival flora measured. Logistic regression analysis indicated that, of all the daily strains investigated, only financial strain was significantly associated with greater attachment and alveolar bone loss (odds ratio, OR = 1.70, 95% CI = 1.09 to 2.65 and OR = 1.68, 95% CI = 1.20 to 2.37, respectively) after adjusting for age, gender, and cigarette smoking. When coping behaviors were evaluated, it was found that those with more financial strain who were high emotion-focused copers (a form of inadequate coping) had a higher risk of having more severe attachment loss (OR = 2.24, 95% CI = 1.15 to 4.38) and alveolar bone loss (OR = 1.91, 95% CI = 1.15 to 3.17) than those with low levels of financial strain within the same coping group, after adjustment for age, gender, and cigarette smoking. Similar results were found among the low problem-focused copers for AL (OR = 2.21, 95% CI = 1.11 to 4.38) and ACH (OR = 2.12, 95% CI = 1.28 to 3.51). However, subjects with high levels of financial strain who reported high levels of problem-based coping (considered adequate or good coping) had no more periodontal disease than those with low levels of financial strain, suggesting that the effects of stress on periodontal disease can be moderated by adequate coping behaviours. They concluded that psychosocial measures

of stress associated with financial strain and distress manifest as depression, are significant risk indicators for more severe periodontal disease in adults in an age-adjusted model in which gender (male), smoking, diabetes mellitus, B. forsythus, and P. gingivalis are also significant risk indicators.

Johannsen A et al. (2005)[43] conducted a study to investigate the influence of anxiety measured by one single question on gingival inflammation and periodontal disease in non-smokers and smokers. They included 144 subjects within 30–40 years of age, with untreated periodontal disease divided into an aggressive periodontitis (AP)-group and a chronic periodontitis (CP)-group and 26 healthy controls. After the clinical examination the subjects answered an uncomplicated question regarding anxiety in everyday life, as well as smoking habits. The results of this study suggested that self-reported anxiety was associated with an adverse affect on the gingiva and that anxiety seemed to be associated with increased severity of periodontal disease in smokers. An association between smoking, anxiety and periodontal disease was reported in this study and reported the possibility that anxiety and stress would adversely affect the immune response and, therefore reduce resistance to periodontal disease.

Hilgert et al.(2006)[44] performed a cross sectional study where they evaluated the extent and the severity of chronic periodontitis and its association with the levels of cortisol and the scores of an inventory of stress symptoms in a population aged 50 years or older. 235 individuals were asked to answer the Lipp's Inventory of Stress Symptoms for adults, three saliva samples were collected for cortisol analysis, and were examined for evaluation for periodontitis. Based on logistic regression, cortisol levels were positively associated with the following outcomes: means of clinical

attachment level (CAL) > = 4 mm [OR =5.1, 95% CI (1.2, 20.7)]; 30% of sites with CAL >= 5 mm [OR = 6.9, 95%CI (1.7, 27.1)]; and 26%of sites with probing depth >= 4 mm [OR = 10.7, 95% CI (1.9, 54.1)] . The results suggested that cortisol levels were positively associated with the extent and severity of periodontitis.

Rosania et al. in (2009)[45] conducted a cross-sectional pilot study to explore the associations between psychologic factors, markers of periodontal disease, psychoneuroimmunologic variables, and behaviour. This study included 45 periodontal patients who were asked to complete composite health, chronic stress, depression, and demographic questions, and salivary cortisol was measured by radioimmunoassay. Stress, depression and salivary cortisol scores were found to correlate signficantly with severity of periodontitis and the number of missing teeth, when controlling for age, family history and brushing frequency. Moreover, patients who reported neglecting their oral care during stressful or depressed periods exhibited the greatest clinical attachment loss and highest number of missing teeth. Thus they concluded that stress and depression may be associated with periodontal destruction through behavioral and physiologic mechanisms.

Ansai T et al. (2009)[46] investigated the associations between salivary cortisol levels and dehydroepiandrosterone (DHEA) and periodontitis in never-smokers and smokers of elderly subjects in 171 subjects. They found a significant association between the salivary steroid hormones cortisol and DHEA and periodontitis severity in community-dwelling elderly subjects who had never smoked. They stated that assessment of hormone levels may be a useful screening method for periodontitis, though limited to never-smokers.

Goyal S et al. (2011)[47] explored the role of psychosocial stress influencing the periodontium with the use of a questionnaire data and serum cortisol level. 47 subjects divided into chronic periodontitis group and stressed group. Their stress level was evaluated using a standard questionnaire method (social readjustment rating scale). Plaque index (PI), gingival index (GI), periodontal disease index (PDI) and serum cortisol level were also measured. Spearman's rank correlation coefficient and unpaired 't' test found a strong correlation between cortisol and PDI; and cortisol and PI and between stress, cortisol, PI, GI and PDI. Also a statistically significant correlation was found between cortisol and smoking.

Rai B et al. (2011)[48] in a pilot study explored the associations among periodontal disease, psychologic factors, and salivary markers of stress, psychoneuroimmunologic variables, and health behaviours. They analysed stress scores and salivary stress markers chromogranin A, cortisol, alpha-amylase and beta-endorphin which they found to significantly correlate with clinical parameters of periodontal disease in 100 adult patients with periodontitis. Salivary cortisol and beta-endorphin significantly associated with tooth loss and periodontal clinical parameters, after adjusting for stress variables. Moreover, the greatest tooth loss was observed in those patients who neglected to brush their teeth during stressful periods. This study suggested that stress might be associated with periodontal disease through physiologic and behavioral mechanisms.

Refulio Z et al. (2013)[49] in a cross sectional study evaluated association among stress, salivary cortisol levels, and chronic periodontitis among 70 systemically healthy nonsmoking patients. 25 males and 45 females of age range 30 to 65 years of which

36 patients with chronic periodontitis and 34 without CP were recruited for the study. The stress and anxiety levels were assessed by Zung's self-rating depression and anxiety scale about their stress, depression, and anxiety levels. The clinical measurements probing pocket depth, clinical attachment levels, bleeding on probing and tooth mobility were used to evaluate the disease severity. The salivary cortisol levels were evaluated using a highly sensitive electrochemiluminescence immunoassay. They reported that all the patients with CP and one periodontally healthy subject were diagnosed with depression. Patients with moderate CP had statistically significantly higher levels of SCL than subjects with a diagnosis of slight CP. Also, subjects with severe CP showed the same outcome as compared to those with slight CP. In addition, 46 subjects presented high SCL whereas 24 had a normal level. CP was found to be correlated with the SCL, with an OR of 4.14 (95% CI, 1.43 to 12.01). They concluded that patients with a high salivary cortisol levels and depression may show an increased risk for CP.

Nayak SU et al. (2013)[50] evaluated gingival crevicular fluid (GCF) and salivary cortisol levels in 45 anxious and nonanxious patients with chronic periodontitis. The patients were divided into 3 groups – Group 1-Control, Group 2- chronic periodontitis without anxiety, Group 3-Chronic periodontitis with anxiety .State – Trait anxiety inventory and Hamilton Anxiety rating scale were used to assess the anxiety levels of all the subjects. Clinical measures such as plaque index (PI), gingival index (GI), probing pocket depth (PPD) and clinical attachment level (CAL) were recorded. GCF and unstimulated whole saliva samples were collected, and cortisol levels were determined using ELISA. PI, GI, PPD, and CAL were higher in Group 3. Cortisol level was significantly higher in Group 3. A positive correlation was found among

salivary and GCF cortisol and CAL in Group 3. This study demonstrated a possible link between chronic periodontitis and anxiety and that GCF and salivary cortisol can be used as potential marker for chronic periodontitis.

Wong et al. (2014)[51] examined the effect of acute tobacco abstinence on cortisol levels in regular smokers, and whether abstinence-induced changes in cortisol levels were correlated with various signs and symptoms of the tobacco withdrawal syndrome in 77 smokers. The patients attended two counterbalanced sessions one following 12–20 h of abstinence and the other following ad lib smoking. At both sessions, salivary cortisol levels were measured at three time points. Additionally, a battery of self-report questionnaires, physiological assessments, and cognitive performance tasks were administered to measure signs and symptoms of tobacco withdrawal. They observed that salivary cortisol levels were significantly lower during the abstinent session versus the non-abstinent session. No significant associations were found between abstinence-induced changes in cortisol and other tobacco withdrawal measures.

Jaiswal R et al. (2016)[52] investigated the association between psychological stress and serum cortisol levels in patients with chronic periodontitis in 40 patients equally divided into healthy controls and stressed patients with chronic periodontitis. After the clinical examination (PPD, CAL, OHI-S index) psychological stress estimation was done by a questionnaire. The serum cortisol was estimated biochemically using the enzyme-linked immunosorbent assay method. OHI-S and serum cortisol levels of all the subjects were compared using independent sample t-test and a statistically significant difference in mean OHI-S and serum cortisol levels between healthy and

periodontitis subjects was observed. A positive relation between serum cortisol level and PPD as well as serum cortisol and CAL was observed implying that with increase in serum cortisol, PPD and CAL also increases. They inferred that routine serum cortisol assessment may be a reasonable and a valuable investigative indicator to rule out stress in periodontitis patients as it should be considered as an imperative risk factor for periodontal disease.

Rohini G et al. (2015)[53] estimated and compared the serum cortisol levels in periodontally diseased patients and periodontally healthy individuals in 45 subjects divided as Group I- aggressive periodontitis patients (n = 15), Group II- Chronic periodontitis patients (n = 15), and Group III Healthy controls (n = 15). Serum samples were collected from each of the groups and cortisol levels were determined using cortisol immunoassay kit. The cortisol levels were higher in Group I compared to the other groups. On comparison of mean cortisol levels among the groups, the values were statistically significant between Group I and Group III. Group I showed a significant negative correlation between cortisol levels and GI.

Shende AS et al. (2016)[54] performed a pilot study to evaluate the relationship between stress and periodontal disease. This study included 50 chronic periodontitis subjects. The clinical parameters including plaque index (PI), probing depth (PD), and clinical attachment level (CAL) were assessed. The assessment of stress based on the Zung self rating depression and anxiety scale, the scores of which were correlated with the periodontal findings**.** The number of subjects showing depression and anxiety were significantly less and the severity of depression and anxiety was mild in them. The clinical parameters (PI, PD, CAL) showed no significant differences among the

subjects with varying levels of stress. They observed no statistical significance for stress to be contributing toward the periodontal disease.

B) STUDIES ON ASSOCIATION OF SMOKING, IL-1β AND CHRONIC PERIODONTITIS

Gorska R et al. (2003)[55] assessed the relation between clinical parameters and concentrations of the key (IL-1β, TNF-a, IL-2, IFN-g, IL-4, IL-10) cytokines, important in the initiation and progression of periodontal diseases, within inflamed gingival tissues and serum samples from patients with severe chronic periodontitis. Twenty-five patients with severe chronic periodontitis, with probing depths (PD) >5 mm, and 25 periodontally healthy persons were included in the study. After clinical examination gingival tissue biopsies were collected from one active site of each patient and from healthy individuals, and blood samples were withdrawn on the day of tissue biopsy. The concentrations of cytokines were determined by ELISA and the relationship between their profiles in situ and in circulation with clinical parameters were analysed. They found that the concentrations of IL-1β, TNF-α, IL-2, IFN-γ were, 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, the levels of IL-4 and IL-10 in both kinds of samples obtained from patients and controls were generally low or even undetectable, and remained, on average, on the same level. However, the frequency of IL-4 and IL-10 was much higher in healthy gingival tissues. High concentrations of TNF-α, IFN-γ and IL-2 and,

especially, a high ratio of IL-1β/IL-10 and TNF-α/IL-4 found in tissue biopsies from periodontitis patients, strongly correlated with the severity of periodontitis.

Miller CS et al. (2006)[56] conducted a cross-sectional study to determine if salivary biomarkers specific for three aspects of periodontitis—inflammation, collagen degradation and bone turnover—correlate with clinical features of periodontal disease. Unstimulated whole expectorated saliva was collected from 57 adults divided into case (with moderate-to-severe periodontal disease) and control (healthy) groups. After the collection of saliva periodontal clinical parameters including PPD, CAL, BOP were recorded. Concentrations of salivary biomarkers IL-1β, MMP-8 and osteoprotegrin (OPG) for each subject were analysed using enzyme immunosorbent assays (ELISAs). They found that mean levels of IL-1β and MMP-8 in saliva were significantly higher in case subjects than in controls. Elevated salivary levels of MMP-8 or IL-1β significantly increased the risk of periodontal disease (odds ratios in the 11.3-15.4 range). Combined elevated salivary levels of MMP-8 and IL-1β increased the risk of experiencing periodontal disease 45-fold, and elevations in all three biomarkers correlated with individual clinical parameters indicative of periodontal disease. They inferred that salivary IL-1β may be a sensitive marker of periodontal inflammation and is easier, non invasive, rapid, requires no special expertise as compared to GCF.

Tobon-Arroyave et al. (2008)[57] assessed the concentration of the proinflammatory cytokine IL-1β in saliva of periodontally diseased and healthy patients and their relationship with the periodontal status. Unstimulated whole saliva samples from patients with chronic periodontitis ($n = 30$), aggressive periodontitis ($n = 18$) and

healthy controls (n = 18) were obtained for the study. The periodontal status of each subject was assessed by criteria based on probing depth, clinical attachment loss and the extent/severity of periodontal breakdown. The levels of IL-1β were measured in saliva samples with a high sensitivity enzyme-linked immunosorbent assay (ELISA). Although no significant difference ($P = 0.624$) was found for salivary IL-1β levels between periodontitis groups, they were significantly greater ($P < 0.01$) than those detected for healthy controls. Furthermore, Spearman correlation analysis showed statistically significant correlations ($P < 0.01$) between data from salivary IL-1β levels and clinical measurements. The findings of this study reemphasized the importance of whole saliva as sampling method in terms of immunological purposes in periodontal disease and suggest that the elevated IL-1β concentration may be one of the host-response components associated to the clinical manifestations of periodontal disease.

Vahabi et al. (2011)[58] investigated the possible correlation between interleukin-1β, IL-6, and tumor necrosis factor-α as immunologic mediators and gingival clinical parameters in chronic and aggressive periodontitis. After recording clinical parameters, gingival tissue specimens from 12 chronic and 14 aggressive active sites, harvested from interproximal areas during their routine periodontal surgeries, were cultured. The cytokines present in the culture media were quantified using enzyme-linked immunosorbent assay (ELISA). No significant differences were found between the three cytokine concentrations in aggressive and chronic periodontitis. There were no correlations between cytokine concentrations and clinical parameters. There were direct statistical correlations between IL-6 and TNF-α in both periodontitis types ($p<0.05$) and direct statistical correlations between IL-1β and TNF-α only in chronic

periodontitis ($p<0.05$). No significant difference was observed between chronic and aggressive groups as to cytokine concentrations.

Williamson et al. (2012)[59]compared multiple biomarkers (27 cytokines) including IL-1β, IL-1 receptor agonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, eotaxin, basic fibroblast growth hormone, growth-colony stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interferon-inducible protein 10, MCP-1, macrophage inflammatory proteins (MIPs)-1α, MIP-1β, platelet-derived growth factors BB, TNF-α, and vascular endothelial growth factor in the saliva of healthy adults in plasma samples, passive drool saliva samples, and filter paper saliva samples in 50 healthy adults. Demographic data and three samples were obtained from each subject: saliva collected on filter paper over 1minute, saliva collected by passive drool over 30 seconds, and venous blood (3 mL) collected by venipuncture. Cytokines were assayed using Bio-Rad multiplex suspension array technology. Descriptive statistics and pairwise correlations were used for data analysis. The most consistent and highest correlations were between the passive drool and filter paper saliva samples, although relationships were dependent on the specific biomarker. Correlations were not robust enough to support substitution of one collection method for another. There was little correlation between the plasma and passive drool saliva samples.

Toker H et al. (2012)[60] evaluated the impact of smoking on the relationship between interleukin-1 (IL-1β) and oxidation in patients with periodontitis and response to nonsurgical periodontal therapy. 30 patients with generalized chronic periodontitis (15 smokers and 15 nonsmokers) and 10 periodontally healthy controls were evaluated for

this study. IL-1β level, total oxidant status (TOS) and total antioxidant status (TAS) were recorded in GCF. PPD, CAL, GI and PI and bleeding on probing were also measured. The GCF and clinical parameters were recorded at baseline and 6 wk after periodontal treatment. The study showed statistically significant improvement of clinical parameters in both smokers and nonsmokers after periodontal treatment. Moreover, the baseline IL-1β levels were significantly higher in smokers compared with nonsmokers ($p < 0.05$). After periodontal treatment, the IL-1β levels were significantly reduced in both smokers and nonsmokers ($p < 0.05$). There were no significant differences in TOS and TAS between periodontitis patients and healthy controls at baseline and 6 week after periodontal treatment. The level of IL-1β in gingival crevicular fluid was positively correlated with TOS in both smokers and non-smokers. The results confirm that periodontal therapy has an effect on IL-1β levels in gingival crevicular fluid, but not on TOS and TAS.

Ghurabi BH (2013)[61] studied the serum cytokine profile in chronic periodontitis subjects and the influence of cigarette smoking on the serum levels of cytokines. 60 subjects with chronic periodontitis equally divided into 30 smokers and 30 non-smokers and 30 healthy control individuals were included in this study. Serum samples were separated from the whole blood of all the patients. The levels of cytokines were determined by an enzyme linked immunosorbent assay. The study showed statistically significant elevation in levels of IL-1β, IL-8 and IL-17 in chronic periodontitis patients compared to healthy control. They concluded that serum levels of IL-1β, IL-8 and IL-17 reflects the activity of periodontal destruction, and the enhanced production of these inflammatory cytokines (IL-1β and IL-17) and

chemokine (IL-8) in the presence of smoking may have clinical consequences. This study confirmed that smoking aggravates periodontitis.

Mousavijazi M et al. (2013)[62] investigated the association between psychological stress and elevation of inflammatory mediators related to periodontal disease in 25 patients with chronic periodontitis and 25 patients with aggressive periodontitis. 25 healthy subjects without any evidence of periodontal disorder were also randomly selected as the control group. They analysed IL-6 and IL-1β from gingival crevicular fluid. The Kettle stress questionnaire was also used to determine stress severity. They observed that IL-1β was significantly higher, but IL-6 was only slightly higher. The median score of stress was higher in aggressive periodontitis than the chronic disorder and also in the two periodontal disease groups than the healthy subjects. Among clinical parameters, CAL and PPD were positively correlated with the GCF IL-1β level. There were strong positive relationships between stress severity and in both aggressive and chronic periodontitis; however stress did not influence GCF contents of IL-6. They concluded that psychological stress has a pivotal role in the stimulation of inflammatory processes via IL-1β increase in aggressive and chronic periodontitis.

Gaphor et al. (2014) [63] tested the hypothesis that whether there is any relationship between salivary IL1 β levels and clinical findings in smokers and non-smokers and to assess usefulness of IL-1β for diagnosis of periodontal severity. The sample comprised 80 male volunteers (40 smokers and 40 non-smokers); aged 25-40 year old. Periodontal condition was assessed using plaque index, bleeding index and clinical attachment level and bone loss. The assay of IL-1β was performed by ELISA. The reported mean value of plaque index was significantly higher among non smoker,

and smoker periodontitis (1.72, and 1.87, respectively) than non-smoker, and smoker healthy groups (0.44, and 0.64 respectively) (P=0.000). The mean value of bleeding on probing was significantly higher among non-smoker and smoker periodontitis (0.22, and 0.06 respectively) than non-smoker and smoker healthy groups (0.04, and 0.01 respectively) (P=0.000). The mean value of IL-1β was significantly higher among smoker and non-smoker periodontitis (525.8 pg/ml, 357 pg/ml, respectively) than group of smoker and non-smoker healthy persons (124 pg/ml, 81.5 pg/ml, respectively) (P=0.000). Smokers with periodontitis reported significantly higher CAL than non-smokers with periodontitis (3.02 and 2.5, respectively) (p value ≤ 0.05). The bone loss was significantly higher among smokers with periodontitis than non-smoker with periodontitis (2.6, 2.14, respectively) (P value=0.04). The correlation between salivary IL-1β and PI, BOP, CAL, bone loss was significant (r=0.773, 0.335, 0.941, 0.939 respectively) (correlation <0.001). They concluded that positive association exists between periodontal diseases and smoking, salivary IL-1β was positively associated with clinical signs of periodontal disease and it appears to serve as biomarker of periodontitis.

Kaushik R et al. (2011)[64] performed a study to assess and compare the salivary IL-1β levels in patients with chronic periodontitis before and after periodontal phase I therapy and periodontally healthy controls. 28 patients with moderate-to-severe generalized chronic periodontitis and 24 age, race, and ethnicity-matched controls were enrolled for this study. Saliva samples were collected and clinical parameters recorded were clinical attachment loss (CAL), probing depth, bleeding on probing, periodontal index, and gingival index. Clinical evaluation and sample collection were repeated 1month after periodontal phase I therapy in patients with periodontitis.IL-1β

levels were assessed using enzyme-linked immunosorbent assay. They found that mean IL-1β levels in patients with periodontitis at baseline (1,312.75 pg/mL) were significantly higher (P<0.0001; eight-fold) than in controls (161.51 pg/mL). Although treatment in patients with periodontitis resulted in significant reduction in IL-1β levels (mean: 674.34 pg/mL; P =0.001), they remained significantly higher (P <0.0001; fourfold) than control levels. They found significant correlations between IL-1β levels and all clinical parameters (P <0.01) except percentage sites with clinical AL >2 mm (P >0.05). They inferred that IL-1β levels increases in the saliva of patients with chronic periodontitis, which gets reduced after phase I therapy, suggesting a close association between salivary IL-1β and periodontitis.

Hussain and Ali (2014)[65] evaluated the levels of interleukin-1β (IL-1β) in GCF and Serum of patients with gingivitis and chronic periodontitis and explored whether the effect of IL-1 β was due to its local production. 90 males divided as 50 with chronic periodontitis (CP), 25 gingivitis and 15 healthy controls. The Chronic periodontitis patients were subdivided into 2 subgroups according to their mean of pocket depth: subgroup I (PPD 4-6 mm) and subgroup II (PPD≥ 6 mm). The serum and GCF samples were collected from all the patients. The collected GCF volume was determined by using a Periotron. The concentration of interleukin -1β in gingival crevicular fluid and serum was quantified by ELISA. They found that the mean concentration of crevicular interleukin 1β (pg/µl) was higher in Chronic periodontitis group (275.61±60.63) than in gingivitis group (174.04±57.09) and in control group (72.96 ±27.82). The concentration of serum IL-1β (pg/µl) was found equal in chronic periodontitis group (193.74±88.14) and gingivitis group (193.44±33.37) while in control group it was (172.20±34.92). Moreover, all the clinical parameters were

higher in subgroup II than in subgroup I. The descriptive statistics for serum and crevicular IL-1β concentration (pg/µl) was elevated in subgroup II in comparison with subgroup I.A high significant difference in the concentration of IL-1β in GCF compared to serum concentration in chronic periodontitis and control group was found between subgroups, while a significant difference was found in Gingivitis group. The findings of the present study indicated that the concentration of crevicular IL-1β was higher in Chronic periodontitis group than in gingivitis group and in control group and can be consider as monitor marker which give information about periodontal disease progression. The IL-1β level in serum was very low in comparison to its level in GCF, this difference may be due to that IL-1β is produce locally and acts on the local environment.

C. STUDIES ON ASSOCIATION OF SMOKING AND CHRONIC PERIODONTITIS

Haffajee AD and Socransky SS (2001)[66] investigated and examined clinical features of periodontal disease and patterns of attachment loss in 289 adult periodontitis subjects who were current, past or never smokers. A questionnaire was used to obtain smoking history. Measures of plaque accumulation, overt gingivitis, bleeding on probing, suppuration, probing pocket depth and probing attachment level were taken. Subjects were subset according to smoking history into never, past and current smokers and for certain analyses into age categories <41, 41–49, >49. They found that current smokers had significantly more attachment loss, missing teeth, deeper pockets and fewer sites exhibiting bleeding on probing than past or never smokers. Current smokers had greater attachment loss than past or never smokers whether the subjects had mild, moderate or severe initial attachment loss. Increasing age and smoking

status were independently significantly related to mean attachment level and the effect of these parameters was additive. Mean attachment level in non-smokers <41 years and current smokers >49 years was 2.49 and 4.10 mm respectively. Stepwise multiple linear regression indicated that age, pack years and being a current smoker were strongly associated with mean attachment level. Full mouth attachment level profiles indicated that smokers had more attachment loss than never smokers particularly at maxillary lingual sites and at lower anterior teeth suggesting the possibility of a local effect of cigarette smoking.

Persson L et al. (2001)[67] investigated the influence of tobacco smoking in GCF levels of elastase, Lactoferrin (LF),α-1-antitrypsin(α-1-AT) and α-2 macroglobulin(α-2-MG) in chronic periodontitis in 15 smokers and 17 non-smokers . The elastase activity was measured with a chromogenic low molecular substract and the LF, α-1-AT and α-2-MG with ELISA. They found that with regard to severe lesion, smokers had significantly lower concentration of α-1-AT and α-2-MG than non-smokers. With regards to moderate lesions, smokers tended to exhibit a lower concentration of α-2-MG but the difference was not statistically significant. When moderate and severe lesions were compared smokers exhibited no gradual increase with disease severity in contrast to non-smokers who showed significantly increased levels of LF and α-2-MG in severe as compared to moderate lesions. Thus, the authors proposed a new mechanism of smoking inhibiting inflammatory response by interfering in protease inhibitors.

Hashim et al. (2001)[68] performed a longstanding prospective cohort study where he examined periodontal attachment loss in 914 young adults and based on longitudinal

smoking histories at ages 15, 18, 21 and 26 years. They determined that smokers had three times the likelihood to develop one or more sites with attachment loss of 4mm or more. The prevalence of loss of attachment of >4mm was 19.4%. Among those who smoked at ages 15, 18, 21 and 26, it was 33.6%, and, after controlling for sex, self-care and dental visiting, they were nearly three times as likely to have one or more sites with >4mm loss of attachment. These investigators concluded that chronic exposure to smoking was a strong predictor of periodontal disease prevalence in young adults.

Kamma JJ et al. (2004)[69]performed a cross sectional study to evaluate the influence of cigarette smoking on the gingival crevicular fluid (GCF) levels of interleukin (IL)-1β, IL-4, IL-6 and IL-8 in aggressive or early onset periodontitis (EOP) patients and in healthy controls (H), psychosocial stress being considered as modifying factor .65 EOP and 35 periodontally healthy individuals were interviewed about their smoking habits and their stressful social events. Clinical examination included the assessment of plaque index (PI), bleeding on probing (BOP), clinical attachment level (CAL) and probing pocket depth (PPD). GCF was collected using durapore strips, from four sites per patient, randomly selected in each quadrant. The total amounts of IL-1β, IL-4, IL-6 and IL-8 were measured in a total of 400 samples using ELISA. They found that all clinical parameters were significantly higher in the EOP group compared to the H group. There were no significant differences between EOP smokers and EOP non-smokers with regard to plaque accumulation, CAL and PPD of the sampling sites, whereas mean CAL and PPD of the diseased sites were greater in EOP smokers than in EOP nonsmokers. In addition, EOP smokers seemed to have significantly less BOP and greater bone loss compared to EOP non-smokers. Significant interactions

between ''EOP'' and ''smoking'' were present for total amounts of IL-1β and IL-4. IL-1β, IL-6 and IL-8 showed significant main effects with healthy smokers and healthy non-smokers, respectively. More specifically EOP smokers were statistically affected by stress. They concluded that smoking influences host-related factors including cytokine network.

Badrick E et al. (2007)[70] performed a cross sectional study to assess the relationship between smoking status and salivary cortisol. The study population consisted of 3103 men (1514 never-smokers, 1278 ex-smokers, and 311 smokers) and 1128 women (674 never-smokers, 347 ex-smokers, and 107 smokers). Smoking status, average number of cigarettes smoked, and additional covariates were documented. Saliva samples were collected and salivary cortisol levels were measured using a commercial immunoassay with chemiluminescence detection. They observed that smoking status was significantly associated with increased salivary cortisol release throughout the day (P <0.001) this was apparent for the cortisol awakening response ($P < 0.001$) when examined separately. Compared with never-smokers, smokers had higher release of total cortisol ($P = 0.002$), whereas no difference was observed between never-smokers and ex-smokers ($P = 0.594$): There was no significant relationship between number of cigarettes smoked and total cortisol release. However, a difference was observed for the cortisol awakening response: mean release by tertiles of cigarettes smoked (nanomoles per liter): high, 13.49; medium, 9.58; low, 8.49.

Gautam DK et al. (2011)[71] performed a cross sectional study to evaluate the periodontal health status among cigarette smokers and non cigarette smokers, and oral hygiene measures.400 male patients were divided into smokers and non-smokers. The

CPI score was recorded and a questionnaire including questions on oral hygiene habits and smoking habits was answered by the patients. The findings in the present study showed that smokers with periodontal disease had less clinical inflammation, gingival bleeding and deeper periodontal pockets when compared with non-smokers. This study proved that smoking is a major environmental factor associated with accelerated periodontal destruction.

Tymkiw KD et al.(2011)[34] performed a study to compare the expression of 22 chemokines and cytokines in gingival crevicular fluid (GCF) from 20 smokers and 20 non-smokers with periodontitis and 12 periodontally healthy control subjects. GCF samples were collected and cytokines analyzed utilizing a commercial multiplexed fluorescent bead-based immunoassay. Compared to healthy control subjects, GCF in subjects with chronic periodontitis contained significantly higher amounts of IL-1α, IL-1β, IL-6, IL-12 (p40) (pro-inflammatory cytokines); IL-8, MCP-1, MIP-1α, RANTES (chemokines); IL-2, IFN-γ, IL-3, IL-4 (Th1/Th2cytokines); IL-15 (regulator of T-cells and NK cells). Smokers displayed decreased amounts of pro-inflammatory cytokines (IL-1α, IL-6, IL-12 (p40)), chemokines (IL-8, MCP-1, MIP-1, RANTES) and regulators of T-cells and NK cells (IL-7, IL-15). Periodontitis subjects had significantly elevated cytokine and chemokine profiles. Smokers exhibited a decrease in several pro-inflammatory cytokines and chemokines and certain regulators of T-cells and NK-cells. Thus they demonstrated immunosuppressant effects of smoking which may contribute to an enhanced susceptibility to periodontitis.

Suzuki et al. (2016)[37] investigated the relationships among salivary stress biomarkers, cigarette smoking, and mood states. A total of 49 healthy sixth-year dental students was the study population and Lifetime exposure to smoking was calculated using the Brinkman index (BI).Resting saliva samples were collected, and concentrations of

cortisol, secretory immunoglobulin A (SIgA), interleukin (IL)-1β, interleukin-6, and tumor necrosis factor (TNF)-α were determined. Mood states (tension-anxiety, depression-dejection, anger-hostility, fatigue, confusion, and vigor) over the previous week were assessed using the Profile of Mood States - Brief Japanese Version. Salivary IL-1β levels were significantly higher in smokers than non-smokers (P = 0.044), regardless of the BI or mood state. Higher fatigue scores and lower vigor scores were observed in smokers. They concluded that IL-1β has strong association with the smoking status.

Kolte et al. (2016)[72] performed a cross sectional survey examining the relationship between psychological stress and obesity and periodontal disease in smokers and non-smokers. This study included 90 patients divided as smokers and non-smokers with chronic periodontitis and healthy controls. Socioeconomic data, psycho-social measurements, physical parameters and clinical findings of PPD, CAL, PI and GI were recorded. Intra-group comparison of PPD and CAL in the three anxiety levels (mild, moderate, severe) showed increased periodontal destruction with an increase in anxiety levels, the results being statistically highly significant for PPD differences in smokers (P < 0.0001).The mean PPD and CAL in smoker and non-smoker groups in obese patients was higher as compared to non-obese patients and the differences were highly significant. The results of this study indicated a positive and strong correlation between anxiety, obesity and periodontal disease in smokers and non-smokers and confirmed that smoking further attenuates this association.

Materials and Methods

The present study was undertaken to evaluate the Stress, Serum and Salivary Cortisol and Interleukin-1β levels in Chronic periodontitis in Smokers and Non-smoker patients and healthy controls using Enzyme Linked Immunosorbent Assay (ELISA) and to correlate the concentrations with the stress levels and clinical parameters.

Study Subjects

A total number of 75 patients above 30 years of age visiting the Department of Periodontology, of our institute were recruited in this study. The study design was reviewed and approved by the Institutional Ethics Committee and is in accordance with the Helsinki Declaration. Prior to the initiation of the study an informed consent was obtained from all the patients who willingly agreed to participate in the study.

Study groups

A complete case history including the clinical and radiographic examination was recorded for the selected patients. Cigarette consumption was determined by verbal questioning. Smokers were enrolled if they regularly smoked 10 cigarettes/day, and non-smokers were characterized as not having smoked cigarettes in their lifetime. The same number of smokers and non-smokers were present in the periodontitis groups. The intraoral examination was conducted by a single examiner which included Probing pocket depth (PPD), Clinical attachment level (CAL), Plaque index (PI) [Silness and Loe 1964], Gingival index (GI) [Loe and Silness, 1963], Papillary Bleeding Index (PBI) [Muhlemann H.R. 1977].The stress and anxiety levels of the patients were assessed using the Zung's self –rating depression scale questionnaire. Patients were then categorized into 3 groups of 25 subjects in each depending upon the smoking habits and presence of chronic periodontal disease.

A total of 75 patients were grouped as follows:-

Group I: 25 healthy patients without any signs of periodontal disease.

Group II: 25 Smokers with untreated moderate to severe chronic periodontitis.

Group III: 25 Non-smokers with untreated moderate to severe chronic periodontitis.

Inclusion criteria

Group I

a) Periodontally healthy patients with no signs of periodontal disease were considered as Healthy controls.

b) Patients with no history of smoking.

Group II

a) Current smokers with untreated moderate to severe chronic periodontitis, as assessed by clinical finding of PPD ≥ 5 mm and CAL ≥5mm. (≥30% of teeth affected) and with radiographic evidence of bone loss.

b) Patients with history of smoking at least 10 cigarettes per day for the last 3 years

Group III

a) Non-smokers with untreated moderate to severe chronic periodontitis, as assessed by clinical finding of PPD ≥ 5mm and CAL≥5mm. (≥30% of teeth affected) and with radiographic evidence of bone loss.

b) Patients with no history of smoking.

Males and females were randomly selected in each group.

Exclusion criteria

1. Patients with self reported psychiatric disorders or psychotic medications.
2. Patients with any systemic disease.
3. Pregnant, post menopausal or lactating women.

4. Patients with history of antibiotic, steroidal or any other chemotherapeutic intake or immunosuppressive therapy within 6 weeks.

5. Patients who had undergone any type of periodontal therapy or oral prophylaxis in past 6 months.

6. Patients with acute illness.

Armamentarium

Following material and armamentarium was used for the assessment of clinical parameters and for the collection of blood-

For examination of the patient:

1. Mouth mirror
2. Explorer
3. UNC -15 (Hu-Freidy) periodontal probe.
4. Tweezer
5. Kidney tray
6. Disposable gloves
7. Disposable face mask
8. Surgical drape
9. Cotton rolls
10. Sphygmomanometer and stethoscope.

For drawing blood and collection of saliva

1. Spirit
2. Sterile cotton
3. Disposable syringe and needle (Oneuse 2ml)
4. Plain plastic vials
5. Tourniquet
6. Sterile eppendorf tubes (1.5 ml)

Assessment of periodontal and clinical parameters

1. Probing Pocket Depth (PPD)

It was measured to the nearest higher millimeter using Hu Friedy UNC-15 periodontal probe on 6 sites of all present teeth (distobuccal, buccal, mesiobuccal, distolingual, lingual, mesiolingual). PPD was measured as the distance from the crest of marginal gingival to the depth of the periodontal pocket or gingival sulcus. Patients were considered healthy if they exhibited probing depth ≤ 3mm & there was no clinical attachment loss. Patients were diagnosed with chronic periodontitis if they exhibited PPD ≥ 5mm and Clinical attachment levels of ≥ 5mm at multiple sites.

2. Clinical attachment level (CAL)

It was measured using Hu Friedy UNC-15 periodontal probe on 4 sites (distal, buccal, mesial, lingual/palatal) from the cementoenamel junction (CEJ) to the base of the periodontal pocket of all the present teeth. This was calculated by measuring the distance from CEJ to the gingival margin and subtracting this value from probing depth measurement. Patients were considered healthy if they exhibited no clinical

attachment loss. Patients were diagnosed with chronic periodontitis if they exhibited clinical attachment level ≥ 3mm at multiple sites.

3. Plaque index (PI): (Silness and Loe, 1964)

PI was examined in the scoring units of teeth: distofacial, facial, mesiofacial and lingual surfaces. A mouth mirror and dental explorer were used to assess plaque index.

The criteria for scoring were as follows:

SCORE	CRITERIA
0	No plaque in gingival area
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque was recognized only by running a probe across the tooth surface
2	Moderate accumulation of soft deposits within the gingival pocket and on the gingival margin and/or adjacent tooth surface, which could be seen by the naked eye.
3	Abundance of soft matter within the gingival pocket and/or on the gingival margin and adjacent tooth surface

A plaque index per person was obtained by adding all of the plaque scores and dividing by the number of surfaces examined.

$$\textbf{Plaque Index (PI)} = \frac{\textbf{Total plaque score}}{\textbf{No of surfaces examined}}$$

The following suggested nominal scale was used for patient evaluation.

Scores	Rating
0	Excellent
0.1-0.9	Good
1.0- 1.9	Fair
2.0- 3.0	Poor

4. Gingival index (GI): (Loe and Silness, 1963)

This is a system for assessing the severity of gingivitis in four possible areas. The tissues surrounding each tooth were divided into four gingival scoring units: the distofacial papilla, the facial margin, mesiofacial papilla and the entire lingual gingival margin. A blunt periodontal probe was used (UNC 15) to assess the bleeding potential of the gingival margin according to the following criteria-

SCORE	CRITERIA
0	Normal gingival
1	Mild inflammation, slight change in color, slight edema, no bleeding on palpation
2	Moderate inflammation, redness, edema and glazing, bleeding on palpation
3	Severe inflammation, marked redness and edema, ulcerations, tendency of spontaneous bleeding

The scores of all the surfaces were added and divided by number of surfaces examined which provided the gingival index score per person.

$$\textbf{Gingival Index (GI)} = \frac{\textbf{Total GI scores per tooth}}{\textbf{No. of surfaces}}$$

The numerical score of the gingival index taken into consideration for varying degrees of clinical gingivitis were as follows-

Gingival scores	Condition
0.1 to 1.0	Mild gingivitis
1.1 to 2.0	Moderate gingivitis
2.1 to 3.0	Severe gingivitis

5. Papillary bleeding index (PBI) *(*Muhlemann H.R. 1977)

The PBI was recorded with the blunt periodontal probe by carefully inserting into the gingival sulcus at the base of the papilla on the mesial aspect, then moving coronally to the papilla tip. This was repeated on the distal aspect of the same papilla. The intensity of bleeding thus provoked was recorded on a scale of 0 to 4.

The intensity of any bleeding was recorded as:

Score 0- No bleeding

Score 1-A single discrete bleeding point appears

Score 2-Several isolated bleeding points or a single line of blood appears

Score 3-The interdental triangle fills with blood shortly after probing

Score 4-Profuse bleeding occurs after probing; blood flows immediately into the marginal sulcus.

The scores from all the teeth were added and divided by number of teeth examined which yielded the papillary bleeding index per individual.

$$\textbf{PBI} = \frac{\textbf{Total scores of all teeth}}{\textbf{Total number of teeth examined}}$$

Assessment of depression and stress levels

The patients were asked to complete the **Zung's Self-rating depression scale** about their stress and depression level. The questionnaire was originally designed in English, and it was modified to a bilingual one with questions in both English and Marathi (local language) for better understanding of the questions by the participants. The Zung self-rating depression scale (ZSDS), designed by W.W. Zung, is a short self-administered survey to quantify the depressed status of a patient. There were 20 items on the scale that rated the four common characteristics of depression: The pervasive effect, the physiological equivalents, other disturbances, and psychomotor activities. Ten positively worded and ten negatively worded questions on the scale were scored as 1-4 (a little of the time, some of the time, good part of the time, most of the time). The questions in the questionnaire were related to almost all the relative components of daily life events. The summation of the individual scores of all the questions gave the score for each participant.

The scores range from 25-100.

• 25-49 Normal range

• 50-59 Mildly depressed

• 60-69 Moderately depressed

• 70 and above Severely depressed

Collection of saliva samples

Saliva samples were collected from all subjects between 9 am and 11 am to minimize any circadian rhythm effects. The participants were asked not to eat or drink in the overnight period before collection to avoid contamination of the oral cavity. Also the smokers were refrained from smoking later than 60 minutes before the samples were collected. The patients were not allowed to expose themselves to physical strain later than 60 minutes before sampling and they were instructed to rest lying down during the last 30 minutes. Brushing of the teeth was not allowed during the 60 minutes preceding saliva collection to minimize the risk of blood contamination. Patients were asked to rinse their mouth with distilled water 5 minutes prior to saliva collection. Collection of 1.0-2.0 ml unstimulated whole saliva was performed using sterile tubes with passive drooling method. Patients with removable partial dentures kept them in their mouth during saliva collection. Samples were stored at −20°C and salivary cortisol was assayed within the first month after collection.

Collection of blood samples

For the drawing of blood, the venipuncture from antecubital fossa, using a 20 gauge needle, was performed after the saliva collection in order to avoid stress-induced increase in cortisol concentration. After 20 min of rest for the patient, 5ml of venous

blood sample was drawn in the morning between 9:00 and 11:00 am. Once collected, samples were allowed to clot at room temperature for 20 min. Then the clot was removed by centrifuging at 1500 g for 10 minutes. Using clean pipette the serum was aliquoted into labeled cryovials and immediately stored at –20°C in deep freezer until the final assay.

Laboratory armamentarium for assessment of biochemical parameters (Color plate V)

1. Calibrated, volumetric transfer pipettes with disposable tips capable of dispensing0-5 µl, 5-50 µl, 50-200 µl and 200-1000 µl
2. Sterilized test tubes with test tube stand
3. Distilled water
4. Beakers, Measuring cylinder
5. Absorbent paper
6. Test tubes for standard preparations
7. Covered plastic tubes
8. Sterile gloves
9. Semi-Log graph paper or software for data analysis.
10. Timer.

Laboratory equipment (Color Plate VI)

- -80°C deep freezer (REMI Equipments Pvt. Ltd.)
- Lab centrifuge machine (R-8C, REMI Equipments Pvt. Ltd.)
- Vortex mixer (CM 101, REMI Equipments Pvt. Ltd.)

- ELISA microplate washer (LISA wash, REMI Equipments Pvt. Ltd)
- ELISA microplate reader (LISA Microplate reader, REMI Equipments Pvt. Ltd.)

Assay Procedure

Samples were assayed for salivary and serum cortisol and IL-1β levels using commercially available ELISA (Enzyme linked immune-sorbent assay) DetectX® Cortisol Enzyme Immunoassay Kit for evaluation of salivary and serum cortisol and Krishgen Biosystems KB 1063 Human IL-1βELISA for salivary and serum IL-1β levels . Samples were analyzed according to the instruction manual at the Department of Biochemistry, NKP Salve Institute of Medical Sciences, Nagpur, India. Briefly salivary and serum samples were diluted with dilution buffer in the kit and the amount of cortisol and IL-1β determined. All samples were run in duplicate.

Supplied components in DetectX® Cortisol Enzyme Immunoassay Kit

1. Clear coated 96 well plate (Each well coated with goat anti-mouse IgG)
2. Cortisol standard (Cortisol at 32,000 pg/mL in a special stabilizing solution)
3. DetectX® Cortisol antibody(A mouse monoclonal antibody specific for cortisol.)
4. DetectX® Cortisol conjugate (A cortisol-peroxidase conjugate in a special stabilizing solution)
5. Assay buffer concentrate (A 5X concentrate)
6. Dissociation reagent
7. Wash buffer concentrate (A 20X concentrate)

8. TMB substrate

9. Stop solution (A 1M solution of hydrochloric acid)

Cortisol Assay

The serum and salivary samples were diluted before the assay according the manufacturer instructions. The serum samples were dilute with the Dissociation Reagent (DR) to the final dilution ≥ 1:100. The saliva samples were diluted to ≥ 1:4 with assay buffer supplied.

Assay Principle

The standard preparation and the assay protocol was performed according to the user insert provided by the manufacturer.

Standard Preparation

Six test tubes were labelled as #1 through #6. 450 µL of Assay buffer was pipette into tube #1 and 250 µL into tubes #2 to #6.The pipette tip was prerinsed several times to ensure accurate delivery. 50 µL of the cortisol stock solution was carefully added to tube #1 and vortex completely. 250 µL of the cortisol solution was removed from tube #1 and added to tube #2 and vortex completely. The serial dilutions for tubes #3 through #6 were repeated. The concentration of cortisol in tubes 1 through 6 was made as 3,200, 1,600, 800, 400, 200, and 100 pg/mL. **(Figure 1)**

Total cortisol was measured in serum samples and free cortisol in saliva samples. The cortisol standard was provided to generate a standard curve for the assay and all samples were read off with the generated standard curve. Standards or diluted samples were pipetted into a clear microtiter plate coated with an antibody to capture mouse

antibodies. A cortisol-peroxidase conjugate was then added to the wells. The binding reaction was then initiated by the addition of a monoclonal antibody to cortisol. The immunological reaction occured between the limiting amount of added anti-cortisol monoclonal antibody, the cortisol antigen in the sample or standard, and the limiting amount of added cortisol-peroxidase conjugate. As the concentration of cortisol in the sample increased, the amount of cortisol-peroxidase conjugate bound decreased causing a decrease in signal, and vice versa. The signal was generated from the cortisol-peroxidase bound to the anti-cortisol antibody which itself was bound to the goat anti-mouse IgG coated plates. Excess cortisol-peroxidase did not bind to the plates and was washed out of the well prior to the addition of substrate.

After an hour incubation the plate was washed and substrate was added. The substrate reacts with the bound cortisol-peroxidase conjugate. After a short incubation, the reaction stopped and the intensity of the generated color was detected in a microtiter plate reader at 450 nm wavelength. The concentration of the cortisol in the sample was calculated, after making suitable correction for the dilution of the sample, using software available.

CORTISOL ASSAY PROCEDURE SUMMARY

1. Pipette 50 µL of samples or standards into wells in the plate.

↓

2. Pipette 75 µL of Assay buffer into the non-specific binding (NSB) wells.

↓

3. Pipette 50 µLof Assay buffer into wells to act as maximum binding wells

↓

4. Add 25 µL of the DetectX® Cortisol conjugate to each well using a repeater pipet.

↓

5. Add 25 µL of the DetectX® Cortisol antibody to each well, except the NSB wells

↓

6. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour.

↓

7. Aspirate the plate and wash each well 4 times with 300 µL wash buffer.

↓

8. Add 100 µL of the TMB Substrate to each well, using a repeater pipette.

↓

9. Incubate the plate at room temperature for 30 minutes without shaking.

↓

10. Add 50 µL of the Stop Solution to each well, using a repeater pipette.

↓

11. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.

Calculation of Results

Average of the duplicate OD readings for each standard and sample was calculated. A standard curve was created by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the % B/B0 curve, were multiplied by the dilution factor to obtain neat sample values. For this purpose the online tool from www.myassays.com/arbor-assays-cortisol-enzyme-immunoassay-kit.assay was used to calculate the data. After the cortisol assay was done ELISA for IL-1 β was performed.

Assay Procedure for IL-1 β

Materials Provided

1. Microtiter coated plate (96 wells) – 1
2. Recombinant human IL-1β standard, 90ng/ml, 25µl – 4 vials
3. Human IL-1β Biotin conjugated detection antibody, 100µl – 2 vials
4. Concentrated streptavidin horseradish peroxidase, 300µl - 1 vial
5. Wash buffer (20X) – 25ml
6. Assay diluent (5X) – 10ml
7. TMB substrate – 12ml
8. Stop solution – 12ml

All the reagents were diluted immediately prior to use as instructed by the manufacturer.

Assay Protocol

1. All the reagents were brought to room temperature prior to use. All standards and samples were run in duplicate.
2. 100µl/well of **Standards** and **Samples** were added to the plate. Five two-fold serial dilutions of the 250pg/ml top standard, were performed in separate tubes. Thus, the Human IL-1β standard concentrations became 250 pg/ml,

125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.63pg/ml, 7.81pg/ml and 3.91pg/ml. **(Figure 2)**

Assay Diluent (1X) serverd as the zero standard (0 pg/ml). Plate was sealed and incubated for 2 hours at room temperature (18-25°C).

3. Plate was aspirated and washed 4 times with **Wash Buffer (1X)** and residual buffer was blotted by firmly tapping plate upside down on absorbent paper.

4. 100µl of diluted **Detection Antibody** solution was added to each well. The plate was sealed and incubated for 1 hour room temperature (18-25°C).

5. Plate was washed 4 times with **Wash Buffer (1X)** as in step 3.

6. 100µl of diluted **Streptavidin-HRP** solution was added to each well, then again the plate was sealed and incubated for 30 minutes room temperature (18-25°C)..

7. Plate was washed 4 times with **Wash Buffer (1X)** as in step 3. For this final wash, the wells were soaked in Wash Buffer for 30 seconds to 1 minute for each wash.

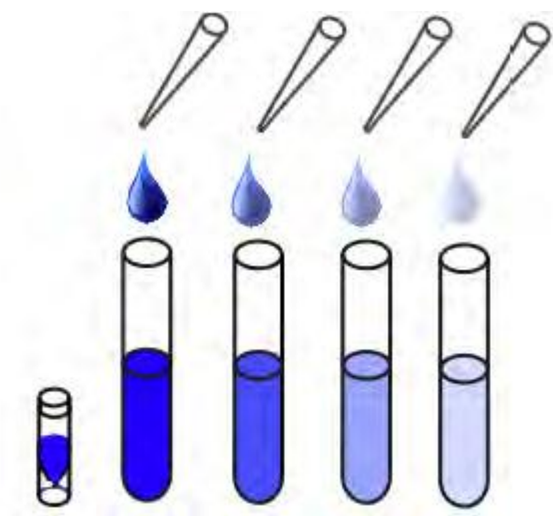
8. Then 100µl of **TMB Substrate** solution was added and the plate was incubated in the dark for 20-minutes. Positive wells turned bluish in color.

9. Reaction was stopped by adding 100µl of **Stop Solution** to each well. Positive wells turned from blue to yellow.

10. The absorbance was read at 450 nm within 30 minutes of stopping reaction.

Calculation of Results

The mean absorbance was determined for each set of duplicate or triplicate standards and samples. The mean absorbance of the zero standards (background) was subtracted from each well. The standard curve was plotted on Semi-Log graph paper, with cytokine concentration on the x-axis and absorbance on the y-axis. The best fit straight line through the standard points was drawn. To determine the unknown cytokine concentrations the unknowns mean absorbance value on the y-axis were found and a horizontal line was drawn to the standard curve. At the point of intersection, a vertical line was drawn to the x-axis and the cytokine concentration was read.



	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Assay Buffer Volume (µL)	**450**	250	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5
Volume of Addition (µL)	**50**	250	250	250	250	250
Final Conc (pg/mL)	3,200	1,600	800	400	200	100

Figure 1: Preparation of standard for cortisol assay

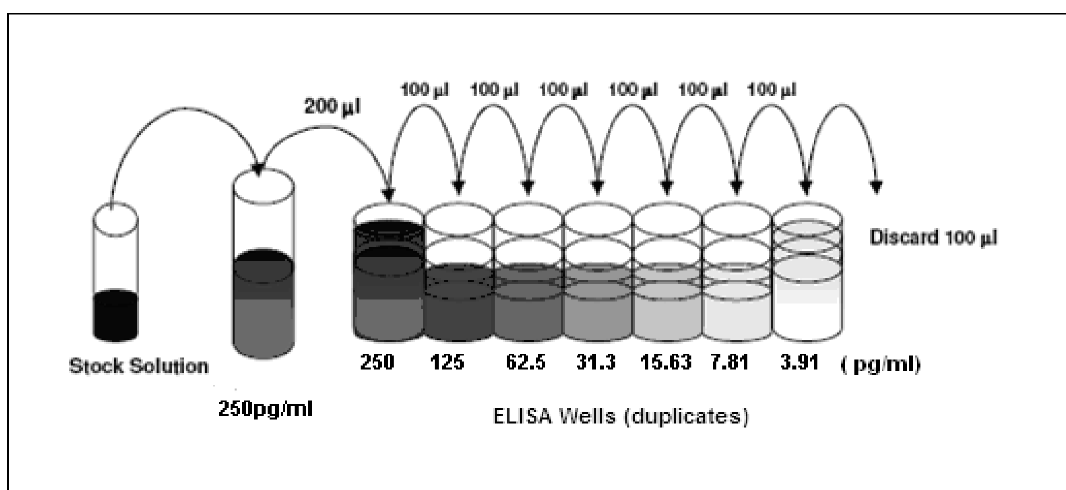
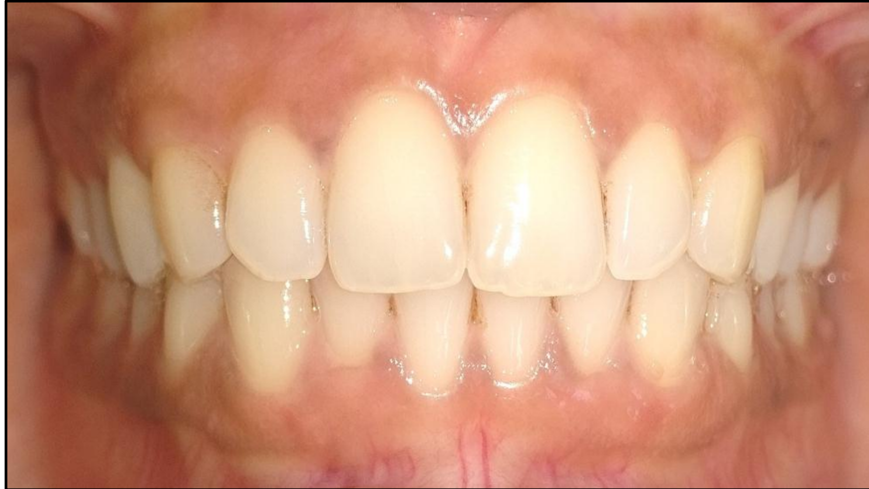


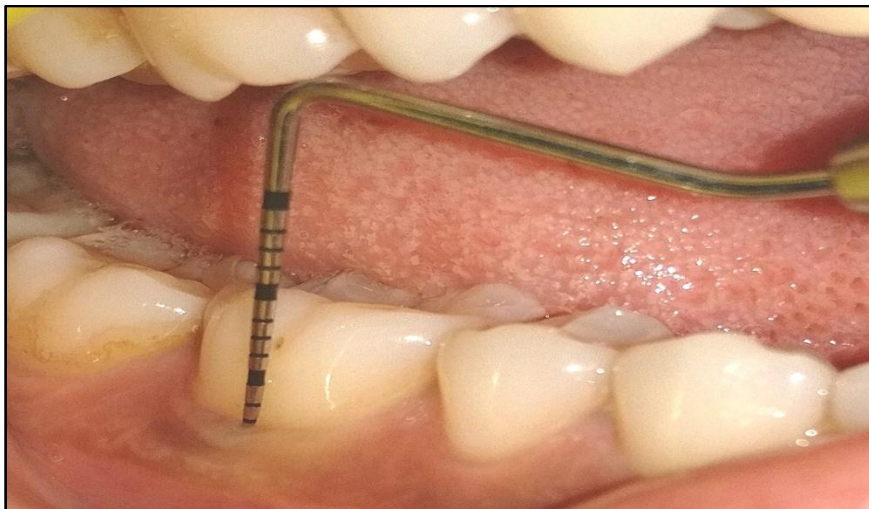
Figure 2: Preparation of standard for IL--1β assay

Color Plate I

Group I (Healthy control)



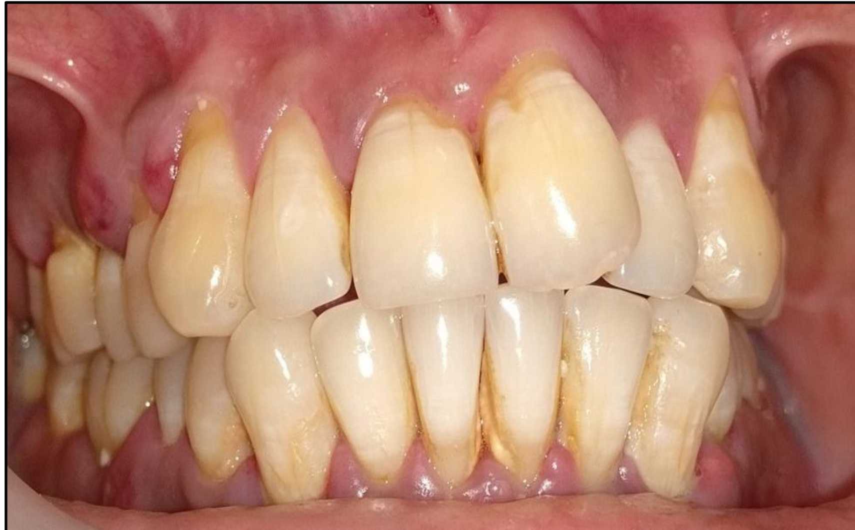
Front view of healthy patient



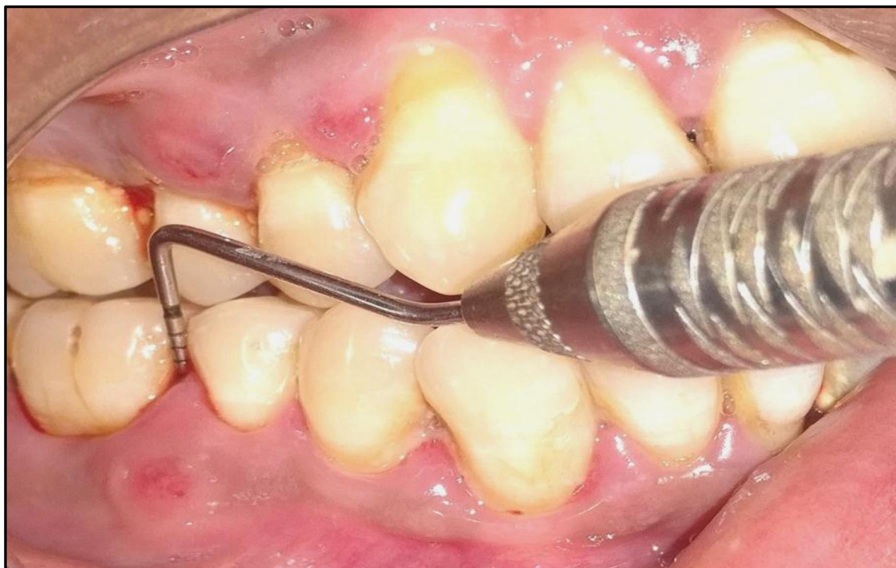
Normal probing depth of 2 mm

Color Plate II

Group II (Smokers with chronic periodontitis)



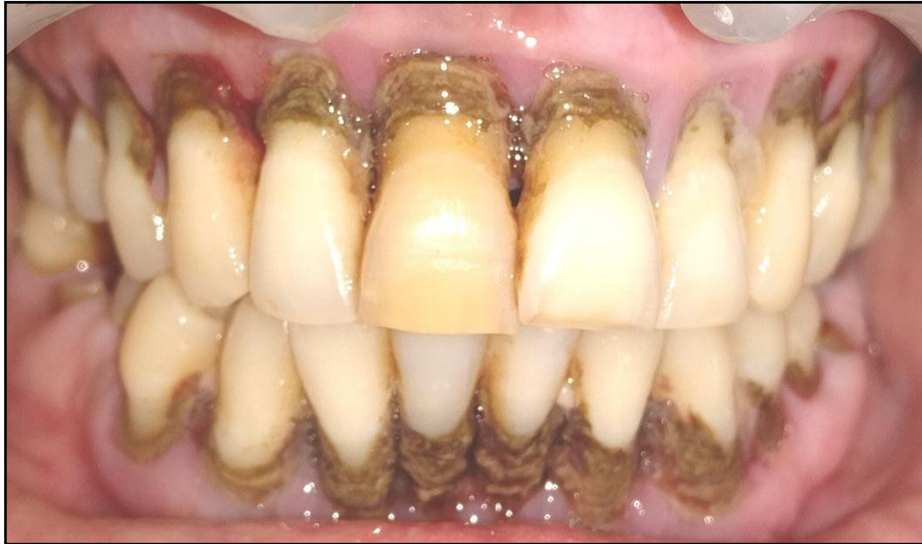
Front view of smoker with chronic periodontitis



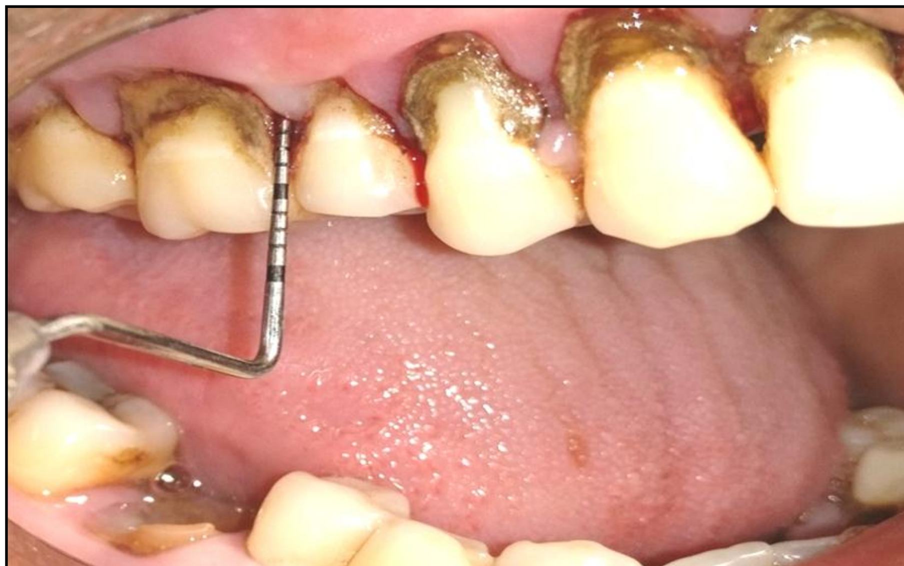
Overall Probing depth > 5mm

Color Plate III

Group III (Non-Smokers with chronic periodontitis)

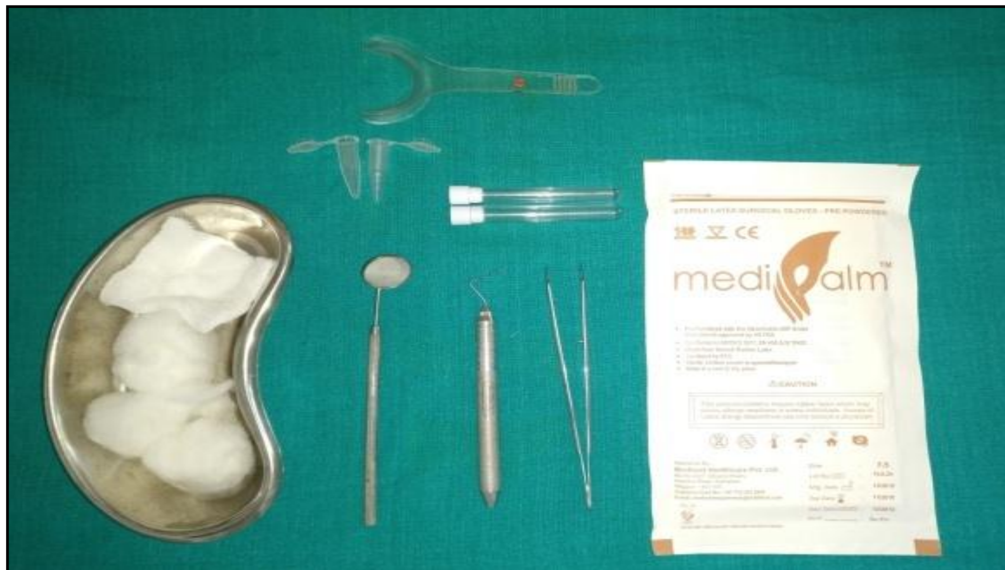


Front view of non smoker patient with chronic periodontitis



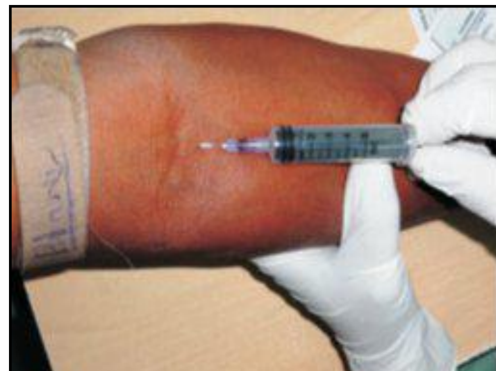
Overall probing depth >5mm

Color Plate IV



Armamentarium for clinical examination and saliva and serum collection

Collection of Saliva sample



Collection of venous blood sample from antecubital fossa

Separated serum from the blood sample

Colour Plate V

Deep freezer

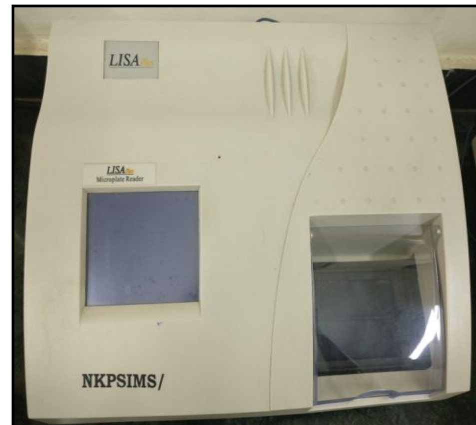
Arbor Assay Human Cortisol ELISA kit and Krishgen Biosystems Human IL-1β ELISA kit and armamentarium for biochemical analysis

Color Plate VI

Centrifuge Machine

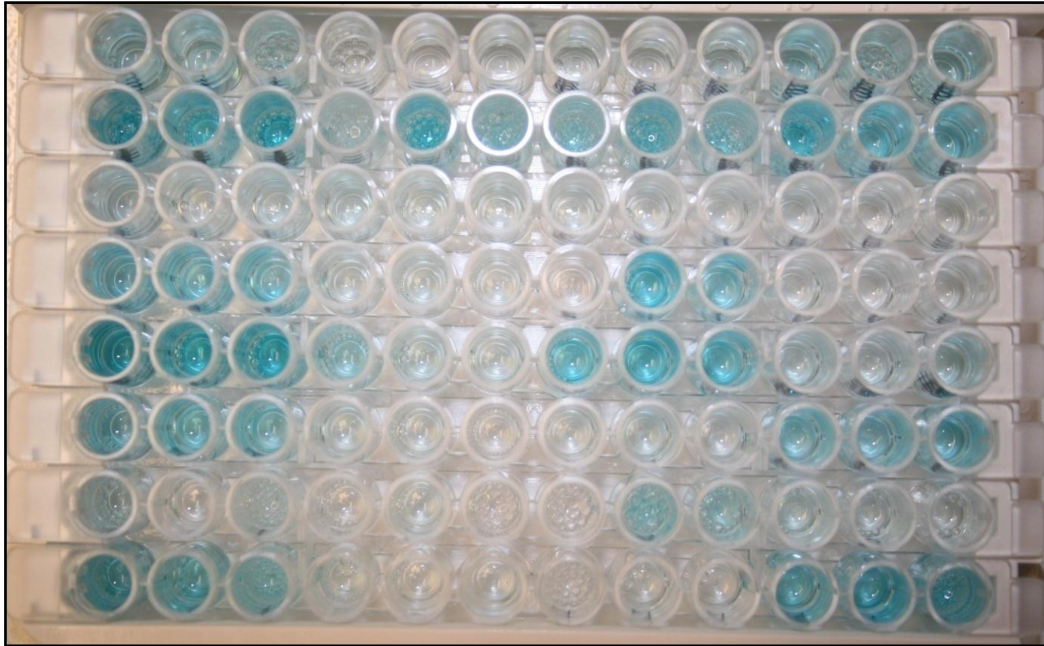
Vortex Mixer

Microplate Washer

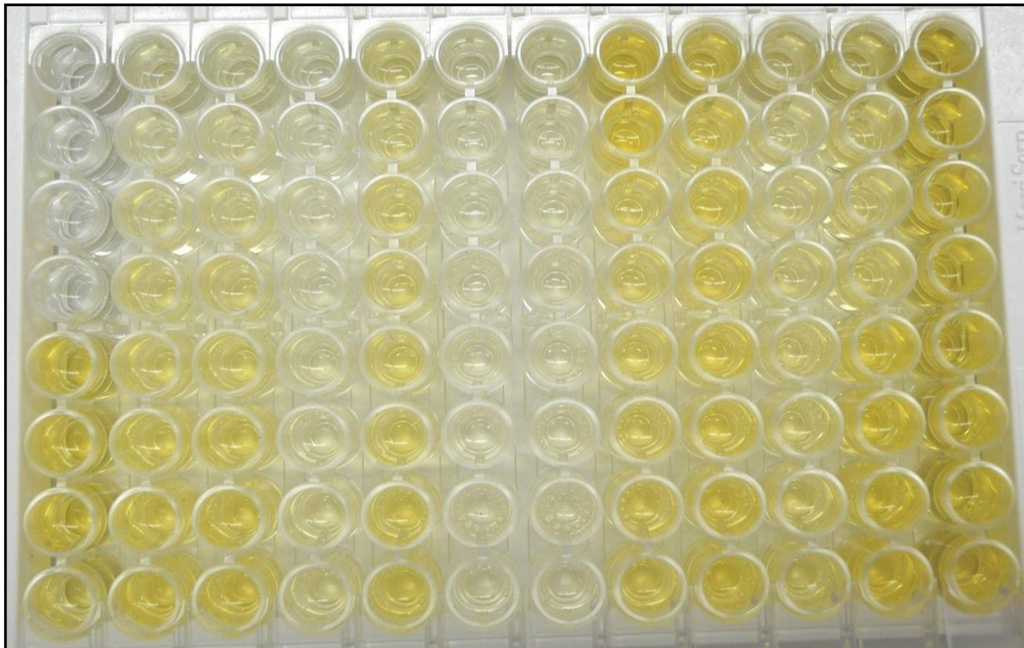


Micro-Plate Reader Machine

Color Plate VII



ELISA plate after adding TMB substrate



ELISA plate after adding stop solution

Results

The present cross sectional study was aimed to evaluate the levels of stress, serum and salivary cortisol and IL-1β in smokers and non-smokers. Various biomarkers have been evaluated in chronic periodontitis till date, amongst which is the stress hormone cortisol and pro-inflammatory cytokine IL-1β. We hypothesized its possible link with chronic periodontitis and variations in their serum and salivary levels in smokers and non-smokers. For this research we had recruited patients which were examined clinically and biochemically for assessing stress and chronic periodontitis and then categorized into three groups. Serum and salivary cortisol and IL-1β levels were assessed using Enzyme linked immunosorbent assay. Incorporation of clinical and biochemical techniques enabled us to fulfill our above objectives.

STATISTICAL ANALYSIS

The data regarding the periodontal parameters Probing pocket depth (PPD), Clinical attachment level (CAL), Plaque Index (PI), Gingival index (GI), Papillary bleeding index (PBI) for patients in all the three study groups viz. Group I- Healthy controls, Group II-Smokers with chronic periodontitis Group III-Non-smokers with chronic periodontitis was obtained. Frequency distribution and descriptive statistics like mean were obtained for periodontal parameters. Also, descriptive statistics obtained were compared across three groups.

The descriptive statistics for the periodontal parameters of patients in all the three study groups are shown in **Table 1.** The intergroup comparison between the periodontal parameters was calculated using One-way ANOVA test. **Table 1** gives the mean and standard deviation of periodontal parameters for patients in all the three groups. It is evident that all the parameters were highly significantly different across groups with p-values < 0.0001. Intergroup comparison, calculated using t test for independent samples between Group II and Group III are depicted in **Table 1 A.**

The mean PPD was highest i.e. 8.03 ± 1.29 mm in Group II followed by 5.44 ± 0.51mm in Group III and 1.37 ± 0.33mm in control Group I. Intergroup comparison between Group II and Group III for probing pocket depth shown in **Table 1A** reveals a highly significant difference with the p value< 0.0001 .

The mean clinical attachment level was also higher in Group II (8.93 ± 1.17mm) as compared to Group III (7.07 ± 0.47mm) and Group I (0.00 ± 0.00 mm). The intergroup comparison for clinical attachment level between Group II and Group III

(Table 1A) illustrate the difference to be statistically highly significant with the p value < 0.0001.

The mean plaque index for all the three groups was found to be 0.64 ± 0.32, 2.56 ± 0.39 and 1.82 ± 0.47 respectively. The intergroup comparison for plaque index between Group II and Group III shows highly significant difference with the p value < 0.0001.

In addition, the gingival index was the highest (2.14 ± 0.61) in Group III as compared to Group II (1.71 ± 0.60) and Group I (0.39 ± 0.21).The intergroup comparison for gingival index between Group II and Group III shows a statistically significant difference with the p value 0.0142 .

Similarly the papillary bleeding index was also greater in Group III (2.24 ± 0.69) as compared to Group II (1.39 ± 0.76) and Group I (0.04 ± 0.04). The intergroup comparison for papillary bleeding index between Group II and Group III shows a statistically significant difference with the p value 0.0001.

Descriptive statistics for cortisol levels in serum and saliva as depicted in **Table 2** in patients from all the three study groups was calculated using One-way ANOVA while the intragroup comparison of serum and salivary levels in each group was calculated using paired t-test as illustrated in **Table 2A. (Graph 1)**

In serum, the mean cortisol level was highest i.e. 17.59 ± 8.53 pg/ml in group II, followed by 15.38 ± 8.90 pg/ml in group III and 13.68 ± 6.20 pg/ml in group I. The difference in the means was statistically insignificant with p-value of 0.228. While in saliva, maximum mean level was obtained in group II i.e. 563.40 ± 236.19 pg/ml,

followed by 417.16 ± 99.67 pg/ml in group III and 19.45 ± 4.03 pg/ml in group I. The difference in the means was statistically highly significant with p-value < 0.0001.

Table 2 (A) depicts pair wise comparison of salivary cortisol levels calculated using Tukey's post-hoc analyses. It was revealed that the difference between group I and II, as well groups I and III were highly significant (p-value < 0.0001) whereas the difference between groups II and III was significant with p-value of 0.023.

Also the comparative analysis was performed for cortisol levels in serum and saliva levels in each group. In all the three groups, the mean levels in saliva were significantly higher than that of serum as indicated by p-values < 0.0001**. (Graph 2)**

Table 3 provides the descriptive statistics for IL-1β levels in serum and saliva in patients from all the three study groups. In serum, the mean levels of IL-1β were maximum in group II i.e. 24.51 ± 26.93 pg/ml, followed by 19.39 ± 6.18 pg/ml in group III and 11.44 ± 3.11 pg/ml in group I. The difference of means was statistically significant with p-value of 0.0187.

A paired comparison of IL-1β levels in serum calculated using Tukey's post-hoc analysis showed that the difference between group I and II was statistically significantly different (p-value 0.0144), while other paired differences i.e. between Group I - Group III and Group II and group III were insignificant with p-value > 0.05 **(Table 3A) (Graph 3)**

On similar lines, analysis was performed on salivary IL-1β levels. The maximum mean level was obtained for group II, i.e.278.95 ± 81.40 pg/ml, followed by 251.35 ± 81.19 pg/ml in group III, and then 160.61 ± 62.23 pg/ml in group I. The difference

between the means was statistically highly significant with p-value < 0.0001 amongst all the three groups. Also the analysis was performed between serum and saliva levels in each group. In all the three groups, the mean levels in saliva were significantly higher than that of serum as indicated by p-values < 0.0001**. (Graph 4)**

The pair wise comparison using Tukey's post-hoc test revealed that when the comparison for salivary IL-1β levels was performed between Group I and Group II statistically highly significant differences was observed with p-value < 0.0001 and between Group I and Group III a significant difference with p-value 0.0002 was observed. Nevertheless, Group II - Group III showed insignificant differences with the p value 0.4039 for the salivary IL-1β levels. **(Table 3A).**

Table 4 provides the descriptive statistics for SDS scored in patients from three study groups. The mean score for group II was maximum i.e. 56.8 ± 9.47, followed by group III with 45.36 ± 9.1 and then group I with 41.04 ± 4.81. The difference in the distribution of scores across groups was statistically highly significantly different with p-value < 0.0001 as obtained using Kruskal-Wallis test. The paired analysis calculated using Wilcoxon rank sum test is illustrated in **Table 4A.** It revealed that the difference between groups I and II was highly significant with p-value < 0.0001, followed by significant difference in groups II and III with p-value of 0.0002 and then groups I and III with p-value of 0.0261 **(Table 4.A)(Graph 5)**

Correlations of SDS scores with serum and saliva cortisol levels were obtained in each group as shown in **Table 5.** In group I, both serum and saliva cortisol levels showed positive correlation with SDS scores with coefficients 0.4870 and 0.3769 with respective p-values 0.0136 and 0.0633. In group II, serum cortisol levels showed

positive relationship with SDS scores with a coefficient of 0.1528 (P-value: 0.4659). Also, saliva cortisol showed positive relationship with the scores with coefficient of 0.4471 (P-value: 0.025). In group III, serum and saliva coefficients showed positive correlation with SDS scores with coefficient values 0.2043 (P-value 0.3274) and 0.5450 (P-value: 0.0048) respectively. Scatter plots showing the relationship are displayed in **Graphs 6 – 11.**

Correlation of different clinical periodontal parameters with serum and saliva cortisol was obtained using Spearman rank correlation and is depicted in **Table 6.** All the periodontal parameters including PPD, CAL, PI, GI, PBI were positively correlated with serum cortisol with correlation coefficients 0.1334, 0.1679, 0.0165, 0.1104, 0.1384 respectively. However, the correlations were poor and statistically insignificant as indicated by P-values > 0.05. On the contrary, the salivary cortisol was found to be significantly and positively correlated with all the clinical periodontal parameters with the p value <0.0001. The correlation coefficients for PPD, CAL, PI, GI, PBI were 0.8031, 0.8197, 0.7542, 0.6189, 0.5928 respectively**. (Graphs 12-21)**

Correlation of cortisol and IL-1β values in serum and saliva was calculated using Spearman Rank correlation coefficient and is illustrated in **Table 7.** Only the correlation between saliva IL-1β and saliva cortisol was higher i.e. 0.4476 compared to other correlations with P-value < 0.0001. Other correlations were poor and statistically insignificant.

Discussion

Periodontal disease is a mixed oral infection initiated by milieu of virulent subgingival bacteria. It is characterized by persistent inflammation, connective tissue breakdown, and alveolar bone destruction. This multifactorial disease is influenced by a variety of risk factors that may affect the onset, severity and progression of disease. Tobacco smoking is one of the principle modifiable environmental risk factor associated with generalised forms of severe periodontitis.[73] Numerous studies have demonstrated that smoking worsens the periodontal disease. Cigarrete smoke contains at least 400 potentially toxic substances including hydrogen cyanide, carbon monoxide (resulting in the formation of carboxyhaemoglobin) free radicals, nicotine, nitrosamines (potent carcinogens) and a variety of oxidant gases causing platelet activation and endothelial dysfunction.[74,75]

Several studies have demonstrated that following non-surgical therapy healing in terms of reduction in gingival bleeding and pocket depth is less favorable in smokers as compared to nonsmokers.[76]A study by **Grossi et al. (1997)[77]**showed that current smokers have less healing and reduction in subgingival Tanarella forsythenssis and Porphyromonas gingivalis after treatment compared to former and non-smokers. This suggested that smoking impairs periodontal healing. **Ah et al. (1994)[78]** reported less reduction in probing depth and gain in clinical attachment in smokers who had been treated by periodontal surgery and thus corroborated the finding that smokers were poor candidates for successful periodontal therapy.

Nicotine from cigarette stimulates the sympathetic ganglia to produce neurotransmitters including catecholamines.[79] These affect the alpha-receptors on blood vessels which in turn causes vasoconstriction. The vasoconstriction of peripheral blood vessels caused by smoking can also effect the periodontal tissue as smokers have less overt signs of gingivitis than nonsmokers and clinical signs of gingival inflammation such as redness, bleeding and exudation are also less evident in smokers.[80] The vasoconstrictive actions of nicotine may be responsible for the decreased gingival blood flow. **Bergstrom and Floderus-Myrhed (1983)**[81] reported that less gingival bleeding in smokers than in non-smokers was not only due to vasoconstriction of gingival vessels, but may also be attributable to the heavier keratinization of the gingivae in smokers.

Cytokines, small polypeptides with a wide spectrum of inflammatory, hemopoietic, metabolic and immunomodulatory properties, are produced by a variety of cells, including the macrophage/monocyte system, dendritic cells, lymphocytes,

neutrophils, endothelial cells and fibroblasts.[82] For example, IL1β, IL6, and tumor necrosis factor alpha (TNF-α) are considered pro inflammatory cytokines. Conversely, IL1 receptor antagonist, IL4, IL10, IL11, and IL13 can suppress the production of inflammatory cytokines.[83] Interleukin (IL)-1 is found in two active forms, IL-1α and IL-1β encoded by separate genes.

Interleukin-1β is the prototypical interleukin-1 cytokine and as such is the most extensively studied cytokine in clinical studies of salivary biomarker in periodontal disease. Interleukin-1β has a multitude of immunological functions including activation of neutrophils, T-and B-cells during infection as well as mediating liver acute phase protein release and febrile responses. In periodontitis, interleukin 1β is associated with neutrophil recruitment and activation of osteoclasts through its ability to induce chemokines and activate osteoclasts. Furthermore, the association between periodontitis and elevated interleukin-1β in gingival crevicular fluid is well established.[84]

Bloemen et al. (2011)[85] showed that just a single exposure of IL-1β can shift the phenotype of osteoblast-like cells from one that favours bone formation to one that supports bone destruction.

The clinical parameters like probing pocket depth, clinical attachment level, plaque index, gingival index, papillary bleeding index etc. are most common and universally used indicators for determining disease status. However, they only provide information about past periodontal tissue destruction and do not elucidate current disease activity nor predict future activity due to low sensitivity and positive predictive value.[86]

Therefore, several molecules have been tried as potential biomarker including enzymes, cytokines, receptors, and other proteins. They have served to be a quick, efficient, and objective diagnostic and monitoring method, with the ability to screen for susceptibility and diagnosis of periodontal disease, evaluate response to treatment, predict future tissue destruction, and identify disease progression.[87] These biomarkers can be found in several biologic fluids, namely the gingival crevicular fluid (GCF) which contains local biomarkers and can potentially provide information at the site level; the blood, serum, or plasma which contain systemic biomarkers and can potentially provide information at the patient level; and saliva which contains both local and systemically derived markers and provides information at the patient level as well. Analysis of saliva gives a better representation of the local pathological changes in the mouth. Saliva contains constituents of GCF, salivary glands, enzymes, hormones, protein molecules etc. Although the GCF has the advantage that it provides information at the site level, GCF collection is rather complicated and more time-consuming than full mouth probing and very less quantity of sample collected making it in-convenient during routine procedures in the dental office.[88]On the other hand, saliva is a biological material that is abundant, the sampling procedure is easy, fast, non-invasive, and more convenient for the patient and clinician. Saliva cannot provide site-specific information; however, it is an easily accessible fluid, which contains local and systemically derived markers of periodontal diseases.[89]

Owing to the advantages and ease of use it is logical to think that salivary and blood diagnostic tests have the potential to be used for diagnosis and monitoring of periodontal disease at the patient level. Taking this into consideration this cross sectional study was formulated to utilize serum and saliva as an avenue for evaluating

stress, which is a systemic condition affecting oral environment especially the periodontal tissues. Ample of literature have associated stress and its hormone cortisol from serum and saliva with chronic periodontitis. Also many studies have evaluated pro-inflammatory cytokine IL-1β in patients with chronic periodontitis. However, to the best of our knowledge no study till date has associated stress hormone cortisol from the serum and saliva with pro-inflammatory cytokine IL-1β in smokers and non-smokers with generalised chronic periodontitis.

In the present study, the data relates the cross sectional examination conducted on 75 patients (40 males and 35 females) divided as –

Group I- Control group (25 periodontally healthy non smoking subjects)

Group II- Test group (25 smokers with chronic periodontitis)

Group II- Test group (25 non-smokers with chronic periodontitis)

The study population constituted of patients with the age 30 years and above so as to rule out the early onset periodontitis cases. Group II (smokers with chronic periodontitis) comprised of only male population.

When the clinical periodontal parameters including probing pocket depth (PPD), clinical attachment level (CAL), plaque index(PI), gingival index (GI),papillary bleeding index (PBI) were compared amongst all the groups it was observed that all there was statistically highly significant difference with p-values < 0.0001. However intergroup comparison amongst Group II and Group III clearly demonstrated that PPD, CAL and PI were significantly more in smokers as compared to non-smokers

group (p value <0.0001).This is in accordance to studies done by **Feldman et al. (1982)**[89] where they found association between six periodontal indices (calculus deposition, plaque accumulation, gingival inflammation, periodontal pocket depth, alveolar bone loss and tooth mobility) and smoking different tobacco products. They reported that cigarette smokers had significantly more calculus deposition, significantly greater pocket depth, accumulated slightly less plaque, and had more alveolar bone loss. Gingival inflammation and tooth mobility did not differ between smokers and nonsmokers, nor between the two smoker groups.

The possible explanations for increased severity of periodontal disease in smokers are the toxic effects of nicotine contained in the cigarette on the periodontium. Nicotine binds to root surface in smokers,[90] and in vitro studies show it can alter fibroblast attachment[91] and integrin expression,[92] and decrease collagen production while increasing collagenase production.[93] The increased periodontal destruction in group II patients is attributed to the impairment of immune system in smokers. Cigarette smoking, nicotine, and its byproducts have a vasoconstrictive effect, not only on peripheral circulation, but on coronary and gingival blood vessels as well. In addition, smoking may reduce the functional activity of leukocytes and macrophages in saliva and GCF, as well as decreasing chemotaxis and phagocytosis of blood and tissue polymorphonuclear (PMN) leukocytes, thereby likely depressing phagocyte-mediated protective responses to periodontal pathogens.[94] Tobacco smoking also reduces the short term oxidation-reduction potentials in dental plaque. Reduced oxygen levels are associated with a decrease in PMN mobility and an increase in the proportion of anaerobic bacteria in dental plaque.[95]

Higher PI in smokers (2.56 ± 0.39) as compared to non-smokers and healthy controls can be attributed to poor oral hygiene prevalent amongst smokers. Also the smokers group exhibited highest SDS score as compared to non smoker group, with mean of 56.8 i.e. mildly depressed which can be attributed to the ignorance in the oral hygiene procedure thereby deteriorating the effects of smoking and stress on periodontium. This finding is in accordance with the study done by **Bergstrom J (1989)**[96] **Genco et al (1999)**[14] **Kolte et al.(2016)**[72].Other investigations have shown little difference in the level of plaque accumulation, comparing smokers with non-smokers. **Calsina et al.(2002)**[97]in their study on effects of smoking on periodontal tissues; found that among cases plaque index did not show differences between smokers and non-smokers. On the other hand, **Scabbia et al.(2001)**[98] in their study showed smokers had significantly more plaque than non-smokers, the possible explanation for this difference may be due to the fact that the PI is dependent on oral hygiene measures adopted by the patients or due to difference in methodology to measure the amount of plaque using disclosing agents.

While the gingival index and the papillary bleeding index were significantly more in non smoker group as compared to smoker group with p values 0.0142 and 0.0001 respectively. This finding would confirm the reduction in clinical signs of inflammation and reduced bleeding on probing due to smoking and disguise the gingival inflammation. This finding is in accordance with study done by **Bergstrom J and Floderus-Myrhed B (1983)**[81] where they found less gingival bleeding in smokers than in non-smokers. The probable cause is vasoconstriction of gingival vessels, but may also be attributable to the heavier keratinization of the gingivae in smokers.

The serum cortisol levels were elevated in smokers as compared to control and non-smokers group. However, the intergroup comparison did not show any significant difference (p value= 0.228) in all the three groups. This might be attributed to the fact that all the included study population was in good systemic health. While the salivary cortisol levels were elevated to 563.40 ± 236.19 pg/ml in group II, and 417.16 ± 99.67pg/ml in group III as compared to group I (19.45 ± 4.03pg/ml). Thus, it can be inferred that saliva exhibits a more efficient and accurate profile of the local changes occurring in the periodontal environment as compared to serum. This finding is in accordance to the cross sectional study performed by **Badrick et al. (2007)[70]** where they evaluated the salivary cortisol levels in current smokers, ex- smokers and never smokers. They reported that salivary cortisol was increased in current smokers, compared with nonsmokers; with no differences observed between ex-smokers and never-smoker and thus suggesting that smoking has a short-term effect on the neuroendocrine system. In another study by **Handa et al. (1994)[99]**middle-aged Japanese male smokers had lower plasma cortisol in the morning than did nonsmokers. When intragroup comparison for serum and salivary values were done a statistically highly significant difference was observed with the p value < 0.0001.

Also ample of literature confirms elevated salivary cortisol in smokers. Other studies that have assessed cortisol from plasma or saliva under resting conditions in the laboratory have shown mixed results, with higher levels in some studies as in **al'Absi et al. (2003)[100]; Baron et al. (1995)[101],** and no differences in others by **Gossain et al. (1986)[102]; Kirschbaum et al. (1994)[103]; Tsuda et al. (1996)[104]** Different methods of data collection and sample timing make it difficult to resolve these discrepancies.

Since cortisol exhibits diurnal variations markedly, the results of the study may also vary accordingly making it difficult to interpret the study.

When a paired comparison for salivary cortisol was calculated with the Tukey's post-hoc analysis it was revealed that the difference between group I and II, as well groups I and III were highly significant, whereas the difference between groups II and III was significant with p-value of 0.023. Thus, the salivary cortisol in smokers with periodontitis was found to be significantly more as compared to non-smokers with chronic periodontitis.

When serum IL-1β values in all the three groups were compared, the mean levels of IL-1β were maximum in group II i.e. 24.51 ± 26.93pg/ml, followed by 19.39 ± 6.18 pg/ml in group III and 11.44 ± 3.11 pg/ml in group I. The difference of means of all the three groups was statistically significant with p-value of 0.0187. The elevated levels of IL-1βin smokers and non-smokers with chronic periodontitis as comapared to controls in our study is consistent with the results of the study done by **Ghurabi (2013)**[61]where the author found statistically significant elevation in levels of IL-1β, IL-8 and IL-17 in chronic periodontitis patients compared to healthy control ($p<0.001$) and elevated serum IL-1βin smokers as compared to non smoker.

The elevated levels detected in our study among smokers could be due to response to LPS of gram negative bacteria which might have been increased in smokers**.** Another explanation for this elevation may be due to nicotine in cigarette smoke that affects the host inflammatory response to oral pathogens by up regulating release of prostaglandin and cytokines leading to accelerated periodontal tissue destruction **(Axelsson, 2005).**[105]

The salivary IL-1β levels were found to be maximum in smokers (278.95 ± 81.40 pg/ml) followed by (251.35 ± 81.19 pg/ml) in non-smokers with the least value for controls (160.61 ± 62.23pg/ml).

Nevertheless, the pair wise comparison group II and group III showed insignificant differences with the p value 0.5008 for serumIL-1β levels and 0.4039 for the salivary IL-1β levels. This is in accordance with the study done by **Kamma et al.(2004)**[69]where they found no significant difference between smokers and non-smokers for GCF IL-1β levels in aggressive periodontitis individuals.

The effect of smoking on cytokines production in periodontal patients has been extensively investigated and conflicting results have been reported.

Bostrom et al. (2000)[106] analyzed GCF levels of IL-1β and its receptor antagonist IL- 1ra with respect to smoking in patients with moderate-to-severe periodontal disease, showed no association between GCF levels of these molecules with smoking.

When nicotine was applied in vitro, on peripheral blood monocytes and lymphocytes and on gingival mononuclear cells of patients with periodontitis, no effect on IL-1β secretion was observed, suggesting that nicotine cannot activate more cells, possibly due to maximal previous stimulation in the periodontitis lesion **(Payne et al. 1996, Bernzweig et al. (1998).**[107,108] Therefore, smoking affects the expression of IL-1β in healthy individuals only.

While the analysis between serum and saliva levels in each group illustrated the mean IL-1β levels in saliva were significantly higher than that of serum it reemphasized the importance of whole saliva as sampling method in terms of immunological purposes

in periodontal disease. This is suggestive of the fact that elevated IL-1β concentration may be one of the host-response components associated to the clinical manifestations of periodontal disease.

The possible explanation of lower serum levels of IL-1β as compared to salivary IL-1β, is that microorganisms colonize in the subgingival area and cause local expression of cytokines, eventually causing tissue breakdown. IL-1β is derived initially from gingival tissues as response to bacterial stimulation, especially by LPS, may mediate inflammatory response in tissues distant from the oral cavity in those patients. Local production of IL-1β may be higher than systemic production because of the bacterial environment in the subgingival area and host defense. Locally produced cytokines find its way into the systemic circulation in chronic periodontitis in due course of time. So adequate amounts of cytokine can be found in local fluids like GCF or saliva as compared to serum.

Pair wise comparison of salivary IL-1β levels showed statistically significant differences between healthy and non-smokers with chronic periodontitis group with p value 0.0002 and highly significant difference with p value < 0.0001 between controls and smokers. This finding is similar to studies done by **Gumus P et al. (2014)**[109]. In **2005 Keles et al.**[110] found that the concentrations of IL-1β were significantly higher in serum and gingival tissue biopsies samples in patients as compared to healthy control. On the contrary **Elkhouli (2011)**[111]mentioned that there were no differences in the levels of IL-1β between patients and healthy control.

The Zung Self-Rating Depression Scale was used to assess the depression score. It was observed that smokers rose up with the highest SDS score of 56.8 and median 60

followed by 47 in non-smokers and 42 in controls. This can be interpreted as smokers were moderately depressed, while non-smokers and healthy controls were in normal range. Paired comparison amongst all the three groups showed highly significant differences.

Correlations of SDS scores with serum and saliva cortisol levels were obtained in each group. Both serum and salivary cortisol in all the groups were found to be positively correlated with the SDS score. However, only the salivary cortisol was found to be statistically significantly correlated with the SDS score in smokers and non-smokers. It is evident that salivary cortisol is in direct relation with the amount of stress. This finding is in accordance with the study done by **Refulio Z et al. (2013)**[49]. In this study, higher scores of self reported depression were associated with more deep pockets in smokers, and with more gingival inflammation in non-smoking subjects without deep pockets. This is in line with a series of previous reports, suggesting that almost any kind of anxiety or stress will affect the condition of the gingiva. (**Marcenes & Sheiham 1992,**[6] **Monteiro da Silva et al. 1998,**[112] **Vettore et al (2003)**[113])

The present study thus confirms the possible linkage between smoking, stress and periodontal disease. Also the correlations amongst the clinical parameters and serum and salivary cortisol levels revealed a positive though poor and non significant association with serum cortisol while a highly significant association with the salivary cortisol. Similar were the findings by **Mudrika et al.(2014)**[114] **and Hilgert et al. (2006).**[44]

Also the correlation between saliva IL-1β and saliva cortisol was found to be positive and highly significant .Similar findings were demonstrated by **Mengel et al. (2002)**[117]in a study with no correlation between the serum levels of immunological mediators (IL-1β, IL-6), glucocorticoids (cortisol) and the registered stress.

The SDS score was found to be maximum in smokers also the serum and salivary IL-1β levels were also higher as compared to those of healthy controls and non-smokers. This can confirm the findings of **Deinzer R et al. (1999)**[115]who reported that academic stress might affect periodontal health by increasing local IL- 1β levels.

Despite of higher SDS scores in smokers the correlations between SDS score and serum cortisol levels did not show any significant differences amongst smokers and non-smokers. Many similar studies were conducted by other authors, using however, different target populations, threshold scores and indices to positively identify the disease.[6,7,34]Studies conducted by other authors[42] also applied different self-report scales as instruments to measure psychological variables (Minnesota Multiphasic Personality Inventory, Modifiers and Perceived Stress Scale, Brief Symptom Inventory) as well as different psychological variables (stress, anxiety, depression). These differences may limit the comparisons between the investigations. Some studies have also tried to correlate psychiatric patients to periodontal disease.[8,116-119]

In the present study, the presence of stress or depression was assessed by a self rating questionnaire system which might be subjected to individual bias related to understanding of the gravity of the stated question and also ability to respond correctly according to the scale and the situation bias may also take place, that is, the

condition of instability of the clinical phenomenon being evaluated. Also it does not allow an assessment of the subjective and behavioural aspects of individuals.

The analysis of the effects of stress (with self-report scales) have on periodontitis may be approached inappropriately in epidemiological studies, for it is difficult to correlate facts of the present or recent past to periodontitis, especially due to the mean age of the onset of disease, its clinical course and chronicity .

This study plays an imperative role in establishing a definitive and distinct relationship and link between the stress, serum and salivary stress biomarkers along with disease activity determinant IL-1β in smokers and non-smokers with chronic periodontitis.

Conclusion

The present study was undertaken to evaluate whether stress, serum and salivary cortisol and IL-1β are associated and found to be increased in smokers and non-smokers with chronic periodontitis

A total of 75 patients were recruited and categorized into 3 groups, with 25 patients in each. Group I being healthy, Group II being smokers with chronic periodontitis and Group III being non-smokers with chronic periodontitis. All the patients were assessed clinically and biochemically for categorization into respective groups. Clinical parameters evaluated were PI, GI, PBI, PPD and CAL. Biochemical parameters included were cortisol and IL-1β levels from serum and saliva samples ELISA test was used to analyse serum and salivary cortisol and IL-1β levels. Stress levels were assessed using Zung self rating depression scale questionnaire.

A significantly high PPD and low GI and PBI were observed in smokers with chronic periodontitis as compared to nonsmokers with chronic periodontitis. A significantly high salivary cortisol values were observed as compared to serum in all the groups. Amongst the chronic periodontitis patients smokers exhibited significantly higher salivary cortisol values.

From the analysis of the results, following observations can be drawn:

1. Salivary cortisol levels are significantly higher than serum cortisol levels in smokers with chronic periodontitis as compared to nonsmokers with chronic periodontitis.

2. Serum IL-1β values are higher in smokers with chronic periodontitis as compared to nonsmokers with chronic periodontitis.

3. Smokers exhibit high stress levels as compared to non-smokers.

4. The stress is positively correlated with the salivary cortisol levels in smokers and non-smokers

5. The salivary cortisol is significantly associated with the periodontal clinical parameters PPD, CAL, PI,GI,PBI.

6. The salivary IL1 β levels are positively correlated with the salivary cortisol levels.

This study had the following limitations:

1. The present study is simply an observational study. However, it is desirable to evaluate the results with interventional periodontal therapy and on a long term basis which will enable us to draw definitive and consistent conclusions.

2. Selection of the subjects was made on the basis of clinical indicators such as PPD & CAL, which do not necessarily reflect active periodontal destruction.

3. The severity of the chronic periodontitis was not differentiated as mild, moderate or severe.

4. Assessor for the assessment of all the clinical parameters and estimation of serum and salivary cortisol and IL-1β was the same and there were no blinded examinations. Therefore, possibility of operator bias to some extent cannot be ruled out.

Bibliography

1. **Breivik T, Thrane, PS, Murison R, Gjermo P.** Emotional stress effects on immunity, gingivitis and periodontitis. **Eur J Oral Sci.** 1996Aug;104(4 (Pt 1)):327-34.

2. **Hans Selye**. Stress without distress**. Science and art**,1982 ed, 1-135.

3. **Cohen S, Kessler RC, Gordon LU**. Measuring Stress: A guide for health and social scientists. **Oxford University Press** 1997;13:67-68.

4. **Johnson BD and Engel D**. Acute necrotizing ulcerative gingivitis. A review of diagnosis, etiology and treatment. **J Periodontol.** 1986 Mar;57(3):141-50.

5. **Croucher R, Marcenes WS, Torres MC, Hughes F, Sheiham, A**. The relationship between life-events and periodontitis: A case–control study. **J Clin Periodontol** 1997Jan;24(1):39-43.

6. **Marcenes WS and Sheiham A.** The relationship between work stress and oral health status. **Soc Sci Med.** 1992Dec;35(12):1511-20.

7. **Green LW, Tryon WW, Marks B, Huryn J.** Periodontal disease as a function of life events stress. **J Human Stress.** 1986Spring;12(1):32-6.

8. **Belting C. and Gupta O**. The influence of psychiatric disturbances on the severity of periodontal disease. **J Periodontol.** 1961;32:219–26.

9. **Webster Marketon JI, Glaser R**. Stress hormones and immune function. **Cell Immunol.** 2008 Mar-Apr;252(1-2):16-26.

10. **Ader R, Cohen N, Felten D.** Psychoneuroimmunology: interactions between the nervous system and the immune system. **Lancet.** 1995 Jan 14;345(8942):99-103.

11. **Black PH**. Central nervous system-immune system interactions: psychoneuroendocrinology of stress and its immune consequences. **Antimicrob Agents Chemother.** 1994Jan; 38(1):1–6.

12. **Glaser R, Kiecolt-Glaser JK**. Stress-induced immune dysfunction: implications for health. **Nat Rev Immunol.** 2005Mar;5(3):243-51.

13. **Warren KR, Postolache TT, Groer ME, Pinjari O, Kelly DL, Reynolds MA**. Role of chronic stress and depression in periodontal diseases. **Perio 2000.** 2014 Feb;64(1):127-38.

14. **Genco RJ, Ho AW, Grossi SG, Dunford RG, Tedesco LA.** Relationship of stress, distress and inadequate coping behaviors to periodontal disease. **J Periodontol.** 1999Jul;70(7):711-23.

15. **Aleksejuniene J, Holst D, Eriksen HM, Gjermo P**. Psychosocial stress, lifestyle and periodontal health. **J Clin Periodontol.** 2002Apr;29(4):326-35.

16. **Genco RJ, Ho AW, Kopman J, Grossi SG, Dunford RG, Tedesco LA.** Models to evaluate the role of stress in periodontal disease. **Ann Periodontol.** 1998 Jul;3(1):288-302.

17. **Groer M, Murphy R, Bunnell W, Salomon K, Van Eepoel J, Rankin B et al.** Salivary measures of stress and immunity in police officers engaged in simulated critical incident scenarios. **J Occup Environ Med.** 2010Jun;52(6):595-602.

18. **Kunz-Ebrecht SR, Mohamed-Ali V, Feldman PJ, Kirschbaum C and Steptoe A**. Cortisol responses to mild psychological stress are inversely associated with proinflammatory cytokines. **Brain Behav Immun.** 2003Oct;17(5):373-83.

19. **Papacosta E. and Nassis GP.** Saliva as a tool for monitoring steroid, peptide and immune markers in sport and exercise science. **J Sci Med Sport.** 2011Sep;14(5):424-34.

20. **Gatti R, Antonelli G, Prearo M, Spinella P, Cappellin E. and De Palo EF.** Cortisol assays and diagnostic laboratory procedures in human biological fluids. **Clin Biochem.** 2009Aug;42(12):1205-17.

21. **Aardal E and Holm AC**. Cortisol in saliva-reference ranges and relation to cortisol in serum **Eur J Clin Chem Clin Biochem.** 1995Dec;33(12):927-32.

22. **Akerstedt T and Levi L**.Circadian rhythms in the secretion of cortisol, adrenaline and noradrenaline.**EurJ Clin Invest.** 1978Apr;8(2):57-8.

23. **Alwan AH.**Determination of Interleukin-1β (IL-1 β) and Interleukin-6(IL6) in Gingival Crevicular Fluid in Patients with Chronic Periodontitis. **IOSR-JDMS** 2015;14:81-90.

24. **Stashenko P, Fujiyoshi P, Obernesser MS, Prostak L, Haffajee AD, Socransky SS.** Levels of interleukin 1 beta in tissue from sites of active periodontal disease**. J Clin Periodontol.** 1991Aug;18(7):548-54.

25. **Lö YJ, Liu CM, Wong MY, Hou LT, Chang WK**. Interleukin 1beta-secreting cells in inflamed gingival tissue of adult periodontitis patients. **Cytokine.** 1999Aug;11(8):626-33.

26. **Dinarello CA**. Biology of interleukin-1**. FASEB J** 1988 Feb;2(2):108-15.

27. **Mengel R, Bacher M, Flores-de-Jacoby L**. Interactions between stress, interleukin- 1b, interleukin-6 and cortisol in periodontally diseased patients. **J Clin Periodontol.** 2002Nov;29(11):1012-22.

28. **Cavaillon JM, Fitting C, Haeffner-Cavaillon N**. Recombinant C5a enhances interleukin 1 and tumor necrosis factor release by lipopolysaccharide-stimulated monocytes and macrophages. **Eur J Immunol.** 1990Feb;20(2):253-7.

29. **Cavaillon JM, Haeffner-Cavaillon N**. Signals involved in interleukin 1 synthesis and release by lipopolysaccharide- stimulated monocytes/macrophages. **Cytokine.** 1990 Sep;2(5):313-29.

30. **Okusawa S, Dinarello CA, Yancey KB, Endres S, Lawley TJ, Frank M. et al.** C5a induction of human interleukin 1. Synergistic effect with endotoxin or interferong. **J Immunol.** 1987Oct 15;139(8):2635-40.

31. **Richards D and Rutherford RB.** The effects of interleukin 1 on collagenolytic activity and prostaglandin-E secretion by human periodontal-ligament and gingival fibroblast. **Arch Oral Biol.**1988;33(4):237–43.

32. **Tatakis DN.** Interleukin-1 and bone metabolism: a review. **J Periodontol.** 1993May;64(5 Suppl):416-31.

33. **Torres de Heens GL, Kikkert R, Aarden LA, van der Velden U, Loos BG**. Effects of smoking on the ex vivo cytokine production in periodontitis. **J Periodontal Res.** 2009Feb;44(1):28-34.

34. **Tymkiw KD, Thunell DH, Johnson GK, Joly S, Burnell KK, Cavanaugh JE,et al.** Influence of smoking on gingival crevicular fluid cytokines in severe chronic periodontitis. **J Clin Periodontol.** 2011Mar;38(3):219-28.

35. **Buchmann AF, Laucht M, Schmid B, Wiedemann K, Mann K, Zimmermann US.** Cigarette craving increases after a psychosocial stress test and is related to cortisol stress responses but not to dependence scores in daily smokers. **J Psychopharmacol.** 2010Feb;24(2):247-55.

36. **Perkins KA, Grobe JE.** Increased desire to smoke during acute stress. **Br J Addict.** 1992Jul;87(7):1037-40.

37. **Suzuki N, Nakanishi K, Yoneda M, Hirofuj T, HaniokaT.** Relationship between salivary stress biomarker levels and cigarette smoking in healthy young adults: an exploratory analysis. **Tob Induc Dis.** 2016 Jun;14:20.

38. **Stein EA, Pankiewicz J, Harsch HH, Cho JK, Fuller SA, Hoffmann RG**, **et al.** Nicotine-induced limbic cortisol activation in the human brain: a functional MRI study. **Am J Psychiatry.** 1998Aug;155(8):1009-15.

39. **Booij SH, Bos EH, Bouwmans ME, van Faassen M, Kema IP, Oldehinkel AJ, et al.** Cortisol and α-amylase secretion patterns between and within depressed and non-depressed individuals. **PloS One.** 2015 Jul6;10(7):e0131002.

40. **Kunz-Ebrecht SR, Mohamed-Ali V, Feldman PJ, Kirschbaum C, Steptoe A.** Cortisol responses to mild psychological stress are inversely associated with proinflammatory cytokines. **Brain Behav Immun.** 2003Oct;17(5):373-83.

41. **Buduneli N.** Pathogenesis and Treatment of Periodontitis: Effects of tobacco smoking on chronic periodontitis and periodontal treatment. **1st ed.Rijeka, Croatia: In Tech;** 2011;81–96.

42. **Moss, ME, Beck JD, Kaplan BH, Offenbacher S, Weintraub JA, Koch GG, et al.** Exploratory case-control analysis of psychosocial factors and adult periodontitis. **J. Periodontol.** 1996 Oct;67(10 Suppl):1060-9.

43. **Johannsen A, Asberg M, Soder P-O, Soder B.** Anxiety, gingival inflammation and periodontal disease in non-smokers and smokers – an epidemiological study. **J Clin Periodontol.** 2005May;32(5):488-91.

44. **Hilgert JB, Hugo FN, Bandeira DR, Bozzetti MC**. Stress, cortisol, and periodontitis in a population aged 50 years and over**. J Dent Res.** 2006 Apr;85(4):324-8.

45. **Rosania AE, Low KG, McCormick CM, Rosania DA.** Stress, depression, cortisol, and periodontal disease. **J Periodontol.** 2009 Feb;80(2):260-6.

46. **Ansai T, Soh I, Ishisaka A, Yoshida A, Awano S, Hamasaki T et al**. Determination of cortisol and dehydroepiandrosterone levels in saliva for screening of periodontitis in older Japanese adults**. Int J Dent.** 2009;2009:280737.

47. **Goyal S, Jajoo S, Nagappa G, Rao G**. Estimation of relationship between psychological stress and periodontal status using serum cortisol level. A clinico-biochemical study. **Indian J Dent Res.** 2011Jan-Feb;22(1):6-9.

48. **Rai B, Kaur J, Anand SC, Jacobs R**. Salivary stress markers, stress, and periodontitis: A pilot study. **J Periodontol.** 2011Feb;82(2):287-92.

49. **Refulio Z, Rocafuerte M, de la Rosa M, Mendoza G, Chambrone L.** Association among stress, salivary cortisol levels and chronic periodontitis. **J Periodontal Implant Sci.** 2013Apr;43(2):96-100.

50. **Nayak SU, Nayak DG, Uppoor AS and Pai KK**. Evaluation of cortisol levels in gingival crevicular fluid and saliva in anxious and non-anxious patients with chronic periodontitis**. Dent Res J (Isfahan).** 2013Jul-Aug;10(4):474–81.

51. **Wong JA, Pickworth WB, Andrew J, Waters AJ , Absi MA, and Leventhal AM** Cortisol levels decrease after acute tobacco abstinence in regular smokers**Hum Psychopharmacol.** 2014Mar;29(2):152-62.

52. **Jaiswal R, Shenoy N and Thomas B.** Evaluation of association between psychological stress and serum cortisol levels in patients with chronic periodontitis – Estimation of relationship between psychological stress and periodontal status. **J Indian Soc Periodontol.** 2016Jul-Aug;20(4):381-385.

53. **Rohini G, Kalaivani S, Kumar V, Rajasekar SA, Tuckaram J, Pandey V.** Estimation and comparison of serum cortisol levels in periodontally diseased patients and periodontally healthy individuals: A clinical-biochemical study. **J Pharm Bioallied Sci.** 2015Aug;7(Suppl 2):S457-60.

54. **Shende AS, Bhatsange AG, Waghmare AS, Shiggaon LB, Mehetre VN, Meshram EP**. Determining the association between stress and periodontal disease: A pilot study. **J Int Clin Dent Res Organ.** 2016;8:111-4.

55. **Gorska R, Gregorek H, Kowalski J, Laskus-Perendyk A, Syczewska M, Madalinski K.** Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. **J Clin Periodontol.** 2003Dec;30(12):1046-52.

56. **Miller CS, King CP, Langub MC, Kryscio RJ, Thromas MV**. Salivary biomarkers of existing periodontal disease: a cross-sectional study.**J Am Dent Assoc.** 2006Mar;137(3):322-9.

57. **Tobon-Arroyave SI, Jaramillo-Gonzalez PE, Isaza-Guzman DM.** Correlation between salivary IL-1β levels and periodontal clinical status. **Arch Oral Biol.** 2008Apr;53(4):346-52.

58. **Vahabi S, Sattari M, Taheraslani M, Bagheban AA.** Correlation between Interleukin-1β, Interleukin-6 and Tumor Necrosis Factor-α and clinical parameters in chronic and aggressive periodontal disease. **J Periodontol Implant Dent.** 2011;3(2):51–56.

59. **Williamson S, Munro C, Pickler R, Grap MJ and Elswick RK**. Comparison of biomarkers in blood and saliva in healthy adults. **Nurs Res Pract.**2012;2012:1-4.

60. **Toker H, Akpınar A, Aydın H, Poyraz O.** Influence of smoking on interleukin-1beta level, oxidant status and antioxidant status in gingival crevicular fluid from chronic periodontitis patients before and after periodontal treatment. **J Periodontal Res.** 2012Oct;47(5):572-7.

61. **Ghurabi BH.** Impact of smoking on the IL-1β, IL-8, IL-10, IL-17 and TNF-α production in chronic periodontitis patients. **J Asian Sci Res.** 2013;3:462-70.

62. **Mousavijazi M, Naderan A, Ebrahimpoor M, Sadeghipoor M.** Association between psychological stress and stimulation of inflammatory responses in periodontal disease. **J. Dent (Tehran).** 2013 Jan;10(1):103-11.

63. **Gaphor SM, Ali SH, Abdullah MJ** Evaluation of salivary Interleukin-1beta (IL-1β) level in relation to the periodontal status in smoker and non smoker individuals**. J Interdiscipl Med Dent Sci.** 2014; 2:120.

64. **Kaushik R, Yeltiwar RK, Pushpanshu K.** Salivary interleukin-1b levels in patients with chronic periodontitis before and after periodontal phase I therapy and healthy controls: A case-control study. **J Periodontol.** 2011Sep;82(9):1353-9.

65. **Hussain A A, Ali BG.** Evaluation of Interleukin 1β levels in gingival crevicular fluid and serum of patients with gingivitis and chronic periodontitis. **IOSR Journal of Dental and Medical Sciences.** 2014;13(11):70-75

66. **Haffajee AD, Socransky SS**. Relationship of cigarette smoking to attachment level profiles. **J Clin Periodontol.** 2001Apr;28(4):283-95.

67. **Persson L, Bergstrom J, Ito H, Gustafsson A**. Tobacco smoking and neutrophil activity in patients with periodontal disease. **J Periodontol.** 2001Jan;72(1):90-5.

68. **Hashim R, Thomson WM, Pack ARC**. Smoking in adolescence as a predictor of early loss of periodontal attachment. **Community Dent Oral Epidemiol.** 2001Apr;29(2):130-5.

69. **Kamma JJ, Giannopoulou C, Vasdekis VGS, Mombelli A.** Cytokine profile in gingival crevicular fluid of aggressive periodontitis: influence of smoking and stress. **J Clin Periodontol.** 2004Oct;31(10):894-902.

70. **Badrick E, Kirschbaum C and Kumari M.** The relationship between smoking status and cortisol secretion. **J Clin Endocrinol Metab.** 2007Mar;92(3):819-24.

71. **Gautam DK, Jindal V, Gupta SC, Tuli A, Kotwal B, Thakur T**. Effect of cigarette smoking on periodontal health status: Comparative cross- sectional study. **J Indian Soc Periodontol.** 2011Oct;15(4):383-7.

72. **Kolte AP, Kolte RA, Lathiya VN**. Association between anxiety, obesity and periodontal disease in smokers and non-smokers: A cross-sectional study. **J Dent Res Dent Clin Dent Prospects.** 2016Autumn; 10(4): 234–240.

73. **Palmer RM, Wilson RF, Hasan AS, ScottDA.** Mechanisms of action of environmental factors--tobacco smoking**. J Clin Periodontol.** 2005;32 Suppl 6:180-95.

74. U.S. Department of Health and Human Services. How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health, 2010.chapter 3,27-102.

75. **Benowitz NL**. Cigarette smoking and cardiovascular disease: pathophysiology and implications for treatment. **Prog in Cardiovasc Dis.** 2003Jul-Aug;46(1):91-111.

76. **Jansson LE, Hagstrom KE**. Relationship between compliance and periodontal treatment outcome in smokers. **J Periodontol.** 2002Jun;73(6):602-7.

77. **Grossi SG, Zambon J, Machtei EE,Schifferle R, Andreana S, Genco RJ etal.** Effects of smoking and smoking cessation on healing after mechanical periodontal therapy. **J Am Dent Assoc.** 1997 May;128(5):599-607.

78. **Ah MK, Johnson GK, Kaldahl WB, Patil KD, Kalkwarf KL**. The effect of smoking on the response to periodontal therapy. **J Clin Periodontol.** 1994Feb;21(2):91-7.

79. **Trauth. JA, Seidler FJ, Ali SF, Slotkin TA**. Adolescent nicotine exposure produces immediate and long-term changes in CNS noradrenergic and dopaminergic function. **Brain Res.** 2001Feb 23;892(2):269-80.

80. **Clark NG, Hirsch RS**. Personalized risk factors for generalised periodontitis. **J Clin Periodontol.** 1995Feb;22(2):136-45.

81. **Bergstrom J, Floderus-Myrhed B**. Co-twin study of the relationship between smoking and some periodontal disease factors. **Community Dent Oral Epidemiol.** 1983Apr;11(2):113-6.

82. **Arai K, Lee F, Miyajima A, Miyataka S, Arqi N, Yokota T.** Cytokines: coordinators of immune and inflammatory responses. **Annu Rev Biochem** 1990;59:783–836.

83. **Graves DT, Cochran D.** The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. **J Periodontol** 2003Mar;74(3):391-401.

84. **Stashenko P, Fujiyoshi P, Obernesser MS, ProstakL, Haffajee AD, Socransky SS.** Levels of interleukin 1 beta in tissue from sites of active periodontal disease. **J Clin Periodontol.** 1991Aug;18(7):548-54.

85. **Bloemen V, Schoenmaker T, de Vries TJ, Everts V.** IL-1beta favors osteoclastogenesis via supporting human periodontal ligament fibroblasts. **J Cell Biochem.** 2011Jul;112(7):1890-7.

86. **Haffajee AD, Socransky SS, Goodson JM.** Clinical parameters as predictors of destructive periodontal disease activity. **J Clin Periodontol.** 1983May;10(3):257-65.

87. **Stathopoulou PG, Buduneli N, Kinane DF**. Systemic biomarkers for periodontitis. **Curr. Oral Health Rep**2015;2(4):218–26.

88. **Kaufman E, Lamster IB**. Analysis of saliva for periodontal diagnosis— a review. **J Clin Periodontol.** 2000Jul;27(7):453-65.

89. **Feldman RS, Bravacos JS, Rose CL**. Association between smoking different tobacco products and periodontal disease indexes. **J Periodontol.** 1983Aug;54(8):481-7.

90. **Cuff MJ, McQuade MJ, Scheidt MJ, Sutherland DE, Van Dyke TE**. The presence of nicotine on root surfaces of periodontally diseased teeth in smokers. **J Periodontol**. 1989 Oct;60(10):564–9.

91. **Tanur E, McQuade MJ, McPherson JC, AlHashimi IH, Rivera Hidalgo F.** Effects of nicotine on the strength of attachment of gingival fibroblasts to glass and nondiseased human root surfaces. **J Periodontol.** 2000 May;71(5):717–22.

92. **Austin GW, Cuenin MF, Hokett SD, Peacock ME, Sutherland DE, Erbland JF, et al.** Effect of nicotine on fibroblast beta 1 integrin expression and distribution in vitro. **J Periodontol.** 2001Apr;72(4):438-44.

93. **Tipton DA, Dabbous MK.** Effects of nicotine on proliferation and extracellular matrix production of human gingival fibroblasts in vitro**. J Periodontol.** 1995Dec;66(12):1056-64.

94. **Palmer RM.** Tobacco smoking and oral health: Review. **Br Dent J.** 1988;164:258-60.

95. **Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, et al.** Assessment of risk for periodontal disease. I. Risk indicators for attachment loss**. J Periodontol.** 1994Mar;65(3):260-7.

96. **Bergstrom J.** Cigarette smoking as risk factor in chronic periodontal disease. **Community Dent Oral Epidemiol.** 1989 Oct;17(5):245-7.

97. **Calsina G, Ramón JM, Echeverría JJ.** Effects of smoking on periodontal tissues**. J Clin Periodontol.** 2002 Aug;29(8):771-6.

98. **Scabbia A, Cho KS, Sigurdsson TJ, Kim CK, Trombelli L.** Cigarette smoking negatively affects healing response following flap debridement surgery. **J Periodontol.** 2001Jan;72(1):43-9.

99. **Handa K, Kono S, Ishii H, Shinchi K, Imanishi K, Arakawa K.** Relationship of alcohol consumption and smoking to plasma cortisol and blood pressure. **J Hum Hypertens.** 1994 Dec;8(12):891-4.

100. **al'Absi M, Wittmers LE, Erickson J, Hatsukami D, Crouse B**. Attenuated adrenocortical and blood pressure responses to psychological stress in ad libitum and abstinent smokers. **Pharmacol Biochem Behav**. 2003Jan;74(2):401-10.

101. **Baron JA, Comi RJ, Cryns V, Brinck-Johnsen T, Mercer NG.** The effect of cigarette smoking on adrenal cortical hormones. **J Pharmacol Exp Ther**. 1995Jan;272(1):151-5.

102. **Gossain VV, Sherma NK., Srivastava L, Michelakis AM, Rovner DR.** Hormonal effects of smoking—II: Effects on plasma cortisol, growth hormone, and prolactin. **Am J Med Sci.** 1986 May;291(5):325-7.

103. **Kirschbaum C, Scherer G, Strasburger CJ.** Pituitary and adrenal hormone responses to pharmacological, physical, and psychological stimulation in habitual smokers and nonsmokers. **Clin Investig.** 1994Oct;72(10):804-10.

104. **Tsuda A, Steptoe A, West R, Fieldman G, Kirschbaum C.** Cigarette smoking and psychophysiological stress responsiveness: effects of recent smoking and temporary abstinence. **Psychopharmacology (Berl).** 1996 Aug;126(3):226-33.

105. **Axelsson P**. External modifying factors involved in periodontal diseases. Diagnosis and risk prediction of periodontal diseases. Karlstad: **Quintessesnce Publishing Co, Inc 2005.**

106. **Bostrom L, Linder LE, Bergstrom J.** Smoking and GCF levels of IL-1b and IL- 1ra in periodontal disease. **J Clin Periodontol.** 2000Apr;27(4):250-5..

107. **Payne JB, Johnson GK, Reinhardt RA, Dyer JK, Maze CA, Dunning DG**. Nicotine effects on PGE2 and IL-1 beta release by LPS-treated human monocytes. **J Periodontal Res.**1996Feb;31(2):99-104.

108. **Bernzweig E, Payne JB, Reinhardt RA, Dyer JK, Patil KD.** Nicotine and smokeless tobacco effects on gingival and peripheral blood mononuclear cells. **J Clin Periodontol.** 1998Mar;25(3):246-52.

109. **Gümüş P, Nizam N, Nalbantsoy A, Özçaka Ö, Buduneli N**. Saliva and serum levels of pentraxin-3 and interleukin-1β in generalized aggressive or chronic periodontitis. **J Periodontol.** 2014 Mar;85(3):e40-6.

110. **Keles G, Acikgoz G, Ayas B, Sakallioglu E, Firatli E.** Determination of systemically and locally induced periodontal defects in rats. **Indian J Med Res.** 2005Mar;121(3):176-84.

111. **Elkhouli A.** The efficacy of host response modulation therapy (omega-3 plus low-dose aspirin) as an adjunctive treatment of chronic periodontitis (clinical and biochemical study). **J Periodontal Res.** 2011 Apr;46(2):261-8.

112. **Monteiro da Silva AM, Newman HN, Oakley DA and O'Leary R.** Psychosocial factors, dental plaque levels and smoking in periodontitis patients. **J Clin Periodontol.** 1998Jun;25(6):517-23.

113. **Vettore MV, Leao AT, Monteiro da Silva AM, Quintanilha RS, Lamarca GA** The relationship of stress and anxiety with chronic periodontitis. **J Clin Periodontol.** 2003 May;30(5):394-402.

114. **Mudrika S, Muthukumar S, Suresh R.** Relationship between salivary levels of cortisol and dehydroepiandrosterone levels in saliva and chronic periodontitis. **J Int Clin Dent Res Organ.** 2014**;**6:92-7.

115. **Deinzer R, Forster P, Fuck L, Herforth A, Stiller-Winkler R, Idel H**. Increase of crevicular interleukin 1b under academic stress at experimental gingivitis sites and at sites of perfect oral hygiene. **J Clin Periodontol.**1999 Jan;26(1):1-8.

116. **Davis CH and Jenkins D**. Mental stress and oral diseases. **J Dent Res.** 1962 Sep;41(5):1045–49.

117. **Monteiro da Silva AM, Oakley DA, Newman HN, Nohl FS, Lloyd HM.** Psychosocial factors and adult onset rapidly progressive periodontitis. **J Clin Periodontol.** 1996Aug;23(8):789-94.

118. **Baker EG, Crook GH, Schwabacher ED**. Personality correlates of periodontal disease**. J Dent Res.** 1961 May;40(3):396–3.

119. **Solis ACO, Lotufo RFM, Pannuti CM, Brunheiro EC, Marques AH, Lotufo-Neto F.** Association of periodontal disease to anxiety and depression symptoms, and psychosocial stress factors. **J Clin Periodontol.** 2004Aug;31(8):633-8.

Tables

Table 1: Descriptive statistics for periodontal parameters for patients in three study groups

Parameters	Groups			P-value*
	I	II	III	
PPD [M ± SD]	1.37 ± 0.33	8.03 ± 1.29	5.44 ± 0.51	< 0.0001 (HS)
CAL [M ± SD]	0.00 ± 0.00	8.93 ± 1.17	7.07 ± 0.47	< 0.0001 (HS)
PI [M ± SD]	0.64 ± 0.32	2.56 ± 0.39	1.82 ± 0.47	< 0.0001 (HS)
GI [M ± SD]	0.39 ± 0.21	1.71 ± 0.60	2.14 ± 0.61	< 0.0001 (HS)
PBI [M ± SD]	0.04 ± 0.04	1.39 ± 0.76	2.24 ± 0.69	< 0.0001 (HS)

**Calculated using One-way ANOVA; HS: Highly Significant*

Table 1A:Comparison of periodontal parameters in patients from group II and group III

Parameters	Groups		P-value*
	II	III	
PPD	8.03 ± 1.29	5.44 ± 0.51	< 0.0001 (HS)
CAL	8.93 ± 1.17	7.07 ± 0.47	< 0.0001 (HS)
PI	2.56 ± 0.39	1.82 ± 0.47	< 0.0001 (HS)
GI	1.71 ± 0.60	2.14 ± 0.61	0.0142 (S)
PBI	1.39 ± 0.76	2.24 ± 0.69	0.0001 (S)

**Calculated using t test for independent samples; HS: Highly Significant, S: Significant, NS: Not Significant*

Table 2: Descriptive statistics for Cortisol levels in serum and saliva in patients from three study groups

Cortisol (pg/ml)	Groups			P-value*
	I	II	III	
Serum [M ± SD]	13.68 ± 6.20	17.59 ± 8.53	15.38 ± 8.90	0.228 (NS)
Saliva [M ± SD]	19.45 ± 4.03	563.40 ± 236.19	417.16 ± 99.67	< 0.0001 (HS)
P-value**	< 0.0001 (HS)	< 0.0001 (HS)	< 0.0001 (HS)	

**Calculated using One-way ANOVA; ** Calculated using paired t-test; NS: Not Significant, HS: Highly Significant*

Table 2A:Paired comparison of Cortisol levels in patients from three study groups insaliva

Cortisol		P- value*
Saliva	Group I - Group II	< 0.0001 (HS)
	Group I - Group III	< 0.0001 (HS)
	Group III - Group II	0.0023 (S)

**Calculated using Tukey HSD test; S: Significant, HS: Highly Significant*

Table 3:Descriptive statistics for IL-1β levels in serum and saliva in patients from three study groups

IL-1β (pg/ml)	Groups			P-value*
	I	II	III	
Serum [M ± SD]	11.44 ± 3.11	24.51 ± 26.93	19.39 ± 6.18	0.0187 (S)
Saliva [M ± SD]	160.61 ± 62.23	278.95 ± 81.40	251.35 ± 81.19	< 0.0001 (HS)
P-value**	< 0.0001 (HS)	< 0.0001 (HS)	< 0.0001 (HS)	

**Calculated using One-way ANOVA; ** Calculated using paired t-test; S: Significant; HS: Highly Significant*

Table 3A: Paired comparison of IL-1β levelsin patients from three study groups

IL-1β		P- value*
Serum	Group I - Group II	0.0144 (S)
	Group I - Group III	0.1932 (NS)
	Group II - Group III	0.5008 (NS)
Saliva	Group I - Group II	< 0.0001 (HS)
	Group I - Group III	0.0002 (S)
	Group II - Group III	0.4039 (NS)

**Calculated using Tukey HSD test; S: Significant, HS: Highly Significant, NS: Not Significant*

Table 4:Descriptive statistics for SDSscores in patients from three study groups

SDS Score	Group			P-value*
	I	II	III	
Mean	41.04	56.8	45.36	< 0.0001 (HS)
SD	4.81	9.47	9.1	
Median	42	60	47	

**Using Kruskal-Wallis test; HS: Highly Significant*

Table 4A:Paired comparison of SDS scores in patients from three study groups

Groups	P-value*
I - II	< 0.0001 (HS)
II - III	0.0002 (S)
I - III	0.0261 (S)

**Calculated using Wilcoxon rank sum test; S: Significant, HS: Highly Significant*

Table 5: Correlation of SDS scores with Cortisol levels in each group

Cortisol Concentration	Levels	Correlation Coefficients*	P- value
Group I			
	Serum	0.4870	0.0136 (S)
	Saliva	0.3769	0.0633 (NS)
Group II			
	Serum	0.1528	0.4659 (NS)
	Saliva	0.4471	0.0250 (S)
Group III			
	Serum	0.2043	0.3274 (NS)
	Saliva	0.5450	0.0048 (S)

**Calculated using Spearman's Rank Correlation; NS: Not significant*

Table 6: Correlation of different periodontal parameters with serum and saliva cortisol

Periodontal parameters	Serum Cortisol		Saliva Cortisol	
	Correlation	P-value*	Correlation	P-value*
PPD	0.1334	0.2539 (NS)	0.8031	< 0.0001 (HS)
CAL	0.1679	0.1499 (NS)	0.8197	< 0.0001 (HS)
PI	0.0165	0.8883 (NS)	0.7542	< 0.0001 (HS)
GI	0.1104	0.3458 (NS)	0.6189	< 0.0001 (HS)
PBI	0.1384	0.2365 (NS)	0.5928	< 0.0001 (HS)

*Obtained using Spearman rank correlation; S: Significant; NS: Not significant

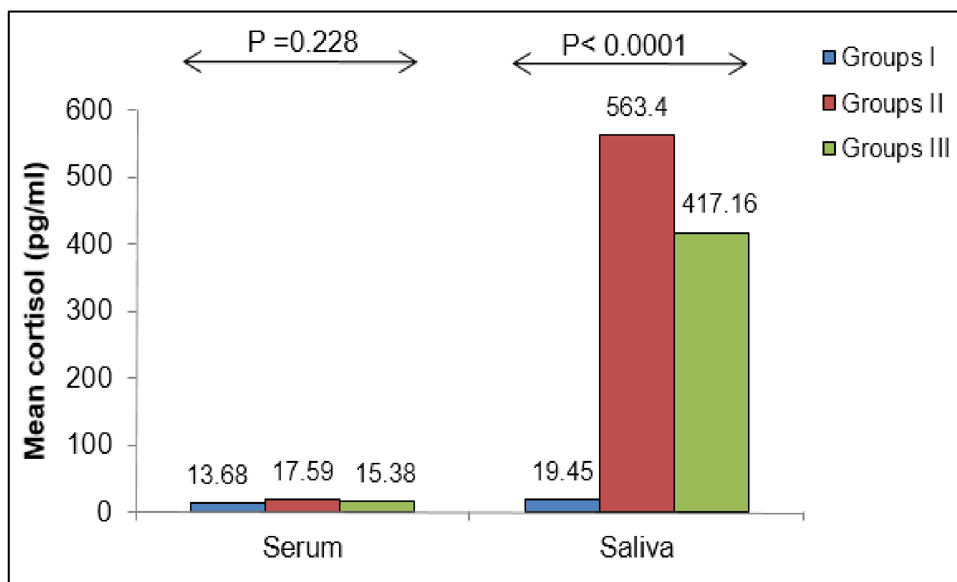
Table 7: Correlation of cortisol and IL-1β values in serum and saliva

		Serum Cortisol	Saliva Cortisol
		Correlation (P-value)	Correlation (P–value)
IL-1β	Serum	-0.0343 (0.7702)	-0.0811 (0.4893)
	Saliva	0.0395 (0.7365)	0.4476 (< 0.0001)

*Obtained using Spearman rank correlation

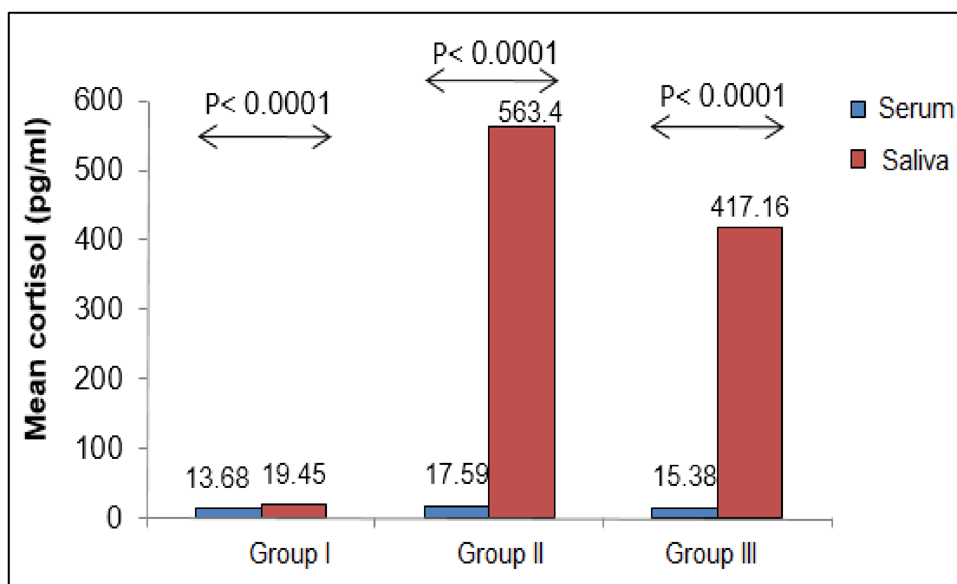
Graph 1

Column chart showing mean cortisol levels in three groups in serum and saliva



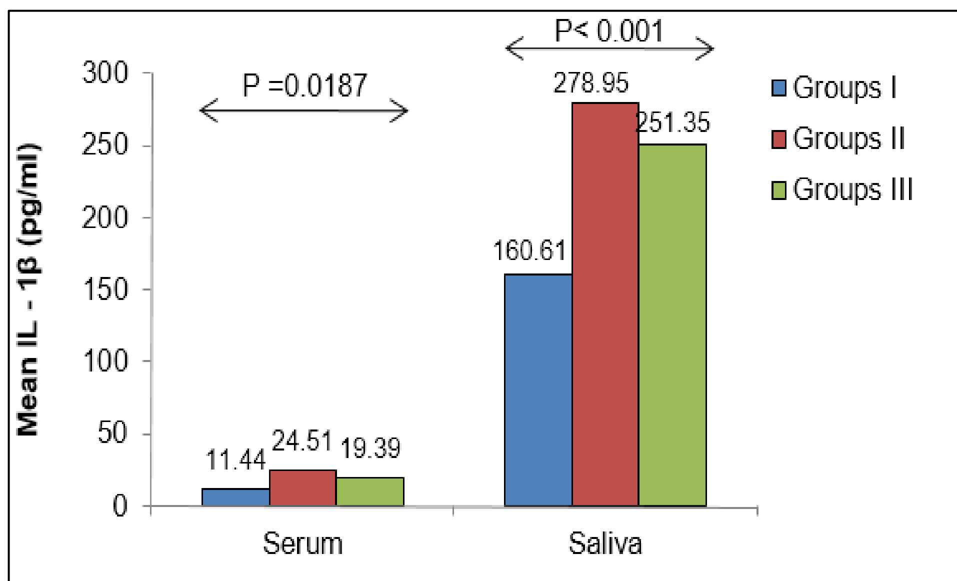
Graph 2

Column chart showing mean cortisol levels in serum and saliva in each group



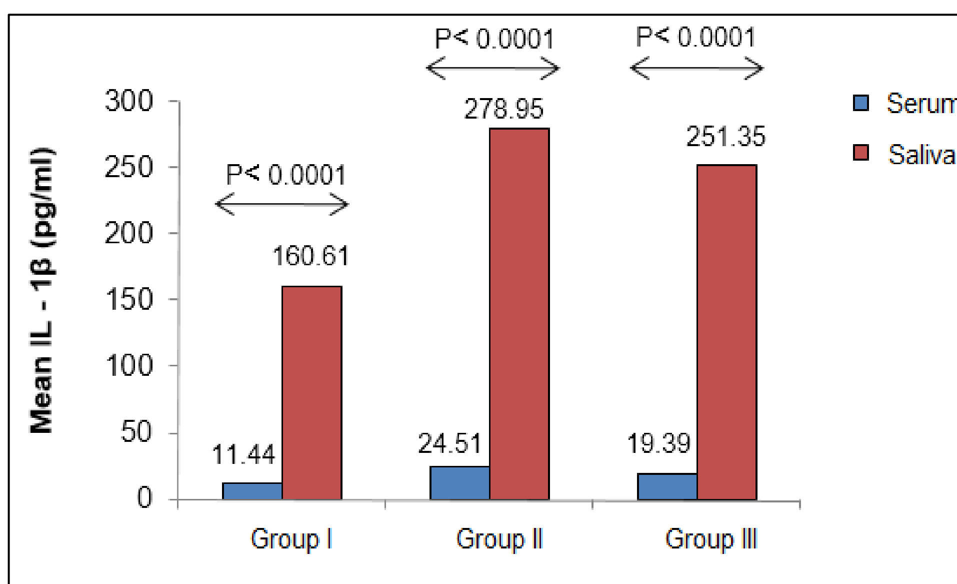
Graph 3

Column chart showing mean IL-1β levels in three groups in serum and saliva



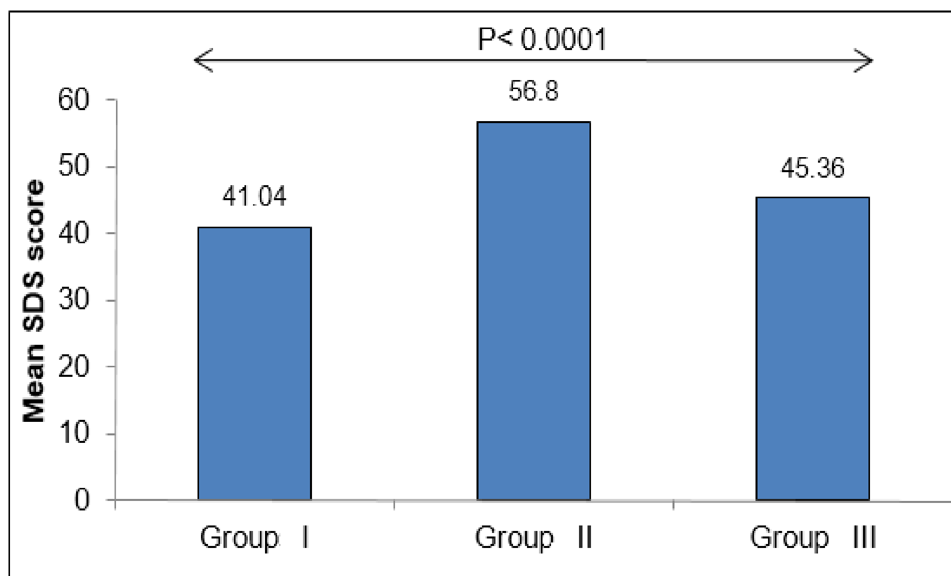
Graph 4

Column chart showing mean IL-1β levels in serum and saliva in three groups



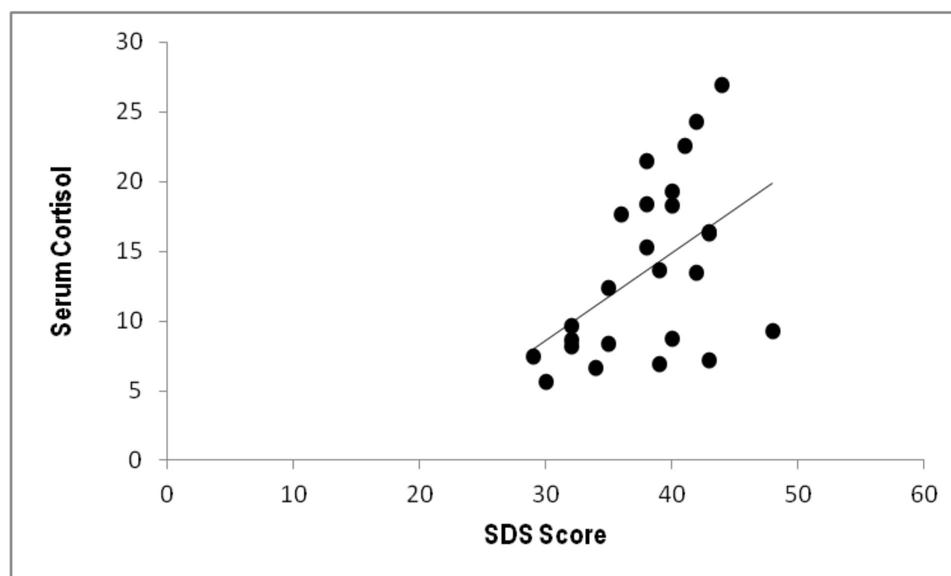
Graph 5

Column chart showing mean SDS scores for three groups



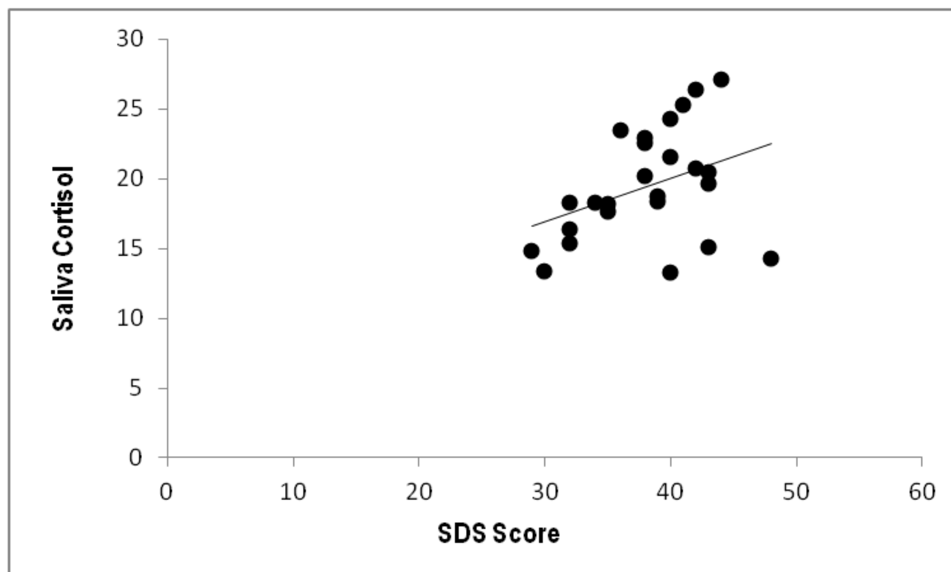
Graph 6

Scatter plot showing relationship between SDS scores and Serum cortisol in Group I



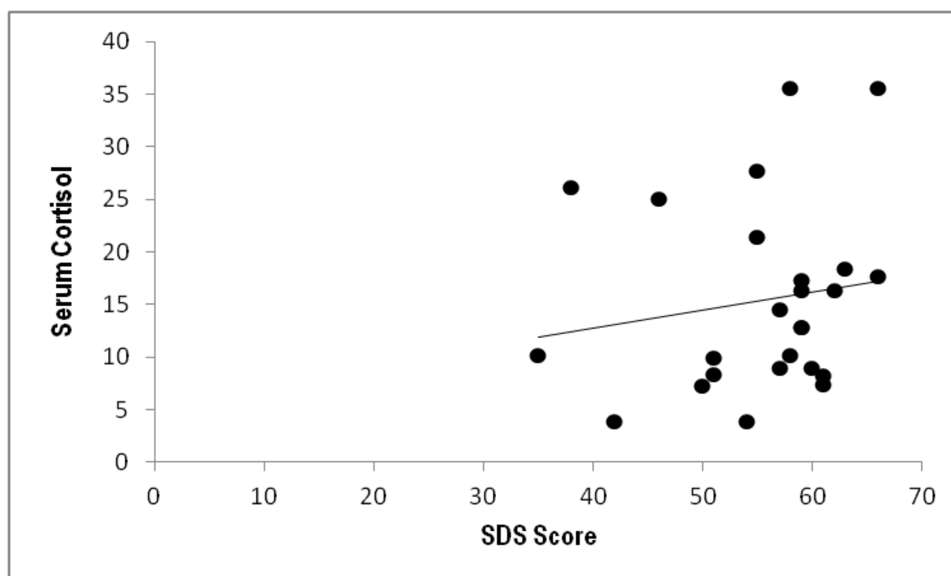
Graph 7

Scatter plot showing relationship between SDS scores and Saliva cortisol in Group I



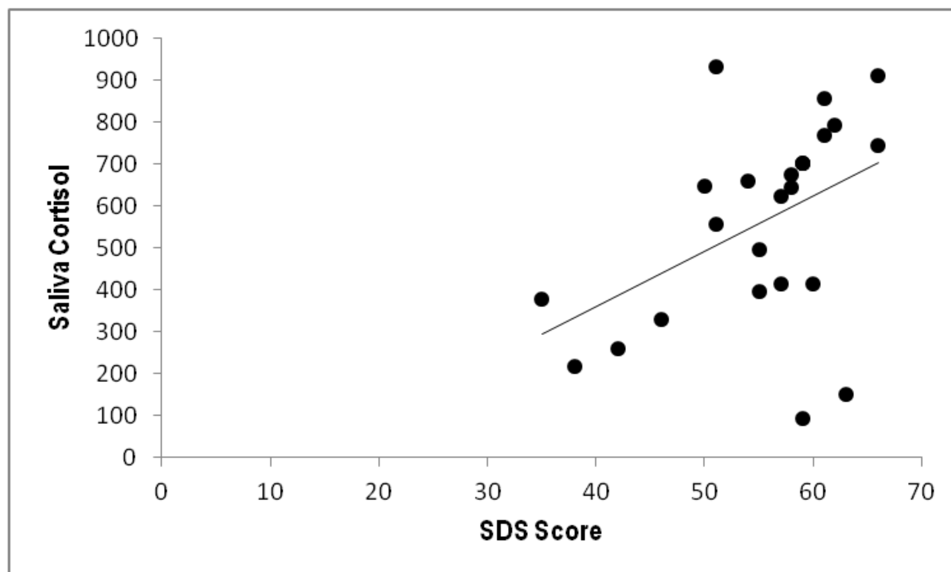
Graph 8

Scatter plot showing relationship between SDS scores and Serum cortisol in Group II



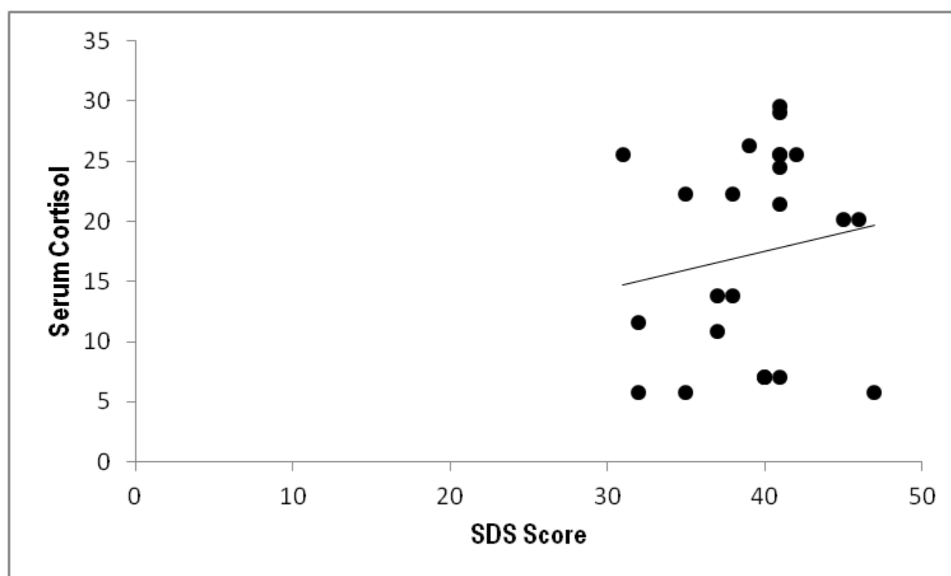
Graph 9

Scatter plot showing relationship between SDS scores and Saliva cortisol in Group II



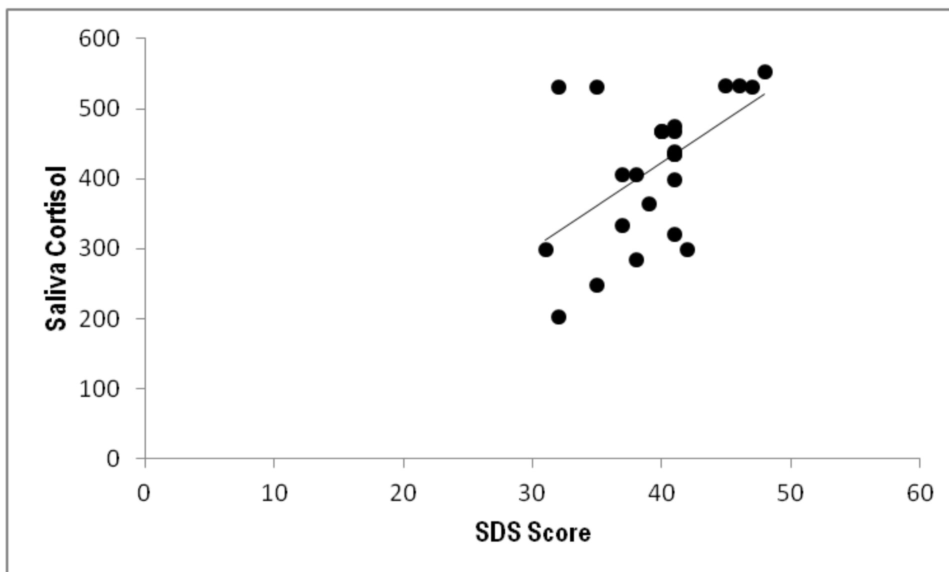
Graph 10

Scatter plot showing relationship between SDS scores and Serum cortisol in Group III



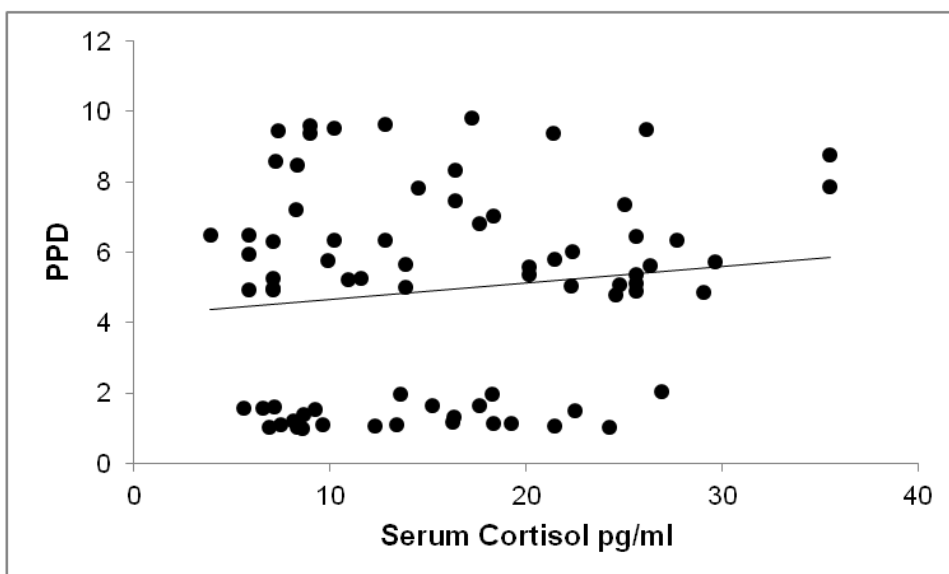
Graph 11

Scatter plot showing relationship between SDS scores and Saliva cortisol in Group III



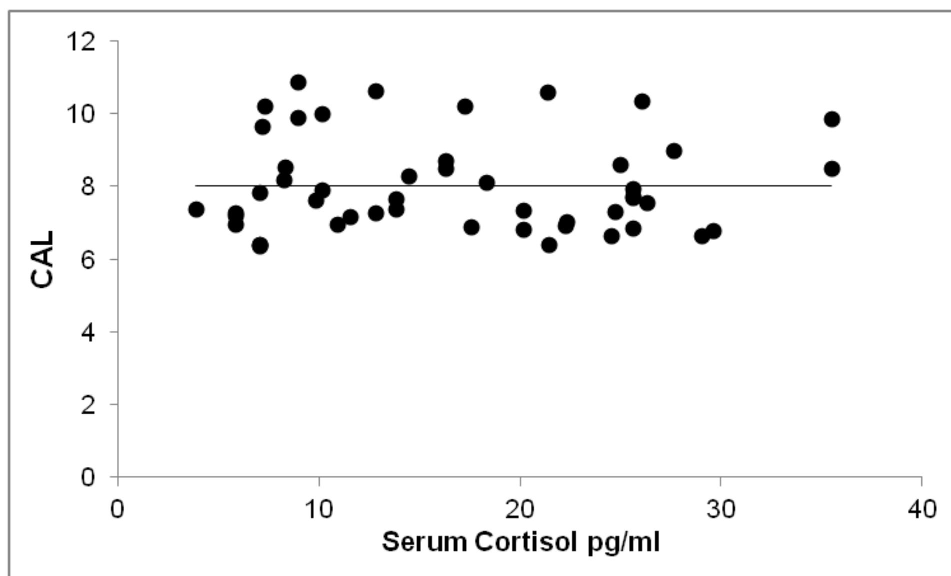
Graph 12

Scatter plot of Serum cortisol and PPD



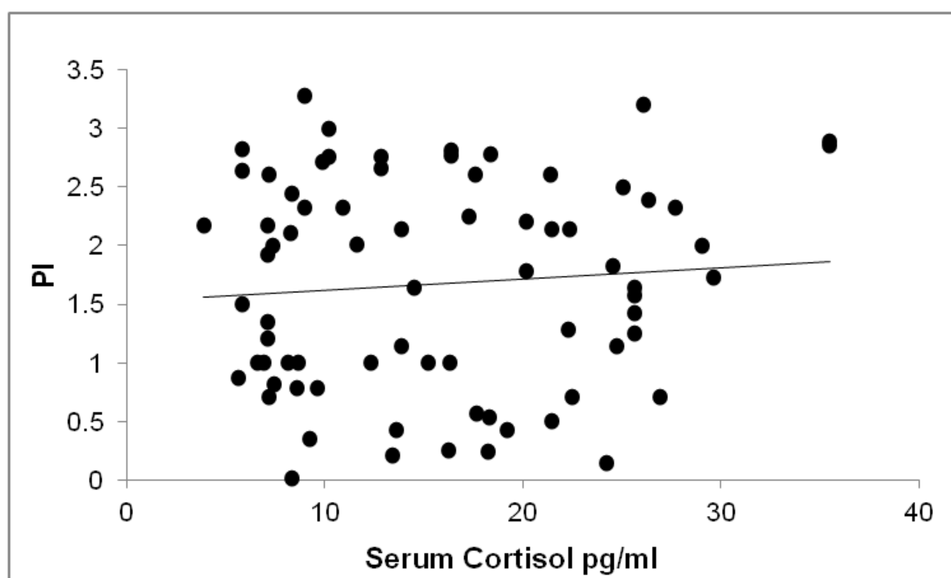
Graph 13

Scatter plot of Serum cortisol and CAL



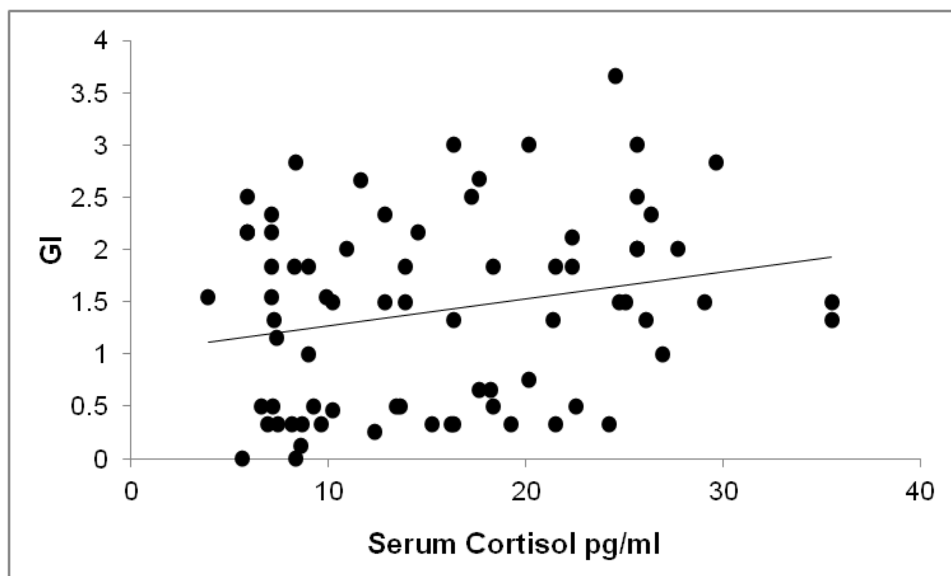
Graph 14

Scatter plot of Serum cortisol and PI



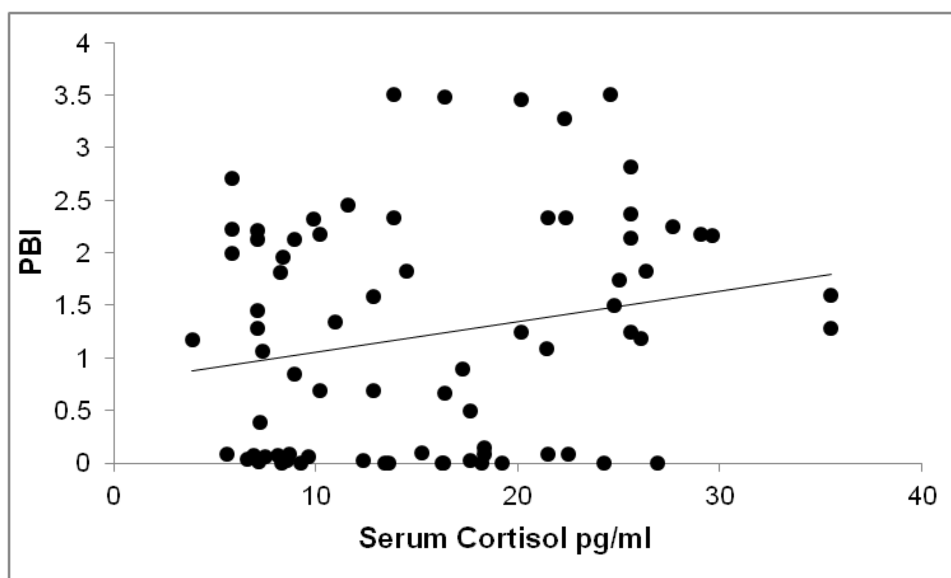
Graph 15

Scatter plot of Serum cortisol and GI



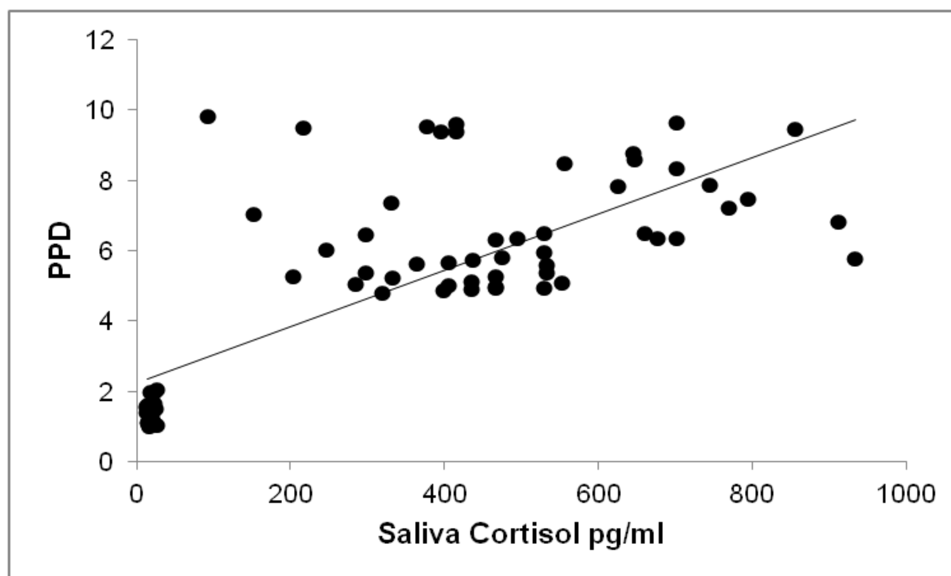
Graph 16

Scatter plot of Serum cortisol and PBI



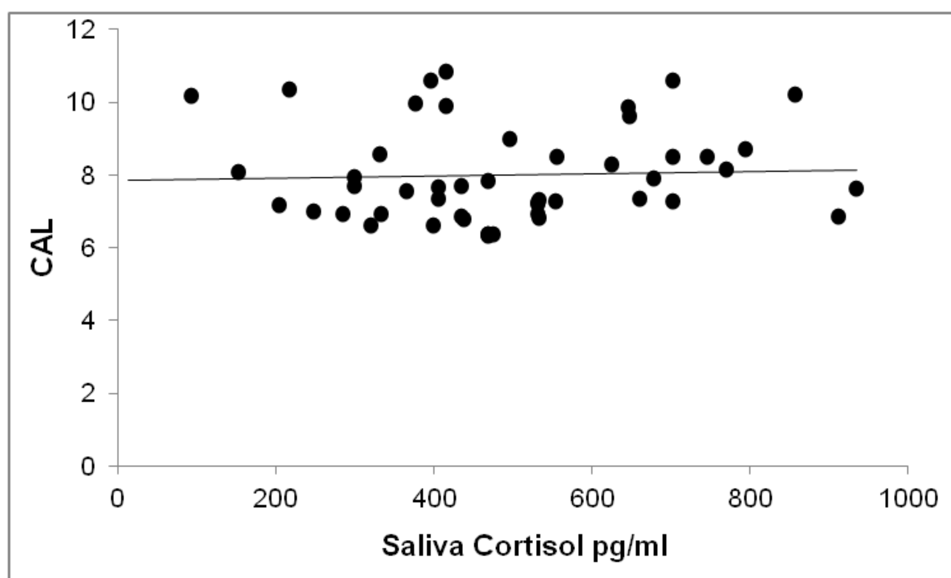
Graph 17

Scatter plot of Saliva cortisol and PPD



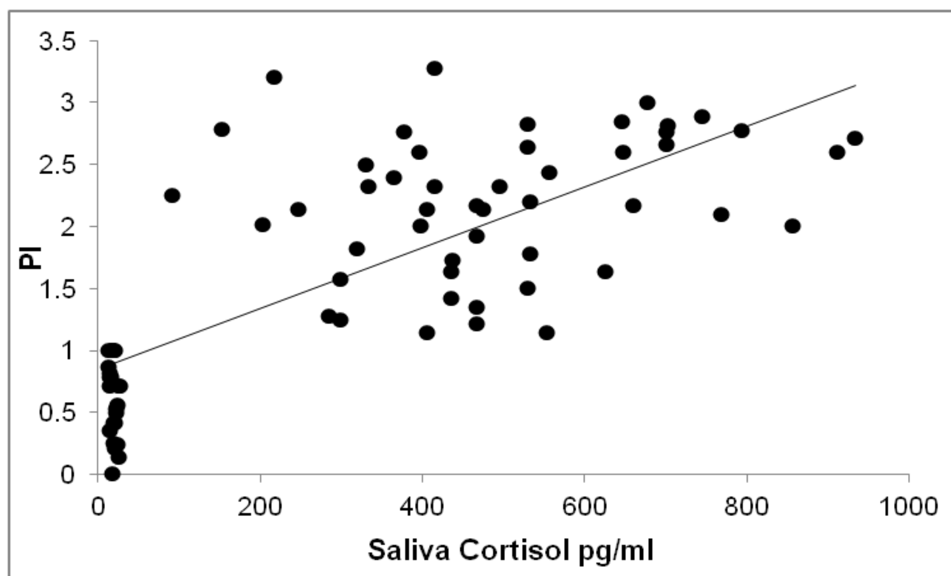
Graph 18

Scatter plot of Saliva cortisol and CAL



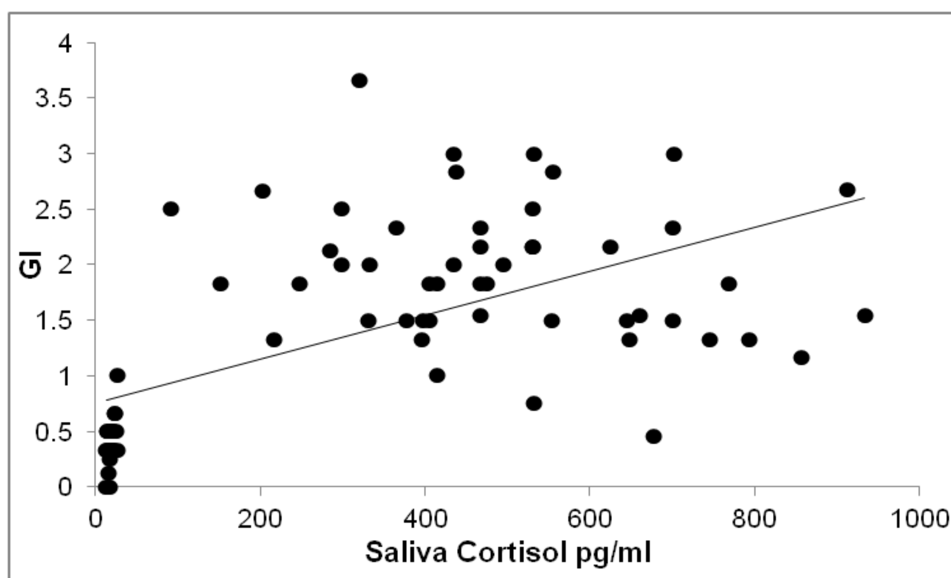
Graph 19

Scatter plot of Saliva cortisol and PI



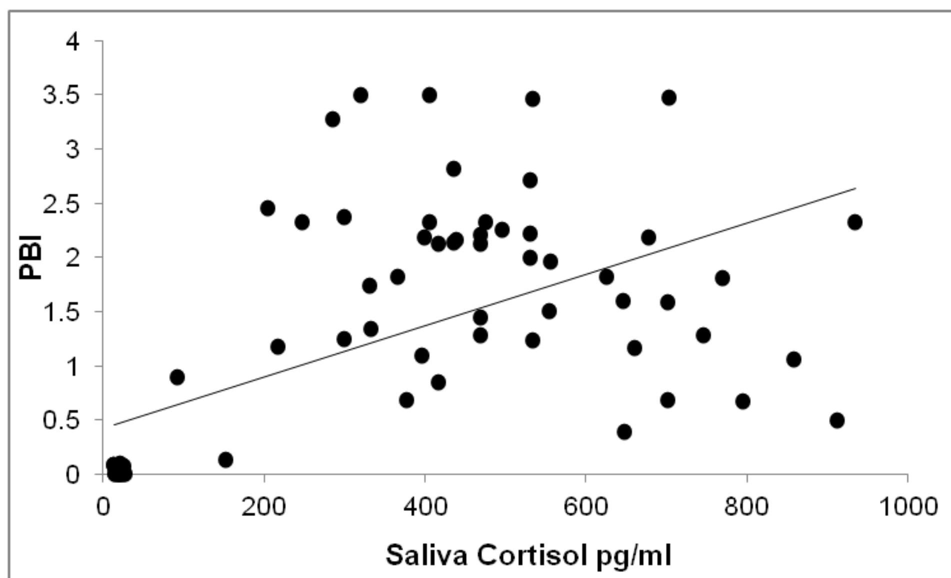
Graph 20

Scatter plot of Saliva cortisol and GI



Graph 21

Scatter plot of Saliva cortisol and PBI



MASTER CHART

Periodontal Clinical Parameters in GROUP I

Sr No.	Probing pocket depth (PPD)	Clinical attachment level (CAL)	Plaque Index (PI)	Gingival Index (GI)	Papillary Bleeding Index (PBI)
1	1.12	0	0.78	0.33	0.06
2	1.13	0	0.82	0.33	0.06
3	1.09	0	1	0.25	0.03
4	1.52	0	0.71	0.5	0.08
5	2.05	0	0.71	1	0
6	1.67	0	1	0.33	0.1
7	1.23	0	1	0.33	0.07
8	1.01	0	0.78	0.125	0.03
9	1.16	0	0.53	0.5	0.09
10	1.64	0	0.56	0.66	0.02
11	1.15	0	0.42	0.33	0
12	1.07	0	0.5	0.33	0.09
13	1.03	0	0.14	0.33	0
14	1.05	0	1	0.33	0.07
15	1.62	0	0.71	0.5	0.01
16	1.59	0	0.87	0	0.09
17	1.03	0	0.01	0	0
18	1.56	0	0.35	0.5	0
19	1.59	0	1	0.5	0.04
20	1.4	0	1	0.33	0.09
21	1.2	0	0.25	0.33	0
22	2	0	0.42	0.5	0
23	1.98	0	0.24	0.66	0
24	1.12	0	0.21	0.5	0
25	1.34	0	1	0.33	0

Periodontal Clinical Parameters in GROUP II

Sr. No	Probing pocket depth (PPD)	Clinical attachment level (CAL)	Plaque Index (PI)	Gingival Index (GI)	Papillary Bleeding Index (PBI)
1	7.23	8.07	2	1.33	1.18
2	9.81	10.18	2.25	2.5	0.9
3	7.03	8.08	2.78	1.83	0.14
4	8.59	9.62	2.6	1.33	0.39
5	9.38	10.58	2.6	1.33	1.09
6	5.79	7.61	2.71	1.54	2.32
7	9.61	9.88	2.32	1	0.85
8	9.54	9.97	2.76	1.5	0.69
9	6.82	6.87	2.6	2.67	0.5
10	8.48	8.51	2.44	2.83	1.96
11	6.37	7.26	2.66	2.33	1.59
12	7.85	8.27	1.64	2.16	1.82
13	8.34	8.48	2.81	3	3.48
14	7.24	8.16	2.1	1.83	1.81
15	9.48	10.2	2	1.16	1.06
16	7.89	8.49	2.89	1.33	1.28
17	6.49	7.34	2.17	1.54	1.17
18	7.48	8.69	2.77	1.33	0.67
19	9.49	10.34	3.2	1.33	1.18
20	7.38	8.57	2.5	1.5	1.74
21	9.65	10.59	2.76	1.5	0.69
22	6.34	8.97	2.32	2	2.25
23	9.38	10.85	3.28	1.83	2.13
24	6.37	7.89	3	0.46	2.18
25	8.79	9.85	2.85	1.5	1.6

Periodontal Clinical Parameters in GROUP III

Sr. No	Probing pocket depth (PPD)	Clinical attachment level (CAL)	Plaque Index (PI)	Gingival Index (GI)	Papillary Bleeding Index (PBI)
1	4.82	6.62	1.82	3.66	3.5
2	5.62	7.54	2.39	2.33	1.82
3	5.04	6.91	1.28	2.12	3.28
4	6.02	7.01	2.14	1.83	2.33
5	5.13	7.69	1.42	2	2.14
6	5.74	6.78	1.73	2.83	2.16
7	4.96	6.34	2.17	1.54	1.45
8	5.82	6.38	2.14	1.83	2.33
9	4.96	6.93	2.82	2.5	2.71
10	6.32	7.82	1.92	1.83	2.21
11	5.24	6.94	2.32	2	1.34
12	5.61	6.81	2.2	0.75	1.24
13	4.98	6.37	1.21	2.33	2.13
14	5.03	7.34	1.14	1.83	2.33
15	6.48	7.93	1.25	2.5	2.37
16	4.91	6.85	1.64	3	2.82
17	5.29	6.38	1.35	2.16	1.28
18	5.37	7.32	1.78	3	3.46
19	6.49	7.24	2.64	2.16	2
20	4.89	6.61	2	1.5	2.18
21	5.38	7.68	1.57	2	1.25
22	5.28	7.16	2.01	2.66	2.46
23	5.97	7.19	1.5	2.16	2.22
24	5.67	7.64	2.14	1.5	3.5
25	5.08	7.29	1.14	1.5	1.5

Cortisol and IL-1β concentration and SDS Score in GROUP I

Sr No.	Cortisol Concentration(pg/ml)		IL-1β Conc(pg/ml)		SDS Score
	Serum	Saliva	Serum	Saliva	
1	9.621	15.32	12.35	369.95	32
2	7.464	14.77	8.1	126.32	39
3	12.32	18.21	12.22	225.36	48
4	22.52	25.32	11.31	248.31	32
5	26.92	27.12	12.35	156.48	31
6	15.24	20.16	9.02	248.18	36
7	8.124	18.3	23.32	153.21	46
8	8.592	16.34	12.11	178.48	43
9	18.34	22.51	10.39	104.37	44
10	17.64	23.45	9.02	105.24	42
11	19.23	21.53	10.51	108.74	44
12	21.48	22.87	12.35	175.16	38
13	24.24	26.34	10.39	152.85	42
14	6.907	18.34	14.51	148.21	39
15	7.157	15.05	6.5	169.34	43
16	5.614	13.32	12.45	119.32	44
17	8.34	17.62	12.35	195.53	35
18	9.24	14.26	10.39	103.5	48
19	6.587	18.26	10.39	98.58	44
20	8.671	13.24	13.6	94.37	40
21	16.23	19.64	9.48	157.36	43
22	13.62	18.75	12.56	187.62	47
23	18.24	24.31	8.9	146.3	41
24	13.42	20.69	12.35	114.32	42
25	16.34	20.48	9.02	128.13	43

Cortisol and IL-1β concentration and SDS Score in GROUP II

Sr No.	Cortisol Concentration(µg/dl)		IL-1β Conc(pg/ml)		SDS Score
	Serum	Saliva	Serum	Saliva	
1	24.57	92.41	131.23	324.7	69
2	26.34	152.36	9.25	254.36	63
3	22.32	647.8	58.11	178.61	67
4	22.37	396	9.48	181.8	59
5	25.62	934.1	13.6	324.7	67
6	29.63	415.8	19.78	312.96	57
7	7.109	377.2	13.25	342.14	55
8	21.48	912.2	16.92	308.61	67
9	5.861	556.2	9.25	331.73	51
10	7.109	701.7	20.12	312.96	43
11	10.93	625.3	13.02	239.72	37
12	20.18	702.6	16.8	324.7	60
13	7.109	769.26	57.19	331.73	61
14	13.88	856.9	16.9	313.53	60
15	25.62	745.67	12.45	313.98	61
16	25.62	660.3	10.74	324.74	65
17	7.109	794.2	16.8	324.7	62
18	20.18	217.6	14.85	293.39	38
19	5.861	331.33	14.28	331.73	46
20	29.05	701.7	20.12	160	58
21	25.62	495.32	62.35	354.84	39
22	11.61	415.8	19.78	110.86	60
23	5.861	677.2	13.25	39.57	59
24	13.88	645.67	12.45	324.7	64
25	24.76	260.3	10.74	312.96	52

Cortisol and IL-1β concentration and SDS Score in GROUP III

Sr No.	Cortisol Concentration(µg/dl)		IL-1β Conc(pg/ml)		SDS Score
	Serum	Saliva	Serum	Saliva	
1	17.26	320.1	8.79	305.29	41
2	18.36	365.2	21.71	271.3	50
3	7.21	285.3	21.08	255.74	50
4	21.4	247.6	13.64	352.55	35
5	9.87	435.1	25.62	330.69	46
6	8.966	438.1	24.31	299.11	51
7	10.2	467.9	19.32	107.54	44
8	17.62	475.4	16.34	325.54	49
9	8.36	530.7	33.28	271.3	53
10	12.84	467.9	19.78	251	54
11	14.51	333.1	16.32	314.9	52
12	16.36	532.8	14.05	220.66	51
13	8.26	467.9	14.8	318.33	55
14	7.36	405.5	13.14	220.26	67
15	35.5	298.8	12.34	138.67	51
16	3.894	435.1	11.58	290.3	41
17	16.36	467.9	12.57	220.66	29
18	26.11	532.8	29.36	294.3	38
19	25.04	530.7	24.31	62.23	40
20	12.84	398.7	18.75	251	32
21	27.69	298.8	21.54	346.83	47
22	8.966	203.6	29.62	299.11	46
23	10.2	530.7	19.64	107.54	39
24	35.5	405.5	20.54	138.67	47
25	3.894	553.8	22.38	290.3	26

Evaluation of Stress, Serum and Salivary Cortisol and Interleukin-1β Levels in Smokers and Non-smokers with Chronic Periodontitis

CASE HISTORY PROFORMA

Name - **OPD No. -**

Age/ Sex - **Date -**

Address - **Phone No -**

Occupation -

Education - Literate / Illiterate

Chief Complaint:

Past Dental History:

Past Medical History:

Family History:

Oral Hygiene Habit:

History of Cigarette Smoking : Yes / No

No. of Cigarettes/ Bidis Smoked Per Day:

Duration of Smoking :

Teeth Present:

Gingival Index (GI)- Loe And Sillness(1963) :

16		

12		

24		

44		

32		

36		

Total Score:

Plaque Index (PI) : (Sillness And Loe 1964)

17	**16**	**15**	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**

47	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**

Score: $\frac{\text{Total scores of all teeth}}{\text{Total number of teeth examined}}$

Probing Pocket Depth (mm):

17	16	15	14	13	12	11	21	22	23	24	25	26	27
47	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**

Clinical Attachment Levels (mm):

17	16	15	14	13	12	11	21	22	23	24	25	26	27
47	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**

Papillary Bleeding Index (PBI) (Muhlemann H.R. 1977)

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38

Total scores of all teeth =

Total number of teeth examined

Zung's Self –Rating Depression Scale Score:

Interpretation :

Biochemical Analysis :

1. **Serum Cortisol Levels:**
2. **Salivary Cortisol Levels :**
3. **Serum Interleukin- 1β Levels:**
4. **Salivary Interleukin- 1β Levels:**

(Confidential)

INFORMED CONSENT FORM

"Evaluation of Stress, Serum and Salivary Cortisol and Interleukin-1β Levels in Smokers and Non-smokers with Chronic Periodontitis"

Mr./Master/Mrs./Miss.____
Resident of: ____
____aged _____ years,
excercising my free will/choice, without any pressure/lure of incentive in any form, hereby give my consent for the project to be conducted by **Dr. Pranjali Bawankar**

I acknowledge the receipt of "patient's information sheet", and also the doctor has informed me about this research project suitably and sufficiently to my satisfaction. I agree to let my X-rays, photographs, blood investigations, other investigations to be taken as required. I agree to take part in this project and will not mix any other projects during the period of this trial. I shall report to the dental hospital or other place where called on given appointment dates and time. I shall inform the doctor on any adverse effects or unusual symptoms noticed by me. I shall co-operate with the doctors and paramedical staff, in all respects. I permit to publishing the results of my participation in this study. I shall not be given any reimbursement or compensation. I have been informed of my right to opt out of this research project at any time without giving any reason for doing so. I hereby record my consent for participation in the said trial.

1	_______________	____________________	__________	_________
	Patient's name	Signature/Thumb print	Date	Date
2	_______________	____________________	__________	_________
	Witness's name	Signature/Thumb print	Date	Date
3	_______________	____________________	__________	_________
	Investigator's name	Signature/Thumb print	Date	Date