EVALUATION OF STRESS, SERUM AND SALIVARY GHRELIN AND CORTISOL LEVELS IN SMOKERS AND NON-SMOKERS WITH STAGE III PERIODONTITIS

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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 andsecreted
41	PMN	Polymorphonuclear neutrophils

INTRODUCTION

Chronic periodontitis is a bacterial induced inflammatory disease that tramples the intricate provocative rapport of the host immunoinflammatory response on inclusion of plaque biofilm, which can lead to subsequent amendments in periodontal tissue homeostasis resulting in degradation of connective tissue and alveolar bone supporting the tooth armature.[1,2] Several modifiable risk factors, such as smoking, cause a spike in systemic inflammatory markers that can further alter gene regulation through a variety of biologic mechanisms.[3] Mounting evidence points to the ability of psychological stress to dysregulate the inflammatory response can trigger the development as well as headway of the disease.[4] The importance of this relationship seems to be most apparent when the adaptive capacities is overwhelmed by environmental demands and events in times of psychological stress, simply referred to as "stress".[5] Stress is well-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 which has been known for more than 40 years to be an important predisposing factor in the development of necrotising periodontal diseases.[6,7,8] This psychosocial stress perhaps interacts with lifestyle factors, such as smoking, in the initiation of periodontal disease.[9]

Impact of Stress on Stage III Periodontitis

The bidirectional association has been observed between stress and inflammation. These processes incorporate gene, neural, endocrine and immune interactions. Animal and human study observations have shown that stress influences the immune system in many ways. Stress raises neuroendocrine hormones such as glucocorticoids and catecholamines. By activating these hormones, stress has detrimental effects on the immune system, including the reduction of lymphocyte populations, lymphocyte proliferation, natural killer cell activity, increased antibody activity and the reactivation of latent viral infections. The limbic-hypothalamic-pituitary-adrenal axis and the sympathetic nervous system are the main neural pathways triggered by physical (i.e. viruses or toxins) and psychological (i.e. major life events, violence or work related factors) stressors.[10,11]

Chronic stress triggers chronic inflammation by activating macrophages, dendritic cells, microglia, adipocytes and endothelial cells that secrete cytokines. Other effects include altered cell signaling, changes in natural killer cell cytotoxicity and changes in T-helper 1/T- helper 2 balance, all of which could lead to the potential for poor immune response to microorganisms and vaccines, susceptibility to infections, reactivation of latent viruses and deficits in wound healing. In the event of stress, the sympathoadrenal system and the neural pathways activated by physical limbic -

hypothalamic-pituitary-adrenal axis may be triggered, all of which may be involved in the development of chronically inflammatory state.[12]

Coelho JMF et al (2020) estimated a positive association between stress and periodontitis suggesting the significance of multidisciplinary treatment in the management of an individual's oral and general health condition.[13]

Chronic stress and depression have been speculated to reduce immune response, resulting in more pathogenic infection and concomitant periodontal tissue destruction. Evidence also suggests 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.[14,15] Although stress can negatively influence many behaviours including oral hygiene, stress plays a key role in the pathophysiology of periodontitis.[16]

Stress, Cortisol and Stage III Periodontitis

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

Cortisol functions as an anti-inflammatory and immunosuppressive hormone by inhibiting the development of T lymphocytes and suppressing the activity of natural killer of cells (NK) or macrophages.[17] Besides these effects, the blood glucose levels are elevated and affects the fat metabolism.[18] It has recently been proposed that salivary-free cortisol provides benefits over serum cortisol. Cortisol is 90%-95%

protein linked, approximately 60% transcortin and 30% albumin in serum.[19] Transcortin binds cortisol with high affinity but low capacity, whereas albumin has a low affinity but an almost infinite capacity for cortisol. Cortisol appears primarily in free form in saliva. It has a concentration of approximately two thirds of unbound cortisol in serum and is well associated with this serum fraction. Approximately 15% of salivary cortisol is bounded to transcortin.[20]

Botelho J. et al (2018)[21] in their systematic review meta-analysis clarified that patients with aggressive periodontitis have higher salivary cortisol levels than patients with chronic periodontitis or healthy controls. Such a variation in salivary cortisol response may have a detrimental effect on the periodontium, leading to the worsening of the burden of aggressive periodontitis.

Cortisol has circadian variations. **Akerstedt T.** and **Levi L. (1978)**[22] recorded that plasma cortisol levels were highest early in the morning and decreased to their lowest points in the evening, often with a minor secondary peak after mid-day meal. There is escalating evidence that the cortisol changes in the first hour after waking is a separate psychobiological process regulated by different mechanisms than cortisol levels over remainder of the day.

Stress, Smoking and Stage III Periodontitis

Cigarette smoking is one of the most impactful environmental risk factors for periodontitis, since it is linked not only to the risk but also to the prognosis. Smoking is the second most modifiable risk factor for periodontal disease after microbial dental plaque.[23] Smokers are more likely to have higher prevalence of probable periodontal pathogens, as well as substantially greater clinical attachment loss and bone loss.[24]

Many facets of acquired as well as innate immunity are affected by smoking. Smoking alters vascular function, neutrophil/monocyte activities, adhesion molecule expression, and antibody development, according to studies on the process by which smoking modifies the host response and ultimately contributes to the progression of periodontal tissue destruction, suggesting that phagocytes may be the foremost cells by which the effect of smoking is mediated.[25]

Previous experimental studies have shown that cigarette craving is increased after acute stress exposure in smokers.[26,27] On the other hand, prolonged exposure to nicotine is thought to maximize the subjective stress levels and intensify a depressed mood by causing changes in neurotransmitter systems and neural pathways implicated in mood regulation.[28] In response to chronic and recurring stressors, bidirectional contact between the HPA axis and immune system is important. Cortisol, an HPA axis effector, peaks in response to nicotine administration and sinks in response to acute tobacco cessation.[29] Salivary cortisol levels are higher in depressed people than in non-depressed people when they are under chronic stress.[30] Proinflammatory cytokines, such as interleukin (IL)-1, interleukin (IL)-6, and tumor necrosis factor (TNF) are thought to activate the HPA axis throughout early inflammation.[31]

The hypothalamic -pituitary – adrenal axis (HPA axis) is the body's "stress regulating system," monitoring the cortisol and other stress-related hormones. The HPA axis is activated as a part of the usual physiological rejoinder to inflammatory, physical, and emotional loads and is intended to shield the host as well as upholding the homeostasis in a challenging environment. In response to stress, the hypothalamic paraventricular nucleus releases corticotropin-releasing hormone, which acts on the

pituitary gland. As a result, pituitary gland secretes adrenocorticotropic hormone, which induces the adrenal cortex to release cortisol into the bloodstream. The supra-chiasmatic nucleus or hypothalamus, the body clock that rhythmically stimulates the HPA axis and thus regulates cortisol release and keeps a certain amount of cortisol in the blood throughout normal circumstances.[32]

Kolte et al, 2016 [33] inspected the impact of smoking in consortium of obesity and periodontal disease and explored a relationship between psychological stress, obesity and periodontitis in smokers and non-smokers and reported a positive and a stout correlation between anxiety, obesity and periodontal disease in smokers and non-smokers. Later **Bawankar P. et al, 2018** [34] in a cross -sectional study revealed that smokers with CP had higher stress and cortisol levels than non-smokers, indicating a favourable interaction between stress and CP with smokers.

Ghrelin and Stage III Periodontitis

Ghrelin is a 28-amino acid polypeptide hunger hormone is secreted predominantly by the oxyntic cells of the stomach wall. It has been identified as the natural ligand for the growth hormone secretagogue receptor, an orphan G-protein-coupled receptor (GHS-R).[35] Apart from systemic secretion, ghrelin is found to be produced and released by cells of the parotid and submandibular salivary glands as well as taste bud cells.[36,37] However salivary secretion indicates a direct modulatory role of ghrelin on taste bud signaling and function, which could lead to improved food taste and appetite for food ingestion.[38]

Des-acylated and acylated are the novel two different forms of ghrelin which are seen to be in tissue and blood. Both variants of ghrelin act as a regulatory factor in growth

hormone secretion, food intake as well as in energy metabolism.[39] Ghrelin must be acylated in order to bind with the growth hormone secretagogue receptor (GHS-R1a) and also to elicit the growth hormone expression as recent research studies suggested that ghrelin's anti-inflammatory activity is strongly associated to its degree of acylation.[35,40,41]

The influence of ghrelin on endocrine function was the primary goal of major research studies performed by **Fukushima N. et al (2005)[42]**, **Kim SW. et al (2005)[43]** and **Maccarinelli G. et al (2005).[44]** These recent verdicts also suggest the evidence of ghrelin having several modulatory effects on immune system as well as bone metabolism. Ghrelin downregulates the lipopolysaccharide (LPS) induced proinflammatory cytokine production including interleukin 1-β (IL1-β) and tumor necrosis factor (TNF-α) and exhibits strong anti-inflammatory activity. In addition to anti-inflammatory activity, ghrelin also stimulates the differentiation and proliferation of osteoblastic cells and enhances bone formation.[45] **Deng et al**.[46] stated that ghrelin significantly upsurges the expression of alkaline phosphatase (ALP), osteocalcin (OSC), and collagen type-1 (all markers of osteoblast differentiation). Furthermore, ghrelin levels were also found to be positively correlated with osteoprotegrin (OPG) levels and negatively correlated with soluble receptor activator nuclear factor kappa-B (sRANKL) levels.[47]

Periodontitis is a chronic inflammatory disease characterized by the loss of connective tissue attachment and bone around the teeth as a result of bacterial accumulation. Although microorganisms are the predominant etiologic agents, chemical mediators of inflammation are primarily responsible for periodontal tissue destruction. Studies performed by **Gorska R. et al.[48] (2003)** and **Pradeep AR. et al.[49] (2009)** have

documented that periodontally diseased patients habitually exhibits significant enhanced levels of proinflammatory cytokines such as IL-1 β, IL-6 and TNF- α in serum levels and these elevated levels of proinflammatory cytokines contribute to alveolar bone loss by inducing RANKL expression while simultaneously suppressing the OPG expression.[50]

Yilmaz G. et al. (2014)[51] scrutinized a link between ghrelin, periodontal parameters, serum cytokines, and bone turnover markers in patients with chronic periodontitis and explored that both the total and acylated ghrelin levels seen to be predominantly elevated in chronic periodontitis group. Similar results were also observed by **Jentsch H. et al. (2017)[52]** where they assessed the acylated and total ghrelin levels in saliva, gingival crevicular fluid as well as serum of periodontally healthy and diseased subjects with respect to different body mass categories. The results revealed the elevated levels of total ghrelin were seen to be in the CP<25 (normal weight) group and for serum and salivary acylated levels, insignificant differences were found in between the groups. **Kaabi et al. (2014)[53]** stated that, in smokers, the serum and salivary ghrelin levels tend to be substantially lower after smoking than before.

As finite sparse studies have been dealing with the role of ghrelin in the pathogenesis of stage III periodontitis, the collective impact of stress, ghrelin and smokers has yet to be determined. Thus, the cumulative impact of these influences is thought to be accountable for advanced periodontal tissue degradation. In the context of above, the current research aims to evaluate the stress, serum and salivary ghrelin and cortisol levels in smokers and non-smokers with stage III periodontitis.

AIMS AND OBJECTIVES

The present study aimed to evaluate the association of stress, serum and salivary cortisol and ghrelin in smokers and non-smokers with stage III periodontitis.

Objectives

1. To evaluate and compare the stress levels in smokers and non-smokers with stage III periodontitis.
2. To evaluate and correlate the serum and salivary cortisol levels in smokers and non-smokers with stage III periodontitis.
3. To evaluate and correlate the serum and salivary ghrelin levels in smokers and non-smokers with stage III periodontitis.
4. To correlate the stress with cortisol and ghrelin levels in smokers and non-smokers with stage III periodontitis.

REVIEW OF 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 Stage III Periodontitis

B. Review of studies on association of Ghrelin and Stage III Periodontitis

C. Review of studies on association of smoking, and Stage III Periodontitis

A. REVIEW OF STUDIES ON ASSOCIATION OF STRESS, CORTISOL LEVELS AND STAGE III PERIODONTITIS

Moss ME et al. (1996)[54] investigated 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. Serum antibody was examined

for three periodontal pathogens (Bacteroides forsythus [IgG Bf], Porphyromonas gingivalis [IgG Pg], Actinobacillus actinomycetemcomitans [IgG Aa] and the interaction between antibody levels and the depression score derived from the Brief Symptom Inventory. Both IgG Pg and IgG Aa were found to be strongly linked to case status. IgG Bf was associated with periodontal disease but only among individuals who had higher depression scores. The smoking status has been linked to the case status. These findings were assessed prospectively by examining factors associated with more extensive disease among 71 cases after 1 year of follow-up. Basic smoking status and IgG Bf among individuals with high baseline depression were found to be associated with more extensive illness. This exploratory analysis was used to identify specific lines of investigation concerning psychosocial measures as important environmental factors for adult periodontitis.

Genco RJ et al. (1999)[55] in a cross-section analysis of 1,426 participants evaluated the relationship between stress, anxiety and dealing with periodontal disease. Participants were asked to complete a set of 5 psychosocial questionnaires that measured psychosocial characteristics and attitudes, including discrete life events and their impact; chronic stress or everyday stress; distress; dealing with styles and strategies; the challenges and uprisings. Clinical assessment of supragingival plaque, gingival bleeding, subgingival calculus, probing depth, clinical attachment level (CAL) ad radiographic alveolar crestal height (ACH) was performed and 8 possible bacterial pathogens from subgingival flora was calculated. Logistic regression analysis showed that, of all the regular strains investigated, only financial strain was substantially correlated with increased bone attachment and alveolar bone loss (odds ratio, OR = 1.70, 95 per cent CI = 1.09 to 2.65 and OR = 1.68, 95 per cent CI = 1.20

to 2.37, respectively) after age, gender and cigarette smoking changes. When coping habits were assessed, it was found that those with higher financial strains who were high emotion-focused copers (a form of ineffective coping) were at higher risk of experiencing more extreme attachment loss (OR = 2.24, 95% CI = 1.15 to 4.38) and a 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 findings were observed between low problem-focused copers for CAL (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 acceptable or good coping) had no more periodontal disease than those with low levels of financial strain, indicating that the impact of stress on periodontal disease may be moderated by sufficient coping behaviours. They concluded that there were psychosocial interventions of stress associated with financial strain and distress manifest as depression, as 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)[56] conducted a study to investigate the effect of anxiety assessed by a single question on gingival inflammation and periodontal disease in smokers and non-smokers. They included 144 subjects in a age range of 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 diagnostic examination, the participants answered an uncomplicated query about anxiety in daily life as well as smoking habits. The findings of the study suggested

that self-reported anxiety was associated with an adverse impact on the gingiva and seemed to be associated with anxiety.

Hilgert et al. (2006)[57] conducted a cross-sectional study in which they examined the extent and severity of chronic periodontitis and its correlation with cortisol levels and an inventory of strss symptoms in a population aged 50 years or older. 235 individuals were asked to respond to the Lipp Stress Symptoms Inventory for adults, saliva samples were obtained for cortisol analysis, and periodontitis was assessed. Based on logistic regression, cortisol levels were positively correlated with the following outcomes: means of clinical attachment (CAL) > = 4mm [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 >= 4mm [OR = 10.7, 95% CI (1.9, 54.1)]. The results suggested that the cortisol levels were positively associated with the extent and severity of periodontitis.

Rosania et al. (2009)[58] performed a cross-sectional pilot study to investigate association between psychologic variables, periodontal disease markers, psychoneuro-immunological measures, and behaviour. The present study included 45 periodontal patients who were asked to complete composite health, chronic stress, depression, demographic questions and radioimmunoassay was used to measure salivary cortisol. Stress, depression and salivary cortisol scores were found to be significantly associated with periodontitis severity as well as number of missing teeth, family history and brushing frequency. In addition, patients who reported lack of oral hygiene during traumatic or depressed periods had the highest clinical loss of attachment and the highest number of missing teeth. Subsequently they concluded that

stress and depression could be associated with periodontal destruction through behavioural and physiological mechanisms.

Ansai T et al. (2009)[59] investigated association between salivary cortisol levels and dehydroepiandrosterone (DHEA) and periodontitis in Japanese elder smokers and non-smokers comprised of total 171 participants. A significant association was seen between the salivary steroid hormone cortisol and DHEA and the periodontitis severity in community-dwelling elderly subjects who had never smoked. These findings suggestive of stress-related hormones are useful risk factors for periodontitis, as they display moderate levels of sensitivity and specificity for periodontitis. Thus they concluded that the measurement of hormone levels may be a beneficial tool for periodontitis, albeit limited to non-smokers.

Goyal S et al. (2011)[60] studied the influence of psychosocial stress affecting periodontium with the use of questionnaire and serum cortisol data. 47 subjects divided into chronic periodontitis and stressed groups. Stress levels were assessed using a standard questionnaire method (social readjustment rating scale). Plaque index (PI), gingival index (GI), periodontal disease index (PDI) and serum cortisol levels were also measured. A strong relationship between cortisol and PDI; cortisol and PI as well as between stress, cortisol, PI, GI and PDI, was found by Spearman's rank correlation coefficient and unpaired t test. A statistically significant link was found between cortisol and smoking.

Rai B et al. (2011)[61] explored the association between periodontal disease, psychologic and salivary stress factors, psychoneuro-immunological variables and health behaviours. Stress scores and salivary stress markers were analysed for

chromogranin A, cortisol, alpha-amylase and beta-endorphin, which were found to be significantly correlated with the clinical parameters of periodontal disease in 100 adult periodontitis subjects. Salivary cortisol and beta- endorphin significantly associated with dental loss as well periodontal clinical parameters after adjustment for stress variables. In addition, the greatest loss of teeth was seen in those patients who had neglected their oral hygiene maintenance. This study revealed that stress may be associated with periodontal disease through physiological and behavioural mechanisms.

Refulio Z et al. (2013)[62] conducted a cross-sectional study and assessed the association of stress, salivary cortisol and chronic periodontitis among 70 systemically healthy non-smoking 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. Stress and anxiety levels were assessed by Zung's self-rating depression and anxiety scale based on stress, depression and anxiety levels. Clinical measurements of pocket depth, clinical attachment, bleeding on probing and tooth mobility were used to quantify the severity of the disease. Salivary cortisol levels (SCL) were measured using a highly sensitive electrochemiluminescence immunoassay. Both patients with CP and one periodontally stable patient were diagnosed with depression. Patients with severe CP had statistically significantly higher SCL levels than those diagnosed with mild CP. In comparison, participants with extreme CP showed the same outcome as those with moderate 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 high salivary cortisol levels and depression may show an increased risk for CP.

Nayak SU et al. (2013)[63] examined gingival crevicular fluid (GCF) and salivary cortisol levels in 45 anxious and non-anxious patients with chronic periodontitis. Patients were further categorized into 3 groups- Group I- 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 determine the anxiety levels of all study participants. Clinical measurements such as plaque index (PI), gingival index (GI), probing pocket depth (PPD) and clinical attachment level (CAL) have been registered. GCF and non-stimulated whole saliva samples were gathered and cortisol levels were determined using ELISA. In Group 3, PI, GI, PPD and CAL seen to be elevated. A strong association was observed between salivary and GCF cortisol and CAL in Group 3. This study demonstrated a possible correlation between chronic periodontitis and anxiety and thus GCF and salivary cortisol can be used as potential marker for periodontitis.

Wong et al. (2014)[64] 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)[65] 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)[66] estimated and compared serum and cortisol levels in periodontally ill and healthy individuals. Total 45 patients were divided into three groups: 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, and cortisol levels were assessed using the cortisol

immunoassay kit. In Group I, cortisol levels were higher when compared to other two groups. When comparing the mean cortisol levels between the groups, the values were statistically significant between Group I and III while Group I showed a significant negative correlation between cortisol levels and GI.

Shende AS et al. (2016)[67] 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.

Teja V. et al. (2018)[68] conducted a clinico-biochemical study to explore the association of stress, salivary cortisol and chronic periodontitis. This cross-sectional study encompasses total 92 patients which were further sorted based on periodontal condition (number of teeth present), plaque index (PI), bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment levels (CAL) and stressed levels and divided patients into following groups: Group 1 (Healthy controls), Group 2 (patients with periodontitis and no stress), Group 3 (patients without periodontitis and with stress) and Group 4 (patients with periodontitis and stress). Stress was assessed by DASS-21, a scale comprised of 21 items used to evaluate depression, anxiety as

well as stress. Saliva samples were collected and cortisol levels were estimated by ELISA. Results revealed that group 4 showed high mean salivary cortisol levels as compared to other groups. Group I had a significant negative correlation of cortisol to BOP, stress to PI, and stress to cortisol level, whereas there is a positive correlation of salivary cortisol levels to PD in group 4 which was statistically insignificant. A significant difference for salivary cortisol levels seen to be among different groups. This study interpreted that salivary cortisol levels were associated with both chronic periodontitis as well as psychological stress, thus increases in inflammation and stress levels further boosts the salivary cortisol levels.

B. REVIEW OF STUDIES ON ASSOCIATION OF SMOKING AND STAGE III PERIODONTITIS

Haffajee AD and Socransky SS (2001)[69] 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)[70] 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)[71] 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)[72] 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 non-smokers. 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)[73] 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 litre): high, 13.49; medium, 9.58; low, 8.49.

Gautam DK et al. (2011)[74] 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)[24] 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.

Kolte et al. (2012)[33] investigated and compared the consequence of smoking on the periodontal status ad salivary composition of whole saliva in patients with clinically healthy periodontium and chronic periodontitis. The study comprised of total 400 subjects equally divided into four groups as follows: Group I – non-smokers with

clinically healthy periodontium; Group II – smokers with clinically healthy periodontium; Group III – non-smokers with chronic periodontitis and Group IV- smokers with chronic periodontitis. Clinical measurements as well as non-stimulated whole saliva samples were obtained. Total protein, calcium, magnesium and phosphorous amount in whole saliva were biochemically tested and revealed that in smokers with periodontitis, biochemical examination of whole saliva, total protein, calcium, magnesium and phosphorous was reduced to 0.430 ± 0.50 gm/dl, 3.47 ± 1.49 mg/dl**,** 0.80±3.87 mEq/L Neo and 18.45 ± 8.77 mg% from 1.70 ± 2.09 gm/dL, 13.89 ± 10.34 mg/dL, 1.26±0.90 mEq/L Neo and 29.23 ± 16.02 mg%, respectively, in non-smokers with healthy periodontal subjects.

Koopman A. et al. (2015)[75] conducted a study and compared the effect of tobacco withdrawal on acetylated and total ghrelin plasma concentrations and enrolled fifty four normal-weighted smokers and 30 healthy non-smokers in a present study. Acetylated and total ghrelin levels were measured in blood plasma drawn two hours after a standardized meal and three hours after smokers smoked their last cigarette. Cotinine plasma concentration, the Fagerstrom Test for Nicotine Dependency (FTND) and the numeral amount of cigarettes smoked per day were recorded to govern the degree of tobacco addiction. The investigators revealed that smokers had slightly higher acetylated ghrelin plasma concentrations than total ghrelin in non-smokers.

Suzuki et al. (2016)[27] 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.

Bawankar P. et al. (2018)[34] conducted a cross-sectional study to perceive the association of serum and salivary cortisol and Interleukin -1β levels in chronic periodontitis with and without smokers as well as its probable role in the pathogenesis of chronic periodontitis (CP). Overall, 75 patients were enrolled in the study which further divided into 3 groups as follows: Group 1: healthy controls (30), Group 2: smokers with CP (30) and Group III: non-smokers with CP (30). The clinical parameters assessed were plaque index (PI), gingival index (GI), probing pocket depth (PPD), clinical attachment levels (CAL) and papillary bleeding index (PBI). The stress and anxiety levels of the patients were judged using the Zung's self-rating depression scale questionnaire incorporated 20 item questionnaires including 10 negatively and 10 positively worded questions. Cigarette consumption was resoluted by verbal questioning and categorized as smokers and non-smokers. Serum and salivary levels of cortisol and IL-1β were analysed using commercially available ELISA kit (enzyme linked immunosorbent assay). Smokers with CP spectacled higher values of PPD, CAL, PI while lower PBI and GI scores were noticed as compared to non-smokers with CP. The salivary cortisol and IL-1β were quite elevated as

compared to serum values in Group 2 than Group 3. The Group 2 patients revealed higher depression scores as compared to Group 3 patients. The depression scores were positively linked with salivary cortisol in Group 2 patients. An elevated stressed as well as cortisol levels were seen to be in CP with smokers as compared to non-smokers thus revealed a positive relationship between stress and smokers with CP.

C. REVIEW OF STUDIES ON ASSOCIATION OF GHRELIN AND STAGE III PERIODONTITIS

Yilmaz G. et al (2014)[51] has investigated a study to evaluate the plasma ghrelin levels in chronic periodontitis subjects and also to inspect a relationship existence between ghrelin, periodontal parameters, serum cytokines and bone turnover markers. Thirty-five chronic periodontitis (CP) and periodontally healthy individuals © were incorporated in this study. Periodontal parameters plaque index (PI), gingival index (GI), percentage of bleeding probing (BOP%), probing depth (PD), clinical attachment levels (CAL), of each tooth were verified. Blood samples were gained to govern the levels of total and acylated ghrelin, interleukin 1 beta (IL-1β), tumor necrosis factor-alpha (TNF- α), the soluble receptor activator nuclear factor kappa B ligand (sRANKL), alkaline phosphatase (ALP) and osteocalcin (OSC). Both total and acylated ghrelin levels were found to be elevated in the CP population, and when comparing gender differences, major differences were only seen in males, while acylated ghrelin levels appeared to decrease in females presenting with CP symptoms. These findings suggest that gender has an effect on ghrelin and its isotypes, and that they have different activity patterns during inflammation.

Groschl M. et al. (2005)[77] explored whether ghrelin is present in human saliva, is developed by salivary glands, and whether it has any possible roles in the oral cavity by gazing its effect on oral keratinocyte proliferation. PCR was used to assess the expression of ghrelin and unique receptor mRNA. Immunoblotting and size-exclusion quick protein liquid chromatography (FPLC) with subsequent RIA were used to identify proteins. Salivary total and bioactive ghrelin were measured using specific RIA's. Immunohistochemistry was used to glance at the distribution of ghrelin in salivary gland cryosections, In primary oral keratinocytes, the effect of ghrelin on the inclusion of 5-bromo-2-deoxyuridine as a measure of cell proliferation was investigated. The existence of ghrelin in saliva and glandular tissues was verified by Western blot analysis. The receptor was found to be functional and was expressed by the glands as well as oral keratinocytes. When total ghrelin concentrations in saliva and serum were compared for healthy people (BMI 18-27 kg/m2), saliva had substantially lower concentrations ($P<0.001$). When oral keratinocytes were incubated with ghrelin, cell proliferations wsa significantly increased ($P<0.001$). Co-incubation with NOX-B11 (50nmol/L), a novel specific inhibitor of acylated ghrelin, completely blocked this effect

Jentsch H. et al (2017)[52] measured acylated and total ghrelin, chemerin, and IL-1 in saliva, gingival crevicular fluid (GCF), and serum of periodontally stable and diseased subjects in relation to various body mass categories. The two major groups (patients with chronic periodontitis and periodontally healthy/ gingivitis subjects) were divided into overweight/obese subjects (BMI ≥25) and normal weight subjects (BMI < 25). The levels of acylated and total ghrelin, chemerin and interleukin-1 (IL-1) in saliva, gingival crevicular fluid, and serum were measured, as well as the levels of subgingival bacteria were analyzed. Reduced ghrelin and

higher chemerin levels in the gingival crevicular fluid can be correlated to periodontal disease and obesity. Unlike IL-1, however, chemerin and ghrelin levels in gingival crevicular fluid do not explicitly indicate periodontal destruction. Gingival inflammation, along with elevated T. denticola counts, have been linked to overweight/obesity in patients without periodontitis.

Kaabi YA. et. al. (2014)[53] examined the direct effect of acute cigarette smoking on the ghrelin hormone produced in saliva to create an additional correlation between cigarette smoking and altered food taste and loss of appetite. Before and after one cigarette, blood and saliva samples were obtained from 30 healthy nonsmoker male subjects. ELISA immunoassay was used to test total ghrelin in serum and saliva. The data revealed a statistically significant decrease in salivary ghrelin after smoking ($P<0.001$). Total ghrelin levels in serum were not affected by smoking ($P=0.1362$). Furthermore, a positive association was found between serum and salivary ghrelin before smoking ($r=0.4143$) and ($P=0.0158$), but this correlation was lost after smoking ($r=0.4143$ and $P = 0.0158$), but this correlation was lost after smoking ($r= 0.1147$ and $P= 0.5461$). Acute cigarette smoking can reduce ghrelin levels in saliva, which may lead to smoker's dull food taste.

Nokhbehsaim M. et al. (2017)[78] explored the expression of the functional ghrelin receptor (GHS-R1a) in periodontal cells and tissues in vitro and in vivo under microbial conditions. Real time PCR, immunocytochemistry and immunofluorescence were used to examine GHS-R1a expression in human periodontal cells challenged with the periodontopathogen Fusobacterium nucleatum, gingival biopsies from periodontally stable and diseased individuals,

and in rats with and without ligature induced periodontitis. In periodontal cells, F. nucleatum caused an initial upregulation and a further downregulation of GHS-R1a. When compared to healthy sites in rat experimental periodontitis, GHS-R1a expression at periodontitis sites was increased during the early stages of periodontitis but significantly decreased later. Periodontally diseased sites had substantially lower GHS-R1a expression than stable sites in human gingival biopsies. Periodontal bacteria influence the expression of the functional ghrelin receptor in periodontal cells and tissues. The anti-inflammatory effects of ghrelin could be reduced in chronic periodontal infections due to the downregulation of the function ghrelin receptor caused by long term exposure to periodontal bacteria, which may contribute to an enhanced periodontal infection.

Ueberberg B. et al. (2009)[79] examined the RNA and protein expression of ghrelin and its receptor GHS-R1a in different human tissues. TaqMan Real-Time RT-PCR wea performed on samples (BD Biosciences) in a single stage. Specific primary antibodies against ghrelin and its receptor were analyzed by using immunohistochemistry on paraffin-embedded tissues. Ghrelin mRNA was found in all human tissues, with the highest concentrations in the liver, pituitary, and small intestine. Ghrelin peptide expression was found in reproductive and endocrine organs (ovary, anterior pituitary, adrenal gland) as well as gastrointestinal tract organs using

immunohistochemistry(stomach, pancreas). GHSR1a mRNA expression was found in 10 out of 24 human organs studied, with the pituitary, adrenal gland and spinal cord showing the highest levels. Immunohistochemistry revealed that the receptor peptide was expressed in the anterior pituitary, thyroid, pancreas and testis as well as parts of the CNS (cerebrum, cerebellum), single cells of bone marrow. The presence of ghrelin and its receptor in endocrine and reproductive organs may indicate new mechanism of regulation in endocrine or reproductive organs.

MATERIALS AND METHODS

The present study was undertaken to evaluate the Stress, Serum and Salivary Cortisol and Ghrelin levels in Stage III 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 90 patients with age range 30-65 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 of 1975 as revised in 2013. Prior to the initiation of the study an informed consent was obtained from those who agreed to participate voluntarily.

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 intraoral examination was conducted by a single examiner and charting of periodontal parameters was done which included Probing pocket depth (PPD), Clinical attachment level (CAL), Plaque index (PI) [Sillness and Loe 1964], Gingival Index (GI) [Loe and Silness, 1963], Papillary Bleeding Index (PBI) [Muhlemann H. R. 1977]. Diagnosis of Stage III periodontitis is based on 2017 World Workshop.[80]

The stress and anxiety levels of the patients were assessed using the Zung's self-rating depression scale questionnaire. Patients were further categorized into three groups. Each group comprised of 30 patients based on the smoking habit and presence of Stage III periodontitis. A total of 90 patients (48 males and 42 females) with the age range 30-65 yrs were grouped as follows:

Group I: 30 Healthy patients without any signs of periodontal disease.

Group II: 30 Non-Smokers with untreated Stage III Periodontitis.

Group III: 30 Smokers with untreated Stage III 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 Stage III periodontitis with interdental (CAL) ≥ 5mm and radiographically bone loss extending to middle third of root and beyond.

b) Patients with no history of smoking

Group III

a) Non-smokers with untreated Stage III periodontitis with interdental (CAL) ≥ 5mm and radiographically bone loss extending to middle third of root and beyond.

b) Patients with history of smoking ≤ 10 cigarettes per day.

Exclusion Criteria:

1. Patients with 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 chemotherapeutics 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 the collection of blood.

For examination of the patient:

1. Mouth mirror
2. Explorer
3. UNC-15 (Hu-Friedy) 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
4. Plain plastic vials
5. Torniquet
6. Sterile Eppendorf tubes (1.5ml)

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 gingiva 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 Stage III 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 stage III periodontitis if they exhibited clinical attachment level ≥ 3mm at multiple sites.

3. Plaque Index (PI): (Sillness and Loe, 1964)[81]

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 Sillness, 1963)[82]

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 gingiva
1	Mild inflammation, slight change in colour, 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

Papillary Bleeding Index (PBI) by Muhlemann[83]

The PBI was recorded with the blunt periodontal probe by carefully inserting into the gingival sulcus at the base of 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

Zung's Self Rating Depression Scale [84]

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 English and Marathi (local language) for better understanding of the questions by the participants. The Zung's 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, a 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 (Color Plate V)

Saliva samples were collected from all subjects between 9 am to 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 -20^0 C and salivary cortisol was assayed within the first month after collection.

Collection of blood samples

For the drawing of blood, the venepuncture 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 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 for 10 minutes. Using clean pipette the serum was aliquoted into labelled cryovials and immediately stored at – 20^0 C in deep freezer until the final assay.

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

1. Calibrated volumetric transfer pipettes with disposable tips capable of dispensing 0-5 µl, 50-200 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

- -80^{0} C deep freezer (REMI Equipments Pvt. Ltd.) **(Color Plate IV)**
- Lab centrifuge machine (R-8C, REMI Equipments Pvt. Ltd.) **(Color Plate VIII)**
- Vortex mixer (CM 101, REMI Equipments Pvt. Ltd.) **(Color Plate VIII)**
- ELISA microplate washer (LISA wash, REMI Equipments Pvt Ltd.) **(Color Plate VII)**
- ELISA microplate reader (LISA Microplate reader, REMI Equipment's Pvt. Ltd.) **(Color Plate VII)**

Samples were assayed for salivary and cortisol and ghrelin levels using commercially available **ELISA** (Enzyme linked immune-sorbent assay) **IMMUNOCONCEPT INDIA PVT. LTD (07AADCI8100K1ZN)**. **DIAMETRA** and **XEMA** Cortisol Enzyme Immunoassay kits for evaluation of salivary and serum cortisol levels and **SINCERE** Ghrelin ELISA for salivary and serum ghrelin levels. Samples were analysed according to the instruction manual at the Department of Biochemistry.

Supplied Components in Xema Serum Cortisol Enzyme Immunoassay Kit

Sr No.	Symbol	Description		Quantity	Colour Code
1	SORB MTP	Cortisol EIA strips, 8x12 wells	polystyrene microwells coated with murine monoclonal to cortisol	1	
2	CAL 1–6	Calibrator set, 0.8 ml each. Theset contains 6 calibrators: 0; 40; 80; 200; 600, 2000 nmol/l	human cortisol diluted in a preselected human serum preservative – 0,01 % Bronidox L, 0,01 %2-Methyl-4-isothiazolin-3-one-hydrochloride; also contains blue dye	6	blue (C1 - colourless)
3	CONTROL	Control serum(0.8 ml)	dilution of preselected human serum, with high content of cortisol with preservative - 0,01 % Bronidox L, 0,01 % 2-Methyl-4-isothiazolin-3-one-hydrochloride, colourless	1	colourless
4	CONJ HRP	Conjugate, 11 ml	aqueous solution of cortisol coupled with horseradish peroxidase diluted on phosphate buffered solution	1	red

			preservative - 0,01 % Bronidox L, 0,01 % 2-Methyl-4-isothiazolin-3-one-hydrochlorideand red dye		
5	SUBSTMB	Substrate solution, 11 ml	ready-to-use single-component tetramethylbenzidine (TMB) solution.	1	colourless
6	BUF WASH21X	Washing solution concentrate 21x,22 ml	aqueous solution of sodium chloride and detergent(Tween 20), contains proClin300 as a preservative	1	colourless
7	STOP	Stop solution	5,0 % vol/vol solution of sulphuric acid	1	colourless
8	N003	Plate sealing tape		2	N/A
9	K210I	Instruction Cortisol EIA		1	N/A
10	K210Q	QC data sheet Cortisol EIA		1	N/A

CORTISOL ASSAY PRINCIPLE (SERUM)

This test is based on competition enzyme immunoassay principle. Tested specimen is placed into the microwells coated by specific murine monoclonal to cortisol-antibodies simultaneously with conjugated cortisol-peroxidase. Cortisol from the specimen competes with the conjugated cortisol for coating antibodies. After washing procedure,

the remaining enzymatic activity bound to the microwell surface is detected and quantified by addition of chromogen-substrate mixture, stop solution and photometry at 450 nm. Optical density in the microwell is inversely related to the quantity of the measured analyte in the specimen.

Assay Procedure

Set the desired number of microstrips into the frame; allocate 14 wells for the calibrators cal 1–6 and control samples control and two wells for each unknown sample.
do not remove adhesive sealing tape from unused strips.

Pipet 25 µl of calibrators CAL 1–6, control samples CONTROL and unknown samples into the wells.

Dispense 100 µl of CONJ HRP into the wells. Cover the wells by plate adhesive tape (included into the kit).

Incubate 60 minutes at 37 °C .

Prepare washing solution by 21x dilution of washing solution concentrate (BUF WASH 21X) by distilled water. Wash the strips 5 times.

Dispense 100 µl of SUBS TMB into the wells

Incubate 10-20 minutes at +18...+25 °C

Dispense 100 µl of STOP into the wells.

Measure OD (optical density) at 450 nm.

Calculation of Results

The mean absorbance values (OD450) for each pair of calibrators and samples will be calculated calibration curve and plotted on graph paper: OD versus cortisol concentration. Manual or computerized data reduction is applicable on this stage. After cortisol (serum) assay was done, ELISA for cortisol (saliva) was performed.

Supplied Components in DIMETRA Saliva Cortisol Enzyme Immunoassay Kit

1. **Microtiter coated plate** (96 wells) – 1

Antibody anti Cortisol adsorbed on microplate

2. **Incubation Buffer** (1 vial, 30 mL)

Phosphate buffer 50 mM, pH 7.4, BSA 1 g/L

3. **Conjugate** (1 vial, 1 mL)

Cortisol conjugated to horseradish peroxidase (HRP)

5. **TMB Substrate** (1 vial, 15 mL)

H2O2-TMB 0.26 g/L

6. **Stop Solution** (1 vial, 15 mL)

Sulphuric acid 0.15 mol/L

7. **10X Conc. Wash Solution** (1 vial, 50 mL)

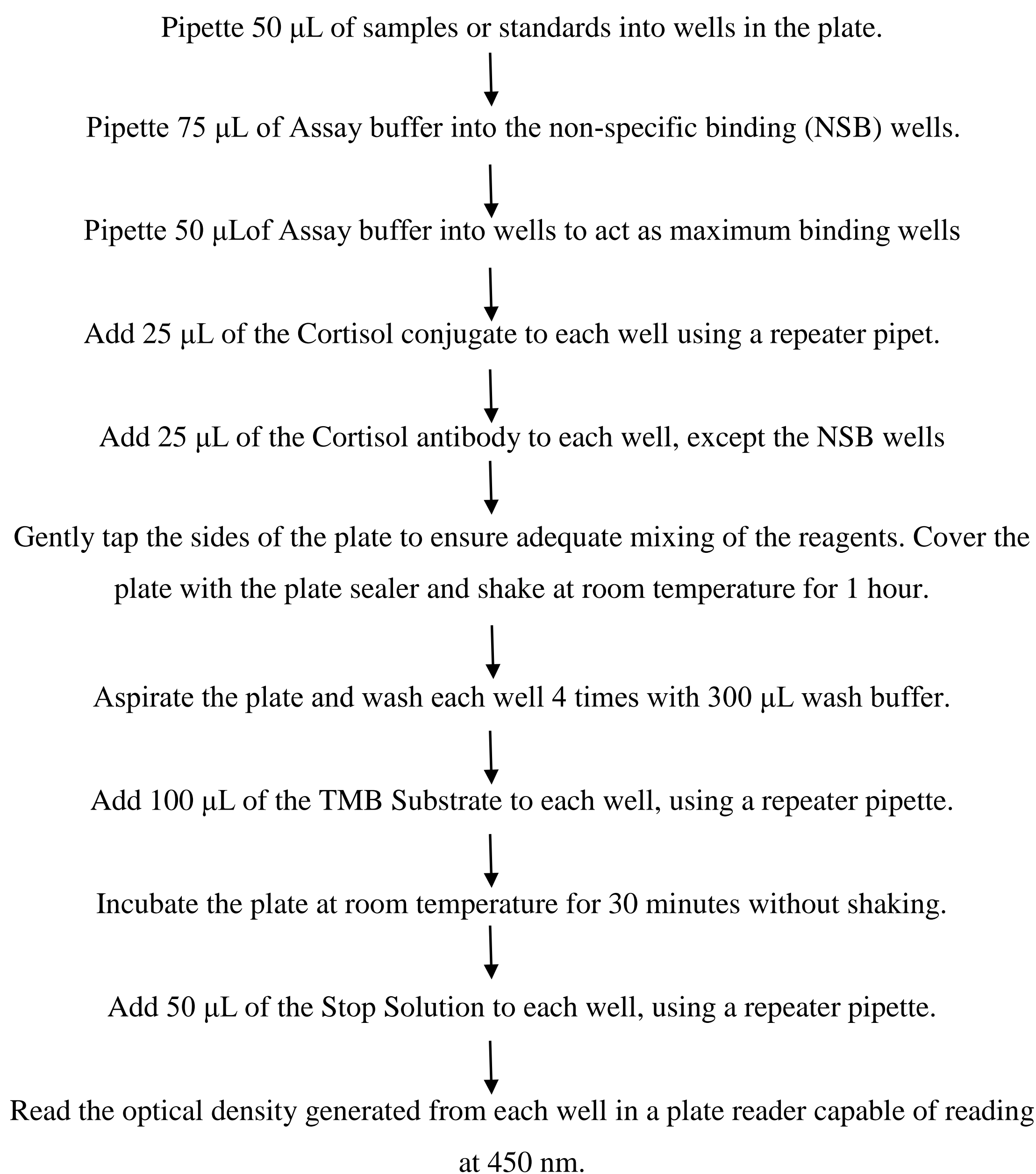
Phosphate buffer 0.2M, Proclin < 0,0015%

Assay Principle

The Cortisol (antigen) in the sample competes with the antigenic Cortisol conjugated with horseradish peroxidase (HRP) for binding to the limited number of antibodies anti Cortisol coated on the microplate (solid phase). After incubation, the bound/free separation is performed by a simple solid-phase washing. Then, the

enzyme HRP in the bound-fraction reacts with the Substrate (H2O2) and the TMB Substrate and develops a blue color that changes into yellow when the Stop Solution (H2SO4) is added. The colour intensity is inversely proportional to the Cortisol concentration of in the sample. Cortisol concentration in the sample is calculated through a calibration curve.

Assay Procedure



Calculation of Results

The values of the samples on the calibration curve will be interpolated to obtain the corresponding values of the concentrations expressed in ng/mL. The sample concentrations obtained, calculated from the % B/B0 curve, were multiplied by the dilution factor to obtain neat sample values. After the cortisol assay was done, ELISA for ghrelin in saliva and serum was performed.

Assay Procedure for Ghrelin

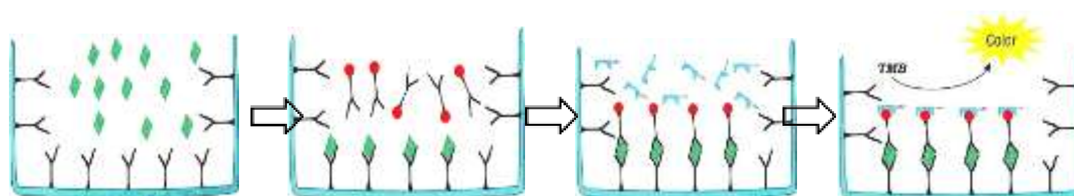
Supplied Components in SINCERE Saliva and Serum Ghrelin Enzyme Immunoassay Kit

1. **Microtiter coated plate** (96 wells) – 1
2. **Wash Solution –** 20ml × 1 bottle
3. **HRP- Conjugate reagent** – 6ml × 1 bottle
4. **Sample diluent** – 6ml × 1 bottle
5. **Chromogen Solution A** - 6ml × 1 bottle
6. **Chromogen Solution B** - 6ml × 1 bottle
7. **Standard 1350 ng/ml** – 0.5 ×1 bottle
8. **Standard diluent –** 1.5 ml × 1 bottle
9. **Stop Solution -** 6ml × 1 bottle

Assay Principle

The kit assay Human GHRL level in the Samples, use Purified Human GHRL antibody to coat microtiter plate wells, make solid-phase antibody, then add Samples

(Containing Human GHRL) to wells, combined Human GHRL antibody which with HRP labeled, become antibody - antigen - enzyme-antibody complex, after washing completely, add TMB substrate solution, TMB substrate becomes blue color at HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change (yellow) is measured spectrophotometrically at a wavelength of 450 nm. The concentration of Human GHRL in the samples is then determined by comparing the O.D. of the samples to the standard curve.



Assay Procedure

1) Preparation of the Standard: Set 10 Standard wells on the ELISA plates coated, label①②③④⑤⑥⑦⑧⑨⑩. Add Standard 100µl to ①②, then add Standard diluent 50µl to ①②, mix ; take out 100µl from①②,and then add it to ③④ separately, then add Standard diluent 50µl to ③④, mix ; then take out 50µl from③④,and discard, then take out 50µl from③④and add to ⑤⑥, then add Standard diluent 50µl to the⑤⑥, mix ; take out 50µl from ⑤⑥ and add to⑦⑧, then add Standard diluent 50µl to the ⑦⑧ ,mix ; take out 50µl from the ⑦⑧ and add to ⑨⑩, add Standard diluent 50µl to⑨⑩, mix , take out 50µl from⑨⑩discard. After dilution, the total

volume in each well is 50 μl, and the concentration is **9 ng/ml, 6 ng/ml, 3 ng/ml, 1.5 ng/ml, 0.75 ng/ml respectively.**

2) Set wells separately: Set blank well (Please do not add Sample and HRP-Conjugate reagent to the blank comparison wells, other each step operation is same), and Testing sample well.

3) Add Sample: Add Sample Diluent 40μl to Testing sample well, then add testing sample 10 μl (**Sample final dilution is 5-fold**). After this add sample to the bottom of pre-coated well, do not touch the well wall as far as possible, and mix gently.

4) Incubate: After closing the plate with Closure plate membrane, incubate for 30 min at 37℃.

5) Prepare the Washing Buffer: 30-fold Wash Solution, diluted 30-fold with Distilled water until 600ml, and reserve.

6) Washing：Uncover Closure plate membrane, discard Liquid, dry by swing, add Washing Buffer to each well, still for 30s then drain, repeat 5 times, dry by pat.

7) Add enzyme：Add HRP-Conjugate Reagent 50μl to each well, **except the Blank well**.

8) Incubate：Operation with 4).

9) Washing：Operation with 6).

10) Color：Add TMB Chromogen Solution A 50ul and then add TMB Chromogen Solution B 50ul to each well, mix gently, evade the light preservation for 15 min at 37℃

11) Stop the Reaction：Add Stop Solution 50μl to each well, to stop the reaction (the blue color change to yellow color immediately).

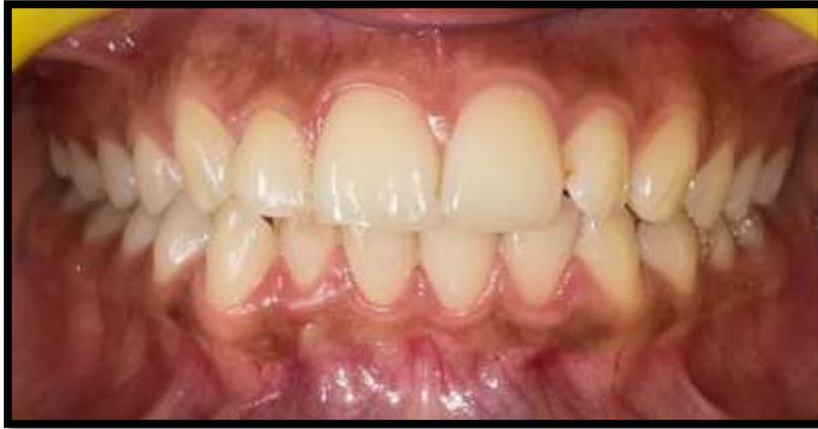
12) Assay：Set the OD of Blank well as zero, read absorbance at 450nm after adding Stop Solution within 15min**.** The judgment of result must take the OD of Microplate reader as a standard, when use the dual-wavelength to assay, reference wavelength is 630nm.

Calculation of Results

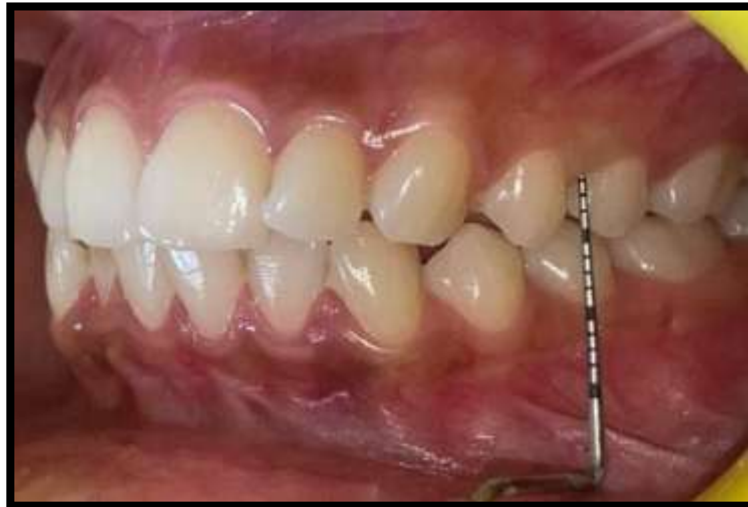
Take the Standard density as the horizontal, the OD value for the vertical, obtain the standard curve, then find out the corresponding density according to the sample OD value, and multiplied by the dilution multiple or calculate the straight line regression equation of the standard curve with the standard density and the OD value ,with the sample OD value in the equation, calculate the sample density, multiplied by the dilution factor, the result is the sample actual density.

COLOR PLATE I

Group I (Healthy Control)



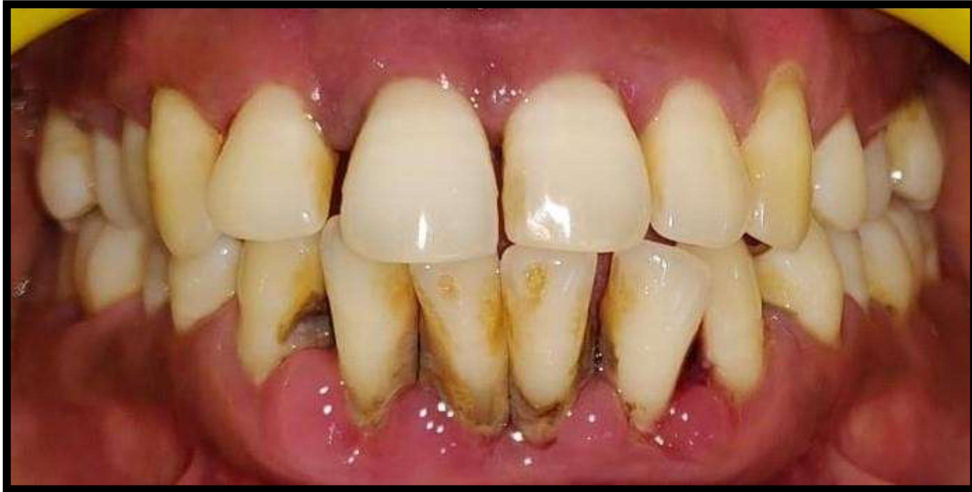
Front view of healthy patient



Normal probing depth of 2mm

COLOR PLATE II

Group II (Non-smokers with Stage III Periodontitis)

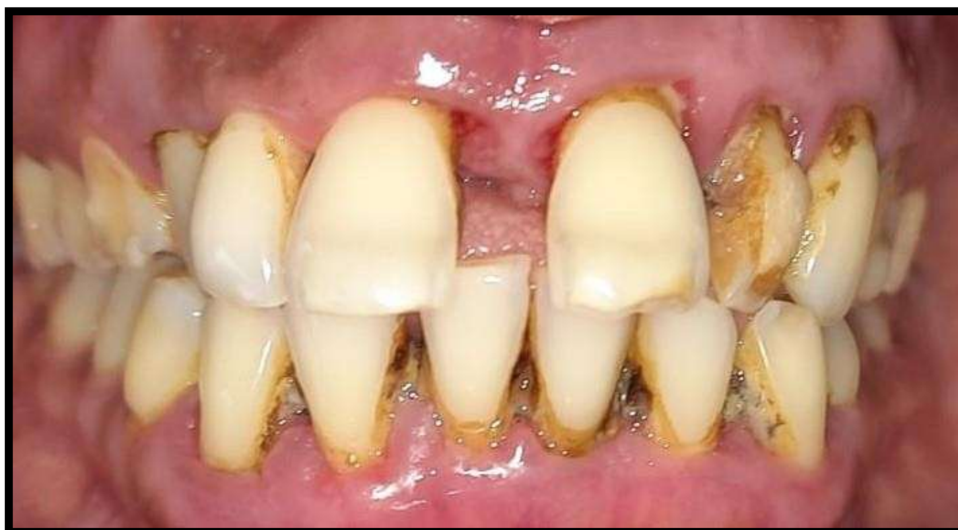


Front view of Non-smokers with Stage III Periodontitis

Overall probing depth ≥ 5mm

COLOR PLATE III

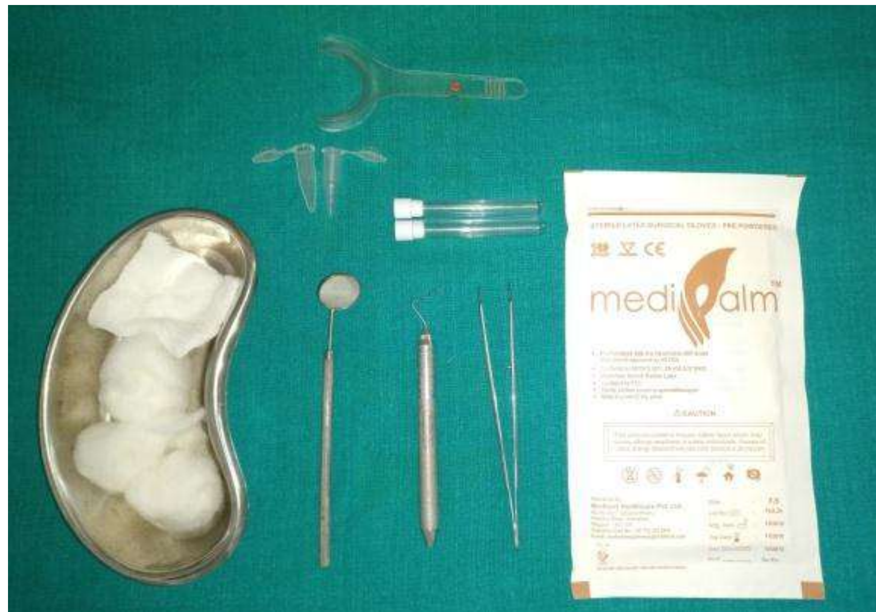
Group III (Smokers with Stage III Periodontitis)



Front view of Smokers with Stage III Periodontitis

Overall probing depth ≥ 5mm

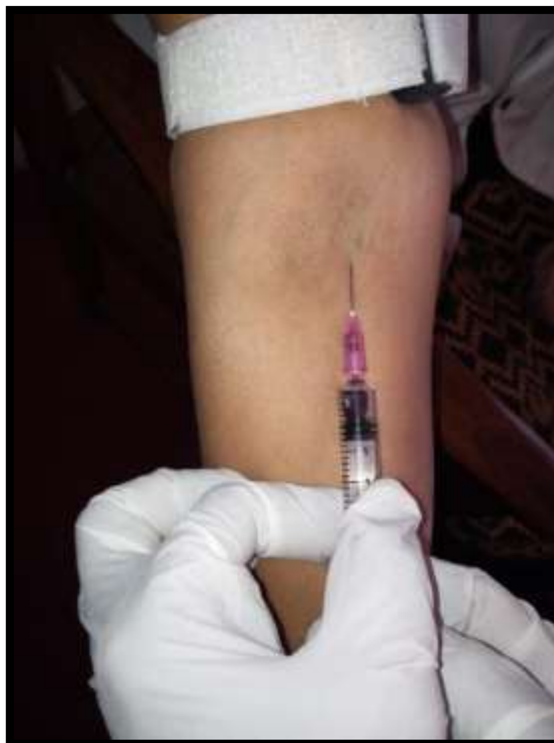
COLOR PLATE IV



Armamentarium for clinical examination and saliva and serum collection

Deep Freezer

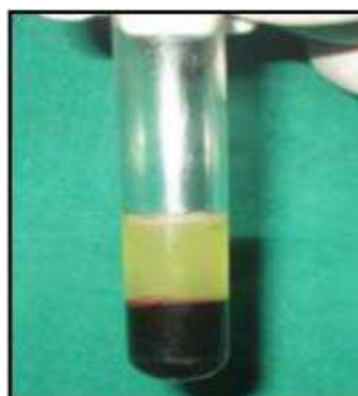
COLOR PLATE V



Collection of venous blood sample from antecubital fossa

/

Collection of Saliva sample

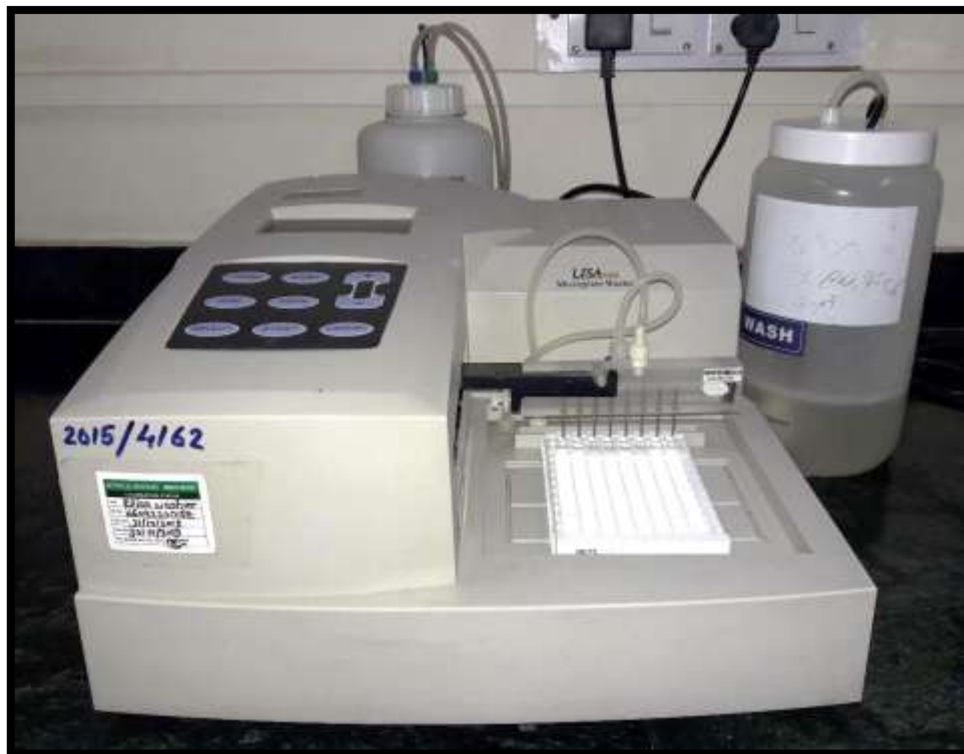


Separated serum from the blood sample

COLOR PLATE VI

Cortisol and Ghrelin ELISA Kits

COLOR PLATE VII



ELISA Washer

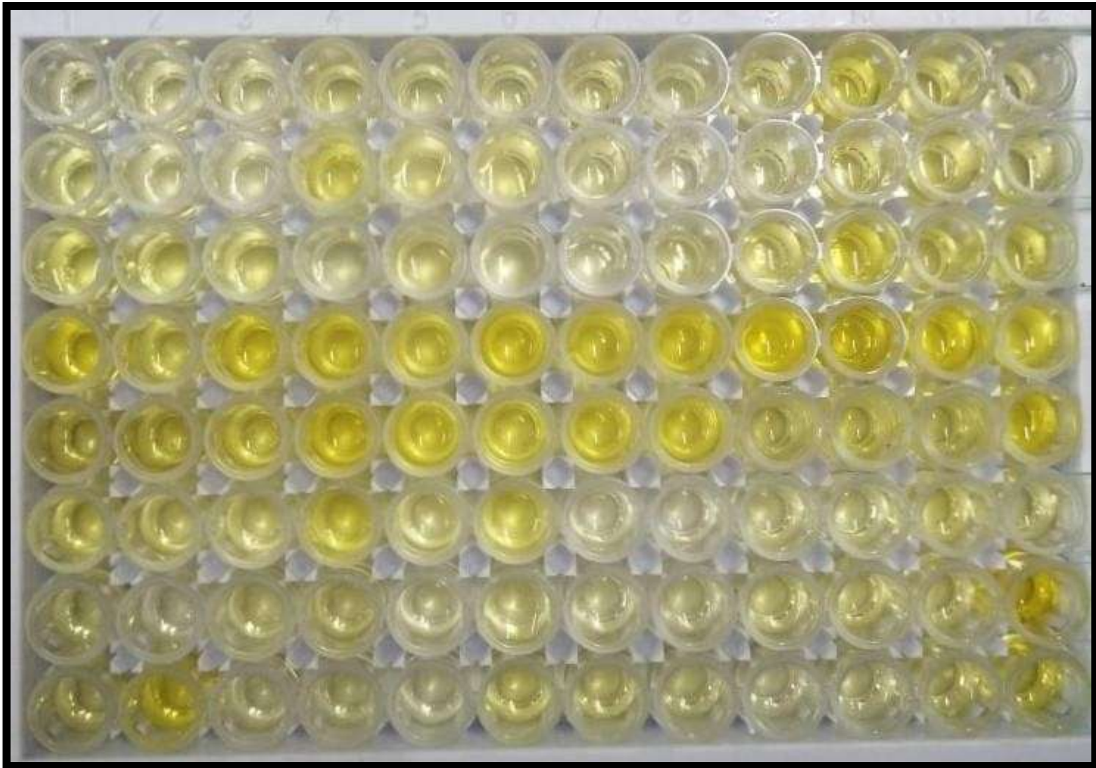
ELISA Reader

COLOR PLATE VIII

Centrifuge Machine

Vortex Mixer

COLOR PLATE IX



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 ghrelin in smokers and non-smokers. Various biomarkers have been evaluated in stage III periodontitis till date, amongst which is the stress hormone cortisol and anti-inflammatory peptide hormone ghrelin. We hypothesized its possible link with stage III 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 stage III periodontitis and then categorized into three groups. Serum and salivary cortisol and ghrelin levels were assessed using Enzyme linked immunosorbent assay. Incorporation of clinical and biochemical techniques enabled us to fulfil our above objectives.

Statistical Analysis:

The descriptive statistics like mean, standard deviation, median and range were obtained for subjects in three study groups. The distribution of subjects as per gender was summarized in terms of frequencies and percentages. The clinical parameters for subjects in three groups were summarized in terms of mean and standard deviation. The PPD was compared across groups using one-way analysis of variance, while CAL was compared between non-smoker and smoker using independent samples t-test. The indices were compared using Kruskal-Wallis test. The pairwise analysis for PPD and CAL was carried out using Tukey's post hoc test, and for indices was carried out using Wilcoxon rank sum test. The cortisol levels in serum and saliva were summarised in terms of mean and standard deviation for each group. The comparison across groups was carried out using one-way analysis of variance (ANOVA), while between serum and saliva was carried out using paired t-test. The post-hoc analysis between groups was performed using Tukey's test. On similar lines, the analysis was performed for ghrelin levels. The SDS scores were also summarized in terms of mean and standard deviation. The difference of means was tested using one-way analysis of variance and the paired differences were tested by Tukey's test. The correlation of SDS scores with serum and saliva cortisol levels was performed using Pearson's correlation coefficient. Also the correlation of clinical parameters with serum and saliva ghrelin levels was performed using Pearson's correlation coefficient in each study group. Also the correlation of cortisol and ghrelin levels was observed in serum and saliva using Pearson's coefficient. All the analyses were performed using SPSS ver 20.0 (IBM Corp, USA) software and the statistical significance was tested at 5% level.

Table 1 gives the mean and standard deviation for various clinical parameters for patients in three study groups. The mean probing pocket depth in healthy controls group was 1.35 (SD: 0.30) mm, while in periodontitis (non-smoker) group was 6.13 (SD: 1.42) mm and in periodontitis (smokers) group was 8.97 (SD: 1.07) mm. The difference of means was statistically highly significant with p-value < 0.0001. The mean clinical attachment level in periodontitis (non-smokers) group was 7.42 (SD: 1.61), while in periodontitis (smokers) group was 10.03 (SD: 1.06); and the difference between the two means was highly significant with p-value < 0.0001. The mean plaque index in control group was 0.71 (SD: 0.3), while in periodontitis (non-smokers) group, it was 2.09 (SD: 0.54) and in the smokers group was 2.71 (SD: 0.46). The difference in the index distribution across groups was statistically highly significant asindicated by p-value < 0.0001. The mean gingival index in control group was 0.44 (SD: 0.35), while in periodontitis (non-smokers) group was 2.78 (SD: 0.56) and in smokers group was 1.89 (SD: 0.64); and the difference of index distribution across groups was statistically highly significant with p-value < 0.0001. The mean papillary bleeding index in control group was 0.05 (SD: 0.06), while in periodontitis (non-smokers) group was 2.97 (SD: 0.86) and in smokers group was 1.63 (SD: 0.82); and the difference in the index distribution was statistically highly significant with p-value < 0.0001.

Table 2 shows the evidence for every clinical parameter, the pair wise mean differences between groups were statistically highly significant with p-value < 0.0001.

Table 3 gives the comparison of clinical parameters between periodontitis (non-smokers) and periodontitis (smokers) groups. It is evident from the table that the mean probing pocket depth, clinical attachment level and plaque index were significantly smaller in non-smokers group as compared to smokers group ($p < 0.0001$). The gingival index and papillary bleeding index were significantly higher in non-smokers group as compared to smokers group ($p < 0.0001$).

Table 4 shows the descriptive statistics like mean and standard deviation for serum and saliva cortisol in three study groups. **Graph 1** shows the mean serum cortisol level in control group was 11.58 (SD: 5.46), while in periodontitis (non-smokers) group was 16.36 (SD: 8.88) and in periodontitis smokers group was 20.78 (SD: 9.23). The difference of means across groups was statistically highly significant as indicated by p-value < 0.0001. Further, the mean saliva cortisol in control group was 23.56 (SD: 6.9, while in periodontitis (non-smokers) group was 399.68 (SD: 107.01) and in smokers group was 399.37 (SD: 189.21). The difference of means across groups was statistically highly significant as indicated by p-value < 0.0001.

Also, the difference between serum and saliva cortisol levels in each study group was statistically highly significant with p-value < 0.0001.

Table 5 provides the pair wise comparison of Cortisol levels in patients from three study groups in serum and saliva. The mean difference of serum Cortisol between control and smoker group was -9.20 [95% CI: -14.15, -4.25] and was statistically highly significant with p-value < 0.0001. Further, for saliva cortisol, the mean difference between control and periodontitis (non-smokers) was -376.12 [95% CI: -453.43, -298.82] and was statistically highly significant with p-value < 0.0001. Also

the mean difference between control and smokers group was -375.82 [95% CI: -453.12, -298.51] and was highly significant with p-value < 0.0001. The other paired differences were statistically insignificant.

Table 6 provides the descriptive statistics for Ghrelin levels in serum and saliva in patients from three groups. **Graph 2** shows the mean serum ghrelin level in control group was 547.56 (SD: 166.53), while in periodontitis (non-smokers) group was 650.25 (SD: 260.86) and in smokers group was 439.05 (SD: 141.79). The difference of means was statistically highly significant with p-value < 0.0001. The mean saliva ghrelin level in control group was 787.29 (SD: 230.29), while in periodontitis (non-smokers) group was 892.40 (SD: 271.65) and in smoker group was 572.76 (SD: 151.87). The difference of means was statistically highly significant with p-value < 0.0001. Also, the difference between serum and saliva ghrelin levels in each study group was statistically highly significant with p-value < 0.0001.

Table 7 provides the pair wise comparison of ghrelin levels in patients from three study groups in serum and saliva. The mean difference of serum ghrelin between periodontitis (non-smokers) and periodontitis (smokers) groups was 211.20 [95% CI: 90.19, 332.20] and was statistically highly significant with p-value < 0.0001. Further, for saliva ghrelin, the mean difference between control and periodontitis (smokers) was 214.53 [95% CI: 76.91, 352.15] and was statistically significant with p-value of 0.001. Also, the mean difference between non-smokers and smoker group was 319.65 [95% CI: 182.02, 457.26] and was highly significant with p-value < 0.0001. The other paired differences were statistically insignificant.

Table 8 gives the descriptive statistics for SDS scores in patients from three study groups. **Graph 3** shows the mean for healthy control group was 42.30 (SD: 4.79), while for periodontitis (non-smokers) group was 46.27 (SD: 8.66) and for periodontitis (smokers) group was 57.13 (SD: 9.38). The difference of means was statistically highly significant with p-value < 0.0001.

Table 9 provides the pair wise comparison of SDS scores in patients from three study groups. The mean difference of SDS between control and periodontitis (smokers) group was -14.83 [95% CI: -19.68, -9.98] and was statistically highly significant with p-value < 0.0001. Further, the mean difference between periodontitis (non-smokers) and periodontitis (smokers) was -10.86 [95% CI: -15.71, -6.01] and was statistically highly significant with p-value < 0.0001. The other paired difference was statistically insignificant.

Table 10 (Graph 4 and 5) provides the correlation of serum and saliva cortisol levels with SDS scores in each study group. In Group I, the correlation between serum cortisol level and SDS was negligible with coefficient 0.094 and statistically insignificant (p=0.62). The correlation of saliva cortisol and SDS scores was low negative (-0.34) and statistically insignificant (p=0.066). In Group II, the correlation of serum cortisol and SDS was negligible (0.158) and statistically insignificant (p=0.405). The correlation of saliva and SDS was low positive (0.454) and was statistically significant with p-value of 0.011. In Group III, the serum cortisol levels showed negligible correlation with SDS (-0.28) and was statistically insignificant (p=0.134). The correlation of saliva cortisol level with SDS was low positive (0.4) and was statistically significant with p-value of 0.028.

Table 11 gives the overall correlation of serum and saliva cortisol levels with serum and saliva ghrelin levels. **(Graph 6 and 7)** shows the correlation between serum cortisol and serum ghrelin was negligible (-0.053) and was statistically insignificant (p=0.62). Also, the correlation between serum cortisol and saliva ghrelin was negligible (-0.079) and was statistically insignificant (p=0.458). The correlation of saliva cortisol with serum ghrelin was negligible (-0.039) and was statistically insignificant (p=0.718). Also, the correlation between saliva cortisol and saliva ghrelin was negligible (-0.097) and was statistically insignificant (p=0.363).

DISCUSSION

Periodontitis is a highly prevalent, multifactorial, chronic inflammatory disease of the periodontium. This dynamic interplay between the microbial activity and the host's inflammatory response, further contributes to connective tissue deterioration and alveolar bone loss.[85] Stress is a series of physiological and psychological responses that a person faces challenging situation. This establishes an interconnection between the stress and periodontitis which is characterized by hormonal modifications and behavioural changes.[86] Chronic stress in association with smoking is thought to have a net negative impact on immune response efficacy, culminating an imbalance between the host and microbial responses.[87] Tobacco usage, primarily in the form of cigarette smoking, is widely acknowledged as the most imperative modifiable risk factor for periodontitis, thereby compromising the periodontal tissue's ability to heal, following a period of disease activity.[88,89] The cigarette smoke primarily composed of at least 400 potentially toxic products, including hydrogen cyanide, carbon monoxide (which

causes formation of carboxyhaemoglobin), free radicles, nicotine, nitrosamines (potent carcinogens and several oxidant gases that induces platelet activation and endothelial dysfunction.[90,91] Gingival bleeding and probing pocket depth both seen to be unfavourable in terms of healing following non-surgical therapy especially in smokers as compared to non-smokers, which has been demonstrated by several studies.[92] A study by **Grossi et al. (1997)**[93] stated that current smokers have less healing and reduction in subgingival Tannerella forsythia and Porphyromonas gingivalis after treatment compared to former and non-smokers.

The nicotine in cigarettes activates the sympathetic ganglia, which produces neurotransmitters such as catecholamines.[94] This triggers the alpha receptors on blood vessels, causing vasoconstriction which can further affect the periodontal tissue as smokers have comparatively less overt signs of gingivitis than non-smokers. [95] The vasoconstrictive action of nicotine may be responsible for the decreased gingival blood flow. **Bergstrom and Floderus-Myrhed (1983)[96]** reported that less gingival bleeding in smokers compared to 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.

Cortisol, a stress hormone released in the adrenal cortex is one of the most essential glucocorticoids which has not only significant anti-inflammatory properties but also has immunosuppressive effects, thus inhibiting the lymphocyte development and inducing lymphatic tissue hyperplasia. Simultaneously an antibody synthesis is impaired which leads to a significant reduction in humoral immune response. Cortisol is seen to be antiphlogistic because of its inhibitory effect on fibroblast proliferation

especially in inflammatory granulation tissue. As a result of these consequences, the release of some proinflammatory cytokines will be suppressed which further lead to reduced immune response caused by cortisol secretion, and thus in this way the homeostasis is disrupted.[97]

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.[98] 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.[99] 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 inconvenient during routine procedures in the dental office.[100] 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 as well as systemically derived markers of periodontal diseases.[101]

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 been associated stress and its hormone cortisol from serum and saliva with stage III periodontitis. Also, many studies have evaluated anti-inflammatory marker ghrelin in stage III periodontitis. However, to the best of our knowledge no study till date has associated stress hormone cortisol from the serum and saliva with anti-inflammatory peptide hormone ghrelin in smoker and non-smokers with stage III periodontitis.

In the present study, the data relates the cross-sectional examination conducted on 90 patients (56 males and 34 females) divided as:

Group I- Control group (30 periodontally healthy subjects)

Group II – Test group (30 non-smokers with Stage III Periodontitis)

Group III – Test group (30 smokers with Stage III Periodontitis)

The study population constituted of patients with the age range of 30 – 65 years so as to rule out the early onset periodontitis cases. Group III (smokers with Stage III periodontitis) comprised of only male population.

The clinical periodontal parameters like PPD, CAL, PI were significantly seen to be elevated in smokers as compared to non-smokers group as indicated by p value <0.0001. This is in accordance to studies done by **Feldman et al. (1982)[101]** found an association between six periodontal indices (calculus deposition, plaque accumulation, gingival inflammation, periodontal pocket depth, alveolar bone loss and tooth mobility) and cigarette smokers with chronic periodontitis. They reported that cigarette smokers had significantly more calculus deposition, significantly greater pocket depth, accumulated slightly less plaque, and had more alveolar bone loss.

The possible explanation 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[102], and in vitro studies show it can alter fibroblast attachment[103], integrin expression and decrease collagen production.[104] The increased periodontal destruction in Group III patients is attributed to the impairment of immune system in smokers. Cigarette smoking, nicotine, and its by-products 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.[105] 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.[106,107]

Higher PI in smokers was observed 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 the mean of 57.13 and 46.27 respectively suggesting that 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)**[15] **Kolte et al. (2016)**[33] **Bawankar P. et al. (2018).**[34] Other investigations have shown little in the level of plaque accumulation, comparing smokers with non-smokers. **Calsina et al. (2002)**[108] in their study on effects of smoking on periodontal tissues; found that plaque index did not show differences between smokers and non-smokers. On the other hand, **Scabbia et al. (2001)**[109] 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 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 GI and the PBI were significantly more in non-smoker group as compared to smoker group with p values< 0.0001 respectively. This observation 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)**[110] 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 seen to be elevated in smoker group followed by non-smoker and control group. Similarly salivary cortisol levels were also seen to be higher in smoker group as compared to non-smoker and healthy group. This verdict suggests that cortisol levels influence the periodontal tissue degradation, which is consistent with the findings of **Badrick et al (2007)**[73] who examined salivary cortisol levels in current smokers, former smokers and non-smokers. The authors explored that salivary cortisol levels were higher in current smokers than in non-smokers, with no differences found between former and non-smokers, implying that smoking has a short-term effect on the neuroendocrine system. Another study by **Handa et al. (1994)**[111] discovered that middle-aged Japanese male smokers had lower morning plasma cortisol levels than that of non-smokers. When intragroup comparison was done for serum and salivary values, a statistically significant difference was observed with p value <0.0001.

Also, an ample of literature confirms elevated salivary cortisol levels 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)**[112]; **Baron et al. (1995)**[113] and no statistical differences was observed in others by **Gossain et al. (1986)**,[114]**Kirschbaum et al. (1994)**[115]and **Tsuda et al. (1996).**[116] 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 Group I and III were highly significant (p value < 0.0001), whereas the difference between Groups II and III was insignificant with p value of 0.999. Thus, the salivary cortisol in smokers with periodontitis was found to be significantly more as compared to non-smokers with stage III periodontitis.

Ghrelin is a peptide hunger hormone which is predominantly secreted by ghrelinergic cells of stomach. In addition to this, it is also an anti-inflammatory biomarker and stimulates the differentiation and proliferation of osteoblastic cells and enhances bone formation.[36] Ghrelin levels are seen to be elevated in ankylosing spondylitis, Crohn's disease, and inflammatory bowel disease while decreased in type II diabetes, obesity and metabolic syndrome.[37,45]

Group II had higher serum levels of total ghrelin than Group I and Group III indicated by p value< 0.0001. These results are in accordance with **Yilmaaz et al. (2014)[51] and H.F.R. Jentch (2017)[52]** where they found elevated total serum ghrelin levels in stage III periodontitis patients as compared to healthy group. This suggests that pro-inflammatory cytokine expression during inflammation might induces the ghrelin expression.[117] On the contrary it was also found that total ghrelin serum levels were seen to be reduced in Group III (smoker) patients. This might attribute to the fact that smoking can significantly reduce the serum ghrelin levels independent of those of saliva. Similarly salivary total ghrelin levels were also seen to be highest in Group II followed by Group I and Group III. The potential reason for this observation is that certain biomolecules and hormones found in blood are often found in saliva, and they

sometimes interact positively; that is when their levels in blood rises, their salivary levels rise as well and vice versa.[118] It is also hypothesized that this effect may be due to nicotine's direct impact on ghrelin secretion from salivary gland or taste bud cells, a process that could lead to smoker's dull food taste. This has finally led to the suggestion that saliva be used as a valuable patient sample instead of blood, since saliva sampling is an easy, fast and non-invasive technique.[119,120]

To the best of our knowledge, this is the first research to test serum and salivary ghrelin levels in smokers with stage III periodontitis in order to determine the additive effect of smoking on ghrelin in patients presenting with stage III periodontitis. According to our results, stage III non-smoker group had showed higher ghrelin concentration levels as compared to stage III smoker and healthy group. **Hataya et al**[121] reported that administration of lipopolysaccharide (LPS) decreased the levels of ghrelin in early phase, but repeated LPS administration caused an increase in ghrelin levels in stage III periodontitis patients. In addition, increased ghrelin levels were reported in inflammatory diseases such as ankylosing spondylitis, inflammatory bowel disease and celiac disease.[122,123,124,] These findings indicate that systemic inflammation influences circulating ghrelin levels. In our study we found that, though the salivary and serum ghrelin levels were increased in stage III periodontitis but the same levels were decreased in smokers presenting with stage III periodontitis. These findings are in accordance with **Kaabi et al (2014)**[53]who investigated the direct impact of acute cigarette smoking on total ghrelin levels in saliva and concluded that acute cigarette smoking can negatively affect ghrelin levels in saliva that might contribute to the dull food taste in smokers.

Pairwise comparison of salivary ghrelin levels showed statistically significant differences between healthy and smokers with stage III periodontitis group with p value < 0.001 and highly significant difference was seen with p value < 0.0001 between smokers and non-smokers with stage III periodontitis group. This finding is similar to studies done by **H.F.R. Jentch et al. (2017).** [52]

The Zung's Self Rating Depression Scale was used to assess the depression score. It was observed that smokers rose up with the highest SDS score of 57.13 and median 9.38 followed by 46.27 in non-smokers and 42.30 in controls. This can be interpreted as smokers were moderately depressed, while non-smokers and healthy controls were in normal range. Paired comparison amongst Group I- Group III and Group II- Group III showed highly significant differences.

Correlation 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).**[62] 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 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.[125,126,127] The SDS score was found to be maximum in smokers, while

serum and salivary ghrelin levels were seen be reduced. Concentrating towards the findings of our study, it can be interpreted that ghrelin levels were inversely proportional to SDS score in case of smokers while directly proportional to the non-smokers with stage III periodontitis.

In the current study, the presence of stress or depression was assessed by a self-rating questionnaire system, which may be subject to individual bias related to understanding the gravity of the stated question and ability to respond correctly according to the scale, as well as situation bias, which refers to the clinical phenomenon in an unstable manner. It also does not provide for the evaluation of an individual's subjective and behavioural characteristics. In epidemiological research, the effects of stress (as measured by self-report scales) on periodontitis may be over-emphasized, since it is difficult to link current and recent events to periodontitis, particularly due to the mean age of the onset of disease, its clinical course and chronicity. This study plays an imperative as well as critical role in establishing a distinct and conclusive relationship and connection between the stress, serum and salivary stress biomarker cortisol, as well as the disease activity determinant ghrelin levels in stage III periodontitis with and without smokers.

CONCLUSION

The present study was undertaken to evaluate whether stress, serum and salivary cortisol and ghrelin are associated and found to be increased in smokers and non-smokers with stage III periodontitis.

A total of 90 patients were recruited and categorized into 3 groups, with 30 patients in each group. Group I being healthy, Group II being non-smokers with stage III periodontitis and Group III being smokers with stage III 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 ghrelin levels from serum and saliva samples ELISA kit was used to analyse serum and salivary cortisol and ghrelin levels. Stress levels were assessed using Zung's self-rating depression scale questionnaire.

A significantly high PPD and low GI and PBI were observed in stage III periodontitis smokers as compared to stage III periodontitis non-smokers. A significantly high salivary cortisol values were observed as compared to serum in all the groups. Amongst the stage III periodontitis patients smokers exhibited significantly higher salivary cortisol values while ghrelin levels found to be lowest. 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 stage III periodontitis as compared to non-smokers with stage III

periodontitis.

2. Serum and salivary ghrelin values are higher in non-smokers with stage III periodontitis as compared to smokers with stage III 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, and PBI.

6. The serum and salivary ghrelin levels directly proportional to stress in stage III periodontitis non-smokers while inversely proportional in stage III periodontitis smokers.

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

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

Parameter	Group						P-value
	Healthy controls (n=30)		Periodontitis (Non-smokers) (n=30)		Periodontitis (Smokers) (n=30)		
	Mean	SD	Mean	SD	Mean	SD	
Probing pocket depth (mm)	1.35	0.30	6.13	1.42	8.97	1.07	< 0.0001*
Clinical attachment level	0.00	0.00	7.42	1.61	10.03	1.06	< 0.0001‡
Plaque index	0.71	0.30	2.09	0.54	2.71	0.46	< 0.0001ǂ
Gingival index	0.44	0.35	2.78	0.56	1.89	0.64	< 0.0001ǂ
Papillary bleeding index	0.05	0.06	2.97	0.86	1.63	0.82	< 0.0001ǂ

*Obtained using one-way analysis of variance; ‡Obtained using t-test for independent samples; ǂObtained using Kruskal Wallis test.

Table 2: Paired comparison of clinical parameters in patients from three study groups

Clinical Parameters			Mean Difference	SD	P-value
PPD	Healthy controls	Non-smoker	-4.77	0.27	< 0.0001
		Smoker	-7.62	0.27	< 0.0001
	Non-smoker	Smoker	-2.84	0.27	< 0.0001
CAL	Healthy controls	Non-smoker	-7.42	0.29	< 0.0001
		Smoker	-10.03	0.29	< 0.0001
	Non-smoker	Smoker	-2.62	0.29	< 0.0001
PI	Healthy controls	Non-smoker	-1.38	0.11	< 0.0001
		Smoker	-1.99	0.11	< 0.0001
	Non-smoker	Smoker	-0.61	0.11	< 0.0001
GI	Healthy controls	Non-smoker	-2.33	0.14	< 0.0001
		Smoker	-1.44	0.14	< 0.0001
	Non-smoker	Smoker	0.89	0.14	< 0.0001
PBI	Healthy controls	Non-smoker	-2.91	0.18	< 0.0001
		Smoker	-1.57	0.18	< 0.0001
	Non-smoker	Smoker	1.34	0.18	< 0.0001

Table 3: Comparison of clinical parameters in patients between Group II and Group III

Parameter	Group				P-value
	Periodontitis (Non-smokers)		Periodontitis (Smokers)		
	Mean	SD	Mean	SD	
Probing pocket depth	6.13	1.42	8.97	1.07	< 0.0001
Clinical attachment level	7.42	1.61	10.03	1.06	< 0.0001
Plaque index	2.09	0.54	2.71	0.46	< 0.0001
Gingival index	2.78	0.56	1.89	0.64	< 0.0001
Papillary bleeding index	2.97	0.86	1.63	0.82	< 0.0001

Table 4: Descriptive statistics for Cortisol levels in serum and saliva in patients from three study groups

	Group						P-value*
	Healthy controls		Periodontitis (Non-smokers)		Periodontitis (Smokers)		
	Mean	SD	Mean	SD	Mean	SD	
Serum cortisol	11.58	5.46	16.36	8.88	20.78	9.23	< 0.0001
Saliva cortisol	23.56	6.96	399.68	107.01	399.37	189.21	< 0.0001
P-value‡	< 0.0001		< 0.0001		< 0.0001		

*Obtained using one-way ANOVA, ‡Obtained using paired t-test

Table 5: Paired comparison of Cortisol levels in patients from three study groups in serum and saliva

Dependent Variable			Mean Difference	P-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Serum cortisol	Healthy controls	Periodontitis (Non-smokers)	-4.78	0.060	-9.73	0.16
		Periodontitis (Smokers)	-9.20	< 0.0001	-14.15	-4.25
	Periodontitis (Non-smokers)	Periodontitis (Smokers)	-4.42	0.089	-9.37	0.52
Saliva cortisol	Healthy controls	Periodontitis (Non-smokers)	-376.12	< 0.0001	-453.43	-298.82
		Periodontitis (Smokers)	-375.82	< 0.0001	-453.12	-298.51
	Periodontitis (Non-smokers)	Periodontitis (Smokers)	0.30	0.999	-77.61	77.00

Table 6: Descriptive statistics for Ghrelin levels in serum and saliva in patients from three study groups

Parameters	Group						P-value*
	Healthy controls		Periodontitis (Non-smokers)		Periodontitis (Smokers)		
	Mean	SD	Mean	SD	Mean	SD	
Serum ghrelin	547.56	166.53	650.25	260.86	439.05	141.79	< 0.0001
Saliva ghrelin	787.29	230.29	892.40	271.65	572.76	151.87	< 0.0001
P-value‡	< 0.0001		< 0.0001		< 0.0001		

*Obtained using one-way ANOVA, ‡Obtained using paired t-test

Table 7: Paired comparison of Ghrelin levels in patients from three study groups in serum and saliva

Dependent Variable			Mean Difference	P-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Serum ghrelin	Healthy controls	Periodontitis (Non-smokers)	-102.69	0.113	-223.70	18.30
		Periodontitis (Smokers)	108.51	0.088	-12.49	229.51
	Periodontitis (Non-smokers)	Periodontitis (Smokers)	211.20	< 0.0001	90.19	332.20
Saliva ghrelin	Healthy controls	Periodontitis (Non-smokers)	-105.11	0.169	-242.73	32.50
		Periodontitis (Smokers)	214.53	0.001	76.91	352.15
	Periodontitis (Non-smokers)	Periodontitis (Smokers)	319.65	< 0.0001	182.02	457.26

Table 8: Descriptive statistics for SDS scores in patients from three study groups

Group	Mean	SD	P-value*
Healthy controls	42.30	4.79	< 0.0001
Periodontitis (Non-smokers)	46.27	8.66	
Periodontitis (Smokers)	57.13	9.38	

*Obtained using one-way ANOVA

Table 9: Paired comparison of SDS scores in patients from three study groups

Comparisons		Mean Difference	P-value	95% Confidence Interval	
				Lower Bound	Upper Bound
Healthy controls	Periodontitis (Non-smokers)	-3.96	0.131	-8.81	0.88
	Periodontitis (Smokers)	-14.83	< 0.0001	-19.68	-9.98
Periodontitis (Non-smokers)	Periodontitis (Smokers)	-10.86	< 0.0001	-15.71	-6.01

Table 10: Correlation of SDS scores with Cortisol levels in each group

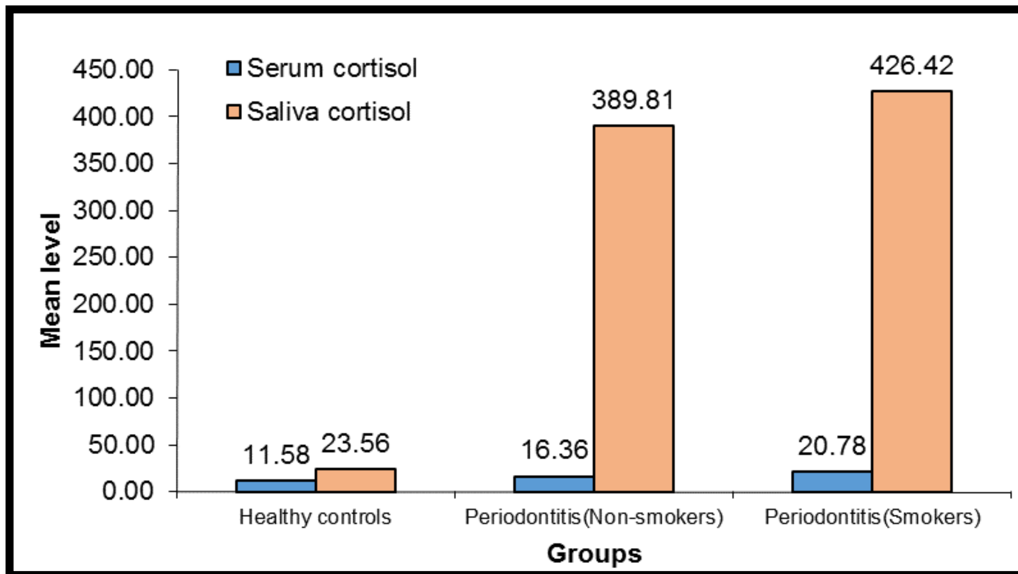
Groups	Levels	Correlation Coefficients	P-value*
Group I	Serum	0.094	0.620
	Saliva	-0.340	0.066
Group II	Serum	0.158	0.405
	Saliva	0.454	0.011 **(S)**
Group III	Serum	-0.280	0.134
	Saliva	0.400	0.028 **(S)**

Table 11: Correlation of Cortisol and Ghrelin in serum and saliva

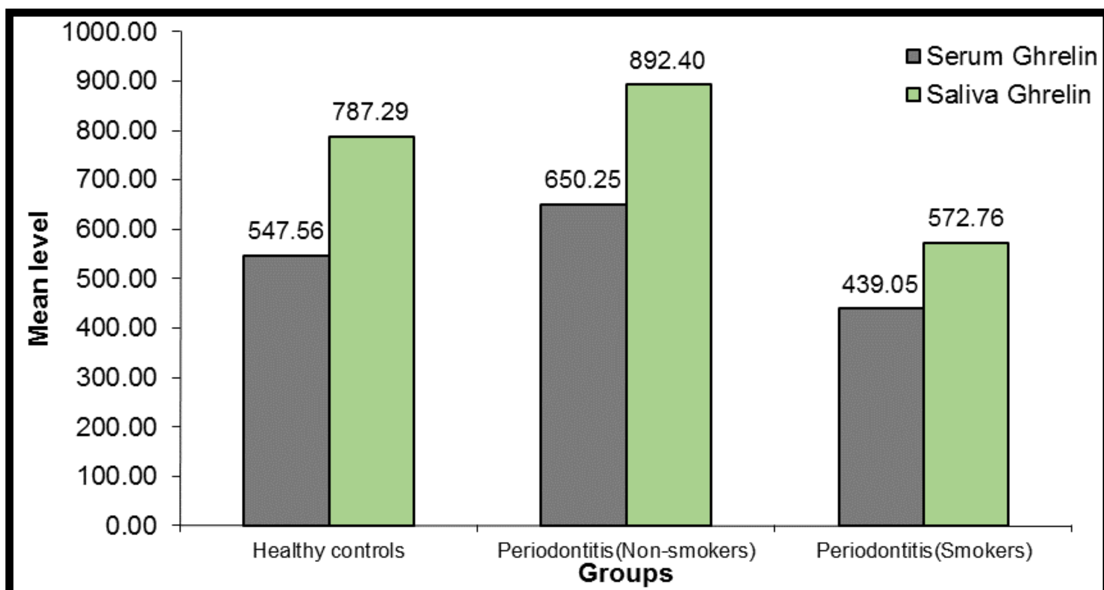
		Serum Ghrelin	Saliva Ghrelin
Cortisol	Serum	-0.053 (0.620)	-0.079 (0.458)
	Saliva	-0.039 (0.718)	-0.097 (0.363)

Graphs

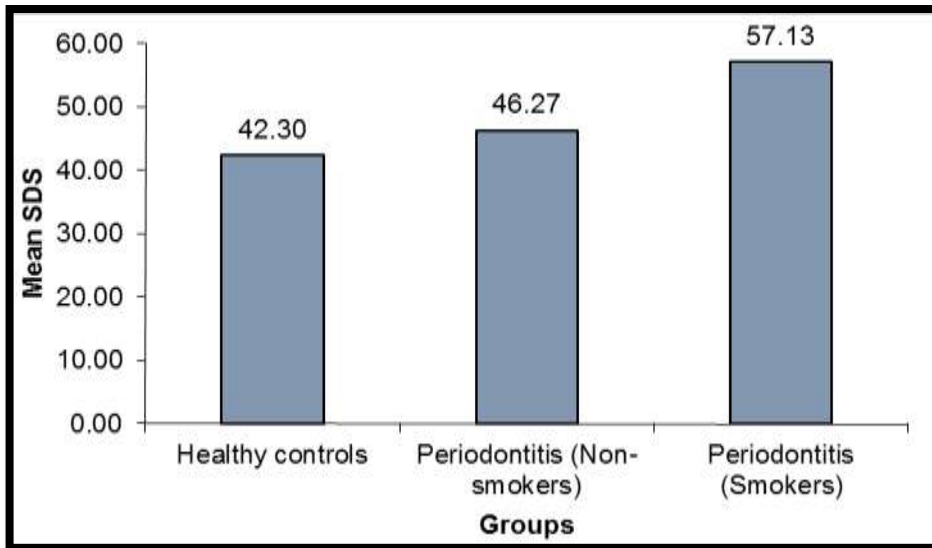
Graph 1: Column chart showing mean cortisol levels in serum and saliva in three groups



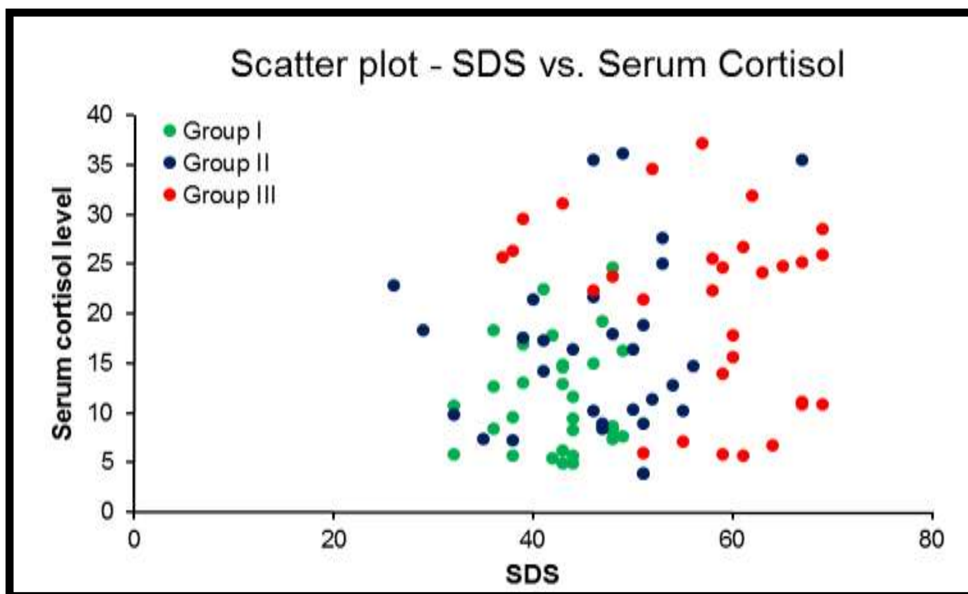
Graph 2: Column chart showing mean Ghrelin levels in serum and saliva in three groups



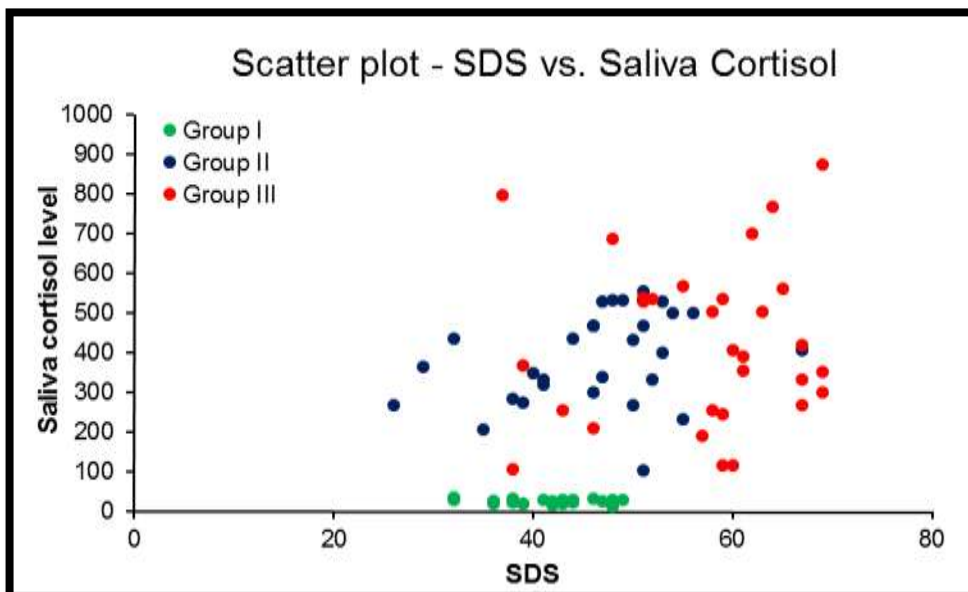
Graph 3: Column chart showing mean SDS for three study groups



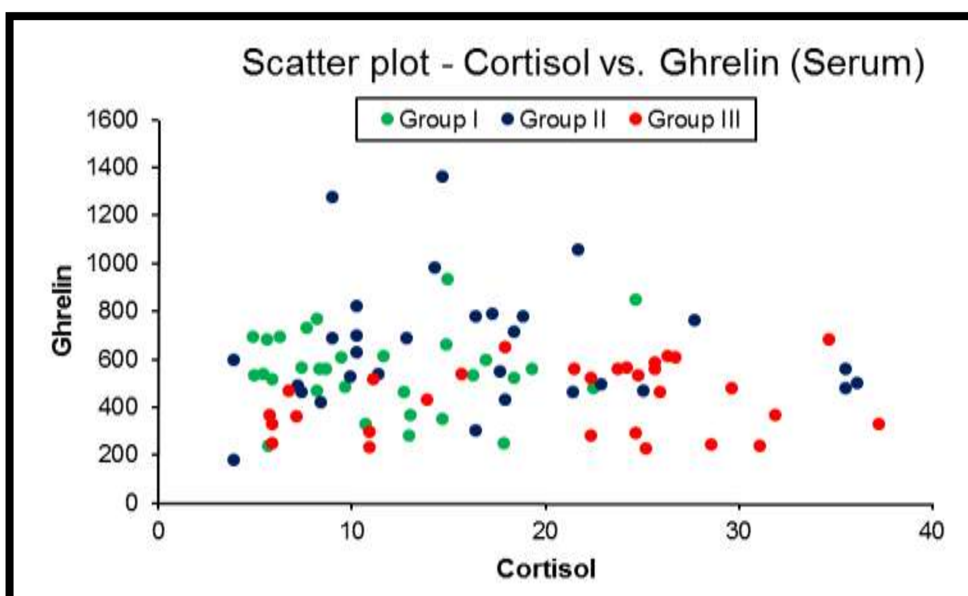
Graph 4: Scatter plot showing relationship of SDS and serum Cortisol in three groups



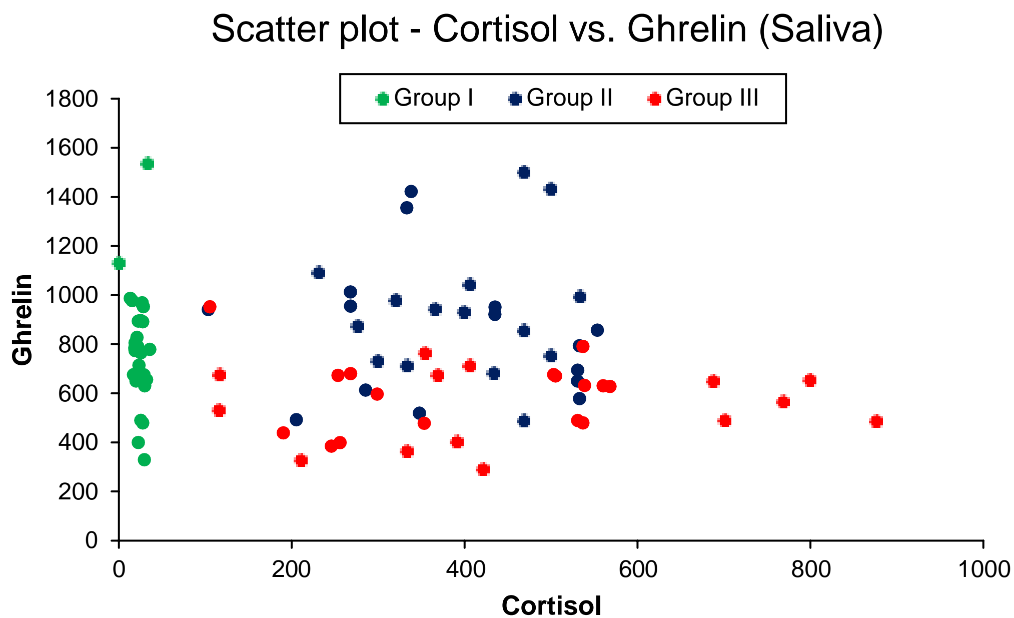
Graph 5: Scatter plot showing relationship of SDS and saliva Cortisol in three groups



Graph 6: Scatter plot showing relationship of Cortisol and Ghrelin in serum in three groups



Graph 7: Scatter plot showing relationship of Cortisol and Ghrelin in saliva in three groups



Master Chart

Periodontal Clinical Parameters in GROUP I

Sr. No.	Probing Pocket Depth	Clinical Attachment Level (CAL)	Plaque Index (PI)	Gingival Index (GI)	Papillary Bleeding Index (PBI)
1	1.13	0	0.82	0.66	0.09
2	1.52	0	0.71	0.33	0
3	1.67	0	0.28	0.33	0
4	1.01	0	0.78	0.33	0.04
5	1.64	0	0.56	0.33	0.09
6	1.07	0	0.5	0.5	0
7	1.05	0	1	0	0
8	1.59	0	0.87	0	0
9	1.56	0	1	0.5	0
10	1.2	0	0.94	0.5	0.1
11	1.98	0	0.42	0.33	0.06
12	1.34	0	1	0.33	0.04
13	1.61	0	0.45	0.5	0
14	1.22	0	0.32	0.66	0.1
15	1.32	0	1	0.75	0.03
16	1.12	0	0.82	0.33	0.02
17	1.09	0	0.71	0	0.06
18	2.05	0	0.94	1.89	0.06
19	1.23	0	0.78	0.63	0.03
20	1.16	0	1	0.124	0.08
21	1.15	0	0.5	0.6	0.3
22	1.03	0	1.56	0.33	0.1
23	1.62	0	0.87	0.33	0.07
24	1.03	0	0.35	0.25	0.03
25	1.62	0	0.21	0.5	0.09
26	1.03	0	0.42	1	0.02
27	1.62	0	1	0.33	0
28	1.03	0	0.45	0.33	0.09
29	1.59	0	0.56	0.125	0.02
30	1.3	0	0.62	0.5	0

Periodontal Clinical Parameters in GROUP II

Sr. No.	Probing Pocket Depth	Clinical Attachment Level (CAL)	Plaque Index (PI)	Gingival Index (GI)	Papillary Bleeding Index (PBI)
1	5.59	6.94	2.34	2	1.38
2	5.61	6.37	2.2	2.92	2.28
3	4.41	5.59	1.21	2.43	2.13
4	5.89	6.96	1.41	1.91	3.73
5	6.48	7.81	2.79	2.6	2.33
6	4.93	5.73	1.93	3.31	3.92
7	6.29	7.32	1.35	2.15	1.28
8	5.39	8.43	1.88	3	3.96
9	6.51	7.67	2.84	2.71	3
10	4.91	5.98	2	2.5	2.69
11	9.38	10.21	2.57	3	2.57
12	5.31	7.52	2.41	3.96	4.46
13	5.97	7.49	1.5	3.16	2.52
14	5.69	6.73	2.14	2.5	3.5
15	4.84	5.65	1.89	3.67	3
16	5.61	6.33	2.68	2.75	3.5
17	5.05	6.65	1.36	2.12	3.85
18	6.02	7.24	2.67	1.93	3.77
19	9.16	11.46	2.56	3	2.14
20	5.94	7.61	1.73	2.44	3.96
21	4.96	5.99	2.17	2.54	3.14
22	6.32	7.81	2.14	2.83	3.16
23	5.24	7.51	2.82	2.5	3.21
24	8.61	9.92	2.92	3.85	2.84
25	5.99	5.29	2.32	2	3
26	5.03	6.23	2.2	2.78	2.32
27	6.49	7.93	1.21	2.96	1.61
28	10.29	11.64	2.67	3.17	4.44
29	6.51	8.21	1.33	3.74	3.27
30	5.43	6.31	1.6	3	2

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	7.24	8.16	2.44	2.83	1.97
2	9.48	10.52	2.66	2.78	1.59
3	8.89	9.94	3.2	2.18	1.82
4	6.49	7.56	2.82	3	3.39
5	7.48	8.71	2.3	1.83	1.79
6	9.49	10.24	2	1.19	1.06
7	8.66	9.89	2.89	1.98	2.28
8	9.65	10.54	3.4	1.54	1.17
9	7.34	8.44	2.84	1.86	0.69
10	9.38	10.65	3.2	1.33	1.18
11	9.37	10.96	2.5	1.5	1.74
12	8.94	9.61	2.76	1.5	0.68
13	10.23	11.79	3.2	2	2.28
14	9.62	10.88	3.28	1.86	2.13
15	10.03	11.59	3	0.47	2.24
16	8.32	9.82	2.89	1.5	1.6
17	9.38	10.43	2	1.33	1.18
18	8.75	9.63	2.25	2.5	0.9
19	9.64	10.95	2.78	1.83	0.39
20	10.58	11.38	2.9	1.35	1.14
21	6.82	7.95	2.5	1.76	1.09
22	8.49	9.56	3.2	1.33	2.32
23	9.59	10.42	2.9	1.65	0.85
24	10.93	11.36	2.44	1	0.69
25	8.34	9.63	3.5	1.5	0.5
26	9.56	10.72	2.64	2.67	1.96
27	9.88	10.36	2.82	2.83	1.59
28	8.89	9.91	2.1	2.33	1.83
29	9.34	9.91	2.17	2.17	3.48
30	8.36	9.49	1.63	3	3.24

Biochemical Parameters in Group I

Sr. No.	CORTISOL CONCENTRATION (pg/ml)		GHRELIN CONCENTRATION (pg/ml)		SDS Score
	SERUM	SALIVA	SERUM	SALIVA	
1	16.89	18.89	598.64	776.83	39
2	7.413	16.98	563.86	675.74	48
3	5.855	35.97	514.94	778.93	32
4	7.668	31..36	732.72	1131.86	49
5	12.67	24.99	466.33	896.45	36
6	14.95	32.95	931.83	1537.69	46
7	14.89	21.11	659.74	826.54	43
8	4.923	26.88	530.99	664.88	44
9	17.855	25.48	247.81	489.21	42
10	5.654	22.56	237.16	398.33	44
11	9.612	31.99	487.76	654.24	38
12	5.431	15.63	541.12	976.45	42
13	12.99	18.91	366.61	804.63	39
14	14.65	23.34	349.83	713.51	43
15	9.47	25.56	609.55	763.92	44
16	16.26	29.93	530.92	630.25	49
17	8.167	27.62	769.61	890.66	48
18	11.592	28.26	611.91	951.78	44
19	18.39	18.88	520.76	772.64	36
20	12.92	29.64	281.68	327.86	43
21	19.28	24.31	560.81	769.93	47
22	22.48	29.05	480.89	674.36	41
23	24.67	13.36	846.69	985.67	48
24	4.9	18.34	692.98	784.12	43
25	8.195	26.79	471.45	968.17	44
26	5.619	21.88	680.76	790.72	38
27	8.34	26.93	560.78	962.81	36
28	10.67	27.86	327.72	478.56	32
29	6.231	22.65	691.93	892.66	43
30	8.671	19.91	558.95	649.33	48

Biochemical Parameters in Group II

Sr. No.	CORTISOL CONCENTRATION (pg/ml)		GHRELIN CONCENTRATION (pg/ml)		SDS Score
	SERUM	SALIVA	SERUM	SALIVA	
1	14.26	333.1	981.67	1355.36	41
2	16.36	432.8	300.91	682.42	50
3	10.26	267.9	820.76	1011.53	50
4	7.36	205.5	461.68	491.26	35
5	35.5	298.8	560.81	732.88	46
6	3.894	467.9	180.89	489.34	51
7	16.36	435.1	780.69	921.05	44
8	36.11	532.8	498.98	793.31	49
9	25.04	530.7	471.45	649.73	53
10	12.84	498.7	690.76	753.62	54
11	27.69	398.8	760.78	931.85	53
12	8.966	103.6	687.72	940.04	51
13	10.2	230.7	700.93	1093.15	55
14	35.5	405.5	480.95	1043.37	67
15	3.894	553.8	596.64	856.92	51
16	17.26	320.1	788.86	979.17	41
17	18.36	365.2	712.94	943.76	29
18	7.21	285.3	489.72	612.49	38
19	21.4	347.6	466.33	518.73	40
20	9.87	435.1	529.83	951.11	32
21	8.966	338.1	1278.74	1421.47	47
22	10.2	467.9	630.99	856.22	46
23	17.62	275.4	547.81	874.36	39
24	8.36	530.7	418.96	693.98	47
25	22.84	267.9	497.77	954.51	26
26	11.36	333.1	541.12	713.64	52
27	18.84	532.8	780.61	994.52	51
28	21.69	467.9	1058.83	1501.16	46
29	14.69	498.8	1359.55	1433.77	56
30	17.894	532.8	429.92	577.41	48

Biochemical Parameters in Group III

Sr. No.	CORTISOL CONCENTRATION (pg/ml)		GHRELIN CONCENTRATION (pg/ml)		SDS Score
	SERUM	SALIVA	SERUM	SALIVA	
1	10.87	353.12	298.64	476.83	69
2	24.18	502.84	563.86	675.74	63
3	11.109	267.93	514.94	678.93	67
4	13.89	115.56	432.72	531.86	59
5	25.89	298.89	466.33	596.45	69
6	37.21	190.15	331.83	437.69	57
7	7.13	567.95	359.74	626.54	55
8	25.19	332.83	230.99	364.88	67
9	5.868	530.71	247.81	489.21	51
10	31.06	255.78	240.96	398.33	43
11	25.67	798.81	587.76	654.24	37
12	15.65	115.66	541.12	676.45	60
13	5.731	390.78	366.61	404.63	61
14	17.88	405.53	649.83	713.51	60
15	26.71	353.86	609.55	763.92	61
16	24.76	560.16	530.92	630.25	65
17	31.88	700.22	369.61	490.66	62
18	26.34	105.33	611.91	951.78	38
19	22.32	253.65	520.76	672.64	58
20	22.37	210.17	281.68	327.86	46
21	25.62	505.13	560.81	669.93	58
22	29.63	367.94	480.89	674.36	39
23	28.54	875.42	246.69	485.67	69
24	24.69	245.76	292.98	384.12	59
25	6.749	767.97	471.45	566.17	64
26	34.63	537.14	680.76	790.72	52
27	21.47	538.85	560.78	630.81	51
28	5.842	536.93	327.72	478.56	59
29	10.87	420.57	232.93	290.66	67
30	23.75	686.84	558.95	649.33	48

Evaluation of Stress, Serum and Salivary Ghrelin and Cortisol Levels in Smokers and Non Smokers with Stage III Periodontitis

A Cross Sectional Study

CASE HISTORY PROFORMA

NAME: **OPD NO:**

AGE/SEX: **DATE:**

ADDRESS: **PHONE NO:**

OCCUPATION:

CHIEF COMPLAINT:

PAST DENTAL HISTORY:

PAST MEDICAL HISTORY:

DRUG HISTORY:

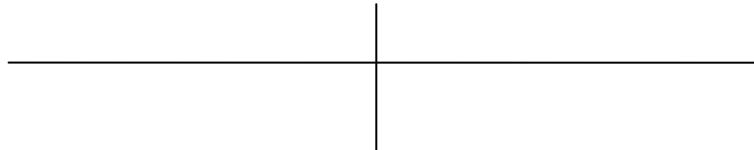
PERSONAL HISTORY :

ORAL HYGIENE HABIT:

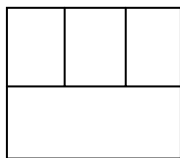
PHYSICAL EXAMINATION:

1. **HEIGHT** =
2. **WEIGHT** =
3. **BODY MASS INDEX** = $\frac{\text{Weight(kg)}}{\text{Height (cm}^2\text{)}}$ =

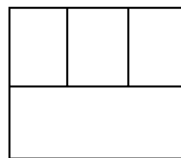
TEETH PRESENT:



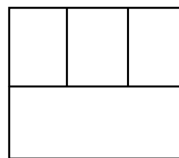
GINGIVAL INDEX ***(Loe&Sillness1963)***



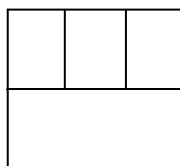
16



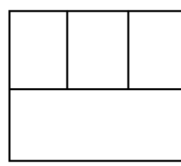
12



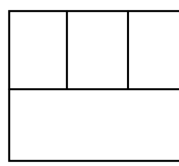
24



44



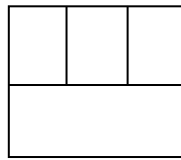
32



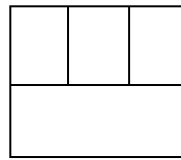
36

SCORE = $\frac{\text{Total scores of all teeth}}{\text{Total no. of sites examined}}$

PLAQUE INDEX



16



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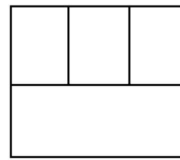

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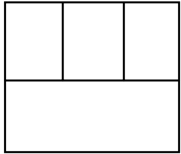


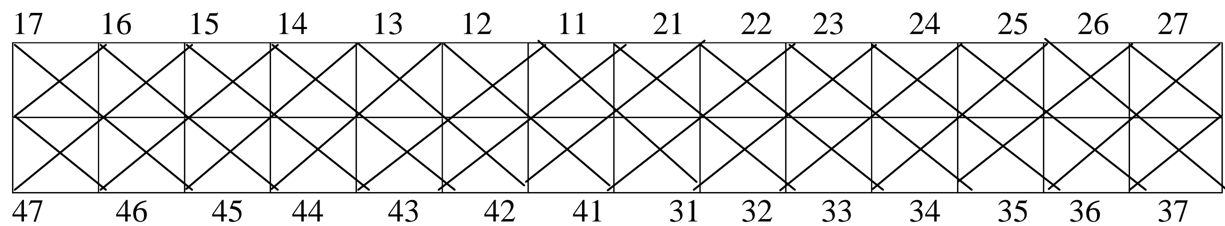
44

32

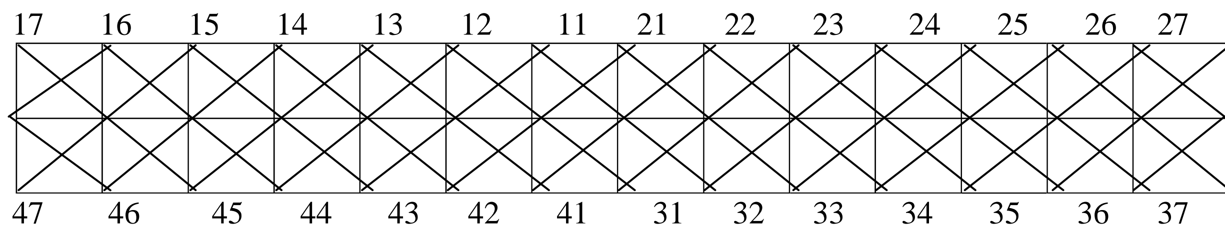
36

SCORE $= \dfrac{\text{Total scores of all teeth}}{\text{Total no.of sites examined}}$

PROBING POCKET DEPTH (mm):



CLINICAL ATTACHMENT LEVELS (mm):



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

Score: $\frac{\text{Totalscoresof all teeth}}{\text{Total numberof teethexamined}} =$

BIOCHEMICAL ANALYSIS :

Biochemical Parameters	Serum	Saliva
Ghrelin (ng/ml)		
Cortisol (ng/ml)		

CLINICAL DIAGNOSIS :

(Confidential)

Informed Consent Form

"Evaluation of Stress, Serum and Salivary Ghrelin and Cortisol Levels in Smokers and Non Smokers with Stage III Periodontitis"

Mr./Master/Mrs./Miss.____(optional)

Resident of: ____

__________________________________aged______years,

exercising 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.** _____________________

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.

____________	____________________	__________	__________
Patient's name	Signature/thumbprint	Date	Time
____________	____________________	__________	__________
Investigator's name	Signature	Date	Time