“COMPARATIVE EVALUATION OF SEMAPHORIN 4D, PEPTIDYLARGININE DEIMINASE 2 AND MATRIX METALLOPROTEINASE-8 LEVELS OF GINGIVAL CREVICULAR FLUID IN PERIODONTALLY HEALTHY AND SEVERE PERIODONTITIS SMOKER AND NON-SMOKER PATIENTS BEFORE AND AFTER NON-SURGICAL PERIODONTAL THERAPY.”

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LIST OF ABBREVIATIONS

Sr. No.	Short Form	Full Form
1	GCF	Gingival crevicular fluid
2	Sema 4A	Semaphorin 4A
3	Sema 4D	Semaphorin 4D
4	PAD	Peptidylarginine Deiminase
5	PPAD	*P. gingivalis* peptidyl-arginine Deiminase
6	RA	Rheumatoid Arthritis
7	MMPs	Matrix metalloproteinases
8	NSPT	Non-surgical periodontal therapy
9	SRP	Scaling and root planning
10	CRP	C-reactive proteins
11	WBC	White Blood Cell
12	PI	Plaque index
13	GI	Gingival index
14	PPD	Probing pocket depth
15	CAL	Clinical attachment level
16	BOP	Bleeding on Probing
17	ELISA	Enzyme-linked immunosorbent assay
18	CHD	Coronary Heart Disease
19	NCHD	Non Coronary Heart Disease
20	OHQoL	Quality Of Life
21	CP	Chronic Periodontitis
22	CVD	Cardiovascular disease
23	FMD	Full-Mouth Disinfection
24	TCI	Tongue-Coating Index
25	LF	Lactoferrin
26	α-1-AT	α-1-antitrypsin
27	α-2- MG	α-2 macroglobulin
28	IL	Interleukin
29	ACPA	Anti-Citrullinated Protein Antibody

Sr. No.	Short Form	Full Form
30	OA	Osteoarthritis
31	SF	Synovial Fluid
32	TIMP	Tissue Inhibitor Of Matrix Metalloproteinases
33	MPO	Myeloperoxidase
34	LPS	Lipopolysaccharide
35	EOP	Early onset periodontitis

INTRODUCTION

A consortium of inflammatory diseases that disrupts the attachment of connective tissue to the teeth along with the supporting bone is known as periodontitis. It is well acknowledged that the presence of pathogenic bacteria capable of producing disease is vital for the onset and progression of periodontitis. The disease progresses with the loss of collagen fibers, apical migration of the junctional epithelium, periodontal pocket formation and alveolar bone resorption. Despite the fact that bacteria causes periodontitis, the response of the host to the pathogenic indisposition is fundamental to the disease's progression[1,2].

Earlier in the 1960s and 1970s, studies aimed at understanding the pathophysiology of periodontal diseases, demonstrating the microbiological specificity of subgingival plaque at periodontitis sites. Later, pathogenesis conceptual models emphasized the impact of genetic and environmental factors, as well as an exacerbated inflammatory host immune response and dysbiotic plaque biofilm, in culminating to periodontal disease's destructive events[3].

Modern literature abridged that periodontal tissue degradation is influenced directly by the combination of bacterial toxic compounds, various systemic diseases, environmental factors and the immune system, which has been triggered by the bacterial infection and consequently, periodontal disease includes both active and quiescent stages of inflammation[4].

GINGIVAL CREVIVULAR FLUID IN PERIODONTITIS

Gingival crevicular fluid (GCF) is a somatic, physiological inflammatory exudate that originates in the gingival sulcus, from the gingival plexus of blood vessels and has been reported since the 19th century. The composition and flow of GCF in response to periodontal disease were further detailed in Waerhaug's classic study. Most researchers often considered GCF as an inflammatory discharge. Data suggests, however, the GCF from clinical healthy tissue is an altered serum transudate that only becomes an inflammatory exudate when disease is present. The understanding that neutrophils move into the periodontal crevice even in health serves to obscure the distinction between GCF as just an inflammatory exudate versus a physiologic transudate, leading to important breakthroughs in periodontology.

During an inflammatory response, a large number of cells as well as inflammatory mediators, including “cytokine”, “proteins”, “proteinases”, “phosphatases”, or “local tissue-degradation products”, were liberated into the GCF. The contents of GCF encompasses electrolytes, relatively small chemical compounds, proteins, cytokines, specific antibodies, bacterial antigens, and enzymes from both the host and the bacteria. The gingival sulcular epithelial lining is permeable to low-molecular-weight compounds, and tissue fluid entrance into the sulcus and pockets, functions as a hypothesized defence mechanism that may play a substantial part in the crevicular environment's homeostasis, as demonstrated by its composition[5].

Microbial colonisation transpires at gingival sites where specific organisms' metabolic, environmental and nutritional demands can be encountered and host defences can be conquered. According to Marsh (1994), disease is caused by groups of organisms rather than single species. Members of such groups may also dynamically adjust in the periodontal pocket/sulcular microenvironment by producing enzymes or other by-products, known as virulence factors that are essential for metabolism and/or physiological status quo maintenance[6].

The majority of medical fields are looking for significant biological potential marker that can indicate the existence of a disease state before it causes significant clinical damage. Due to developments in laboratory techniques and the search for periodontal disease diagnostic indications, GCF has been eluted and analysed exhaustively for the existence of host defence components, particularly molecules from blood, local host tissue, and plaque biofilm. Due to the sheer interactions between the biofilms as well as the cells of the periodontal tissues, GCF appears as an intriguing oral diagnostic fluid due to its simplicity of collection and capacity to sample many locations within the oral cavity at the same time. Also, according to research by Kinney et al (2014), the flow of GCF increases as the severity of gingival inflammation increases and has evidenced as a potential value to evaluate periodontal therapy efficacy[7, 8].

SEMAPHORIN 4 D AND PERIODONTITIS

Numerous debilitating ailments, such as cardiovascular, pulmonary, immunological, neurologic, and cancer, are linked to long-term inflammatory responses to a unique endogenous or exogenous antigen, or to inflammatory responses of unknown origin.

Recent research in the immune system include neuroimmune semaphorins 4A and 4D (Sema4A and SEMA 4D, respectively), as well as their critical regulatory activities in fine-tuning inflammatory processes.

Semaphorins are a collection of membrane-bound and released molecules that operate as axon guides in the nervous system. Owing to their presentation and function of the nervous system in both the neurological and immune systems, Sema4A and SEMA 4D were the very first semaphorin family members revealed to be transcribed on immune cells and had been labeled "immune semaphorins" then later "neuroimmune semaphorins." “Plexin D1”, “B1”, “B2”, and “B3”, as well as the unrelated plexin family “Tim-2” and “Neuropilin 1”, have been discovered to be functional receptors of Sema4A.

Plexin B1 binds to SEMA 4D, which regulates immunological responses and even the bone remodelling process. The invading mononuclear cells and microglia expressed SEMA 4D because one of its receptors, Plexin B1, showing the importance of the SEMA 4D mechanism in disease development. Plexin B1, a potent enhancer of cell survival, migration, and angiogenesis, found transported and triggered by SEMA 4D-mediated activation. Inflammation can aid angiogenesis, and new vessels can aggravate tissue inflammation. In inflammatory illness, aberrant angiogenesis is linked to the creation of lesions in chronic periodontitis, as well as disrupted tissue perfusion, improper ossification, and increased responsiveness to normal or pathological stimuli. So, researchers have piqued interest in them as potential immunotherapy agents in chronic periodontitis[9].

PEPTIDYL ARGININE DEIMINASES AND PERIODONTITIS

Porphyromonas gingivalis (P. gingivalis) causes dysbiosis in a formerly symbiotic microbiome by manipulating the immunological reactions of the host's cells and undermining host-microbe balance in long standing chronic periodontitis. The pathogen's ability to spread to the surrounding tissue is enabled by the breakdown of cellular barriers. Complex diseases such as periodontitis, rheumatoid arthritis (RA), atherosclerosis and alzheimer's disease has substantial association between oral pathogen contributions and disease pathogenesis, despite a lack of precise understanding of the pathways that lead to disease pathogenesis[10]. *P. gingivalis* has a wide range of virulence and potential pathogenic effects and can influence a huge spectrum of organs and disorders. The enzyme "*P. gingivalis peptidyl-arginine Deiminase"* (PPAD) is the virulence factor, which changes bacterial as well as the host proteins by deiminating arginine residues in proteins & peptides and converting them into citrulline. Protein citrullination causes dysregulation of the host's provocative signalling networks by changing the spatial organisation of the protein's original 3D-structure and function.

Due to genes encoding peptidyl arginine deiminases, a group of enzymes, and the host has intrinsic substrates of citrullination (PADs). The human peptidylarginine deiminase family has five isotypes ("PAD 1, 2, 3, 4/5, and 6") that have tissue specific expression. According to Badillo-Soto et al[11], citrullination of synovial proteins is assumed to be dependent on PAD 2 & PAD 4, and both of these enzymes have been detected in the RA synovium. RA patients also showed high levels of antibodies that predominantly bind to PAD 4-citrullinated fibrinogen, according to the study.

Protein citrullination occurs during inflammation, which includes pro-inflammatory stimuli and accelerated cell death. It's unclear how much citrullination takes place intracellularly as a consequence of calcium influx following membrane rupture. Citrullination has a role in a variety of physiological mechanisms, including skin keratinization, immune gene regulation, and other autoimmune and inflammatory illnesses. Both PAD 2 & PAD 4 positive cells displayed macrophage-like morphology, and labelling with PAD 2 or PAD 4 antibodies with an anti-CD68 antibody that most of PAD expressing cells appeared CD68 positive and hence myelomonocytic in origin[12].

MATRIX METALLOPROTEINASES AND PERIODONTITIS

Matrix metalloproteinases (MMPs), which can be detected in GCF, are a structurally related but genetically separate family of proteases that play a critical role through both physiological development as well as tissue remodelling along with pathological inflammatory & malignant tissue destruction. Even though MMPs are responsible for degradation of all extracellular matrix proteins, precise information on their presence, amounts, & activities is thought to be critical for determining active destruction, monitoring, as well as understanding and depiction of varying forms of periodontal disease. MMPs are classified into five categories:

1. “Collagenases (MMP 1, 8 & 13)”
2. “Gelatinases (MMP 2 & 9)”
3. “Stromelysins (MMP 3, 10 & 11)”
4. “Membrane-type MMPs (MMP 14, 15, 16 & 17)”
5. “Others.”

MMPs have the ability to degrade nearly both extracellular matrix as well as basement membrane components, resulting in periodontal tissue loss. Connective tissue remodelling and tissue matrix disintegration are governed by the synthesis of enzymes, activators, inhibitors, cytokines, and growth factors, and also cell–cell & cell–matrix interactions. MMPs, particularly MMP 8 and MMP-9, are primarily produced by neutrophils at infected sites[13].

MMP 8 is the most important collagenase in periodontal gingival connective tissue, accounting for 90–95 % of collagenolytic activity within GCF. Increased GCF collagenase activity and MMP 8levels were found to correlate with the levels of type I collagen degradation products in disease-active/untreated sites versus inactive sites from periodontitis patients and healthy volunteers, overcoming the protective barrier offered by tissue inhibitors of MMP-1 in disease-active/untreated sites versus inactive sites from periodontitis patients and healthy volunteers[14].

Lee et al. 1995 investigated the relationship in both connective tissue degradation & the concentration of active as well as latent MMP 8 expression throughout the inflammatory exudates of human periodontal lesions and found strong in vivo evidence for an active neutrophil collagenase role in the pathological destruction of periodontal connective tissue, suggesting that MMP 8 is a righteous marker for detection of periodontal sites with active periodontal disease[15].

SMOKING AND PERIODONTITIS

Smoking cigarettes is one of the most common environmental triggers causing periodontitis, as it affects both the risk and the prognosis. Smoking is the second most confounder for periodontal disease, after microbial dental plaque[16]. Smokers are more prone to develop potential periodontal infections, clinical attachment loss & bone loss, than nonsmokers. Smoking damages many aspects of acquired and innate immunity[17]. Smoking damages many elements of acquired as well as innate immunity. According to studies on how smoking permutes the host response and eventually culminates in the progression of periodontal tissue destruction, smoking results in impaired vascular function, neutrophil/monocyte interaction, antibody formation, adhesion molecule expression and inflammatory mediator & cytokine release, implying that phagocytes may be the key cells through which the effect of smoking is mediated. By generating alterations in mood-regulating neurobiological and brain pathways, chronic nicotine exposure is thought to increase subjective stress levels thus exacerbate a depressed mood[18].

Non-surgical periodontal therapy (NSPT) is the optimum benchmark in the treatment of periodontitis. Scaling and root planning (SRP) is a technique for mechanically removing bacterial biofilm and deposits, resulting in a healthy local environment and microbiota. Inflamed periodontal tissue are supplanted with collagen-rich, highly perfused connective tissues after NSPT and as s a result, gingival tissues contracts in the apical direction, toward the root surface. Clinical trials have indicated that NSPT is successful in the treatment of periodontitis, with improvements in inflammatory biomarker levels in the GCF, pocket depth, clinical attachment, & inflammatory biomarker levels in the GCF[19].

Previous research provided a correlation between SEMA 4D and peri-implantitis lesions, and PAD 2 and periodontitis, indicating that the pathological aspects of Rheumatoid Arthritis and periodontitis are homologous. But there is scarcity of literature available in association between SEMA 4D, PAD 2 and MMP 8 in association with periodontally healthy and periodontally compromised individuals. Therefore, the present study aims to evaluate and compare the GCF levels of SEMA 4D, PAD 2 and MMP 8 levels in periodontally healthy, severe periodontitis smoker and non- smoker patients before and after non-surgical periodontal therapy.

AIMS AND OBJECTIVES

The study was aimed to evaluate and compare the SEMA 4D, PAD 2 and MMP 8 GCF levels in periodontally healthy, severe periodontitis smoker & non-smoker patients before and after NSPT.

Certain objectives were also associated with this aim:

1. To evaluate the SEMA 4D, PAD 2 and MMP 8 in GCF levels in periodontally healthy, severe periodontitis smoker & non-smoker patients prior to NSPT.

2. To evaluate the SEMA 4D, PAD 2 and MMP 8 in GCF levels in periodontally healthy, severe periodontitis smoker & non-smoker patients after NSPT.
3. To compare the SEMA 4D, PAD 2 and MMP 8 in GCF levels in periodontally healthy, severe periodontitis smoker & non-smoker patients before and after NSPT.

REVIEW OF LITERATURE

For the sake of better understanding, the review of literature has been divided into five parts:

A. Review of studies on effects of NSPT and Periodontitis

B. Review of studies on association of Smoking and Periodontitis

C. Review of studies on association of Semaphorin and Periodontitis

D. Review of studies on association of Peptidylarginine Deiminase and Periodontitis

E. Review of studies on association of Matrix Metalloproteinases and Periodontitis

A. REVIEW OF STUDIES ON EFFECTS OF NON-SURGICAL PERIODONTAL THERAPY AND PERIODONTITIS

Bokhari SAH et al. (2009)[20] conducted a study on effect of NSPT on C-reactive proteins (CRP), fibrinogen, and white blood cell (WBC) counts in individuals with or without coronary heart disease (NCHD). To assess periodontal disease, periodontal markers like as BOP & PPD were evaluated, and all individuals got NSPT, which comprised oral hygiene recommendations as well as sub gingival SRP. The levels of inflammatory markers in the bloodstream were tested before and after periodontal therapy for one month. In patients with CHD or NCHD, the NSPT was observed to result in a significant reduction in BOP and PPD, as well as lower serum inflammatory markers. As a result, the risk of CHD in the treated patients may be decreased.

Kamil W et al. (2011)[21] determined that if individuals with advanced periodontitis, NSPT had an effect on CRP and blood lipid levels. Systemic levels of inflammatory markers were assessed at baseline and 3 months after NSPT in 60 systematically healthy patients who were randomly assigned to the treatment and control groups. The reduction in CRP was significantly, linearly, and directly linked with the reduction in PI, GI, and PPD in the therapy group.

Shah M et al. (2011)[22] assessed oral health related quality of life (OHQoL) of patients with CP by short-form oral health impact profile (OHIP-14) and its improvement after NSPT. The study divided 50 adults in 2 groups, periodontal disease was defined as having at least one proximal site with pocket depth ≥ 4 mm. After NSPT at an interval of 1 week, there was a significant improvement in OHIP-14 scores in the study group.

Bhardwaj S et al. (2015)[23] assessed the effect of periodontal disease and NSPT on plasma Hcy in systemically healthy subjects, based on the GI, PPD and CAL the subjects were divided in two groups namely healthy and CP. Samples were collected of plasma and were quantified at baseline and 12 weeks after SRP FOR Hcy using High performance liquid chromatography with fluorescent detection (HPLC-fld). The data led to the conclusion that NSPT could be used as an adjuvant Hcy lowering therapy, contributing to primary cardiovascular disease (CVD) prevention.

Silveira JO et al. (2016)[24] compared the effects of one-stage full-mouth disinfection (FMD) and traditional quadrant scaling in four weekly sessions (QS) on periodontal cinical parameters and halitosis in patients with advanced chronic periodontitis. The following measurements were taken at baseline and 90 days after therapy for each group: PI, tongue-coating index (TCI), BO, PPD, and CAL. The organoleptic approach was used for halimetry, and gas chromatography was used to assess the amounts of volatile sulphur compounds. The study found that non-surgical periodontal therapy, regardless of protocol, was beneficial in improving individual periodontal clinical state, lowering organoleptic scores and reducing halitosis.

Gul SS et al. (2017)[25], aimed to assess the ability of a novel combination of biomarkers to predict treatment outcome of patients with CP. At baseline, 3 and 6 months following therapy, GCF and subgingival plaque were collected from 77 individuals at three typical sites, one healthy and two diseased. Active enzyme concentrations ("MMP 8, elastase, and sialidase") in GCF, as well as subgingival plaque levels of "*P. gingivalis", "T. forsythia",* and *"F. nucleatum",* were used to predict the outcome measure. The "fingerprint" of GCF enzymes and bacteria revealed here can be used to predict the outcome of nonsurgical periodontal therapy on a site-by-site basis.

Cosgarea R et al.(2018)[26] compared the effect of NSPT on clinical and inflammatory parameters in patients with moderate to severe CP and rheumatoid arthritis (RA) with in CP patients without RA. 18 patients in each of RA-CP and systemically healthy patients with CP were treatment with SRP within 24 h. Clinical parameters, inflammatory markers, and microorganisms in sungingival biofilm were assessed at baseline, 3 and 6 months after NSPT also the disease activity markers of RA and specific antibodies were monitored in RA-CP group. The findings imply that NSPT improves periodontal conditions in CP patients with and without RA, and that eradication of P. gingivalis combined with good oral hygiene may decrease RA disease activity transiently in individuals with RA.

Van der Weijden GAF et al. (2019)[27] evaluated the results of active NSPT in ptients diagnosed with periodontitis. 182 patients with periodontitis received NSPT involving professional oral hygiene instructions, SRP, supra gingival polishing and elective systemic antimicrobial medication. The results were based on full mouth periodontal chart as assessed at time of evaluation. Possible relations with assessed parameters and success of active periodontal therapy were evaluated. Active NSPT in patients with adult periodontitis resulted in approximately one third of the cases in the success end point PPD >5mm. sub-analysis showed that the outcome appeared to be dependent on tooth type, furcation involvement, severity of periodontal disease at intake smoking status.

B. REVIEW OF STUDIES ON ASSOCIATION OF SMOKING AND PERIODONTITIS

Haffajee AD and Socransky SS (2001)[28] 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. 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. 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)[29] 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 CP in 15 smokers and 17 non-smokers. 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.

Hashim et al. (2001)[30] 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 can be considered as a strong predictor of periodontal disease prevalence in young adults.

Kamma JJ et al. (2004)[31] performed a cross sectional study to evaluate the influence of cigarette smoking on the 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. 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.

Gautam DK et al. (2011)[32] 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. When compared to non-smokers, smokers with periodontal disease had less clinical inflammation, gingival bleeding, and deeper periodontal pockets, according to the findings of this study. This study established that smoking is a significant environmental factor linked to periodontal disease.

Tymkiw KD et. al. (2011)[33] performed a study to compare the expression of 22 chemokines and cytokines in GCF from 20 smokers and 20 non-smokers with periodontitis and 12 periodontally healthy control subjects. 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); 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).

Kolte AP et al. (2012)[34] investigated and compared the relationship between psychological stress and obesity and periodontal disease in smokers and non-smokers. 90 patients, equally divided into three groups of non-smokers and periodontally healthy, non-smokers and smokers with untreated moderate-to-severe chronic periodontitis. Socioeconomic data, psychosocial measurements, physical parameters and clinical findings of PPD, CAL, PI and GI were recorded. The intra-group comparison of PPD and CAL in the three anxiety levels showed increased periodontal destruction with an increase in anxiety levels, the results being statistically highly significant for PPD differences in smokers ($P < 0.0001$). The results indicated a positive and strong correlation between anxiety, obesity and periodontal disease in smokers and non-smokers and smoking as further attenuate to this association.

Lappin DF et al. (2013)[35] evaluated the anti-citrullinated protein antibody (ACPA) responses may precede clinical onset of rheumatoid arthritis. Porphyromonas gingivalis peptidylarginine deiminase can citrullinate proteins possibly inducing autoimmunity in susceptible individuals. Determined whether periodontitis, carriage of *P. gingivalis*, smoking and periodontal therapy influence ACPA titres. Serum and plaque samples were collected from 39 periodontitis patients before and after non-surgical periodontal treatment, and from 36 healthy subjects. gingivalis titres were determined by ELISA. In subjects with periodontitis, *P. gingivalis* infection may be responsible for inducing autoimmune responses that characterize rheumatoid arthritis.

C. REVIEW OF STUDIES ON ASSOCIATION OF SEMAPHORIN AND PERIODONTITIS

Kolodkin AL et al. (1993)[36] reported that Semaphorin I (Sema I, formerly known as Fasciclin [Fas] IV) is produced on an epithelial stripe in the limb bud, where it guides two sensory growth cones as they abruptly turn upon encountering this sema I boundary. We reported the finding of two similar viral sequences, as well as the cloning and characterisation of two sema genes in Drosophila and one in humans, all of which generate proteins with conserved Semaphorin domains. Drosophila sema (D-Sema) I is a transmembrane protein, whereas D-Sema II and human Sema III are probable secreted proteins that are related to chick collapsin, which was recently discovered. Subsets of neurons and muscles express D-Sema I and D-Sema II. SemaII is an essential gene in Drosophila that is required for both proper adult behaviour and survival, according to genetic study.

Winberg ML et al. (1998)[37] reported that the Semaphorins are a wide family of secreted and transmembrane proteins, some of which act as axon guidance repellents. Semaphorins are divided into seven groups. Class III Semaphorins bind to Neuropilins, which are neuronal receptors. VESPR, a Plexin family member, is a receptor for a virally encoded Semaphorin in the immune system. Two Drosophila Plexins are identified here, both of which are expressed in the developing nervous system. Plexin A is a neuronal receptor for class I semaphorins (Sema 1a and Sema 1b), and it regulates motor and CNS axon guidance, according to our findings. Plexins, which have complete Semaphorin domains, could be the ancestors of traditional Semaphorins as well as Semaphorin binding partners.

Lallier TE. (2004) [38] examined the periodontal attachment cells (cementoblasts, osteoblasts, and periodontal ligament fibroblasts) are all descended from the same progenitor cell (the cranial neural crest). These cells detach from one another as they differentiate into distinct cell types, forming a laminated structure. Other neural crest derivatives use semaphorins (and their neuropilins and plexin receptors) as cell guidance molecules. The ability of these cells to segregate will be predicted to be correlated with the differential expression of these substances. In culture, human pre-osteoblasts are distinguished from PDL and gingival fibroblasts. These cells also express various semaphorins and plexins. Dermal fibroblasts expressed semaphorins 3D and 7A preferentially, while pre-osteoblasts expressed semaphorin 6B exclusively. Pre-osteoblasts displayed lower amounts of semaphorins 3B, 4C, 5B, and 6C, as well as plexins B1 and C1.

Differential expression of semaphorins and plexins may be involved in regulating cell sorting during the creation and regeneration of the periodontal attachment structure, according to the findings.

Yoshida Y et al. (2015) [39] studied to investigate the role of SEMA 4D in rheumatoid arthritis (RA).Soluble SEMA 4D (sSEMA 4D) levels in serum and synovial fluid were analyzed by enzyme-linked immunosorbent assay. Cell surface expression and transcripts of SEMA 4D were analyzed in peripheral blood cells from RA patients, and immunohistochemical staining of SEMA 4D was performed in RA synovium. Generation of sSEMA 4D was evaluated in an ADAMTS-4-treated monocytic cell line (THP-1 cells). The efficacy of anti-SEMA 4D antibody was evaluated in mice with collagen-induced arthritis (CIA).Levels of sSEMA 4D were elevated in both serum and synovial fluid from RA patients, and disease activity markers were correlated with serum sSEMA 4D levels. SEMA 4D-expressing cells also accumulated in RA synovium. Cell surface levels of SEMA 4D on CD3+ and CD14+ cells from RA patients were reduced, although levels of SEMA 4D transcripts were unchanged. In addition, ADAMTS-4 cleaved cell surface SEMA 4D to generate sSEMA 4D in THP-1 cells.

Chapoval SP et al. (2017) [40] reviewed Semap 4A and 4D in chronic inflammatory diseases and discovered that any debilitating disorders, such as cardiovascular, pulmonary, autoimmune, neurologic, and cancer, are caused by long-term inflammatory processes aimed towards a specific endogenous or foreign antigen, or of unknown etiology. The findings of neuroimmune sema 4A and 4D expression and activity in the immune system, as well as their critical regulatory functions in fine-tuning inflammatory processes, have piqued interest in them as potential immunotherapy agents. The authors overviewed knowledge of Sema4A and SEMA 4D effects in chronic inflammation underlying the disorders.

Bastos MF et al. (2018) [41] evaluated the gene expression levels of sema 3A, 3B, 4A, and 4D in both healthy and diseased implants. Subjects with peri-implantitis presented clinical attachment loss, probing depth ≥ 5 mm, bleeding on probing and/or suppuration, and radiographic bone loss > 4 mm. A real-time polymerase chain reaction was performed, and the gene expression levels of semaphorins in relation to the housekeeping gene were analyzed by using the nonparametric Mann-Whitney test. Thirty-five subjects (16 men, 19 women) with implant-supported restorations, using screw-shaped dental implants with internal or external hexagon were enrolled in this study. Differences between groups in the expression levels of Sem3B were not significant. Advanced peri-implantitis lesions showed higher levels of gene expression for Sem3A and Sem4D and lower levels of Sem4A in comparison to tissues obtained from a healthy dental implant.

Hu S. et al. (2018) [42] reviewed the role of those semaphorins which are a wide family of secreted, transmembrane, or GPI-anchored proteins that signal through their receptors, neuropilins, and plexins as axon guidance cues. The data reveals that they play a role in a wide range of pathophysiological disorders, including atherosclerosis, a vascular inflammatory disease, in addition to guiding. Endothelial dysfunction, leukocyte infiltration, monocyte-macrophage retention, platelet hyperreactivity, and neovascularization have all been shown to play a role in atherosclerosis by eliciting endothelial dysfunction, leukocyte infiltration, monocyte-macrophage retention, platelet hyperreactivity, and neovascularization. The involvement of those semaphorin family members in the formation of atherosclerosis, as well as the molecular relevance of semaphorins to atherogenesis are highlighted.

Veyisoglu G et al. (2019) [43] evaluated and compared the levels of Sema 4D, PAD 2 and MMP 8in GCF in Healthy, Gingivitis and Periodontitis Patients and investigate the effects of periodontal treatment on the levels of these molecules. GCF samples were collected from periodontally healthy controls, patients with gingivitis, and patients with chronic periodontitis, 20 patients in each group. Sema 4D, PAD 2 and MMP 8levels were determined by enzyme-linked immunosorbent assay and concluded that the GCF total amounts of the biomarkers may have a potential to be used as diagnostic tools for periodontal disease.

D. REVIEW OF STUDIES ON ASSOCIATION OF PEPTIDYLARGININE DEIMINASE AND PERIODONTITIS

Liu M et al. (2005)[44] identified and characterized p75 and examine for it in unfertilized oocytes and preimplantation embryos, and investigated the biological significance in fertilisation. With the ABL2 antibody, the protein was immunoprecipitated from ovarian lysates and evaluated by tandem mass spectrometry. Preimplantation development was delayed in 2-cell embryos treated in vitro with the ABL2 antibody or a PAD specific antibody, showing that cortical granule PAD plays a function in preimplantation cleavage and early embryonic development after its release. The PAD is found in mouse oocyte cortical granules, is released extracellularly after the cortical reaction, and remains linked with blastomere surfaces as a peripheral membrane protein until the blastocyst stage of development.

Foulquier C et al. (2007) [45] discovered out which PAD isotypes are expressed in the synovial tissue (ST) of RA patients and are involved in the citrullination of fibrin, which is the main synovial target of Autoantibodies to citrullinated proteins. Reverse transcription-polymerase chain reaction and immunoblotting were used to examine the expression of all PAD isotypes, including the recently described PAD type 6 (PAD-6), first in blood-derived mononuclear leukocytes from healthy donors, and then in ST samples from 16 RA patients and 11 control patients (4 with other arthritides and 7 with osteoarthritis [OA]). PAD 2 and PAD-4 expression levels were linked to the severity of inflammation (cell infiltration, hypervascularization, and synovial lining hyperplasia) in RA patients, and both enzymes were found within or near citrullinated fibrin deposits.

Arandjelovic S et al. (2012) [46] studied the posttranslational modifications affect physiology by altering protein function or influencing immunological identification of self-proteins. PAD 2 activity and substantial protein citrullination are induced when the inflammatory "danger" signal ATP activates the P2X7 purinergic receptor (P2X7). PAD 2 is activated by P2X7 and is sensitive to inhibitors of p38 MAPK and protein kinase C, and PAD 2 regulates the expression of the TNFR2, Adamts-9, and Rab6b transcripts in mast cells. PAD 2 expression in rheumatoid arthritis (RA) synovial tissue is connected to inflammation, and PAD 2 and citrullinated proteins are identified in the synovial fluid of RA patients. So, the findings imply that mast cell activation by P2X7 may contribute to inflammation by allowing PAD 2 and PAD 2 substrates to enter the extracellular space.

Damgaard D et al. (2014) [47] develop an assay for detecting PAD activity, if any, in RA patients' synovial fluid. The PAD activity was measured in synovial fluid samples from five RA patients using an enzyme-linked immunosorbent assay with human fibrinogen as the immobilised substrate for citrullination and anti-citrullinated fibrinogen antibody as the detecting agent. The calcium requirements for PAD 2 and PAD4 half-maximal activities were found to be in the range of 0.35 to 1.85 mM, and synovial fluid calcium levels were shown to be sufficient for the citrullination process to proceed. Citrullination of fibrinogen can occur in cell-free synovial fluid from RA patients, according to an assay with excellent specificity for PAD 2 activity.

Damgaard D et al. (2016) [48] assessed the presence of PAD 2 in the SF of RA and OA patients, as well as the relationship between PAD 2 levels, disease activity, and inflammatory markers in RA. A total of 39 RA patients and 40 OA patients had blood and SF samples taken. Flow cytometry was used to assess TNF-, IL-1, IL-6, IL-8, IL-10, and IL-12. All cytokine levels were higher in SF from RA patients than in SF from OA patients, as were PAD 2 levels and PAD activity. Anti-CCP-positive patients had greater SF PAD 2 levels than anti-CCP-negative individuals. In RA patients, PAD activity in the SF was higher than in OA patients, and it was associated to PAD 2 concentration. In RA patients, extracellular PAD 2 levels in the SF correlate with disease activity. PAD 2 levels are greater in anti-CCP-positive RA patients. In RA patients, extracellular PAD 2 levels in the SF correlate with disease activity. PAD 2 levels in SF are higher in anti-CCP-positive RA patients than in anti-CCP-negative RA and OA patients.

Engström M et al. (2018)[49] examined the leukotoxins produced by actinomycetemcomitans and Mannheimia haemolytica, followed by a triple-blind semi-quantitative analysis using immunohistochemistry. It was found out that there were no correlations between the presence of periodontal pathogens and the expression of citrullinated proteins or PAD enzymes in both epithelium and connective tissue from the different investigated individuals with and without periodontitis, and there were no correlations between the presence of periodontal pathogens and the expression of citrullinated proteins or PAD enzymes in the different investigated individuals with and without periodontitis. In periodontitis, chronic gingival inflammation is linked to increased local citrullination and PAD 2 and PAD 4 expression.

E. REVIEW OF STUDIES ON ASSOCIATION OF MATRIX METALLOPROTEINASES AND PERIODONTITIS

Ingman T et al. (1993) [50] investigated the GCF and saliva of patients with adult periodontitis (AP) and localised juvenile periodontitis (LJP)., the levels of MMP-1, -3, -8, and -9, as well as its endogenous inhibitor, tissue inhibitor of matrix metalloproteinases (TIMP-1), were MMP-1 levels were found to be higher in LJP GCF than in AP and control GCF. TIMP-1 levels were also found to be higher in LJP GCF than in AP and control GCF. In comparison to LJP and control GCF, AP GCF had higher MMP 8levels. The ELISA results show that I PMN MMP 8and MMP-9 are the main collagenase and gelatinase in AP GCF, whereas GCF collagenase in LJP appears to be of the MMP-1 type; (ii) only low levels of TIMP-1, an endogenous MMP inhibitor, are present in AP GCF, emphasising the importance of doxycycline as a possible adjunctive drug in the treatment of AP patients.

Hernández M et al. (2010) [51] studied the levels, molecular forms, isoenzyme distribution, and degree of activation of MMP 8and -14, myeloperoxidase (MPO), and tissue inhibitor of MMP (TIMP)-1 in GCF from patients with progressive periodontitis before and after periodontal therapy. GCF samples from active and inactive sites of periodontitis patients were examined at baseline for GCF levels of MMP-8, MMP-14, MPO, and TIMP-1 using immunofluorometric assays, specific activity assays, and enzyme-linked immunosorbent assays. At baseline, both active and inactive sites had high MMP 8and MPO levels, as well as a robust MPO/MMP 8positive correlation. MPO and MMP 8 levels decreased after treatment, with the exception of active locations, where MMP 8 changes were not significant.

In individuals with chronic periodontitis, GCF levels and correlations between MPO and MMP 8are linked to progression episodes and therapy responses.

Konopka L et at. (2012) [52] investigated the effects of scaling and root planing on the levels of interleukin (IL)-1, IL-8, and MMP 8in gingival crevicular fluid from patients with chronic periodontitis, as well as clinical parameters. The clinical measures were a plaque index, gingival index, pocket depth and clinical attachment loss. At baseline, 1 and 4 weeks following scaling and root planing treatment, periodontal parameters as well as gingival crevicular fluid humoral factor levels were assessed in the control group and chronic periodontitis patients.The nonsurgical therapy resulted in a significant improvement in periodontal indices as well as a significant reduction in gingival crevicular fluid levels of IL-1, IL-8, and MMP-8.

Yakob M et al. (2013) [53] analyzed a link between the presence of site-specific subgingival microbes and MMP 8and MMP-9 levels in GCF. The patient group included 56 people with periodontitis, while the control group included 43 people who did not have periodontitis. *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, and *T. denticola* were all detected using the polymerase chain reaction. The sick group had considerably more denticola in the test sites than the control group. In three of the four test sites, denticola was linked to an elevation in MMP-9 levels. The presence of subgingival microbes in GCF, notably T. denticola, seems to elicit a host response in the test sites, with elevated MMP 8and MMP-9 release.

Leppilahti JM et al (2014) [54] studied distinct GCF matrix metalloproteinase-8 patterns in smokers and non-smokers with CP and see if baseline GCF MMP 8levels can predict categorically assessed treatment outcomes. A total of 15 patients with CP were included in the study (five non-smokers and 10 smokers). Receiver operating characteristic curves were used to assess the capacity of baseline MMP 8levels to predict categorical treatment results. In both smokers and non-smokers, GCF MMP 8response patterns could be clustered into two separate site profiles. The categorical treatment result was significantly influenced by baseline MMP 8levels in smoking sites. The behaviour of MMP 8levels during the maintenance phase is substantially predicted by baseline GCF MMP 8levels. High baseline MMP 8levels at smoking sites imply a poor treatment response.

Sorsa T et at. (2016)[55] Matrix metalloproteinase-8 is a promising candidate biomarker for chair-side/point-of-care diagnostics using oral fluid (gingival crevicular fluid, peri-implant sulcular fluid, and saliva) and mouthrinse to predict, diagnose, and determine the progression of episodic periodontitis and peri-implantitis, as well as to monitor treatments and medications. MMP-8, alone or in combination with IL 1β and *P. gingivalis,* can be used to calculate a cumulative risk score at the subject level as a successful diagnostic tool, particularly in large-scale public health surveys where a thorough periodontal examination is not possible.

de Morais EF et al. (2018) [56] systematically reviewed the scientific literature on MMP 8expression in gingival crevicular fluid and SF in periodontal disease patients, as well as its utility as a putative biomarker in the diagnosis of periodontal disease.

Studies discussing the use of MMP 8 in the diagnosis of periodontal disease that examined its usefulness as a biomarker for periodontal disease were selected. MMP 8concentrations were shown to be considerably greater in patients with periodontal disease compared to controls, as well as in patients with more advanced stages of periodontal disease. The findings of greater MMP 8concentrations in periodontal disease patients compared to controls suggest that MMP 8could be used as a supplement in the diagnosis of periodontal disease.

Heikkinen AM et al. [57] evaluated the periodontal indexes (bleeding on probing, visible plaque index, root calculus, and probing depth), smoking by pack-years, periodontal bacteria *Aggregatibacter actinomycetemcomitans*, *P. gingivalis, T. forsythia, P. intermedia, P. nigrescens, and T. denticola*, and salivary periodontal biomarkers were(active MMP-8, polymorphonuclear leukocyte elastase and total protein, albumin, immunoglobulin A, immunoglobulin G, and immunoglobulin M.

MATERIALS AND METHODS

The goal of this interventional study was to determine the levels of GCF Sema 4D, PAD 2, and MMP 8in periodontally healthy, severe periodontitis smokers, and non-smokers before and after non-surgical periodontal therapy in periodontally healthy, severe periodontitis smokers, and non-smokers using Enzyme Linked Immunosorbent Assay (ELISA).

Study Subjects:

In this study, sixty patients between the ages of 18 and 60 were recruited from our Institute's Department of Periodontology. The Institutional Ethics Committee examined and approved the study design, which complies with the Helsinki Declaration. Those who chose to participate willingly provided informed consent prior to the start of the trial.

Sample size estimation:

Referring to the study by Veyisoglu G. et al. (2019)[43], the authors reported mean levels of SEMA 4D, PAD 2 and MMP 8in GCF in control, gingivitis and chronic periodontitis patients. These data were used to estimate the effect size. Based on the means for SEMA 4D across three patient groups, the effect size was 0.4843, while for PAD 2 was 0.3735 and for MMP 8was 0.5539.

The proposed study also has three groups namely: healthy, severe periodontitis non-smoker and sever periodontitis smoker. An effect size of 0.45 was considered appropriate, which resulted into a sample size of **51 (17 per group)** subjects to provide the desired effect with 80% power and 95% confidence level.

The formulation used was as follows:

The null hypothesis was:

H_0: $\mu_1 = \mu_2 = \mu_3$

i.e. the mean parameter values are equal across groups against the alternative that

H_1: $\mu_i \neq \mu_j$ ($i \neq j$ =1, 2, 3)

Accordingly, the sample size was obtained using formula:

$$n = \frac{(z_{1-\alpha/2\tau} + z_{1-\beta})^2}{ES^2}$$

where τ is the number of possible comparisons (3_{C_2} = 3 in the present case), $z_{1-\alpha/2\tau}$ (2.395) is the standardized value for 5% error and for 3 paired comparisons, $z_{1-\beta}$ (0.841) is the value for 80% power and ES is the effect size.

Statistical analysis

The descriptive statistics for the levels of three parameters will be obtained in terms of mean and standard deviation across study groups. The comparison of means will be carried out using *one-way analysis of variance* (ANOVA). The paired comparisons will be performed using *Tukey's post-hoc test*. All the analyses will be performed using SPSS ver 20.0 (IBM Corp) software and the statistical significance will be tested at 5% level.

Study groups

A dental and medical history was recorded for the selected patients and an intraoral examination was conducted by a single examiner. Subjects were then categorized into 3 groups of 20 subjects in each on the basis of periodontal parameters including Plaque index (PI) [Silness and Loe 1964][58], Gingival index (GI) [Loe and Silness, 1963][59], papillary bleeding index (PBI) score[60], Probing Pocket Depth (PPD) and Clinical Attachment Level (CAL).

1. **Group I (Periodontally healthy patients)**

Periodontally healthy patients with no clinical attachment loss (CAL), probing pocket depth (PPD) < 3 mm, and PBI score < 1.

2. **Group II (Non-Smoker)**

Non-Smoker patients with untreated stage III periodontitis, with PPD ≥ 5 mm and CAL ≥ 4mm, and radiographic evidence of interproximal bone loss.

3. Group III (Smoker)

Smoker patients with untreated stage III periodontitis, with PPD ≥ 5 mm and CAL ≥ 4mm, and radiographic evidence of interproximal bone loss. Patients with history of smoking at least 10 cigarettes per day for the last 3 years.

Diagnosis of periodontitis was based on according to the new Classification for Periodontal and peri-implant diseases and conditions in 2017 by American Academy of Periodontology[61].

Patient selection criteria

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) Non-smokers with untreated moderate to severe chronic periodontitis, as assessed by clinical finding of PPD ≥ 5mm and CAL ≥ 4mm. (≥30% of teeth affected) and with radiographic evidence of bone loss.

b) Patients with no history of smoking.

Group III

a) Current smokers with untreated moderate to severe chronic periodontitis, as assessed by clinical finding of PPD ≥ 5 mm and CAL ≥4 mm. (≥30% of teeth affected) and with radiographic evidence of bone loss.

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

Males and females were randomly selected in each group.

Exclusion criteria

1. Patients who had received periodontal treatment within the last 6 months.
2. Patients with any systemic diseases.
3. Patients who had or are on immunosuppressive chemotherapy.
4. Use of antibiotics within the last 6 months and anti-inflammatory drugs within the last 3 months.
5. Pregnant and lactating females.

Armamentarium

Following material and armamentarium was used for the assessment of clinical parameters and the collection of GCF.

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

For collection of GCF sample

1. 5μl micro capillary pipette
2. Sterile Eppendorf tubes (1.5ml)
3. Sterilized cotton rolls.

For evaluation of SEMA 4D, PAD 2, MMP 8levels in the gingival crevicular fluid

1. Human Semaphorin 4D (SEMA 4D) ELISA Kit
2. Human Peptidyl Arginine Deiminase Type II (PADI2) ELISA Kit
3. Human Matrix metalloproteinase 8/ Neutrophil collagenase (MMP-8) ELISA Kit

Assessment of periodontal and clinical parameters

1. Plaque index (PI): (Silness and Loe, 1964)[58]

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”

2. Gingival index (GI): (Loe and Silness, 1963)[59]

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 (William’s graduated) 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 color, slight edema, no bleeding on palpation"

"2"	"Moderate inflammation, redness, edema and glazing, bleeding on palpation"
"3"	"Severe inflammation, marked redness and edema, ulcerations, tendency of spontaneous bleeding"

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

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

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"

3. Papillary Bleeding Index (Muhlemann H.R 1977)[60]

A periodontal probe (UNC 15) was carefully inserted into the gingival sulcus at the base of the papilla on the mesial aspect, and then moved coronally to the papilla tip. This was repeated on the distal aspect of the same papilla. The intensity of any bleeding thus provoked was recorded on a 0-4 scale.

"Score Criteria"

0- "No bleeding"

1- "A single discrete bleeding point appears"

2- "Several isolated bleeding points / a single fine line of blood appears"

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

4- "Profuse bleeding occurs after probing; blood flows immediately into marginal sulcus"

Calculations

The papillary bleeding index score per person was obtained by totaling all of the papillary bleeding scores and dividing by the number of papillae examined.

4. Probing Pocket Depth (PPD)

It was measured using UNC 15 graduated periodontal probe on 4 sites of all present teeth. Patients were considered healthy if they exhibited probing depth < 3mm & there was no clinical attachment loss. Patients were diagnosed with chronic periodontitis if they exhibited PPD ≥ 5mm and Clinical attachment loss ≥ 5mm at multiple sites.

5. Clinical Attachment Level (CAL)

It was measured using UNC 15 graduated periodontal probe on 4 sites from the cementoenamel junction to the base of the pocket of all the present teeth. Patients were considered healthy if they exhibited no clinical attachment loss. Patients were diagnosed with chronic periodontitis if they exhibited clinical attachment level ≥ 5mm at multiple sites.

Laboratory armamentarium for assessment of biochemical parameters.

1. Calibrated volumetric transfer pipettes with disposable tips capable ofdispensing 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 VI)**
- Vortex mixer (CM 101, REMI Equipments Pvt. Ltd.) **(Color Plate VII)**
- ELISA microplate washer (LISA wash, REMI Equipments Pvt Ltd.) **(Color Plate VIII)**
- ELISA microplate reader (LISA Microplate reader, REMI Equipment's Pvt. Ltd.) **(Color Plate VIII)**

- **Assessment of Biochemical parameters**

Site selection and GCF collection:

Only one site per patient was selected on day 1 as a sampling site in Stage III periodontitis patients, non- smokers and smokers (group 2 & 3), whereas in healthy group multiple sites (3-5 sites per patients) with an absence of inflammation were sampled to ensure the collection of an adequate amount of GCF.

GCF was collected by placing the micro capillary pipette at the entrance of gingival sulcus and gently touching the gingival margin. A standardized volume was collected using calibration on white colour-coated 1-5μl calibrated volumetric micro-capillary pipettes.

Each sample collection was allotted a maximum of 10 minutes and sites that did not express any GCF within the allotted time were excluded. This was to ensure atraumatism and micropipettes that were suspected to be contaminated with blood and saliva were excluded from the study. Collected GCF samples were immediately transferred to airtight plastic vials (eppendorf tubes) and stored at -20°C until assayed. Healthy samples were collected at baseline and used for the analysis comparison between groups at both before and after non-surgical periodontal therapy.

Evaluation of SEMA 4D, PAD 2, MMP 8from GCF

Samples were assayed for SEMA 4D, PAD 2, MMP 8levels using commercially available ELISA (Enzyme linked immune-sorbent assay) Kinesis Dx ELISA Kits, Los Angeles, USA. Samples were analyzed according to the instruction manual at the Department of Biochemistry. Briefly GCF samples were diluted with dilution buffer in the kit and the amount of SEMA 4D, PAD 2, MMP 8was determined. All samples have been run in duplication.

Supplied Components in SEMA 4D, PAD 2, MMP 8Enzyme Immunoassay Kit

1. Microtiter Coated Plate (96 wells) – 1 no
2. SEMA 4D Ab Biotin Conjugate, 1 ml along with Standard 64 ng/mL – 0.5 ml
3. PADI2 Ab Ab Biotin Conjugate, 1 ml along with Standard 1600 pg/mL – 0.5 ml
4. HUMAN MMP 8Ab Biotin Conjugate, 1 ml along with Standard 12.8 ng/mL – 0.5 ml
5. Streptavidin: HRP Conjugate - 6 ml
6. Wash Buffer (30X) – 20 ml
7. Standard Diluent – 3 ml
8. Substrate A – 6 ml
9. Substrate B – 6 ml
10. Stop Solution – 6 ml

Additional materials required

1. Microplate reader capable of measuring absorbance at 450 nm.

2. Adjustable pipettes to measure volumes ranging from 50 µl to 1000 µl.

3.100 ml and 1 liter graduated cylinders.

4. Absorbent paper.

5. Distilled or deionized water.

6. Wash bottle or automated microplate washer.

7. Log-log graph paper or computer and software for ELISA data analysis.

8. Tubes to prepare standard or sample dilutions.

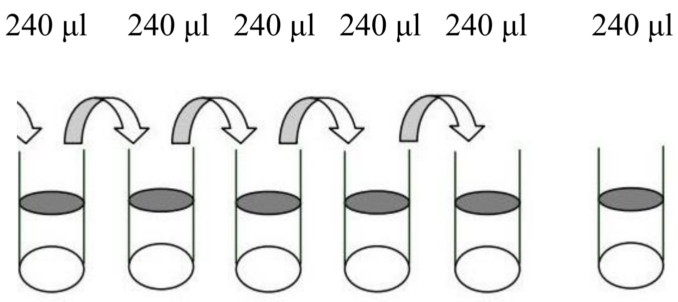
9. Incubator.

Reagent preparation for GCF samples

1. All reagents and samples are brought to room temperature (18 - 25ºC) before use.

2. To make 1X Wash Solution, add 10 ml of 30X Wash Buffer in 290 ml of DI water.

3. For standard preparation: a vial of 120µl Original Standard was briefly spun to which 120µl Standard diluent was added to prepare a 32 ng/ml standard. The solution was thoroughly dissolved by a gentle mix. Pipetting of 120µl standard into each tube was done. The stock standard solution was used to produce a dilution series (shown below). Each tube was thoroughly mixed before the next transfer.



		Std5	Std4	Std3	Std2	Std1
Diluent Volume	Original Standard +Standard diluent	240 µl	240 µl	240 µl	240 µl	240 µl
Conc.	64ng/ml	32ng/ml	16ng/ml	8ng/ml	4ng/ml	2ng/ml

Assay procedure

1. All reagents and samples were brought to room temperature (18 - 25ºC) before use.
2. Remove the number of strips required for the assay.
3. Pipette out 50 µl of Standards and 40 µl Samples into the respective wells as mentioned in the work list. Note do not add the sample, Biotin Conjugate and Streptavidin-HRP to the blank well.
4. Pipette out 10 µl of Biotin Conjugate into each sample well. Do not pipette into the blank and standards wells.

5. Pipette out 50 µl of Streptavidin-HRP Conjugate into each sample and standards well. Do not pipette into the Blank well.
6. Cover the plate and incubate for 1 hour at 37 °C in the incubator.
7. Aspirate and wash plate 4 times with 1X Wash Buffer and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. All the washes should be performed similarly.
8. Then add Substrate A 50 µl, then Substrate B 50 µl to each well including Blank well. Gently mixed, incubate for 10 min at 37 ℃ in dark.
9. Pipette out 50 µl of Stop Solution. Wells should turn from blue to yellow in colour.
10. Read the absorbance at 450 nm within 15 minutes after adding the Stop Solution blanking on the zero standards.

Assay Procedure Summary

All reagents and samples were brought to room temperature (18 - 25ºC) before use.

↓

Pipette out 50 µl of Standards and 40 µl Samples into the respective wells

↓

Pipette out 10 µl of Biotin Conjugate into each sample well.

↓

Pipette out 50 µl of Streptavidin-HRP Conjugate into each sample and standards well.

↓

Cover the plate and incubate for 1 hour at 37 °C in the incubator.

↓

Aspirate and wash plate 4 times with 1X Wash Buffer.

↓

Add Substrate A 50 µl to each well.

↓

Add Substrate B 50 µl to each well.

↓

Gently mixed, incubate for 10 min at 37 ℃ in dark.

↓

Pipette out 50 µl of Stop Solution.

↓

Read the absorbance at 450 nm within 15 minutes after adding the Stop Solution blanking on the zero standards.

SEMAPHORIN 4D ASSAY PRINCIPLE

This is sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human SEMA 4D in samples. Addition of standard or sample to microtiter well which is pre-coated with Human SEMA 4D monoclonal antibody and addition of biotin labeled Human SEMA 4D antibodies, followed by addition of HRP conjugate to form immune complex. Unbound HRP will get removed by washing step after incubation. Then addition of Substrate A and B, develops blue color during incubation period and reaction will get stop after addition of stop solution with development of yellow color. The concentration of the Human SEMA 4D of sample is directly proportional to the yellow color developed in well and will be positively correlated.

Calculation of Results

Taking the standard density as the horizontal axis, OD values in the vertical axis and drawing the standard curve on graph paper. Finding the corresponding concentration according to the sample OD value from the sample curve (the result is the sample concentration) or calculate the straight line regression equation of the standard curve with the standard concentration and the OD value, with the sample OD value in the equation, thus, calculating the sample concentration.

PEPTIDYL ARGININE DEIMINASE TYPE II ASSAY PRINCIPLE

This is sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human PADI2 in samples. Addition of standard or sample to microtiter well which is pre-coated with Human PADI2 monoclonal antibody and addition of biotin labeled Human PADI2 antibody, followed by addition of HRP conjugate to form immune complex. Unbound HRP conjugate will get removed by washing step after incubation. Then addition of Substrate A and B, develops blue color during incubation period and reaction will get stop after addition of stop solution with development of yellow color. The concentration of the Human PADI2 of sample is directly proportional to the yellow color developed in well and will be positively correlated.

Calculation of Results

Take the standard density as the horizontal axis, OD values in the vertical axis and draw the standard curve on graph paper. Find out the corresponding concentration according to the sample OD value from the sample curve (the result is the sample concentration) or calculate the straight line regression equation of the standard curve with the standard concentration and the OD value, with the sample OD value in the equation, calculate the sample concentration.

HUMAN MATRIX METALLOPROTEINASE 8 ASSAY PRINCIPLE

This is sandwich enzyme-linked immunosorbent assay (ELISA) to assay the level of Human MMP 8in samples. Addition of standard or sample to microtiter well which is pre-coated with Human MMP 8monoclonal antibody and addition of biotin labeled Human MMP 8antibodies, followed by addition of HRP conjugate to form immune complex. Unbound HRP will get removed by washing step after incubation. Then addition of Substrate A and B, develops blue color during incubation period and reaction will get stop after addition of stop solution with development of yellow color. The concentration of the Human MMP 8of sample is directly proportional to the yellow color developed in well and will be positively correlated.

Calculation of Results:

Take the standard density as the horizontal axis, OD values in the vertical axis and draw the standard curve on graph paper. Find out the corresponding concentration according to the sample OD value from the sample curve (the result is the sample concentration) or calculate the straight line regression equation of the standard curve with the standard concentration and the OD value, with the sample OD value in the equation, calculate the sample concentration.

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

Recall at 3 months in GROUP II and GROUP III

Probing depth at baseline in Group II

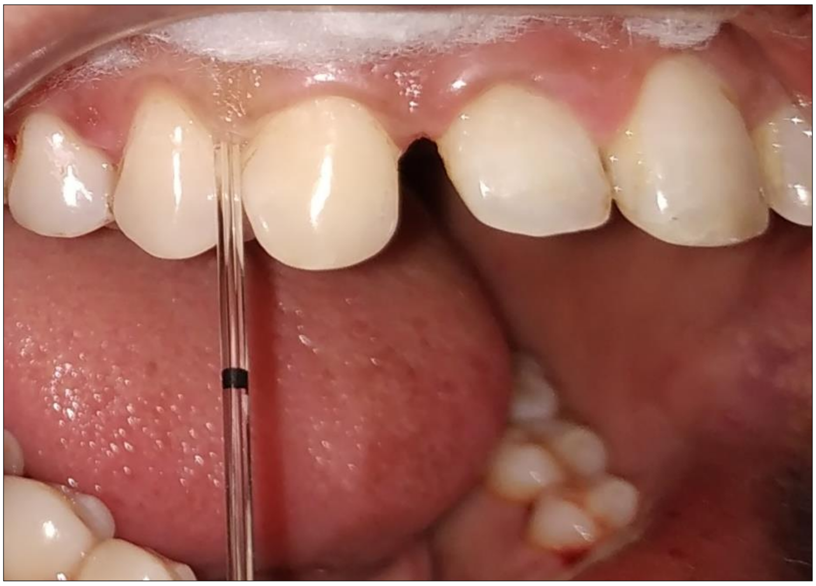
Probing depth at 3 months in Group II

Probing depth at baseline in Group III

Probing depth at 3 months in Group III

COLOR PLATE V

Armamentarium for clinical examination and GCF collection

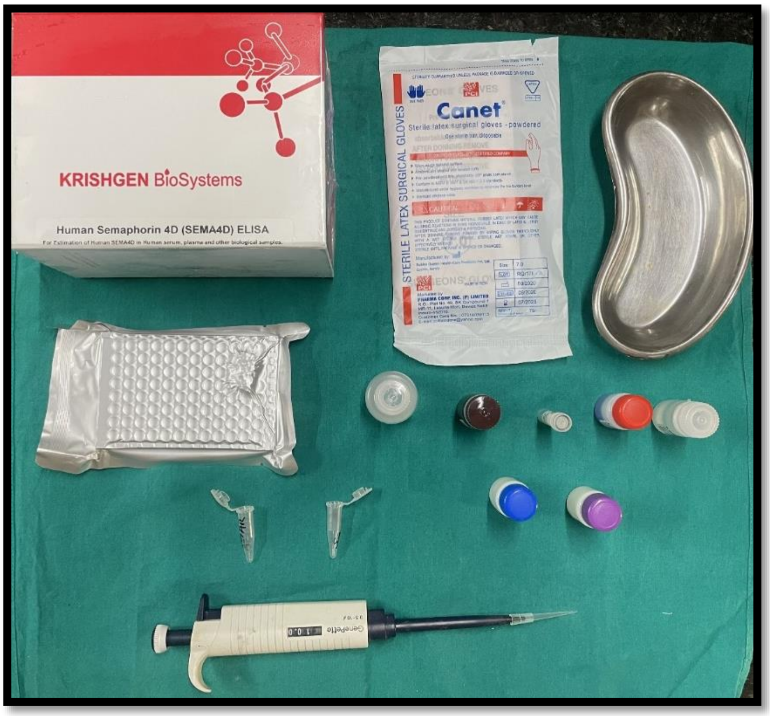


Collection of GCF samples

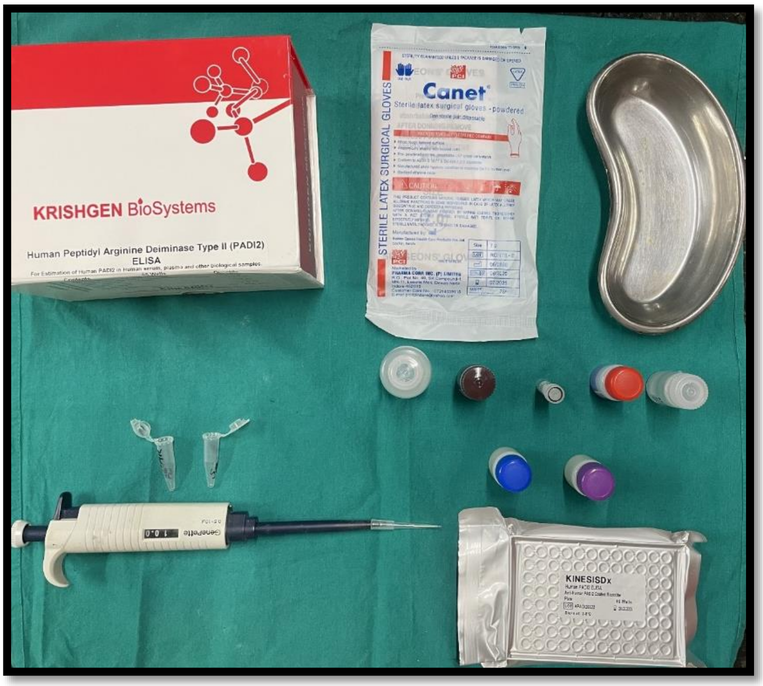
COLOR PLATE VI

Deep Freezer

COLOR PLATE VII



SEMA 4D ELISA Kits



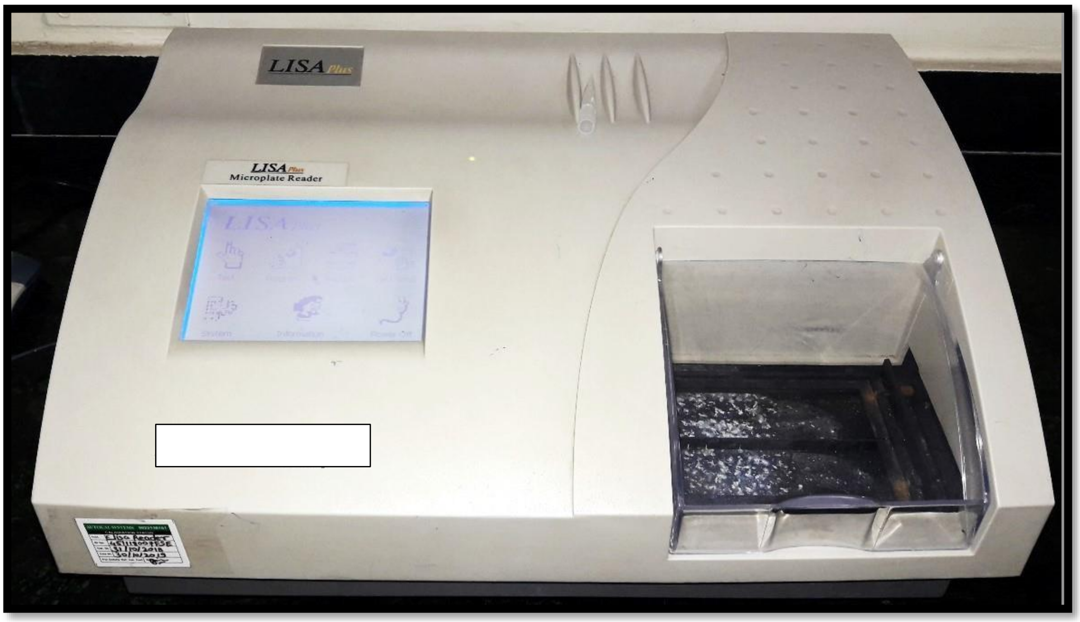
PAD 2 ELISA Kits

MMP 8 ELISA Kits

Vortex Mixer

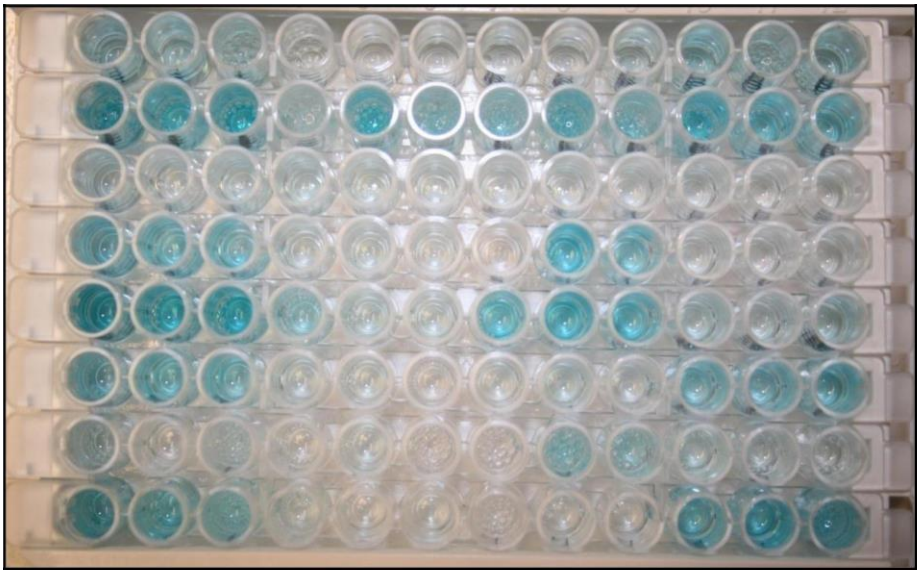
COLOR PLATE VIII

ELISA Washer

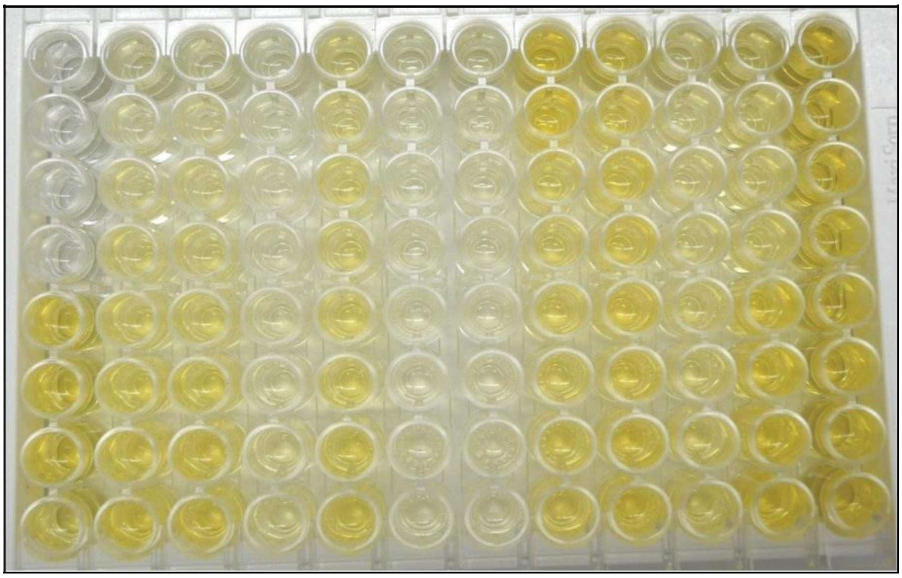


ELISA Reader

COLOR PLATE IX



ELISA plate after adding TMB substrate



ELISA plate after adding stop solution

RESULTS

The present study compared SEMA 4D, PAD 2 and MMP 8levels of GCF in periodontally healthy and severe periodontitis Smoker and non-smoker patients before and after Non-surgical periodontal therapy. Paired t-test was applied to assess the difference from baseline to 3 months, unpaired t-test was applied to assess the difference between two groups. One-way ANOVA test assessed the difference between three groups at baseline and 3 months which was followed by post-hoc Tukey test. The results obtained are presented below:

Table 1

The table represents distribution of patients with respect to gender in control and intervention groups. In healthy patients group; females were 11 (55%) and males were 9 (45%). In non-smokers with severe periodontitis group, females were 11 (55%) and males were 9 (45%). In smokers with severe periodontitis group, males were 20 (100%).

Graph 1

The bar diagram represents distribution of patients with respect to gender in control and intervention groups. The x-axis represents groups and the y-axis represents percentage of males and females in each group.

Table 2

The table represents distribution of patients with respect to age in control. In the age group of 18-22 years, there were 7 (35%) patients, in 23-27years age group, there were 6 (30%) patients and in the age group of 28-32 years, there were 7 (35%) patients.

Graph 2

The bar diagram represents distribution of patients with respect to age in control group. The x-axis represents groups and the y-axis represents percentage of patients in each age group. Patients were more in the age group of 18-22 years.

Table 3

The table represents distribution of patients with respect to age in intervention groups. In the age group of 38-42 years, there were 6 (30%) patients in non-smokers with severe periodontitis group and 1 (5%) patient in smokers with severe periodontitis group. Likewise, 43-47 years had 3 (15%) with non-smokers and 5 (25%) with smokers. 48-52 years age group had 5 (25%) non-smokers and 6(30%) with smokers, 53-57 years age group had 3 (15%) with smokers and 5 (25%) with non-smokers and 58-62 years age group had 3 (15%) with smokers and 3 (15%) with non-smokers.

Graph 3

The bar diagram represents distribution of patients with respect to age in intervention group. The x-axis represents groups and the y-axis represents percentage of patients in each age group. In the age group of 38-42 years the patients were more in the non-smokers with severe periodontitis group while in other age groups they were less or comparable.

Table 4

The table represents mean values of parameters for GI, PI, PPD, CAL and PBI in control group. Mean GI was 0.41 ± 0.19 with values ranging from 0.05 to 0.79. Mean PI was 0.61 ± 0.31 with values ranging from 0.03 to 1.19. Mean PPD was 1.33 ± 0.32 mm with values ranging from 0.58 to 2.02 mm. Mean CAL was 00.0 mm and mean PBI was 0.03 ± 0.02 % with values ranging from 0.00 to 0.09 %.

Graph 4

The bar diagram represents mean and standard deviation of the parameters assessed among healthy patients. The x-axis represents parameters; GI, PI, PPD, CAL and PBI and the y-axis represents the mean values of these parameters.

Table 5

The table represent mean values of parameters for GI, PI, PPD, CAL and PBI and difference in the values between at 3 months from baseline in non-smokers with severe periodontitis group. At baseline, the GI was 2.15 ± 0.19 which decreased 1.10 ± 0.19 to at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 1.04, t=1142 and p<0.0001. At baseline, the PI was 1.83 ± 0.19 which decreased to 0.54 ± 0.18 at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 1.29, t=184.71 and p<0.0001. At baseline, PPD was 6.86 ± 0.19 mm which decreased to 4.10 ± 0.19 mm at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 2.76 mm, t=1841 and p<0.0001. At baseline, CAL was 7.07 ± 0.19 mm which decreased to 4.88 ± 0.23 mm at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 2.20 mm, t=147 and p<0.0001. At baseline, PBI was 2.25 ± 0.19 which decreased to 0.28 ± 0.15 at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 1.97 %, t=106.92 and p<0.0001. The GI, PI, PPD, Cal and PBI score s significantly reduced from baseline to three months in non-smokers with severe periodontitis group.

Graph 5

The bar diagram represents mean values of GI, PI, PPD, CAL and PBI parameters at baseline and 3 months among non-smokers with severe periodontitis group. The x-axis represents parameters; GI, PI, PPD, CAL and PBI and the y-axis represents the mean values of these parameters.

Table 6

The table represent mean values of parameters for GI, PI, PPD, CAL and PBI and difference in the values between at 3 months from baseline in smokers with severe periodontitis group. At baseline, the GI was 1.72 ± 0.19 which decreased to 0.74 ± 0.18 at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 0.98, t=197 and p<0.0001. At baseline, the PI was 2.58 ± 0.19 which decreased to 0.60 ± 0.19 at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 1.98, t=791 and p<0.0001. At baseline, the PPD was 8.06 ± 0.19 mm which decreased to 5.60 ± 0.19 mm at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 2.46 mm, t=547.67 and p<0.0001. At baseline, CAL was 8.94 ± 0.19 mm which decreased to 6.85 ± 0.19 mm at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 2.09 mm, t=419 and p<0.0001. At baseline, PBI was 1.39 ± 0.19 which decreased to 0.25 ± 0.02 % at 3 months. There was a statistical significant difference present from baseline to 3 months with mean difference of 1.14 %, t=27.23 and p<0.0001. The GI, PI, PPD, CAL and PBI scores significantly reduced from baseline to three months in smokers with severe periodontitis group.

Graph 6

The bar diagram represents mean values of GI, PI, PPD, CAL and PBI parameters at baseline and 3 months among smokers with severe periodontitis group. The x-axis represents parameters; GI, PI, PPD, CAL and PBI and the y-axis represents the mean values of these parameters.

Table 7

The table represents difference in the control and intervention groups for GI, PI, PPD, CAL and PBI parameters at baseline. A significant difference was present between the groups for GI with F=431.10 and p<0.0001. A significant difference was present between the groups for PI with F=346.25 and p<0.0001. A significant difference was present between the groups for PPD with F=4351.80 and p<0.0001. A significant difference was present between the groups for CAL with F=7461.87 and p<0.0001. A significant difference was present between the groups for PBI with F=973.38 and p<0.0001.Smokers with CP exihibited higher values of PI, PPD, CAL while lower PBI, and GI scores as compared to non-smokers with CP.

Graph 7

The bar diagram represents the variation in the GI, PI, PPD, CAL and PBI parameters between healthy patients groups, non-smokers with severe periodontitis group and smokers with severe periodontitis group. The x-axis represents parameters; GI, PI, PPD, CAL and PBI and the y-axis represents the mean values of these parameters at baseline.

Table 8

The table represents difference between the groups using post-hoc Tukey test at baseline. A significant difference was present between all the three groups with respect to GI, PI, PPD, CAL and PBI. The difference for GI ranged from 0.42 to 1.73 with $p<0.0001$. For PI, the difference between the groups ranged from 0.75 to 1.96 with $p<0.0001$. For PPD, the mean difference ranged from 1.20 to 6.72 mm with $p<0.0001$. For CAL, the mean difference ranged from 1.87 to 8.93 mm with $p<0.0001$. For PBI, the mean difference ranged from 0.86 to 1.35 % with $p<0.0001$.

Table 9

The table represents difference in the intervention groups for GI, PI, PPD, CAL and PBI parameters at 3 months. A significant difference was present between the groups for GI with mean difference of 0.36 and $p<0.001$. No significant difference was present between the groups for PI with mean difference of 0.06 and $p=0.301$. A significant difference was present between the groups for PPD with mean difference of 1.50 mm and $p<0.0001$. A significant difference was present between the groups for CAL with mean difference of 1.97 mm and $p<0.0001$. A significant difference was present between the groups for PBI with mean difference of 0.03 % and $p = 0.070$. Smokers with CP exihibited higher values of PI, PPD, CAL while lower PBI, and GI scores as compared to non-smokers with CP.

Graph 8

The bar diagram represents the variation in the GI, PI, PPD, CAL and PBI parameters between non-smokers with severe periodontitis group and smokers with severe periodontitis group. The x-axis represents parameters; GI, PI, PPD, CAL and PBI and the y-axis represents the mean values of these parameters 3 months. The values were higher in the smoker followed by non-smokers with severe periodontitis.

Table 10

The table represents mean biochemical parameters in control group. The mean SEMA 4D was 200.91 ± 1.49 ng/mL with the values ranging from 198.61 to 204.23. ng/mL. The mean PAD 2 was 4300.30 ± 111.01 pg/mL with the values ranging from 4093.79 to 4507.55 pg/mL. The mean MMP 8 was 11.93 ± 0.75 ng/mL with the values ranging from 10.55 to 12.88 ng/mL.

Table 11

The table represents mean biochemical parameters in non-smokers with severe periodontitis group at baseline and 3 months. The mean SEMA 4D at baseline was 373.11 ± 1.42 ng/mL and at 3 months was 272.38 ± 1.49 ng/mL. The mean PAD 2 at baseline was 5225.95 ± 111.01 pg/mL and at 3 months was 5054.58 ± 111.01 pg/mL. The mean MMP 8 at baseline was 35.90 ± .89 ng/mL and at 3 months was 26.58 ± .75 ng/mL.

Table 12

The table represents mean biochemical parameters in smokers with severe periodontitis group at baseline and 3 months. The mean SEMA 4D at baseline was 465.76 ± 1.48 ng/mL and at 3 months was 279.99 ± 1.50 ng/mL. The mean PAD 2 at baseline was 5525.34 ± 111.00 pg/mL and at 3 months was 5138.16 ± 111.01 pg/mL. The mean MMP 8 at baseline was 40.67 ± 0.89 ng/mL and at 3 months was 31.35 ± 0.75 ng/mL.

Table 13

The table represents difference in the biochemical parameters from baseline to 3 months in non-smokers with severe periodontitis group. SEMA 4D scores significantly reduced from baseline to 3 months with a mean difference of 100.73 ng/mL, t=767.2 and p<0.0001. PAD 2 scores significantly reduced from baseline to 3 months with a mean difference of 171.36 pg/mL, t=31157 and p<0.0001. The MMP 8 scores significantly reduced from baseline to 3 months with a mean difference of 9.32 ng/mL, t=128.47 and p<0.0001.

Table 14

The table represents difference in the biochemical parameters from baseline to 3 months in smokers with severe periodontitis group. SEMA 4D scores significantly reduced from baseline to 3 months with a mean difference of 186.86 ng/mL, t=1414.89 and p<0.0001. PAD 2 scores significantly reduced from baseline to 3 months with a mean difference of 387.17 pg/mL, t=12441.38 and p<0.0001. The MMP 8 scores significantly reduced from baseline to 3 months with a mean difference of 9.32 ng/mL, t=128.46 and p<0.0001.

Table 15

The table represents difference in the biochemical parameters between the three groups at baseline. There was a significant difference in SEMA 4D between the group with F=17173.43 and p<0.0001. There was a significant difference in PAD 2 between the group with F=661.92 and p<0.0001. There was a significant difference in MMP 8 between the group with F=6513.65 and p<0.0001. The SEMA 4D, PAD 2 and MMP 8 were more in smokers with severe periodontitis group followed by non-smokers with severe periodontitis group and least among healthy patients.

Table 16

The table represents difference in the biochemical parameters between the three groups at baseline using pot hoc Tukey test. The mean difference between the groups at baseline for SEMA 4D ranged from 92.64 to 264.84 ng/mL. For PAD 2, the mean difference ranged from 299.38 to 1225.03 pg/mL with p<0.0001. The difference for MMP 8 was from 4.77 to 28.74 ng/mL with p<0.001. A statistical significant difference was present between the groups for all the biochemical parameters at baseline.

Table 17

The table represents difference in SEMA 4D at 3 months between the three group. There was a statistical significant difference in SEMA 4D biochemical parameter between healthy patients, non-smokers with severe periodontitis and smokers with severe periodontitis at 3 months with F=16980.56 and p<0.0001. SEMA 4D was higher in smokers with severe periodontitis followed by non-smokers with severe periodontitis ad least among healthy patients.

Table 18

The table represents difference in SEMA 4D between the groups at three months using post hoc Tukey test. There was a significant difference between healthy patients and non-smokers with severe periodontitis with a mean difference of 71.46 ng/mL and $p<0.0001$, between healthy patients and smokers with severe periodontitis with a mean difference of 79.07 ng/mL and $p<0.0001$ and between non-smokers and smokers with severe periodontitis with a mean difference of 7.61 ng/mL and $p<0.0001$. SEMA 4D was significantly more among smokers with severe periodontitis followed by non-smokers with severe periodontitis and least among healthy patients.

Table 19

The table represents difference in PAD 2 at 3 months between the three groups. There was a statistical significant difference in PAD 2 biochemical parameter between healthy patients, non-smokers with severe periodontitis and smokers with severe periodontitis at 3 months with $F=345.66$ and $p<0.0001$. SEMA 4D was higher in smokers with severe periodontitis followed by non-smokers with severe periodontitis ad least among healthy patients.

Table 20

The table represents difference in PAD 2 between the groups at three months using post hoc Tukey test. There was a significant difference between healthy patients and non-smokers with severe periodontitis with a mean difference of 754.28 and $p<0.0001$, between healthy patients and smokers with severe periodontitis with a mean difference of 837.86 and $p<0.0001$ and between non-smokers and smokers with severe periodontitis with a mean difference of 83.58 and $p = 0.049$. PAD 2 was significantly more among smokers with severe periodontitis followed by non-smokers with severe periodontitis and least among healthy patients.

Table 21

The table represents difference in MMP 8 at 3 months between the three groups. There was a statistical significant difference in MMP 8 biochemical parameter between healthy patients, non-smokers with severe periodontitis and smokers with severe periodontitis at 3 months with F=3617.35 and p<0.0001. SEMA 4D was higher in smokers with severe periodontitis followed by non-smokers with severe periodontitis ad least among healthy patients.

Table 22

The table represents difference in MMP 8 between the groups at three months using post hoc Tukey test. There was a significant difference between healthy patients and non-smokers with severe periodontitis with a mean difference of 14.65 ng/mL and p<0.0001, between healthy patients and smokers with severe periodontitis with a mean difference of 19.42 ng/mL and p<0.0001and between non-smokers and smokers with severe periodontitis with a mean difference of 4.77 ng/mL and p<0.0001. MMP 8 was significantly more among smokers with severe periodontitis followed by non-smokers with severe periodontitis and least among healthy patients.

Overall, with respect to the parameters like GI, PI, PPD, CAL and PBI; the scores significantly reduced from baseline to 3 months in both non-smokers with severe periodontitis group and smokers with severe periodontitis group. The difference between the parameters at baseline was significant with higher scores among smokers with severe periodontitis group followed by non-smokers with severe periodontitis group and the least among the healthy patients. Similarly, at 3 months, the difference in the PI, PPD, CAL was significantly higher between the groups with higher scores among smokers with severe periodontitis group while lower PBI, and GI scores as compared to non-smokers with severe periodontitis group and the least among the healthy patients.

With respect to biochemical parameters, the SEMA 4D, PAD 2 and MMP 8 significantly reduced from baseline to 3 months among smokers with severe periodontitis group and non-smokers with severe periodontitis group. At baseline the difference among the three groups was significant. Similarly, at 3 months there was a significant difference in biochemical parameters, the SEMA 4D, PAD 2 and MMP 8 with higher values among smokers with severe periodontitis group followed by non-smokers with severe periodontitis group and the least among the healthy patients.

DISCUSSION

Periodontitis is a supremely prevalent, multifactorial, chronic inflammatory disease of the periodontium and prolonged inflammation, connective tissue disintegration along with alveolar bone destruction are the hallmarks of periodontitis. The dynamic interaction between microbial activity and the inflammatory response of the host also contributes to the deterioration and degeneration of the tissues[1,2]. This complicated disease's origin, severity, and course are influenced by a variety of risk factors. Tobacco use is one of the most significant modifiable environmental triggers associated with severe periodontitis. Smoking has been demonstrated in several studies to aggravate periodontal disease. Cigarette smoke contains at least 400 potentially harmful compounds, include hydrogen cyanide, carbon monoxide (which forms carboxyhaemoglobin), free radicals, nicotine, nitrosamines (strong carcinogens), and a range of oxidant gases that promote platelet activation and endothelial dysfunction. Gingival bleeding and PPD have both been shown by the US Department of Health & Human Services to be unfavorable in regards of healing following non-surgical treatment, particularly in smokers as compared with untreated [62]. According to Grossi et al. (1997)[63], current smokers had poorer healing and decrease in *T. forsythia* & *P. gingivalis* following treatment than former and non-smokers.

The nicotine in cigarettes activates the sympathetic ganglia, which produces neurotransmitters such as catecholamines[64]. This activates the alpha receptors present on the blood vessels, producing vasoconstriction, which can further impact periodontal tissue since smokers have less overt indications of gingivitis than nonsmokers[65]. Nicotine's vasoconstrictive impact may be to blame for the reduced gingival blood flow. Bergstrom & Floderus-Myrhed (1983)[66] found that decreased gingival hemorrhage in smokers comparison to non was not just due to gingival vascular vasoconstriction, but may also be owing to smokers' higher keratinization of the gingivae.

A systemic immune response has been associated to periodontal infection. The inflamed epithelium, which serves as an easy entrance point for oral microorganisms, is one putative mechanism through which periodontitis might trigger a systemic inflammatory response. A potent bacterial endotoxin termed lipopolysaccharide (LPS) may be transferred throughout the body from the a periodontal lesion, and several pro-inflammatory mediators like IL-1,& 6, TNF-, MMP-8, and others are released in systemic circulation and also have an influence on distant organ systems.

Many severe diseases, notably cardiovascular, pulmonary, immunological neurologic and cancer, have been related to long-term inflammatory responses to a single endogenous or exogenous antigen, or even to inflammatory responses of unknown etiology. When considering a relationship among semaphorins and periodontium, Spencer et al. demonstrated that a subset of semaphorins were actively governed in PDL, as well as they stated that modifications in semaphorins could play a crucial part in periodontal remodelling, impacting angiogenesis or PDL cell invasion in to the injury sites[67].

P. gingivalis has a broad array of virulence and pathogenic potential, and can affect a wide range of organs and diseases. The virulence factor "P. gingivalis peptidyl-arginine Deiminase" (PPAD) deimininates arginine residues in proteins and peptides and transforms them to citrulline, hence altering both bacterial and host proteins. Protein citrullination causes dysregulation of the host's inflammatory signalling networks by disrupting the spatial structure of a protein's original 3D-structure & function. Inflammation, which includes pro-inflammatory stimulus plus rapid cell death, causes protein citrullination[12].

MMPs are a structurally similar but genetically separate family of proteases involved in both physiological tissue development reorganization, as well as pathologic inflammatory & malignant tissue destruction. MMP 8 is the most abundant collagenase within gingival connective tissue, accounting for 90–95 percent of GCF collagenolytic activity.

PPD, CAL, BI, PI, GI and other clinical parameters are the most frequently and extensively used indicators for assessing disease status. However, due to low sensitivity and positive predictive value, they only provide information on previous periodontal tissue damage and do not indicate present disease activity or anticipate future activity. As a result, cytokines, enzymes and other proteins have all been tested as possible biomarkers. They have proven to be an effective, rapid, diagnostic and monitoring tool, having the ability to screen for periodontal disease susceptibility, diagnose the condition, assess treatment response, forecast future tissue loss and track disease development. These biomarkers can be found in several biologic fluids such as GCF, blood, serum or plasma and saliva. As GCF collection is non-invasive, it has been intensively investigated in the quest for possible periodontal disease diagnostic biomarkers. GCF proves to be an appealing oral diagnostic fluid due to its ease of collection and ability to sample numerous sites concurrently as a result of the interaction of GCF with the bacterial biofilm and the cells of periodontal tissues[28, 68].

GCF is an inflammatory exudate that flows from the periodontal pocket (or gingival sulcus). Non-inflamed areas possess low volumes, which tend to grow as the gingival and periodontal tissues become more inflamed. GCF is a complex mixture of chemicals originating from serum as well as locally generated inflammatory and tissue turnover/breakdown mediators. It also comprises bacterial and cellular components (neutrophils). GCF protects the host against bacteria by having a physical protective effect (dilution of bacteria and their products, plus fluid outflow) and transporting antibacterial chemicals into the pocket.

The amount of GCF flowing from a site is influenced by the level of ulceration of the sulcular/pocket epithelium, as well as the degree of inflammation in the tissues. The volume of GCF and degree of inflammation, on clinical evaluation were found to be substantially correlated and it has been documented that the flow of GCF is proportional to the severity of inflammation, thereby emphasizing its significance as an assessment tool.

Thus, SEMA 4D, PAD 2, and MMP 8 are inflammatory markers that have been linked to inflammatory diseases as well as periodontitis. So the current study was designed with the hypothesis that there is a difference in SEMA 4D, PAD 2, and MMP 8 levels in GCF in periodontally healthy, severe periodontitis smokers and non - smokers patients before and after non-surgical periodontal treatment.

The study included 60 patients, both genders, split into three groups of 20 each:

“Group I: Periodontally Healthy patients.”

“Group II: Non -Smoker Patients with stage III periodontitis.”

“Group III: Smoker Patients with stage III periodontitis.”

The diagnosis of Severe Periodontitis was made based on the American Academy of Periodontology's revised classification for Periodontal & periimplant diseases and disorders published in 2017.

In the current study, participants in all groups received NSPT. At baseline & 3 months following phase I treatment, clinical measures such as PI, GI, PPD, CAL, and PBI were measured.

Patient's ages ranging from 18 to 60 were included in the study. Group III had only men (smokers having Stage III periodontitis). Smokers showed significantly greater PPD, CAL, and PI levels than nonsmokers. Feldman et al. (1982)[69] discovered a connection between chronic periodontitis and six periodontal indicators in cigarette smokers (calculus deposit, plaque accumulation, gingival inflammation, PPD, alveolar bone loss, and tooth mobility

Cigarette smokers had shown much more deposition of calculus, PPD, plaque accumulation, as well as alveolar bone loss, according to a previous studies. The toxic effects of nicotine in cigarettes on the periodontium could be possible explanation for the higher severity of periodontal disease amongst smokers. In smokers[70], nicotine binds to the root surface, and in vitro studies demonstrate that it can affect fibroblast attachment[71], integrin expression, and collagen synthesis[72]. The impaired immune system in smokers is responsible for the increased periodontal damage in Group III patients. Cigarette smoking, nicotine, and its by-products have a vasoconstrictive effect on the coronary and gingival vasculature, including the peripheral circulation. Furthermore, smoking has been shown to diminish the functional activity of leukocytes & macrophages in saliva and GCF, as well as decrease chemotaxis and phagocytosis of blood and tissue polymorphonuclear (PMN) leukocytes, lowering phagocyte-mediated protective responses to periodontal pathogens[73]. Tobacco smoking also inhibits the oxidation-reduction potentials in tooth plaque in the short term. A reduction in PMN mobility and also an increase in the number of anaerobic bacteria in dental plaque are linked to low oxygen levels[74].

SEMA 4D is a semaphorin that plays an important function in the immune system. It would be the first semaphorin to be found as a T-cell activation signal as well as a function in B-cell aggregation. SEMA 4D levels in blood, synovial tissues, & fluids were found to be considerably greater in RA patients than in healthy controls, with such a clear relationship among these levels as well as disease activity ratings in RA patients. SEMA 4D has been demonstrated to increase inflammation by increasing TNF- and IL-6 production[39]. Bastos et al[41] discovered that samples taken from progressive peri-implantitis diseases (bone loss 4 mm and PD 5 mm coupled by BOP and/or suppuration) would have more SEMA 4D than clinical specimens from healthy implants. In our study, the GCF total levels of SEMA 4D in the Group II & Group III at baseline 373.11 ± 1.42 ng/mL and 467.76 ± 1.42 ng/mL respectively which reduced to 272.38 ± 1.49 ng/mL and 279.99 ± 1.49 ng/mL after NSPT at 3 months. Given the comparable aetiology of periodontitis and peri-implantitis, our findings were consistent with those of Bastos et al[41] NSPT has been shown in the literature to improve periodontal clinical status and lower serum inflammatory markers in patients having advanced chronic periodontitis & improve patient quality of life, as recently demonstrated by some clinical trials, which reinforces our findings[20,22,24].

According to these findings, SEMA 4D could play an important role in both periodontal inflammation and destruction. According to several research, SEMA 4D has been connected to B-cell proliferation and the production of proinflammatory cytokines by human monocytes, as well as specific antibodies by B cells[75,76]. These findings could also explain why the GCF total levels of SEMA 4D in Groups III and II were greater than in Group I. The presence of SEMA 4D in the healthy group may indicate a condition of subclinical gingivitis with no clinical indications of inflammation.

SEMA 4D was also found to be substantially associated to different malignancies, significantly identified in the plasma of individuals with & neck cancer. Lung cancer, the third most frequent malignancy, has been related to cigarette smoking for several decades. Plexin B1 inhibition experiments revealed that the ligand SEMA 4D increases the invasive capacity of cancerous cells. The activation of the different pathways by SEMA 4D/PlexinB1 signalling increased cell invasiveness. The smoking-related mechanisms of neoplasia, demonstrates that smoking induces the mitotic genes involved in development of cancers. These findings point to a link between high SEMA 4D expression and decreased rates of cancer patient survival[77].

PAD 2 expression was also found to be linked to the severity of inflammation (cell infiltration, hypervascularization, and hyperplasia of the synovial lining)[45]. PAD 2 levels in RA patients' synovial fluid were connected with disease severity, circulating CRP, & ACPA levels, according to Damgaard et al. 2016[48]. In studies related to periodontology, it has been revealed that periodontitis stroma exhibits a higher presence of citrullinated protein (80%) when compared to control stroma (33%) Furthermore According to the findings, the creation of citrullinated proteins inside the periodontal stroma seems being an inflammation-dependent process[78]. Another study discovered that untreated periodontitis patients had greater ACPA antibody titer than healthy controls[35].

According to Engstrom et al., there had been increased expression of PAD 2 in periodontitis patients' gingival connective tissue relative to the periodontal healthy subjects, but comparable levels of PAD 2 inside the gingival epithelium of the 2 groups[79]. Given the similarities in the pathophysiology of RA and CP, a near certainty to this phenomenon is contemplated. According to the findings, citrullination is a natural process in periodontal epithelium and PAD 2 detection in the healthy periodontium group could be linked to this condition or a subclinical gingivitis lesion.

Because *P. gingivalis* possesses a functioning PAD enzyme that is very humanlike PADs, infection could induce antibody formation against human PADs. PAD 2 levels in CP patients were likewise greater in the cigarette groups in comparison to a nonsmoker group[78]. Because smoking raises the likelihood of periodontitis, a host might well be exposed to a larger burden of *P. gingivalis*, leading in a continual antigen trigger in comparison to nonsmokers. In our study, for group III, the mean baseline PAD 2 levels were 5525.34 ± 111.00 pg/mL which reduced to 5138.10 ± 111.01 pg/mL at 3 months. For group II, the mean baseline levels were 5225.95 ± 111.01 pg/mL which reduced to 5054.58 ± 111.01 pg/mL at 3 months. PAD 2 was significantly more among smokers with severe periodontitis followed by non-smokers with severe periodontitis and least among healthy patients. The PAD 2 levels were reduced after phase 1 periodontal therapy, along with significant improvements in clinical parameters. This finding could be considered as additional evidence for PAD 2's participation in periodontal inflammation. Consequently, SEMA 4D and PAD 2 appeared to be linked only to the severity of inflammation and not to the depth of the sulcus/pocket.

Several studies have shown that autoimmunity has a role in periodontal disease. The bulk of observations show antibodies against host components, mainly collagen, but antibodies against DNA and aggregate immunoglobulin G were also recorded. SEMA 4D and PAD 2 levels were observed to be considerably increased in RA patients with an autoimmune illness[37,38]. Tobacco smoking is linked to a number of autoimmune disorders, including systemic lupus erythematosus, crohn’s disease, and multiple sclerosis, and also one of the environmental variables that predisposes to RA[80]. Tobacco smoking causes citrullination of tissue proteins, which is considered to be the fundamental mechanism by which smoking causes and worsens auto-immune diseases[81]. Another mechanism through which this hazardous effect of smoking occurs was postulated by Kobayashi et al. (2008)[82]. These researchers have found that tobacco smoking triggers an inflammatory cytokine response, particularly IL-1β and TNFα. Hydrocarbons (the major ingredient of tobacco smoke, This inflammatory reaction, which is most evident in synovial fluid, is driven by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Furthermore, smokers' lungs have higher PAD expression and activity. Because PAD is located intracellularly as well as citrullinated proteins were discharged into to the extracellular after apoptosis, this might be the case. PAD enzymes have been shown to produce citrullinated proteins in periodontitis tissue samples[78]. These findings reinforce the findings of our study and could endorse the concept that autoimmunity has a role in periodontal disease. As a result of the relationship between PAD 2 levels and the severity of inflammation in periodontal pockets. This enzyme might be utilised to assess the degree of inflammatory changes in periodontal pockets as well as the activity of the RA illness.

MMP 8 is a key biomarker in the breakdown of connective tissue that happens in periodontitis. MMP 8 is found as a dormant pro-enzyme inside the GCF of shallower periodontal pockets, but it is converted to active metabolite in deeper periodontal pockets. MMP 8 levels have been found to be consistently high in periodontal at risk of irreversible tissue damage[83]. In our study, for group III, the mean baseline MMP 8 levels were 40.67 ± 0.89 ng/mL which reduced to 31.35 ± 0.75 ng/mL after NSPT at 3 months. For group II, the mean baseline levels were 35.90 ± 0.89 ng/mL which reduced to 26.58 ± 0.75 ng/mL at 3 months which were in accordance with Romero AM et al., who observed that MMP-8 levels inside the GCF of CP patients decreased considerably following NSPT[88].

Furthermore, total MMP 8 levels in the GCF were significantly greater in the CP smoking group than those in the non-smoker. Liu KZ et al. got comparable findings in 2006, that are compatible with the current investigation, which found higher MMP 8 levels in smokers' GCF compared to nonsmokers' with CP[84].

In comparison, Mäntylä P et al in 2006,[83] found GCF MMP-8 concentration at baseline to be 3997 31 ng/mL, while Taalab, M.R et al in 2021,[90] found MMP 8 concentration to be 2.00 1.60 ng/mL. Two investigations found comparable GCF MMP-8 concentrations of 306.34 ± 255.97 ng/mL [90] and 331.50 ± 299.70 ng/mL[91].

As according Heikkinen et al. smoking has a dose-dependent effect on biomarker results. Former smokers had been found to have comparable amount of active MMP 8 (aMMP 8) as nonsmokers. Obesity was also discovered to be a confounding factor. When body mass index data were taken into consideration throughout the study, aMMP 8 levels in nonsmokers did not stay statistically significant. The values, however, were unaffected in the case of men smokers[57].

In contrast to these investigations, Passoja et al. & Miller et al. found no significant link between smoking and higher aMMP 8 levels in their independent saliva and GCF analyses, respectively[85,86].

According to the findings of a research conducted by Gursoy et al[87], aMMP 8 levels were greater in nonsmoking patients with periodontitis than controls, and the only statistically meaningful parameter in smokers was TIMP-1 level, which could discriminate among periodontitis patients and controls.

Subgingival bacteria, namely T. denticola, appeared to increase salivary albumin, total total protein in saliva, as well as MMP 8 levels in GCF. T.denticola and T. forsythia are likewise suspected of inducing a cascade-type host response in GCF, with increased MMP 8 release and activation. Proteases generated from *T. denticola* and *P. gingivalis* (dentosilin and gingipain, respectively) may activate & convert latent pro-MMP 8 to aMMP-8 proteolytically and effectively.

We assessed overall MMP 8 (active and latent) in this study and found a strong positive connection between SEMA 4D and MMP 8. As a result, SEMA 4D may be linked to periodontal tissue damage (e.g., collagen and extracellular matrix).

The "biologic systems model" was developed by Offenbacher et al[93] as a diagnostic periodontal disease categorization approach. The model is based on medical and dental results, and also contributing biologic characteristics. According the underlying "biologic phenotypes," the biofilm and the host's immune and inflammatory response are thought to be at the biofilm-gingival interface. The biologic system model itself is built on a framework of components, beginning with recognition of subject-level exposures conversing with genetic and epigenetic factors as well as progressing to also include molecular and cellular processes as well as inflammatory markers in order to describe diverse clinical phenotypes of periodontal disease prediction and diagnosis.

The findings suggest that GCF SEMA 4D, PAD 2, and MMP 8 biomarkers can be used as predictors of periodontal disease development. These biomarkers may be used in larger patient populations to predict periodontal disease development or stability, as well as to identify susceptible sites for future destruction.

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SUMMARY AND CONCLUSION

The current study was conducted to assess the levels of GCF SEMA 4D, PAD 2, and MMP 8 found to be increased in smokers having stage III periodontitis

A total of sixty subjects were enrolled and divided into three groups with 20 patients in each group. Group I included healthy individuals who showed no symptoms of periodontal disease, Group II included nonsmokers with stage III periodontitis, and Group III included smokers (patients with history of smoking at least 10 cigarettes per day for the last 3 years) with stage III periodontitis. All the subjects were assessed clinically and biochemically for categorization into respective groups.

Clinical parameters evaluated were PI, GI, PBI, PPD, and CAL. A significantly high PPD and Smokers with chronic periodontitis had lower GI and PBI than nonsmokers having chronic periodontitis.

Biochemical parameters included were SEMA 4D, PAD 2, MMP 8levels from GCF samples and ELISA kit was used to analyze the respective levels.

A significantly high SEMA 4D, PAD 2, MMP 8 when comparing smokers with chronic periodontitis to non-smokers with chronic periodontitis, values were found to be similar.

The measurement of PPD, CAL, and radiographic alveolar bone loss are being used as periodontal diagnostic procedures. Only parameters assessing established periodontal tissue loss are evaluated by these diagnostic procedures. Despite their ease of use, these metrics do not give a real-time evaluation of the status and have minimal predictive value. Further qualitativeas well as quantitative diagnostic assessment approaches based on serum, saliva, and GCF have been developed for illness diagnosis and prediction. Despite breakthroughs in periodontal disease diagnosis & prognosis, just a few longitudinal studies were done to uncover biomarkers that predict periodontal disease before radiographic and clinical signs.

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

1. The use of GCF as biomarkers to predict future disease progression is firmly encouraged by our findings.
2. Given the positive link between SEMA 4D levels and the severity of various diseases, it may be a valuable predictive biomarker for disease development and diagnostic marker in evaluation of amount of inflammation in side periodontal pockets.

4. The relationship among PAD 2 levels and the severity of inflammation in periodontal pockets, this enzyme could be used to determine the disease activity.
5. The important participation of MMP 8 in tissue destruction and progression of periodontal disease can be established.

6. After NSPT, SEMA 4D, PAD 2 and MMP 8 GCF levels significantly reduced amongst both the groups indicating resolution of severity of the disease.
7. Smokers with CP demonstrated greater values of PPD, CAL, PI while lower PBI and GI scores when compared to non-smokers having CP.

Merits of the study

Due to the complexity of periodontitis, a single biomarker is insufficient to indicate total alterations in periodontal tissue. As a result, employing SEMA 4D, PAD 2, & MMP 8 biomarkers to make a diagnosis periodontitis proven to be more accurate and beneficial. SEMA 4D, PAD 2, and MMP 8 biomarkers could be useful:

1. To identify an individual at risk for developing periodontal disease.

2. To determine prognosis, improve patient acceptance and optimize treatment outcome in patients requiring extensive periodontal or implant therapy.

3. To reduce the overall cost required for severe periodontal diseases, as the disease will be diagnosed and treated at initial stage with assessment of future periodontal disease site.

Limitations of the study

Following are the limitations of the study:

1. There may be possibility of contamination of GCF while collecting the samples.
2. Relatively small number of participants, despite statistically appropriate sample size.
3. Only baseline and 3 months follow up periods were considered.
4. Any unknown confounding factors.

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TABLES

Table .1- Distribution of patients with respect to gender.

Gender	Group I		Group II		Group III	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Females	11	55.0	11	55.0	0	00
Males	9	45.0	9	45.0	20	100
Total	20	100.0	20	100.0	20	100

Table .2- Distribution of patients with respect to age in **Group I**

Age	Frequency	Percentage
18-22 years	7	35.0
23-27 years	6	30.0
28-32 years	7	35.0

Table .3- Distribution of patients with respect to age in intervention groups

Age	Group II		Group III	
	Frequency	**Percentage**	**Frequency**	**Percentage**
38-42 years	6	30.0	1	5.0
43-47 years	3	15.0	5	25.0
48-52 years	5	25.0	6	30.0
53-57 years	3	15.0	5	25.0
58-62 years	3	15.0	3	15.0

Table 4- Descriptive data in **Group I.**

Parameters	N	Minimum	Maximum	Mean	Std. Deviation
GI	20	0.05	0.79	0.41	0.19
PI	20	0.03	1.19	0.61	0.30
PPD (mm)	20	0.58	2.02	1.33	0.31
CAL (mm)	20	0.00	0.00	0.00	0.00
PBI (%)	20	0.00	0.09	0.03	0.025

Table 5- Descriptive data in **Group II** at baseline and 3 months

Parameters	**Time period**	**N**	**Minimum**	**Maximum**	**Mean**	**Std. Deviation**	**Mean difference**	**t-value**	**Significance (p)**
GI	Baseline	20	1.79	2.52	2.15	0.19	1.04	1142.0300	**<0.0001***
	3 months	20	0.74	1.48	1.10	0.19			
PI	Baseline	20	1.47	2.21	1.83	0.19	1.29	184.71	**<0.0001***
	3 months	20	0.27	0.91	0.54	0.18			
PPD (mm)	Baseline	20	6.50	7.24	6.86	0.19	2.76	1841.00	**<0.0001***
	3 months	20	3.74	4.48	4.10	0.19			
CAL (mm)	Baseline	20	6.71	7.45	7.07	0.19	2.20	147.00	**<0.0001***
	3 months	20	4.52	5.26	4.88	0.23			
PBI (%)	Baseline	20	1.89	2.63	2.25	0.19	1.97	106.92	**<0.0001***
	3 months	20	0.02	0.54	0.28	0.15			

*Significance at p<0.05

Significant decrease in parameters is present from baseline to 3 months.

Table 6- Descriptive data in **Group III** at baseline and 3 months

Parameters	**Time period**	**N**	**Minimum**	**Maximum**	**Mean**	**Std. Deviation**	**Mean difference**	**t-value**	**Significance (p)**
GI	Baseline	20	1.36	2.10	1.72	0.19	0.98	197.00	**<0.0001***
	3 months	20	0.38	1.12	0.74	0.18			
PI	Baseline	20	2.22	2.96	2.58	0.19	1.98	791.00	**<0.0001***
	3 months	20	0.24	0.98	0.60	0.19			
PPD (mm)	Baseline	20	7.70	8.44	8.06	0.19	2.46	547.67	**<0.0001***
	3 months	20	5.24	5.98	5.60	0.19			
CAL (mm)	Baseline	20	8.58	9.32	8.94	0.19	2.09	419.00	**<0.0001***
	3 months	20	6.49	7.23	6.85	0.19			
PBI (%)	Baseline	20	1.03	1.77	1.39	0.19	1.14	27.23	**<0.0001***
	3 months	20	0.20	0.29	0.25	0.022			

*Significance at p<0.05

Significant decrease in parameters is present from baseline to 3 months.

Table 7- Difference in the parameters at baseline between the groups

Parameters	Groups	Mean	F	Significance (p)
GI	**Group III**	1.72	431.10	**<0.0001***
	Group II	2.15		
	Group I	0.40		
PI	**Group III**	2.58	346.25	**<0.0001***
	Group II	1.83		
	Group I	0.61		
PPD (mm)	**Group III**	8.06	4351.80	**<0.0001***
	Group II	6.86		
	Group I	1.33		
CAL(mm)	**Group III**	8.94	7461.87	**<0.0001***
	Group II	7.07		
	Group I	0.00		
PBI (%)	**Group III**	1.39	973.38	**<0.0001***
	Group II	2.25		
	Group I	0.03		

Table 8- Post hoc test for difference in parameters between the groups at baseline

Parameters	Groups		Mean Difference	Significance (p)
GI	**Group III**	**Group II**	1.73	**<.0001***
		Group I	1.31	**<.0001***
	Group II	**Group III**	1.73	**<.0001***
		Group I	0.42	**<.0001***
	Group I	**Group III**	1.31	**<.0001***
		Group II	0.42	**<.0001***
PI	**Group III**	**Group II**	1.21	**<.0001***
		Group I	1.96	**<.0001***
	Group II	**Group III**	1.21	**<.0001***
		Group I	0.75	**<.0001***
	Group I	**Group III**	1.96	**<.0001***
		Group II	0.75	**<.0001***
PPD (mm)	**Group III**	**Group II**	5.52	**<.0001***
		Group I	6.72	**<.0001***
	Group II	**Group III**	5.52	**<.0001***
		Group I	1.20	**<.0001***
	Group I	**Group III**	6.72	**<.0001***
		Group II	1.20	**<.0001***
CAL(mm)	**Group III**	**Group II**	7.06	**<.0001***
		Group I	8.93	**<.0001***
	Group II	**Group III**	7.07	**<.0001***
		Group I	1.87	**<.0001***
	Group I	**Group III**	8.93	**<.0001***
		Group II	1.87	**<.0001***
PBI (%)	**Group III**	**Group II**	2.21	**<.0001***
		Group I	1.35	**<.0001***
	Group II	**Group III**	2.21	**<.0001***
		Group I	0.86	**<.0001***
	Group I	**Group III**	1.35	**<.0001***
		Group II	0.86	**<.0001***

*Significance at $p<0.05$

A significant difference is present between the groups at 3 months for the parameters

Table .9- Difference in the parameters at 3 months between the groups

Parameters	Groups	Mean	Mean difference	Significance (p)
GI	**Group III**	0.74	0.36	**<0.0001***
	Group II	1.10		
PI	**Group III**	0.60	0.06	0.301
	Group II	0.54		
PPD (mm)	**Group III**	5.60	1.50	**<0.0001***
	Group II	4.10		
CAL(mm)	**Group III**	6.85	1.97	**<0.0001***
	Group II	4.88		
PBI (%)	**Group III**	0.25	0.03	0.070
	Group II	0.28		

Table 10-Mean biochemical parameters in **Group I**

Biochemical parameters	N	Minimum	Maximum	Mean	Std. Deviation
SEMA 4D (ng/mL)	20	198.61	204.23	200.91	1.49
PAD 2 (pg/mL)	20	4093.79	4507.55	4300.30	111.01
MMP8 (ng/mL)	20	10.55	12.88	11.93	0.75

Table 11- Mean biochemical parameters in **Group II** group

Biochemical parameters	Time period	N	Minimum	Maximum	Mean	Std. Deviation
SEMA 4D (ng/mL)	Baseline	20	370.66	376.28	373.11	1.42
	3 months	20	270.08	275.70	272.38	1.49
PAD 2 (pg/mL)	Baseline	20	5019.44	5433.20	5225.95	111.01
	3 months	20	4848.07	5261.83	5054.58	111.01
MMP 8 (ng/mL)	Baseline	20	34.16	37.47	35.90	0.89
	3 months	20	25.20	27.53	26.58	0.75

Table 12- Mean biochemical parameters in **Group III**

Biochemical parameters	Time period	N	Minimum	Maximum	Mean	Std. Deviation
SEMA 4D (ng/mL)	Baseline	20	463.30	468.92	465.76	1.42
	3 months	20	277.69	283.31	279.99	1.49
PAD 2 (pg/mL)	Baseline	20	5318.82	5732.58	5525.34	111.00
	3 months	20	4931.65	5345.41	5138.16	111.01
MMP 8 (ng/mL)	Baseline	20	38.93	42.24	40.67	0.89
	3 months	20	29.97	32.30	31.35	0.75

Table 13- Difference in biochemical parameters in **Group II** from baseline to 3 months

Biochemical parameters	Time period	Mean	Mean difference	t-value	Significance (p)
SEMA 4D (ng/mL)	Baseline	373.11	100.73	767.23	**<0.0001***
	3 months	272.38			
PAD 2 (pg/mL)	Baseline	5225.95	171.36	31157	**<0.0001***
	3 months	5054.58			
MMP 8 (ng/mL)	Baseline	35.90	9.32	128.47	**<0.0001***
	3 months	26.58			

*Significance at p<0.05

A statistical significant difference is present from baseline to 3 months for SEMA 4D. PAD 2 and MMP 8 biochemical parameters in Group II. The biochemical values significantly decreased at 3 months from the baseline.

Table 14- Difference in biochemical parameters in **Group III** from baseline to 3 months

Biochemical parameters	Time period	Mean	Mean difference	t-value	Significance (p)
SEMA 4D (ng/mL)	Baseline	465.76	185.86	1414.89	**<0.0001***
	3 months	279.99			
PAD 2 (pg/mL)	Baseline	5525.34	387.17	12441.38	**<0.0001***
	3 months	5138.16			
MMP 8 (ng/mL)	Baseline	40.67	9.32	128.46	**<0.0001***
	3 months	31.35			

*Significance at p<0.05

A statistical significant difference is present from baseline to 3 months for SEMA 4D. PAD 2 and MMP 8 biochemical parameters in Group III. The biochemical values significantly decreased at 3 months from the baseline.

Table 15- Difference in biochemical parameters between the groups at baseline

Parameters	**Groups**	**Mean**	**F**	**Significance (p)**
SEMA 4D (ng/mL)	Group I	200.91	17173.43	**<0.0001***
	Group II	373.11		
	Group III	465.76		
PAD 2 (pg/mL)	Group I	4300.30	661.92	**<0.0001***
	Group II	5225.95		
	Group III	5525.34		
MMP8 (ng/mL)	Group I	11.93	6513.65	**<0.0001***
	Group II	35.90		
	Group III	40.67		

Table 16- Post hoc test for difference in parameters between the groups at baseline

Parameters	**Groups**		**Mean Difference**	**Significance (p)**
SEMA 4D (ng/mL)	**Group I**	Group II	172.19	**<.0001***
		Group III	264.83	**<.0001***
	Group II	Group I	172.19	**<.0001***
		Group III	92.64	**<.0001***
	Group III	Group I	264.83	**<.0001***
		Group II	92.64	**<.0001***
PAD 2 (pg/mL)	**Group I**	Group II	925.65	**<.0001***
		Group III	1225.03	**<.0001***
	Group II	Group I	925.65	**<.0001***
		Group III	299.38	**<.0001***
	Group III	Group I	1225.03	**<.0001***
		Group II	299.38	**<.0001***
MMP8 (ng/mL)	**Group I**	Group II	23.97	**<.0001***
		Group III	28.74	**<.0001***
	Group II	Group I	23.97	**<.0001***
		Group III	4.77	**<.0001***
	Group III	Group I	28.74	**<.0001***
		Group II	4.77	**<.0001***

*Significance at p<0.05

A statistical significant difference is present in biochemical parameters between the groups at baseline

Table 17- Difference in biochemical parameters between control and intervention groups for SEMA 4D

Groups	**Mean**	**F**	**Significance (p)**
Group I	200.91	16980.56	**<.0001***
Group II	272.38		
Group III	279.99		

*Significance at p<0.05

A statistical significant difference is present in SEMA 4D biochemical parameter between Group I, Group II and Group III at 3 months.

Table 18- Post hoc test for SEMA 4D

Groups		**Mean Difference**	**Significance (p)**
Group I	Group II	71.46*	**<.0001***
	Group III	79.07*	**<.0001***
Group II	Group I	71.46*	**<.0001***
	Group III	7.61*	**<.0001***
Group III	Group I	79.07*	**<.0001***
	Group II	7.61*	**<.0001***

*Significance at p<0.05

A statistical significant difference is present in SEMA 4D between:

Group I & Group II

Group I & Group III

Group II & Group III

SEM 4D was:

Group III > Group II > Group I

Table 19- Difference in biochemical parameters between control and intervention groups for PAD 2

Groups	Mean	F	Significance (p)
Group I	4300.30	345.66	**<.0001***
Group II	5054.58		
Group III	5138.16		

*Significance at p<0.05

A statistical significant difference is present in PAD 2 biochemical parameter between Group I, Group II and Group III at 3 months.

Table 20- Post hoc test for PAD 2

Groups		Mean Difference	Significance (p)
Group I	Group II	754.28	**<.0001***
	Group III	837.86	**<.0001***
Group II	Group I	754.28	**<.0001***
	Group III	83.58	**.049***
Group III	Group I	837.86	**<.0001***
	Group II	83.58	**.049***

*Significance at p<0.05

A statistical significant difference is present in PAD 2 between:

Group I & Group II

Group I & Group III

Group II & Group III

PAD 2 was:

Group III > Group II > Group I

Table 21- Difference in biochemical parameters between control and intervention groups for MMP 8

Groups	Mean	F	Significance (p)
Group I	11.93	3617.351	**<.0001***
Group II	26.58		
Group III	31.35		

*Significance at p<0.05

A statistical significant difference is present in MMP 8 biochemical parameter between Group I, Group II and Group III at 3 months.

Table 22- Post hoc test for MMP 8

Groups		**Mean Difference**	**Significance (p)**
Group I	Group II	14.65*	**<.0001***
	Group III	19.42*	**<.0001***
Group II	Group I	14.65*	**<.0001***
	Group III	4.77*	**<.0001***
Group III	Group I	19.42000*	**<.0001***
	Group II	4.77000*	**<.0001***

*Significance at $p<0.05$

A statistical significant difference is present in MMP 8 between:

Group I & Group II

Group I & Group III

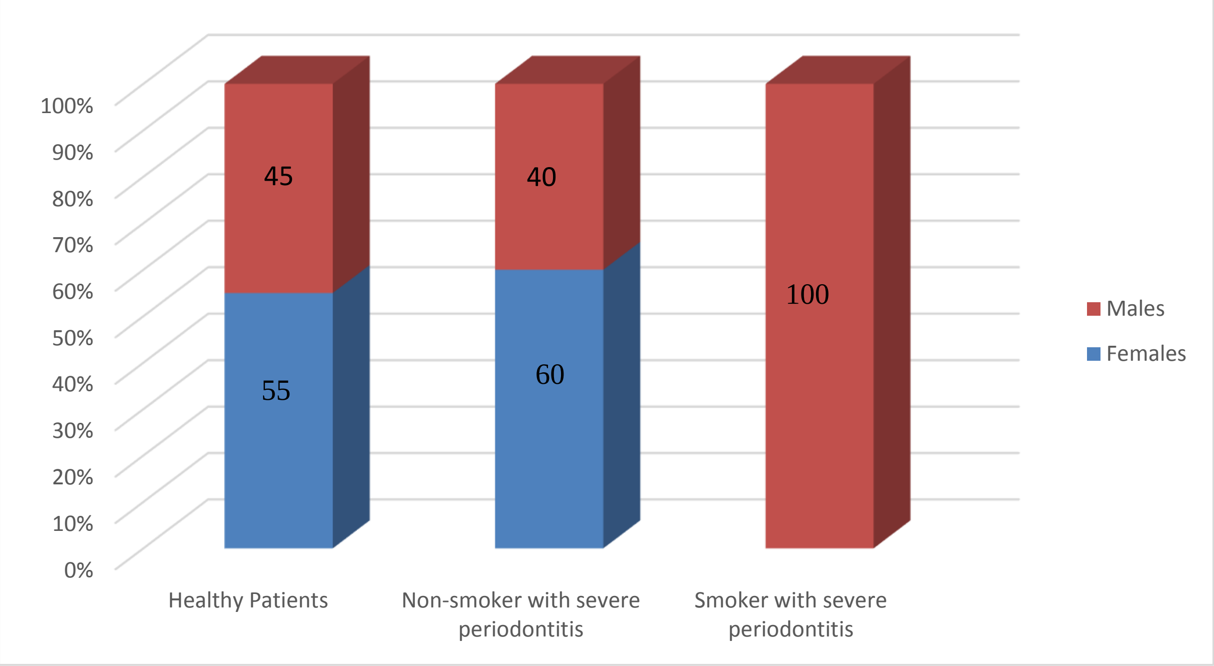
Group II & Group III

MMP 8 was:

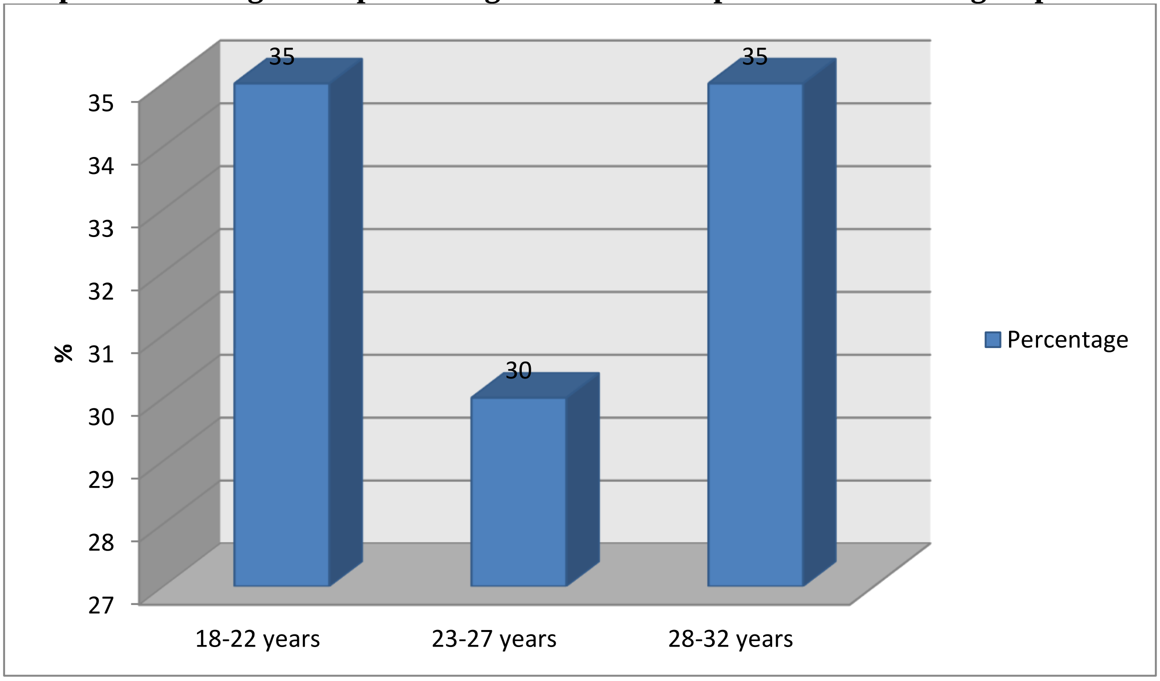
Group III > Group II > Group I

GRAPHS

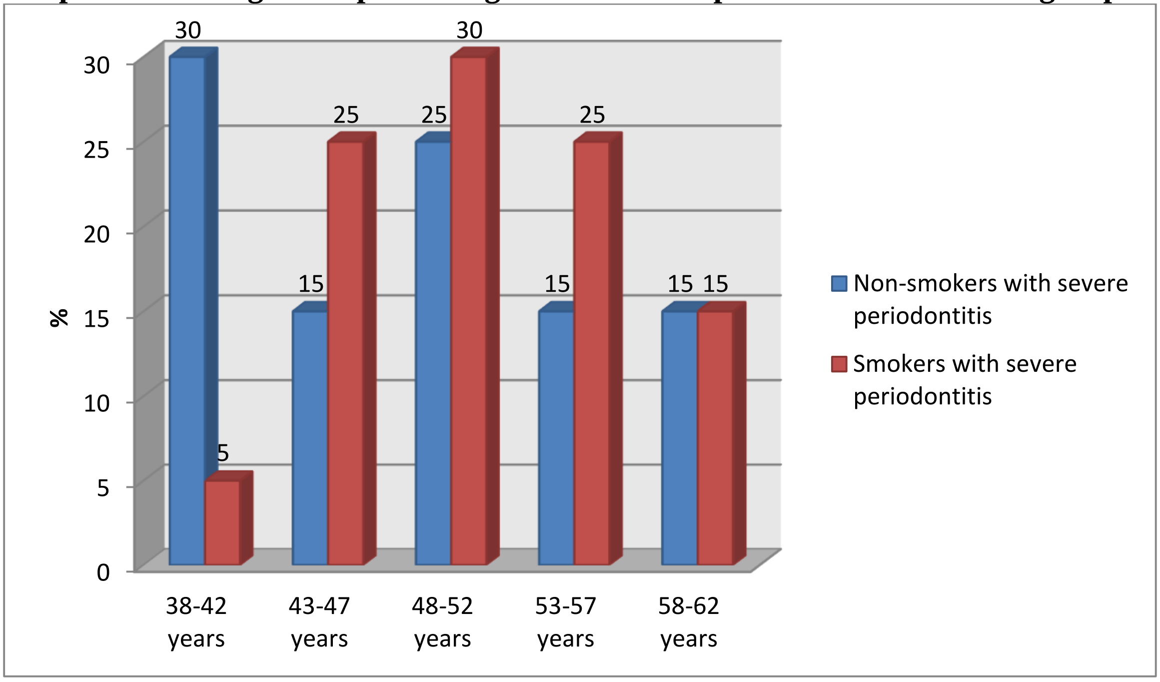
Graph 1- Bar diagram representing distribution of patients in control and intervention groups with respect to gender



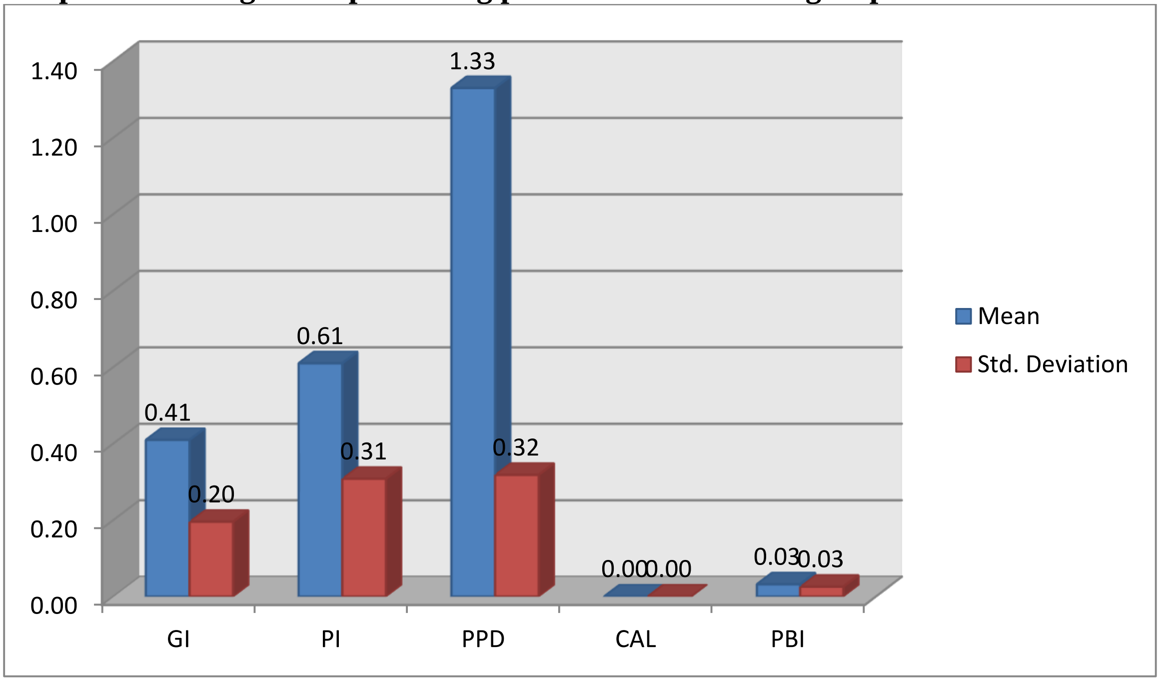
Graph .2- Bar diagram representing distribution of patients in control group with respect to age



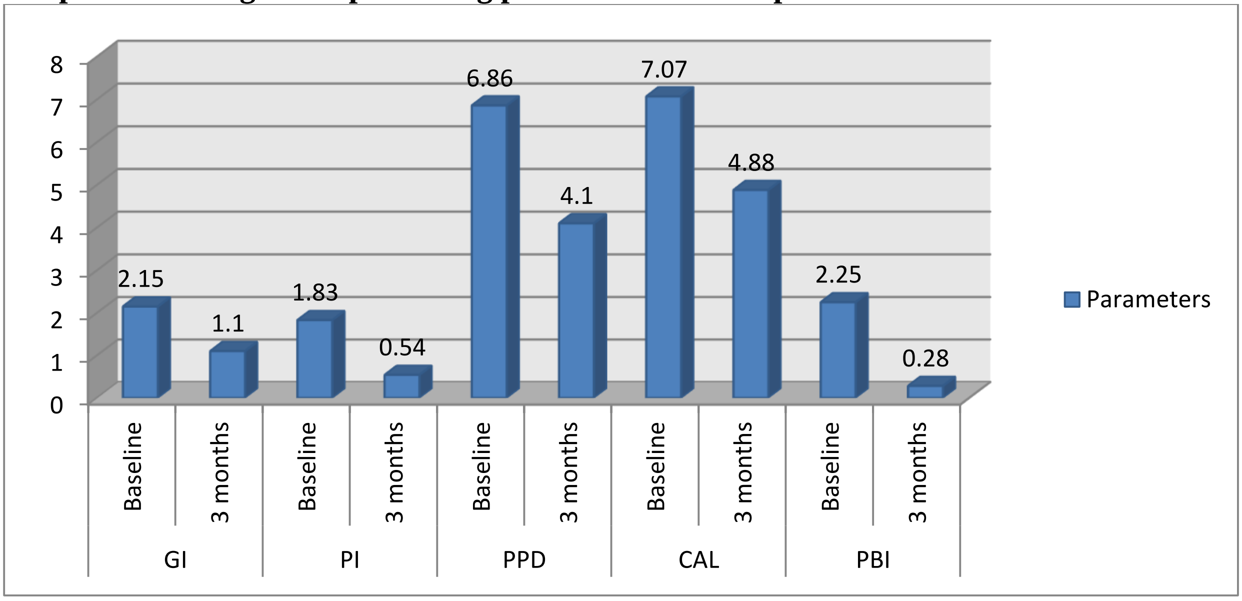
Graph .3- Bar diagram representing distribution of patients in intervention groups with respect to age



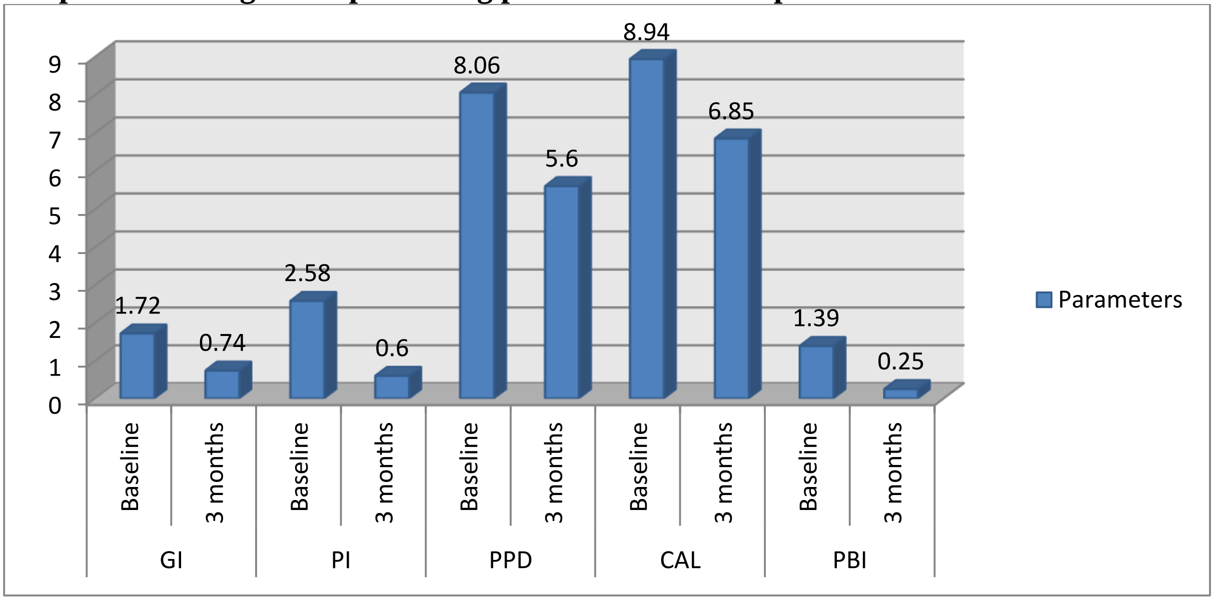
Graph 4- Bar diagram representing parameters in control group



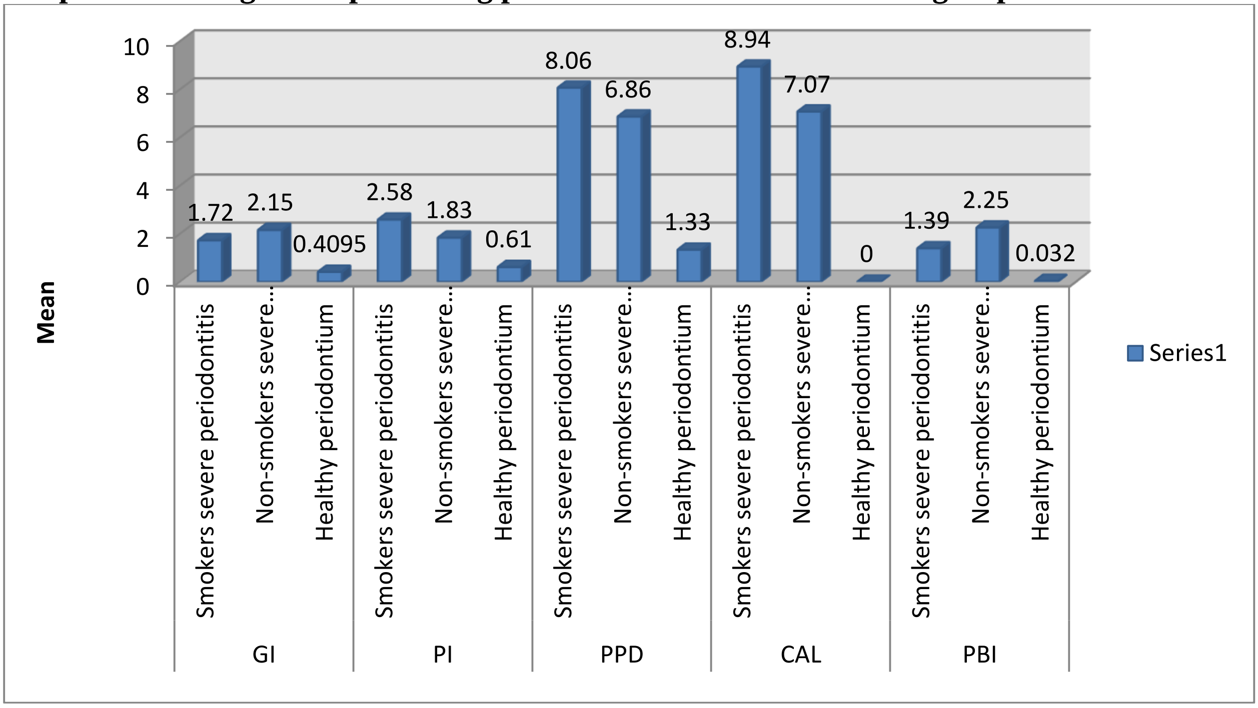
Graph 5- Bar diagram representing parameters in Group II



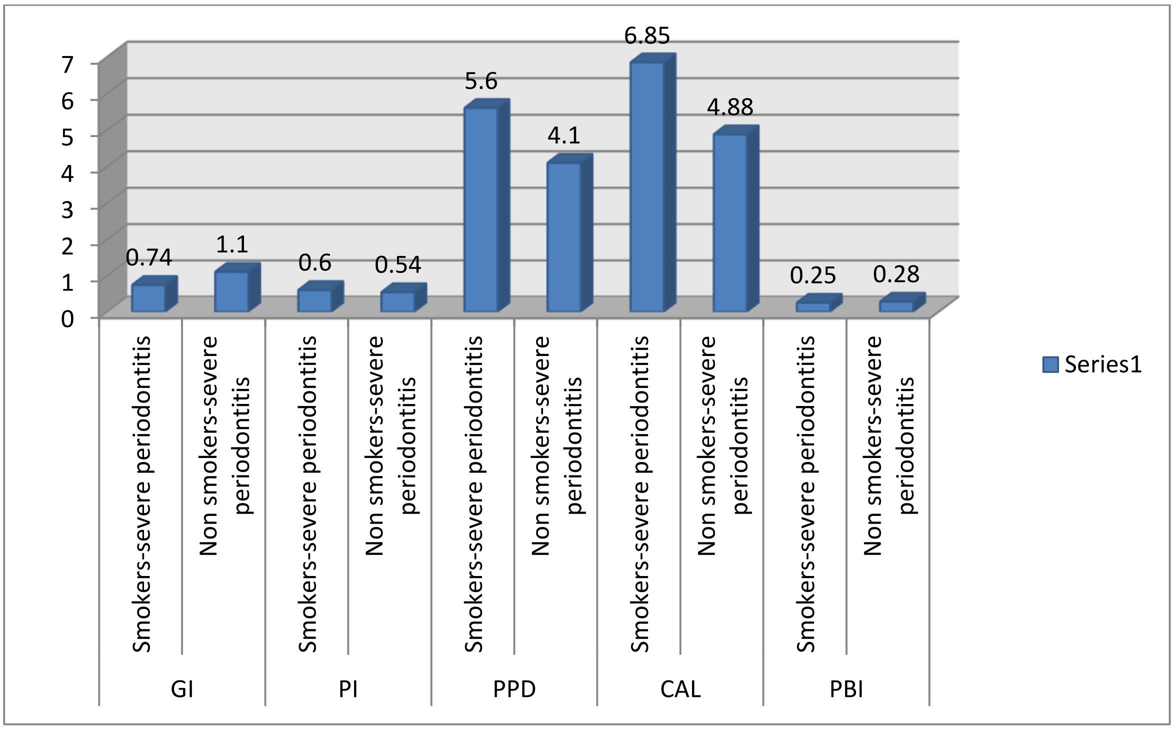
Graph 6- Bar diagram representing parameters in Group III



Graph 7- Bar diagram representing parameters at baseline in three group



Graph 8- Bar diagram representing parameters at 3 months in the groups



Master Chart

Periodontal Clinical and Biochemical Parameters in GROUP I

Sr. No.	Age	Gender	GI	PI	PPD (mm)	CAL (mm)	PBI (%)	SEMA 4D (ng/mL)	PAD 2 (pg/mL)	MMP 8 (ng/mL)
1	19	M	0.30	0.90	1.39	0.00	0.01	200.94	4393.68	11.34
2	21	M	0.43	0.46	1.22	0.00	0.09	200.07	4192.81	12.47
3	20	M	0.51	0.97	1.69	0.00	0.06	201.15	4293.89	10.55
4	29	F	0.05	0.61	1.45	0.00	0.01	199.69	4194.43	12.32
5	24	F	0.41	0.03	1.29	0.00	0.05	201.05	4093.79	11.68
6	22	M	0.23	0.86	1.16	0.00	0.06	202.79	4195.53	12.50
7	28	F	0.66	0.38	1.63	0.00	0.00	204.23	4433.97	11.93
8	18	F	0.59	0.56	1.40	0.00	0.03	201.16	4450.90	10.86
9	27	F	0.61	0.52	2.02	0.00	0.01	203.18	4338.92	12.88
10	29	F	0.17	0.47	1.29	0.00	0.01	200.74	4233.48	11.44
11	25	M	0.79	0.88	0.58	0.00	0.01	202.36	4335.10	12.83
12	23	M	0.30	0.57	1.11	0.00	0.03	201.87	4234.61	12.34
13	27	F	0.29	0.34	1.45	0.00	0.03	198.75	4297.49	11.33
14	22	F	0.57	0.59	1.43	0.00	0.06	200.03	4232.77	12.61
15	29	F	0.51	0.04	0.87	0.00	0.06	198.97	4443.71	10.55
16	28	M	0.22	0.97	1.70	0.00	0.06	200.78	4308.52	12.26
17	30	M	0.46	0.79	1.47	0.00	0.01	202.02	4209.76	12.50
18	26	F	0.59	0.31	1.06	0.00	0.03	200.15	4408.89	12.63
19	21	F	0.15	0.76	1.04	0.00	0.01	198.61	4206.35	11.19
20	28	M	0.35	1.19	1.35	0.00	0.01	199.81	4507.55	12.39
AVERAGE			**0.41**	**0.61**	**1.33**	**0.00**	**0.03**	**200.918**	**4300.31**	**11.93**

Periodontal Clinical Parameters in GROUP II

Sr. No	Age	Gender	GI		PI		PPD (mm)		CAL (mm)		PBI (%)	
			BASELINE	3 Months	BASELINE	3 Months	BASELINE	3 Months	BASELINE	3 Months	BASELINE	3 Months
1	39	M	2.04	0.99	1.72	0.42	6.75	3.99	6.96	4.77	2.14	0.05
2	51	F	2.17	1.12	1.85	0.55	6.88	4.12	7.09	4.90	2.27	0.18
3	47	M	2.25	1.20	1.93	0.63	6.96	4.20	7.17	4.98	2.35	0.07
4	52	F	1.79	0.74	1.47	0.31	6.50	3.74	6.71	4.52	1.89	0.02
5	42	F	2.15	1.10	1.83	0.53	6.86	4.10	7.07	4.88	2.25	0.16
6	56	F	1.96	0.92	1.65	0.35	6.68	3.92	6.89	4.70	2.07	0.02
7	51	F	2.40	1.35	2.08	0.78	7.11	4.35	7.32	5.13	2.50	0.41
8	47	M	2.33	1.28	2.01	0.71	7.04	4.28	7.25	5.06	2.43	0.34
9	51	M	2.34	1.30	2.03	0.73	7.06	4.30	7.27	5.08	2.45	0.36
10	39	F	1.91	0.86	1.59	0.29	6.62	3.86	6.83	4.64	2.01	0.02
11	49	F	2.52	1.48	2.21	0.91	7.24	4.48	7.45	5.26	2.63	0.54
12	55	M	2.04	0.99	1.72	0.42	6.75	3.99	6.96	4.77	2.14	0.05
13	40	F	2.03	0.98	1.71	0.41	6.74	3.98	6.95	4.76	2.13	0.04
14	39	M	2.31	1.26	1.99	0.69	7.02	4.26	7.23	5.04	2.41	0.32
15	58	M	2.25	1.20	1.93	0.63	6.96	4.20	7.17	4.98	2.35	0.26
16	38	F	1.96	0.91	1.64	0.34	6.67	3.91	6.88	4.69	2.06	0.03
17	45	F	2.19	1.15	1.88	0.58	6.91	4.15	7.12	4.93	2.30	0.21
18	60	M	2.33	1.28	2.01	0.71	7.04	4.28	7.25	5.06	2.43	0.34
19	55	F	1.89	0.84	1.57	0.27	6.60	3.84	6.81	4.62	1.99	0.13
20	58	M	2.09	1.04	1.77	0.47	6.80	4.04	7.01	4.82	2.19	0.10
AVERAGE			**2.15**	**1.10**	**1.83**	**0.54**	**6.86**	**4.10**	**7.07**	**4.88**	**2.25**	**0.18**

Biochemical Parameters in Group II

Sr No.	SEMA 4D (ng/mL)		PAD 2 (pg/mL)		MMP 8 (ng/mL)	
	BASELINE	**3 Months**	**BASELINE**	**3 Months**	**BASELINE**	**3 Months**
1	372.99	272.41	5319.33	5147.96	35.31	25.99
2	372.12	271.54	5118.46	4947.09	36.44	27.12
3	373.20	272.62	5219.54	5048.17	34.52	25.20
4	373.74	271.16	5120.08	4948.71	36.29	26.97
5	373.10	272.52	5019.44	4848.07	35.65	26.33
6	374.84	274.26	5121.18	4949.81	36.47	27.15
7	376.28	275.70	5359.62	5188.25	35.90	26.58
8	374.21	272.63	5376.55	5205.18	34.83	25.51
9	374.23	274.65	5264.57	5093.20	36.85	27.53
10	372.79	272.21	5159.13	4987.76	35.41	26.09
11	374.41	273.83	5260.75	5089.38	36.80	27.48
12	373.92	273.34	5160.26	4988.89	36.31	26.99
13	370.80	270.22	5223.14	5051.77	35.30	25.98
14	372.08	271.50	5158.42	4987.05	36.58	27.26
15	371.02	270.44	5369.36	5197.99	34.52	25.20
16	372.83	272.25	5234.17	5062.80	36.23	26.91
17	374.07	273.49	5135.41	4964.04	37.47	27.15
18	373.20	271.62	5334.54	5163.17	36.60	27.28
19	370.66	270.08	5132.00	4960.63	34.16	25.84
20	371.86	271.28	5433.20	5261.83	36.36	27.04
AVERAGE	**373.12**	**272.39**	**5225.958**	**5054.588**	**35.90**	**26.58**

Periodontal Clinical Parameters in GROUP III

Sr. No	Age	Gender	GI		PI		PPD (mm)		CAL (mm)		PBI (%)	
			BASELINE	3 Months	BASELINE	3 Months	BASELINE	3 Months	BASELINE	3 Months	BASELINE	3 Months
1	40	M	1.61	0.63	2.47	0.49	7.95	5.49	8.83	6.74	1.28	0.23
2	52	M	1.74	0.76	2.60	0.62	8.08	5.62	8.96	6.87	1.41	0.25
3	43	M	1.82	0.84	2.68	0.70	8.16	5.70	9.04	6.95	1.49	0.26
4	52	M	1.36	0.38	2.22	0.24	7.70	5.24	8.58	6.49	1.03	0.25
5	46	M	1.72	0.74	2.58	0.60	8.06	5.60	8.94	6.85	1.39	0.23
6	56	M	1.54	0.56	2.40	0.42	7.88	5.42	8.76	6.67	1.21	0.23
7	51	M	1.97	0.99	2.83	0.85	8.31	5.85	9.19	7.10	1.64	0.26
8	47	M	1.90	0.92	2.76	0.78	8.24	5.78	9.12	7.03	1.57	0.28
9	57	M	1.92	0.94	2.78	0.80	8.26	5.80	9.14	7.05	1.59	0.24
10	45	M	1.48	0.50	2.34	0.36	7.82	5.36	8.70	6.61	1.15	0.25
11	50	M	2.10	1.12	2.96	0.98	8.44	5.98	9.32	7.23	1.77	0.27
12	59	M	1.61	0.63	2.47	0.49	7.95	5.49	8.83	6.74	1.28	0.23
13	49	M	1.60	0.62	2.46	0.48	7.94	5.48	8.82	6.73	1.27	0.23
14	60	M	1.88	0.90	2.74	0.76	8.22	5.76	9.10	7.01	1.55	0.29
15	52	M	1.82	0.84	2.68	0.70	8.16	5.70	9.04	6.95	1.49	0.25
16	54	M	1.53	0.55	2.39	0.41	7.87	5.41	8.75	6.66	1.20	0.20
17	45	M	1.77	0.79	2.63	0.65	8.11	5.65	8.99	6.90	1.44	0.28
18	57	M	1.90	0.92	2.76	0.78	8.24	5.78	9.12	7.03	1.57	0.23
19	55	M	1.46	0.48	2.32	0.34	7.80	5.34	8.68	6.59	1.13	0.27
20	58	M	1.66	0.68	2.52	0.54	8.00	5.54	8.88	6.79	1.33	0.24
AVG			**1.72**	**0.74**	**2.58**	**0.60**	**8.06**	**5.60**	**8.94**	**6.85**	**1.39**	**0.25**

Biochemical Parameters in Group III

Sr. No	SEMA 4D (ng/mL)		PAD 2 (pg/mL)		MMP 8 (ng/mL)	
	BASELINE	3 Months	BASELINE	3 Months	BASELINE	3 Months
1	465.63	280.02	5618.71	5231.54	40.08	30.76
2	464.76	279.15	5417.90	5030.67	41.21	31.89
3	465.84	280.23	5518.92	5131.75	39.29	29.97
4	466.38	278.77	5419.46	5032.29	41.06	31.74
5	465.74	280.13	5318.82	4931.65	40.42	31.10
6	467.48	281.87	5420.56	5033.39	41.24	31.92
7	468.92	283.31	5659.00	5271.83	40.67	31.35
8	466.85	280.24	5675.93	5288.76	39.60	30.28
9	466.87	282.26	5563.95	5176.78	41.62	32.30
10	465.43	279.82	5458.51	5071.34	40.18	30.86
11	467.05	281.44	5560.13	5172.96	41.57	32.25
12	466.56	280.95	5459.64	5072.47	41.08	31.76
13	463.44	277.83	5522.52	5135.35	40.07	30.75
14	464.72	279.11	5457.80	5070.63	41.35	32.03
15	463.66	278.05	5668.76	5281.57	39.29	29.97
16	465.47	279.86	5533.55	5146.38	41.00	31.68
17	466.71	281.10	5434.79	5047.62	42.24	31.92
18	465.84	279.23	5633.92	5246.75	41.37	32.05
19	463.30	277.69	5431.38	5044.21	38.93	30.61
20	464.50	278.89	5732.58	5345.41	41.13	31.81
AVERAGE	**465.76**	**279.998**	**5525.34**	**5138.168**	**40.67**	**31.35**

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:

TEETH PRESENT:

PARAMETERS

GINGIVAL INDEX *(Loe & Sillness1963*) – At Baseline

16	12	24
44	32	36

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

GINGIVAL INDEX *(Loe & Sillness1963*) – At 3 Months

16	12	24
44	32	36

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

PLAQUE INDEX *(Sillness & Loe, 1964)* – At Baseline

16	12	24
44	32	36

<u>SCORE:</u> $\frac{\text{Total scores of all teeth}}{\text{Total number of teeth examined}}$

PLAQUE INDEX *(Sillness & Loe, 1964)* – At 3 Months

16	12	24
44	32	36

<u>SCORE:</u> $\frac{\text{Total scores of all teeth}}{\text{Total number of teeth examined}}$

PROBING POCKET DEPTH (mm) – At Baseline:

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

PROBING POCKET DEPTH (mm) – At 3 Months:

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

CLINICAL ATTACHMENT LEVELS (mm) – At Baseline:

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

CLINICAL ATTACHMENT LEVELS (mm) – At 3 Months:

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

PAPILLARY BLEEDING INDEX (PBI) (Muhlemann H.R. 1977) – At Baseline

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

PAPILLARY BLEEDING INDEX (PBI) (Muhlemann H.R. 1977) – At 3 Months

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

Clinical Diagnosis:

Biochemical Analysis:

PARAMETERS	**GROUP I**	**GROUP II**		**GROUP III**	
		PRE (Baseline)	**POST (3 Months)**	**PRE (Baseline)**	**POST (3 Months)**
SEMA4D(ng/ml)					
PAD2(pg/ml)					
MMP 8(ng/ml)					

(Confidential)

Informed Consent Form

"Comparative evaluation of Semaphorin 4D, Peptidylarginine Deiminase 2 and Matrix Metalloproteinase-8 Gingival crevicular fluid levels in periodontally healthy, Severe periodontitis Smoker and non-smoker patients before and after Non-surgical periodontal therapy**"**

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 Aishwarya Ikhar.**

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