"EVALUATION AND COMPARISON OF INTERLEUKIN-35 LEVELS IN GINGIVAL CREVICULAR FLUID OF PERIODONTALLY HEALTHY, GINGIVITIS AND SEVERE PERIODONTITIS PATIENTS BEFORE AND AFTER NON-SURGICAL PERIODONTAL THERAPY: A CLINICAL CONTROLLED TRIAL".

Dissertation Submitted to

Maharashtra University of Health Sciences, Nashik

In the Partial Fulfillment of Regulations

for the Award of the Degree of

MDS

IN

PERIODONTICS

BRANCH II

2022

Appendix 'B'

"EVALUATION AND COMPARISON OF INTERLEUKIN-35 LEVELS IN GINGIVAL CREVICULAR FLUID OF PERIODONTALLY HEALTHY, GINGIVITIS AND SEVERE PERIODONTITIS PATIENTS BEFORE AND AFTER NON-SURGICAL PERIODONTAL THERAPY: A CLINICAL CONTROLLED TRIAL".

By

DR. ASHWINI JADHAV

Under Guidance of

DR. SUREKHA RATHOD

Through

VSPM's DENTAL COLLEGE & RESEARCH CENTRE,

HINGNA, NAGPUR

Dissertation Submitted to Maharashtra University of Health Sciences, Nashik In the Partial Fulfillment of Regulations for the Award of the Degree of

MDS

IN

PERIODONTICS

BRANCH II

2022

Appendix 'C'

CERTIFICATE

This is to certify that the work contained in the dissertation entitled

"EVALUATION AND COMPARISON OF INTERLEUKIN-35 LEVELS IN GINGIVAL CREVICULAR FLUID OF PERIODONTALLY HEALTHY, GINGIVITIS AND SEVERE PERIODONTITIS PATIENTS BEFORE AND AFTER NON-SURGICAL PERIODONTAL THERAPY: A CLINICAL CONTROLLED TRIAL".

has been prepared by **DR. ASHWINI JADHAV**

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

MDS in Periodontics.

Her work on the subject has been checked by me from time to time. I am satisfied regarding the authenticity of her observations, clinical material and experimentation in this dissertation and it conforms to the standards of Maharashtra University of Health Sciences, Nashik.

I have great pleasure in forwarding it to Maharashtra University of Health Sciences, Nashik.

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Appendix 'D'

CERTIFICATE

This is to certify that

"EVALUATION AND COMPARISON OF INTERLEUKIN-35 LEVELS IN GINGIVAL CREVICULAR FLUID OF PERIODONTALLY HEALTHY, GINGIVITIS AND SEVERE PERIODONTITIS PATIENTS BEFORE AND AFTER NON-SURGICAL PERIODONTAL THERAPY: A CLINICAL CONTROLLED TRIAL".

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partial fulfillment of regulations for the award of degree of

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I hereby declare that the dissertation entitled

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has been prepared by me under direct guidance and supervision of ***DR. SUREKHA RATHOD*** *in partial fulfilment of regulations for the award of the degree of* ***MDS in Periodontics****, Maharashtra University of Health Sciences, Nashik and has not been submitted previously for the award of any diploma or degree from any University.*

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DR. ASHWINI JADHAV

PLACE : NAGPUR

EVALUATION AND COMPARISON OF INTERLEUKIN-35 LEVELS IN GINGIVAL CREVICULAR FLUID OF PERIODONTALLY HEALTHY, GINGIVITIS AND SEVERE PERIODONTITIS PATIENTS BEFORE AND AFTER NON-SURGICAL PERIODONTAL THERAPY: A CLINICAL CONTROLLED TRIAL

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LIST OF ABBREVIATIONS

SR. NO.	SHORT FORM	FULL FORM
1.	ALP	Alkaline phosphatase
2.	AgP	Aggressive periodontitis
3.	BL	Bone loss
4.	BOP	Bleeding on probing
5.	CD4	Cluster of differentiation 4
6.	CD8	Cluster of differentiation 8
7.	CD25	Cluster of differentiation 25
8.	CD138	Cluster of differentiation 138
9.	CD38	Cluster of differentiation 38
10.	CVD	Cardio Vascular Disease
11.	CP	Chronic periodontitis
12.	CRP	C-reactive proteins
13.	CHD	Coronary Heart Disease
14.	DM	Diabetes Mellitus
15.	DM2	Type 2 diabetes mellitus
16.	EBi3	Epstein-Barr virus induced gene 3
17.	Er, Cr: YSGG	Erbium, Chromium: Yttrium, Scandium, Gallium, Garnet
18.	ERK	extracellular signal-regulated kinase
19.	ELISA	Enzyme-linked Immunosorbent assay
20.	Foxp3	Forkhead box P3
21.	FPG	fasting plasma glucose
22.	FMD	full-mouth disinfection
23.	G1 phase	Growth 1phase
24.	GCF	Gingival crevicular fluid
25.	GI	Gingival index
26.	gp130	Glycoprotein-130
27.	HPDLCs	Human Periodontal Ligament Cells
28.	HbA1c	Hemoglobin A1c
29.	hs-CRP	highly sensitive C-reactive protein
30.	Hcy	Homocysteine

31.	HPLC-fld	High performance liquid chromatography with fluorescence detection
32.	IFN-γ	Interferon-gamma
33.	IL-12	Interleukin – 12
34.	IL-12R1	Interleukin – 12 receptor 1
35.	IL-12R2	Interleukin – 12 receptor 2
36.	IL-12Rβ1	Interleukin – 12 receptor
37.	IL-12Rβ2	Interleukin – 12 receptor
38.	IL-12p80	Interleukin – 12 p80
39.	IL27p28	Interleukin – 12 p28
40.	IL17A	Interleukin – 17A
41.	IL12A,	Interleukin – 12A
42.	IL-23R	Interleukin – 23 receptor
43.	IL-1	Interleukin-1
44.	IL-6	Interleukin – 6
45.	IL-8	Interleukin – 8
46.	IL-12p35	Interleukin – 12p35
47.	IL-12p40	Interleukin – 12p40
48.	IL-1α	Interleukin-1 alpha
49.	IL-1β	Interleukin-1 beta
50.	IL-4	Interleukin – 4
51.	IL-10	Interleukin-10
52.	IL-13	Interleukin-13
53.	IL-17	Interleukin – 17
54.	IL-27	Interleukin – 27
55.	IL-35	Interleukin-35
56.	iTreg	inducible regulator T cells
57.	iTr35	interleukin (IL) -35-producing T cells
58.	NSPT	Non-surgical periodontal therapy
59.	LN	Lupus nephritis
60.	MMP9	Matrix metalloprotenaise 9
61.	NF-B	nuclear factor –B
62.	NCHD	Without coronary heart disease
63.	nTreg	natural regulator T cells

64.	OHIP-14	Oral hygiene impact profile-14
65.	OHQoL	Oral health-related quality of life
66.	PGE2	Prostaglandin E2
67.	PBI	Papillary Bleeding Index
68.	PI	Plaque Index
69.	PPD	Probing pocket depth
70.	PMN	Polymorphonuclear neutrophils
71.	PD	Probing depth
72.	PPD	Probing pocket depth
73.	P-IL-37	IL-37 coproducing plasma cells
74.	P-IL-35	IL-35 coproducing plasma cells
75.	PBMNCs	Peripheral blood mononuclear cells
76.	qPCR	Quantitative polymerase chain reaction
77.	QS	four weekly sessions
78.	RANTES	Regulated on activation ,normal T cell expressed andsecreted
79.	RNA	Riboneuclic acid
80.	RT-PCR	Reverse transcriptase chain reaction
81.	rIL-35	Recombinant human Interleukin- 35
82.	RANKL	receptor activator of nuclear factor" (NF)- κB ligand
83.	RA	rheumatoid arthritis
84.	SRP	Scaling and root planning
85.	sCD14	Soluble Cluster of differentiation 14
86.	SSc	Systemic sclerosis
87.	SLE	Systemic Lupus Erythematosus
88.	TNF	Tumor necrosis factor
89.	TNF-α	Tumor necrosis factor-alpha
90.	Th1	T- helper1
91.	Th2	T- helper2
92.	Th3	T- helper3
93.	Treg	regulator T cells
94.	Tr1	regulator T cells 1
95.	TCI	tongue-coating index
96.	TC	total cholesterol
97.	TG	triglyceride

98.	Th17	T- helper 17
99.	WBC	white blood cell
100.	UNC	University of North Carolina
101.	25(OH)D3	25-HydroxyVitamin –D3

INTRODUCTION

Periodontitis include the features such as gingival inflammation, periodontal pocket formation, Clinical attachment loss (CAL) and alveolar bone loss (BL). Various histologic findings of periodontitis are loss of collagen fibers and infiltration of various inflammatory cells, location of junctional epithelium apical to the cemento-enamel junction. [1] Periodontal disease is mostly caused by the contact of the host tissue to the microflora that is attached to the teeth in the form of a biofilm, mentioned to as dental plaque, eventually these microorganisms together with the impaired host immune response increase the growth of specific microbes which leads to destruction of tissue and finally tooth loss.[2]

Bacterial biofilms are also linked to a reversible condition known as gingivitis, which can be resolved clinically in about a week after resuming proper dental hygiene procedures thanks to the host immune response. Various biological markers including cytokines are produced by B cells, T cells, macrophages and neutrophils as part of the immune response. It is thought to be a doorway to periodontitis.[3] Although this inflammatory response resolves quickly,

preventing tissue injury, insufficient resolution and failure to restore tissue homeostasis leads to neutrophil-mediated destruction and persistent inflammation.[4]

Bacterial virulence factors produce either direct host tissue degradation or the discharge of biologic mediators from host tissue, both of which contribute to host tissue demise. Inflammatory mediators such as collagen-degrading enzymes, elastase-like enzymes, trypsin-like proteases, amino peptidases, dipeptidylpeptidases, and proteinases, cytokines, and prostaglandins are produced by periodontal microbes and lead to tissue destruction. By the action of bacterial lipopolysaccharide there leads to release of Monocytes, polymorph nuclear leukocytes (PMN), macrophages, and other cells which release interleukin-1 (IL-1), tumour necrosis factor (TNF), and Prostaglandin E2 (PGE2). This TNF appears to have a significant role in periodontal tissue destruction, and PGE2 seems of having partially liable for periodontitis-related bone loss.[5]

Reciprocal interactions between diverse cell kinds, like leucocytes, endothelial cells, fibroblasts, and epithelial cells, govern periodontal inflammation. Cytokines have gotten a lot of attention among the many immunological and inflammatory mediators that are recognized. Pro-inflammatory and anti-inflammatory cytokines regulate periodontal tissue loss, remodeling, and repair in the pathophysiology of periodontal disease. [6] Triggering or inhibition of the intracellular signaling cascades is possible due to these cytokines which are solvable proteins which attach to definite receptors on target cells. T cells secrete pro-inflammatory and anti-inflammatory cytokines, modulate the immune response at sites of inflammation.[7]

T-lymphocytes are divided into subsets based on whether CD4 or CD8 molecules are expressed on their cell-surface. Based on the cytokine production patterns, CD4+ T- cells (T-helper cells) were initially classified into two subsets, T- helper1 (Th1) and T- helper-2.[8] T

cells have the ability to both boost and inhibit the inflammation. Furthermore, there are regulator T (Treg) cells that depressed the immune reaction and they are distinct from T helper cells. Treg cells are further divided into a inducible Treg (iTreg) cells and natural T reg (nTreg); immunological homeostasis suppression is mediated by this nTreg cells that are CD4+ CD25+ T cells that arise and move from the thymus. T-effector cells are responsible for the peripheral production of iTreg cells. iTreg cells based on their cytokine production shows the production of Tr1, Th3, as well as interleukin (IL)-35-producing T (iTr35) cells are the three subclasses iTreg cells. Tr1 and Th3 are responsible for the release of IL-10 and transforming growth factor (TGF).[7]

For development a CD4+ T cells into Th1 cells, as well as generation of interferon (INF)-γ interleukin (IL)-12 plays a significant role. Interleukin-13 (IL-13), IL-12, Interleukin-27 (IL-27), and IL-35 all of these belong to cytokine family IL-12. These four members exist all as a heterodimeric cytokines, with 1 chain (p19, p28, or p35) and the other (p40 or Epstein-Barr virus induced gene 3(EBi3)) that signs via an distinct couplings of 5 receptors (IL-12R1, IL-12R2, IL-23R, gp130, and WSX-1).[9] Another Heterodimeric cytokine whose subunits are structurally related to type I cytokines is IL-12p70 made up of p40 and p35. The p 40 subunit consists of the IL-12Rβ1 and IL-12Rβ2 chains which is homologous to the homologous IL-12p70 receptor. It can also be released as homodimers (IL-12p80) or monomers, which then signal through a protein IL-12Rβ1,2,p19 which was found based on its IL-6 or IL-12p35 homology, and it remained demonstrated to be combine to IL12p40 subunit to form IL-23, a heterodimeric cytokine. The IL-23 receptor is made up of 2 subunits: the IL-12Rβ1 chain and the IL-23R subunit. EBi3 has been discovered as a homologue of IL-12p40, resulting in the formation of IL-27. WSX1 and gap130 make up the IL-27 receptor. EBi3 has been shown to interact with IL-12p35 to produce IL-35, a cytokine.[10,11]

IL-35 as, Forkhead box P3 $(Foxp3)^+$ Treg cell immunosuppressive/anti-inflammatory cytokine, is needed to regulate the activity optimum. For Maintaining the peripheral immune system; modulation of T effector cell proliferation; suppression of Th17 cell development and IL-17 generation are all functions of IL-35. This shows its association with immunological and chronic infectious diseases like periodontitis.[12] T lymphocytes and CD4+ CD25+ T cells, as well as phenotypic markers of Tregs such Foxp3 have increased frequency of appearance in the inflammatory infiltrate of gingival tissues, and individuals with chronic periodontitis. These findings suggest that Tregs are present in chronic lesions and are likely to have a role for controlling the local immune response in chronic periodontitis.[13]

IL-35, a newer cytokine that is anti-inflammatory is made up of a chain of IL-27β, Ebi3 and IL-12α chain p35, is the latest adherent of IL-12 family. It's produced by Treg cell populations that has a strong suppressive effect and is an effective inhibitory cytokine. IL-35 stops the proliferation of the T-cell by arresting the cells in the G1 phase of mitosis without causing apoptosis. iTr35 cells are formed by the help of IL-35, which are Treg cell subgroups. The suppression of target T cells by n Treg cells turns them into iTr35 cells, donating to modulatory atmosphere in inflammatory areas.[14]

To distinguish between periodontally healthy sites and gingivitis and peridontitis sites, many clinical and radiographic approaches are used. The problem with these clinical and radiographic approaches is that they identify changes much later, i.e., alveolar bone loss or gain is not detected on them until there is 30 to 50% of the bone mineral elimination. It's too late to start treatment until then, and the patient will need substantial treatment, such as nonsurgical periodontal therapy and regenerative periodontal surgical operations.[15, 16]

In the biological sciences, disease biomarkers play an increasingly essential role in diagnosis, therapy monitoring, and drug discovery. Biomarkers must be able to detect disease

progression early and provide more reliable medication efficacy evaluations. Inflammatory mediator levels in biological fluids have been found to be a good indication of inflammatory activity in numerous investigations. Hence while studying the pathophysiology of the periodontal diseases we look at the biochemical and immunologic indicators in the GCF to calculate the future disease development and amount of tissue destruction. Various oral fluid biomarkers such as proteins of enzymes and immunoglobulins, host cells, phenotypic markers, hormones, bacteria and bacterial metabolites, ions, and volatile substances have been explored for periodontal diagnosis.[17]

GCF can be obtained non-invasively from the gingival sulcus/pocket as it is a plasmatic extravagate fluid displaying a strong diagnostic potential that reflects periodontal destructive processes, and as a source of metabolic activity-related variables in marginal periodontal disease. GCF is a complex biofluid that travels from the microcirculation over inflamed periodontal tissue. It contains several bacteria- or host-derived compounds that can be employed as a site-specific indicator of disease activity.[18,19] Thus detection of these biomarkers make GCF an appropriate diagnostic tool to measure the amounts of inflammatory mediators are produced and released during periodontal disease progression.[19]

GCF collection is a painless technique, and analysis of specific elements in GCF provides quantifiable cellular metabolism that represents periodontal health. As a result, efforts to create novel diagnostic tools have largely centred on GCF.[17] Studies on the content and composition GCF has provided with necessary information for a better understanding of the disease process and raise hopes for the development of accurate diagnostic tools that could overcome current clinical limits. Various components of GCF like that of the connective tissue degradation end products, acute phase proteins, immune components, and other inflammatory mediators have all been studied in GCF for this reason, making the

development of biochemical diagnostic assays based on their presence in GCF more appropriate.[20]

The microbiome and cytokine composition in GCF in healthy and periodontitis patients, was studied by Zhou et al. and came to the conclusion that the immune response and oral dysbiosis play a key role in periodontitis aetiology. Another study by Pei et al. in which he collected GCF from healthy and periodontitis patients and found that the alteration in the metabolic product and oral dysbiosis observed in GCF can be a good predictor of periodontal disease diagnosis, management and prognosis.[21] The expression/production of the cytokine in periodontal tissues is likely reflected in GCF cytokine production. As a result, looking into the expression of IL-35 in GCF could help researchers better understand the periodontal disease process.

Non-surgical periodontal therapy (NSPT) is the gold standard in treatment of periodontitis. To mechanically remove bacterial biofilm and deposits, scaling and root planning (SRP) is used, resulting in a local environment and microbiota that is in harmony with periodontal health. After NSPT inflamed periodontal tissue is replaced by a very perfused and collagen-rich connective tissues. Gingival tissue shrinks in the apical direction, towards the root surface, as a result of this. NSPT has shown to cause improvements in inflammation, pocket depth, clinical attachment, and inflammatory biomarker levels in the GCF and also has been demonstrated its efficacy in many clinical trials.[22]

Researches have proven that an anti-inflammatory cytokine i.e the IL-35 lowers the immune response by increasing the Treg cells and downregulating the Th17 cells. [23] This points to IL-35 having a beneficial impact in chronic inflammatory diseases like periodontitis. The manifestation of IL-35 m RNA in gingiva of aggressive periodontitis and chronic periodontitis patients was evaluated by Kalburgi NB et al. and demonstrated it has a possible

part in periodontal diseases pathogenesis as there were found increased expression of IL-35 in chronic and aggressive periodontitis.[24] Koseuglu et al (2015) assessed IL-35 levels in GCF, saliva, and plasma of the periodontal disease patients and health patients. There were found significantly higher levels IL-35 concentration in the GCF of the healthy group than the gingivitis and periodontitis groups. Thus GCF IL-35 levels were decreasing with the increase in inflammatory status. So, the authors concluded that IL-35 might be contributing significantly in suppressing of the periodontal disease related inflammation and maintaining the health of the periodontium.[7] Similarly another study by Kaustubh TS et in 2017 demonstrate the level of IL-35 by collected GCF samples from healthy, gingivitis and periodontitis individuals and showed that there were elevated levels of IL-35 in Gingivitis group when compared to the Periodontitis group this shows there levels decrease with the increase in inflammation.[25]

In the literature the studies have shown increased as well as decreased levels on IL-35 in chronic periodontitis state, there is no clear demarcation whether these levels are do approximately increase or at what level of depletion of IL-35 thus the inflammation progress to chronic periodontitis. Thus in the study the levels of IL-35 were evaluated in periodontitis and gingivitis patients before and after the NSPT and compared the levels from before NSPT and after NSPT.

AIM AND OBJECTIVES

The aim of the present study was to evaluate and compare IL-35 levels in GCF of periodontally healthy, gingivitis and Stage III periodontitis group before and after Non-surgical periodontal therapy with the following objectives.

1) To evaluate and compare the GCF Interleukin 35 levels in periodontally healthy, gingivitis and Stage III periodontitis group before non-surgical periodontal therapy.
2) To evaluate and compare the GCF Interleukin 35 levels in periodontally healthy, gingivitis and Stage III periodontitis group after non-surgical periodontal therapy.
3) To compare GCF Interleukin 35 levels before and after non-surgical periodontal therapy in periodontally healthy, gingivitis and Stage III periodontitis group.

4) To compare the GCF Interleukin 35 levels in periodontally healthy sites, gingivitis sites and periodontitis sites in Stage III periodontitis group before and after non-surgical periodontal therapy.

REVIEW OF LITERATURE

Periodontitis is a chronic inflammatory disease that plays a major role in the breakdown of tooth supporting structures.[1]During periodontal disease process healthy gingival gets converted to gingivitis and then to periodontitis. However, it cannot be predicted that when healthy gingiva will get converted to gingivitis and when gingivitis will get converted to periodontitis.[2] The immune response during periodontitis includes the infiltration of neutrophils, macrophages, B cells, and T cells and cytokines and other mediators. Several biomarkers have been evaluated in periodontally healthy, gingivitis and periodontitis subjects.[3] Interleukins have been implicated in various disease processes due to their vast pro- and anti-inflammatory effects. Various anti-inflammatory lnterleukins have been studied for their possible use as diagnostic tool for periodontitis. Major lacuna in this area is that most of the studies have been carried out on anti-inflammatory cytokines that regulate the inflammatory response by their action on Th1, Th2 cells (IL-4, IL-10, IL-13 etc.) and very

few studies are directed towards anti-inflammatory interleukins acting on Treg cells (IL-35).[26]

Treatment of periodontal diseases involves the elimination of inflammation, arresting the progression of periodontal disease, improve esthetics and create an environment conductive to maintain health. NSPT is the cornerstone of periodontal therapy and the first recommended approach to the control of periodontal infections. It is defined as plaque removal, plaque control, suprgingival and subgingival scaling root planning (SRP) and adjunctive use of chemical agents. In periodontitis, NSPT reduces inflammation, pocket depth, and increases Clinical attachment levels (CAL). In periodontal maintenance patients, also NSPT reduces inflammation and disrupts the bacterial biofilm which has a crucial role in disease causation and recurrence. It also helps in maintenance or stability of pocket PD and CAL.[27] Along with the reduction in inflammation there is a change in the levels of the GCF inflammatory biomarkers after NSPT. So in the present NSPT was use as a treatment option in the periodontitis and gingivitis patients.

Role of IL35 in periodontal disease conditions-

Kalburgi NB et al. 2013 The production of IL-35 mRNA of patients with chronic and severe periodontitis was studied. This study involved 60 patients, 20 of whom had chronic periodontitis, 20 of whom had aggressive periodontitis, while 20 of whom were healthy. The expression of IL-35 mRNA in gingival tissue samples was investigated using "Reverse transcriptase chain reaction" (RT-PCR). IL-35 mRNA expression was found to be maximum for chronic periodontitis, preceded with aggressive periodontitis, & lowest in healthy persons, according to the data. This shows the IL-35 may play a role in the progression of periodontitis.[24]

Mitani A et al. 2015 For chronic periodontitis as well as healthy people, the expression and production of IL-35, IL-17, and IL-27 within GCF & gingival tissues taken from the mesial, central, & distal sides of the teeth in this study. ELISA was used to examine IL-35 not just in GCF but also in gingival tissue, which included sulcus/pocket epithelium and underlying connective tissue, and Quantitative polymerase chain reaction (qPCR) was utilised to examine EB13, IL12A, IL17A, and IL27p28. Periodontitis patients had higher levels of IL-35 and IL-17 expression than healthy people, while IL-27 was not found in considerable amounts in periodontal tissue. The researchers found both IL-35 & IL-17 may play a role in the development of periodontitis.[28]

Koseoglu S et al. 2015 IL-35 levels in plasma, GCF, & saliva of periodontitis patients and healthy individuals were evaluated by Koseoglu S et al. The specimens were tested from patients of gingivitis, CP and healthy individuals were tried to compare to a healthy and gingivitis groups, and the total amount of IL-35 was found to be higher in the CP groups. The healthy had much greater saliva or GCF IL-35 levels than the other groups. The importance of IL-35 in periodontal inflammation control and periodontal maintenance of health is demonstrated by these studies.[7]

Cai S et al. 2017 established the interrelationships between IL-35, Soluble Cluster of differentiation 14 (sCD14), and IL- 23 aberrant expression in GCF and blood serum. After NSPT, Thirty chronic periodontitis patients & 30 healthy volunteers were analysed, and it was discovered that IL-35, IL-23, and sCD14 from GCF and blood had a favourable link with the severity of CP. In terms of CP severity, IL-35 levels were linked to sCD14 but not to IL-23. As per findings, IL-35 may endow sCD14 with the ability to stimulate an immune defense cell-mediated inflammatory process that contributes to the development of CP.[29]

Jin Y et al. 2017 The researchers looked at the expression of IL-35 in CP patients' "peripheral blood mononuclear cells" (PBMNCs) with periodontal tissues. As a healthy control, periodontal tissues, GCF, & peripheral blood were taken either CP patients and those with impacted teeth. The IL-35 subunit mRNA was quantified in PBMCs utilizing reverse transcription-quantitative polymerase chain reaction, while GCF and sera were assessed using ELISA. When healthy & CP samples were compared, overall IL-35 component mRNA levels in CP were significantly higher. Both their GCF and serum, CP patients reported higher amounts of IL-35 protein. An elevated amount of IL-35, according to this study, defends from periodontal disease by maintaining homeostasis and lowering inflammation.[12]

Okada K et al. 2017 the impacts of IL-35 on Th17 cells were investigated. The T cell population with in PBMCs from three CP participants and three healthy individuals was studied using flow cytometry. Th17 cells were cultured either with or without Recombinant human Interleukin- 35 (rIL-35) after being generated from PBMCs that used a cytokine cocktail, while IL-17A production was assessed using an ELISA kit. Applying rIL-35 to Th17 cells significantly reduced IL-17A production, according to the findings. This suggests that IL-35 could have an anti-inflammatory impact in periodontitis by directly reducing IL-17 expression.[30]

Kaustubh TS et al. 2017 the levels of IL-35 with in GCF in chronic gingivitis in CP patients were investigated. The samples were taken from 15 individuals with CP and 15 patients with gingivitis. According to ELISA research, a chronic gingivitis group had the highest levels of

Il-35 than the control group, suggesting that IL-35 levels decreased as inflammation levels increased, implying that it may have an anti-inflammatory role.[25]

Ustun K et al. 2018 In order to treat CP and to see effect on GCF IL-1, IL-6, and IL-35 levels, researchers was using an erbium, chromium: yttrium, scandium, gallium, garnet (Er,

Cr: YSGG) laser like a supplement to scaling and root planning. All periodontal parameters were significantly reduced in both groups after periodontal therapy, however there were no significant differences in GCF volume, IL-6, IL-1, and IL-35 levels between the two groups, although there was a considerable drop after baseline following periodontal treatment.[31]

Raj et al. 2018 Using serum and GCF, researchers estimated and compared interleukins-35 levels throughout healthy, gingivitis, CP individuals, as well as evaluated the effects of NSPT on IL-35 levels in CP patients. While analyzing the results, they discovered increased IL-35 levels in CP group before NSPT when compared to the healthy group as well as the CP group after NSPT.[23]

Jing et al. 2019 When comparing the gingiva in chronic periodontitis patients to the gingiva of healthy people, immunohistochemistry revealed considerably higher levels of IL-35 and IL-37. In CP gingival tissues, "CD138+ CD38+ plasma cells" are shown to be the most prevalent immune cell type, and these cells generated IL-35 and IL-37. Finally, the findings suggest that IL-35, IL-37 coproducing plasma cells (P-IL-37 & P-1L-35/IL-37) coexist among plasma cell subgroups within CP lesions, and these two types of plasma cells may regulate periodontitis progression by directly reducing osteoclast production and so restricting alveolar bone loss.[32]

Maboudi et al. 2019 the serum was used to look at the levels of IL-35 and IL-23 in type 2 diabetes and CP patients. Patients were separated into four groups: healthy individual i.e patients neither CP and type 2 Diabetes Mellitus DM, patients lacking CP and with type 2 DM, patients without type 2 DM and with CP, and patients with CP and type 2 DM. It was discovered that level for IL-23 - 35 serum did not differ significantly. The findings showed that neither type 2 diabetes nor chronic pain impacted serum levels of IL-23 and IL-35.[33]

Shindo et al 2019 the effect of IL-35 on the production of IL-6 and IL-8 in "human periodontal ligament cells" (HPDLCs) induced with IL-17A was investigated. The generation of IL-6 and IL-8 of IL17 A-stimulated HPDLCs were suppressed by IL-35. The western blot study revealed that IL-35 reduced phosphorylation of "extracellular signal-regulated kinase" (ERK) and nuclear factor (NF)-B p35 in IL-17A-stimulated HPDLCs. This shows that IL-35 generated by regulatory T cells can slow the progression of periodontitis by lowering IL-6 and IL-8 levels induced by IL-17A.[34]

Castantini et al. 2020 the amounts of vitamin D & inflammatory mediators in periodontitis patients' saliva were measured. ELISA was used to assess salivary cytokine such as 25-HydroxyVitamin –D3 (25(OH) D3), TGF, IL-35, IL-17A, and Matrix metalloprotenaise 9 (MMP9). When comparing periodontitis patients to healthy controls, researchers discovered higher levels of TGF, IL-35, IL-17A, and MMP9.[35]

Cafferata et al. 2020 investigated if IL-35 inhibits alveolar bone resorption in periodontitis via regulating the Th17/Treg imbalance He looked at mice who had periodontitis and were given IL-35 either locally or systemically to treat it. A control group of mice was employed that had not been treated and did not have periodontitis. The findings revealed that IL-35 inhibited alveolar bone resorption for mice with induced periodontitis, as well as a lower detection of Th17 cells and production of Th17-related cytokines for periodontitis mice and a larger quantity for Treg lymphocytes as well as production of Treg-related cytokines.[36]

Kamiya et al. 2020 the effect of IL-35 upon osteoclastogenesis, which is important in the pathophysiology of rheumatoid arthritis & periodontitis, was investigated. Using western blot analysis, overall effect of the IL-35 upon "receptor activator of nuclear factor" (NF)- κB ligand RANKL-stimulated signaling pathways was evaluated. Tatrate-resistant acid phosphate staining, hydroxyapatite resorption assay, & "quantitative polymerase chain

reaction" were used to evaluate the increased osteoclastogenesis and osteoclastic differentiation in RAW264 (RAW) cells induced via receptor activator of RANKL and IL-35. It was discovered both IL-35 and RANKL stimulate osteoclastogenesis in a synergistic manner, suggesting that IL-35 may play a novel and crucial function in inflammatory bone disorders such periodontitis and RA.[37]

Hetta et al. 2020 the researchers looked at the levels of circulatory, pro-inflammatory, and anti-inflammatory cytokines, as well as B regs, in individuals with periodontitis. ELISA was used to measure the levels of the anti-inflammatory cytokine like IL-10, IL-35, and TGF-β & pro-inflammatory cytokines as IL-1β, IL-6, and TNF-β in the serum. When comparing stage 2 periodontitis to healthy patients, higher proportions of B reg were found, and also the amounts of cytokine in the serum have been significantly higher in periodontitis patients than in healthy patients, with a significant positive correlation between B reg and IL-35, IL-10, and TGF- levels. This implies that elevated B reg and serum cytokine levels in periodontitis patients are closely linked to disease progression.[38]

Schmidlin et al. 2021 in a comprehensive study, researchers looked at the role for IL-35 in the aetiology of periodontal disorders. The review contained 15 publications that met the inclusion criteria and addressed on pathobiology of periodontal diseases. The presence of IL-35 in saliva, GCF, serum, as well as gingival biopsies from patients suffering with inflammatory periodontal disorders was established, and the findings of two investigations demonstrated that NSPT could modulate IL-35 expression in chronic periodontitis. This review indicated that IL-35 plays an undeniable function in the pathobiology of inflammatory disorders including periodontitis.[39]

IL-35 in systemic diseases

Ning et al. 2015 The researchers looked at levels of serum IL-35 among patients with "rheumatoid arthritis" (RA) vs healthy controls, and looked at how they correlated with RA clinical markers. An ELISA kit was used to detect serum IL-35 levels in 100 patients & 50 healthy controls. In comparison with healthy controls, RA patients showed significantly lower amounts of IL-35 in their blood. In a correlation research, IL-35 was found to have a substantial negative connection with age, Rheumatoid factor, & neutrophil percent. The researchers discovered the IL-35 plays a protective role in the genesis of RA.[40]

Tang et al. (2018) To explore the role of IL-35 in the genesis of systemic sclerosis (SSc), researchers looked at serum IL-35 levels before and after the procedure, as well as its correlation with clinical parameters and the frequency of various CD4+ T cells in participants with SSc. Flow cytometry was used to characterise Th1, Th2, Th17, as well as T regs with in peripheral blood of 49 SSc patients & 20 healthy controls. An ELISA kit was used to assess serum IL-35 levels. And they discovered that IL-35 levels were considerably higher in SSc patients compared to healthy controls, but that they were much lower after 3 months of treatment. Lower Th1 cell percentages were detected in SSc patients, but greater Th2 and Th17 cell percentages are found, leading to reduced Th1/Th2 ratios & increased Th17/T regs ratios. This suggests that greater blood IL-35 levels are linked to the onset of SSc and the degree of pulmonary fibrosis among SSc patients.[41]

He et al. 2018 The concentrations of serum IL-35 in Systemic Lupus Erythematosus (SLE) patients with and without nephritis, as well as their clinical values, were determined in 120 SLE patients, 80 of which had "Lupus nephritis" (LN) and 40 of whom had SLE without nephritis. The researchers discovered that SLE patients with nephritis exhibited lower blood

levels of IL-35 than inactive SLE individuals without nephritis, meaning that IL-35 could be used as a biomarker of renal involvement in LN patients.[42]

Ye et al. 2019 The proportions of circulating regulatory B cell subsets, as well as plasma IL-35, IL-10, IL-17A, tumour necrosis factor (TNF)-, and interferon (IFN)-, were assessed and compared in 47 Chinese patients with a diagnosis of Systemic lupus erythematosus (SLE) as well as 20 healthy controls. They discovered greater levels of blood IL-10, IFN-γ, TNF-α, & IL-17, and a lower proportion of IL-35+B cells & IL-10+ B cells amongst total blood B cells & a decreased level of plasma IL-35 after assessing them. These results imply both IL-35+Bregs & IL-35 may help to prevent the start and progression of SLE.[43]

Yayla et al. 2020 the researchers looked at the relationship between serum IL-35 and clinical results in SSc patients. This study included 70 patients with SSc & 29 healthy volunteers. Serum IL-35 levels were significantly greater in SSc patients than in healthy controls, but there was no significant relationship between IL-35 with organ involvement. There was also a negative association between IL-35 and the medsger disease severity score. They also discovered that IL-35 levels were higher in participants having SSc, and not in SSc patients with pulmonary fibrosis. [44]

Effects of Non-surgical periodontal therapy.

Bokhari et al. In 2009, researchers investigated the impact of NSPT on the quantity of "C-reactive proteins" (CRP), fibrinogen, & white blood cell (WBC) levels in participants with or without "coronary heart disease" (CHD) (NCHD). To assess periodontal disease, periodontal markers including as "bleeding on probing" (BOP) & probing depth (PD) have been recorded, as well as all individuals got NSPT. Oral hygiene tips were offered, and also subgingival scaling & root planing. Inflammatory markers with in bloodstream were measured before and after one month of periodontal therapy. For patients with CHD and

NCHD, the NSPT was observed to result in a significant reduction in BOP and PD, as well as decreased blood inflammatory markers.[45]

Kamil et al. 2011 NSPT's effect on CRP and blood lipid level in patients with locally advanced periodontitis was investigated. The systemic quantity for inflammatory markers was measured at baseline & 3 months after NSPT in 60 systemically healthy participants who were randomly divided into the experimental and comparison groups. The decrease in PI, GI, & PD in the therapy group was significantly, linear, and directly connected to the decline in CRP.[46]

Shah et al. 2011 determine the short-form oral health impact profile (OHIP-14) was used to measure the oral health-related quality of life (OHQoL) of patients with chronic periodontitis and its improvement following 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.[47]

Moeintaghavi et al. 2012 The effects of NSPT on metabolic control in patients with type 2 diabetes mellitus (DM2) were investigated. Forty patients with DM2 & chronic periodontitis have been randomly divided into two groups: the test group received "full mouth scaling & root planning", while the control group received no periodontal treatment. CAL, "fasting plasma glucose" (FPG), Hemoglobin A1c (HbA1c), "total cholesterol" (TC), triglyceride (TG), & cholesterol levels were measured at baseline as well as compared to data collected three months later. The test group's HbA1c dropped from baseline to follow-up. FPG, GI, PPD, & CAL increased with in control group throughout the same time period, while HbA1c did not alter significantly.[48]

Bhardwaj et al. 2015 Based on the GI, PD, and CAL, researchers looked at the influence of periodontal disease & NSPT on plasma "homocysteine" (Hcy) in systemically healthy subjects. Healthy and CP subjects were divided into two groups. At baseline and 12 weeks after SRP for Hcy, plasma samples were obtained and analysed utilizing "high performance liquid chromatography with fluorescence detection" (HPLC-fld). NSPT could be employed like an adjuvant Hcy reducing therapy, adding to primary Cardio Vascular Disease (CVD) prevention, according to the findings.[49]

Silveira et al. 2016 In people with advanced chronic periodontitis, researchers examined the effects of one-stage "full-mouth disinfection" (FMD) vs traditional quadrant scaling in four weekly sessions (QS) upon periodontal clinical markers and halitosis. The following measurements were taken at baseline & 90 days after therapy for each group: PI, "tongue-coating index" (TCI), BOP, PD, and CAL. Halimetry was done using an organoleptic technique, and the quantities of "volatile sulphur compounds" were measured using gas chromatography. NSPT, irrespective of protocol, was found to be effective in enhancing individual periodontal clinical status, lowering organoleptic ratings levels over time, as well as reducing halitosis, according to the study.[50]

Al-Hamoudi et al. 2017 In chronic periodontitis patients with and without and without obesity, researchers compared the efficacy of "one-stage full-mouth disinfection" (FMD) & evaluated the effect of "phase 1 Periodontal Therapy" on periodontal parameters, whole salivary resistin, and interleukin-6 (IL-6) levels. At baseline and 6 months after SRP, the levels of BOP, PD, whole salivary resistin, and IL-6 were assessed using an enzyme-linked immunosorbent assay. In both obese & non-obese CP patients, SRP has been shown to help reduce periodontal inflammation.[51]

Cosgarea et al. 2018 Patients having moderate - to - severe chronic periodontitis & rheumatoid arthritis (RA-CP) were compared to CP patients without RA to see how NSPT affected clinical and inflammatory markers. Within 24 hours, 18 patients with RA-CP and systemically sound patients having CP were treated with SRP. In the RA-CP group, clinical data, inflammatory markers, & microorganisms in the subgingival biofilm were evaluated during baseline, 3 & 6 months after NSPT, as well as RA disease activity markers & specific antibodies. According to the findings, NSPT improve periodontal disease among CP individuals with & without RA, and inhibition of P. gingivalis in conjunction with proper oral hygiene may temporarily reduce periodontal disease in patients with RA.[52]

Jeyasree et al. 2018 In individuals with chronic generalised periodontitis, the effect of NSPT on clinical parameters was studied, as well as the qualitative amount or quantity of "alkaline phosphatase" (ALP) in saliva & serum following the initial Initial stage of periodontal therapy. Clinically evaluated parameters such as the "Simplified oral hygiene index" (OHI-S), GI, PD, and CAL were analysed, and saliva and blood samples were collected for spectrometry analysis of ALP amount. The study found that after phase I periodontal therapy, i.e. SRP, the amount of serum and salivary ALP decreases significantly, while clinical parameters improve.[53]

Van der Weijden et al. 2019 In this study, 182 patients with periodontitis underwent active NSPT, which included professional oral hygiene maintenance therapy instructions, scaling & root planing, supra gingival polishing, and specialised systemic antibacterial medicine as needed. The results were based on the completion of a full mouth periodontal chart just at time of the evaluation. The researchers looked at the link between the parameters examined and the efficacy of aggressive periodontal therapy. In patients having adult periodontitis, active NSPT resulted in around 1/3 of cases having a PPD >5mm success end point.

According to a sub-analysis, tooth type, furcation presence, & periodontal disease severity, as well as smoking, appeared to influence the outcome.[54]

Yue et al. 2020 In a comprehensive study, researchers looked into whether NSPT can help patients on "haemodialysis "(HD) and/or "peritoneal dialysis" reduce systemic inflammation and enhance metabolism (PD). Randomized controlled studies were found by searching electronic databases. This research includes five RCTs. After NSPT, dialysis patients with periodontitis had significantly lower levels of "highly sensitive C-reactive protein" (hs-CRP) than those with untreated periodontitis after less than or equivalent to 2 months. At the 3- and 6-month follow-ups, no significant differences in IL-6 & albumin levels were identified following NSPT. The results indicate that NSPT can reduce blood hs-CRP levels in HD &/or PD patients in a modest way, but it had no effect on IL-6 or Albumin levels.[55]

MATERIALS AND METHODS

GCF IL-35 levels were measured in periodontally healthy participants, periodontitis patients, & gingivitis patients before & after NSPT in this interventional study.

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

Study groups

1. For the selected patients, dental & medical history was taken, as well as intraoral examination was performed by a single examiner. Periodontal characteristics such as Plaque index (PI) [Silness & Loe 1964] [56], Gingival index (GI) [Loe & Silness, 1963][57], "papillary bleeding index" (PBI) score[58], "Probing Pocket Depth" (PPD), & "Clinical Attachment Level" (CAL) were used to divide the individuals into three groups of 20 each.

Group I (Periodontally healthy individuals) (Color Plate I)

Patients with no clinical attachment loss, a PPD of less than 3 mm, and a PBI score of less than 1 were considered as periodontally healthy.

Group II (Gingivitis patients) (Color Plate II)

Patients with clinical evidence of gingival inflammation and yet no clinical attachment loss, PPD of less than 3 mm, and a PBI score greater than 1 were considered as gingivitis**.**

Group III (Stage III Periodontitis patients) (Color Plate III)

"Stage III Periodontitis" (Severe Periodontitis) patients were diagnosed according to the clinical signs of periodontitis.

The detailed Classification for Periodontal & Peri-implant Diseases & Conditions published by the "American Academy of Periodontology" in 2017 will be used to diagnose periodontitis.[59]

Patient selection criteria

Inclusion Criteria

Patients had to be over the age of 18 and have at-least 20 natural teeth to be considered. Patients with gingivitis who have clinical evidence of gingival inflammation but again no clinical attachment losses, a probing pocket depth (PPD) of less than 3 mm, and a PBI score greater than 1. Patients were classified as having stage III periodontitis i.e the severe periodontitis when they were with PPD $\geq$ 5 mm and CAL $\geq$ 4 mm.

Exclusion criteria

1. Systemic illnesses that may affect periodontal disease.
2. Alcoholics and tobacco (in whatever form).
3. Females who are pregnant or nursing.

4. Periodontal treatment in the previous six months.

5. Anti-inflammatory and antibacterial medications taken in the previous six months

Armamentarium (Color plate IV)

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

For examination of the patient:

1. Mouth mirror

2. University of North Carolina (UNC)-15 periodontal probe

3. Tweezer

4. Dental Explorer

5. Disposable gloves

7. Kidney tray

8. Cotton swab.

9. Surgical drape

For collection of GCF sample

1. 5µl micro capillary pipette
2. Eppendorf tube
3. Sterilized cotton rolls.

For evaluation of IL-35 levels in the gingival crevicular fluid

1. IL-35 ELISA kit

Assessment of periodontal and clinical parameters

1. Plaque index (PI): (Silness and Loe, 1964)[56]

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	Plaque film attaching to a free gingival margin & adjacent tooth region. Only by passing a probing from across tooth surface could the plaque be identified.
2	Moderate buildup of soft deposits in the gingival pocket, on the gingival margin, and/or on the adjacent tooth surface, visible to the naked eye.
3	An excessive amount of soft deposits inside the gingival pocket &/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)[57]

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.

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

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)[58]

On the mesial face of the papilla, a periodontal probe (UNC 15) is carefully inserted into the gingival sulcus at base of papilla, and afterwards advanced coronally to papilla tip. On the distal part of the same papilla, the same thing is repeated. On a scale of 0 to 4, the severity of any bleeding that resulted was recorded.

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

- Calibrated, volumetric transfer pipettes with 0-5 µl range, 5-50 µl range, 50-200 µl range and 200-1000 µl range
- Sterilized test tubes with test tube stand
- Distilled water
- Beakers, Measuring cylinder
- Sterile gloves
- Absorbent paper
- Test tube for standard preparation
- Covered plastic tubes
- Semi-log graph paper or software for data analysis
- Timer

Laboratory equipment

- -80°C deep freezer (REMI Equipments Pvt. Ltd.) **(Color plate V)**
- Vortex mixer (CM 101, REMI Equipments Pvt. Ltd.) **(Color plate VI)**
- ELISA Washer (LISA wash reader, REMI Equipments Pvt. Ltd.) **(Color plate VII)**
- ELISA reader (LISA Microplate reader, REMI Equipments Pvt. Ltd.)**(Color plate VII)**

Assessment of Biochemical parameters

Site selection and GCF collection:

Only one site per patient was selected on day 1 as a sampling site in Gingivitis groups (group 2), 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.

In Stage III Periodontitis group3 samples will be collected, one sample will be taken from healthy site, one from site showing signs of periodontitis and one from the site showing signs of gingivitis. 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.

Evaluation of IL-35 from GCF

Samples were assayed for IL-35 levels using commercially available ELISA (Enzyme linked immune-sorbent assay) Kinesis Dx IL-35 ELISA Kit. 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 IL-35 was determined. All samples have been run in duplication.

Reagent (Color Plate VI)

1. Microtiter Coated Plate (96 wells) – 3 no

2. Human IL-35 Biotin Conjugated Detection Antibody, 1 ml – 3 vial

3. Standard 64ng/ml – 0.5 ml

4. Streptavidin: HRP Conjugate - 6 ml

5. Wash Buffer (30X) – 20 ml

6. Standard Diluent – 3 ml

7. Substrate A – 6 ml

8. Substrate B – 6 ml

9. 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

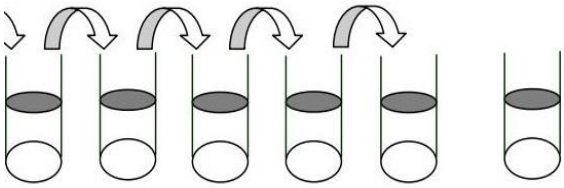
Reagent preparation for GCF samples

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.

240 µl 240 µl 240 µl 240 µl 240 µl



		Std5	**Std4**	**Std3**	**Std2**	**Std1**
Diluent Volume	Original Standard +Standard diluent	240 µl	240 µl	240 µl	240 µl	240 µl

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.

IL-35 ASSAY PRINCIPLE

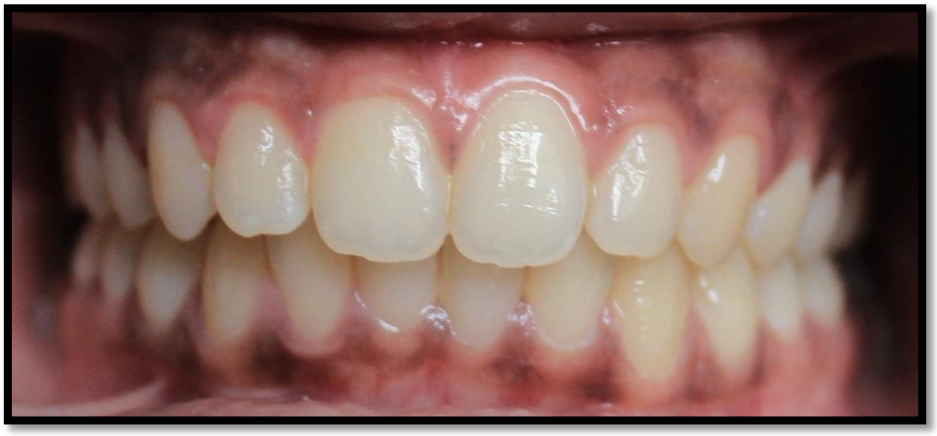
The method employs sandwich ELISA technique. Monoclonal antibodies are pre-coated onto microwells. Samples and standards are pipetted into microwells and Human Interleukin 35, IL-35 present in the sample are bound by the antibodies. Biotin labeled antibody is added and followed by Streptavidin-HRP is pipetted and incubated to form a complex. After washing microwells in order to remove any non-specific binding, the substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Human Interleukin 35, IL-35 in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph Paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Human Interleukin 35, IL-35 concentrations, find the unknown's Mean Absorbance value on the Yaxis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Human Interleukin 35, IL-35 Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit is best recommended for automated results.

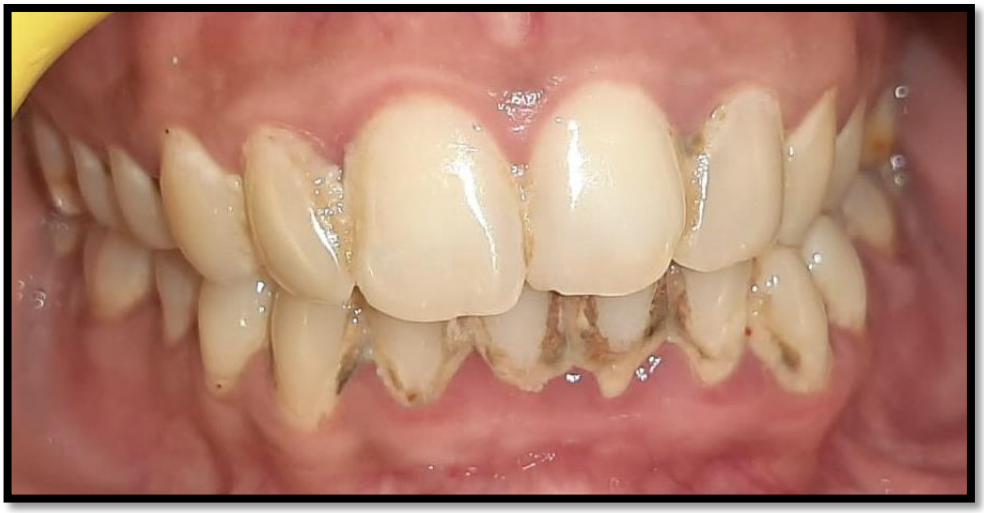
COLOR PLATE I

Group I (Periodontally Healthy Individuals)



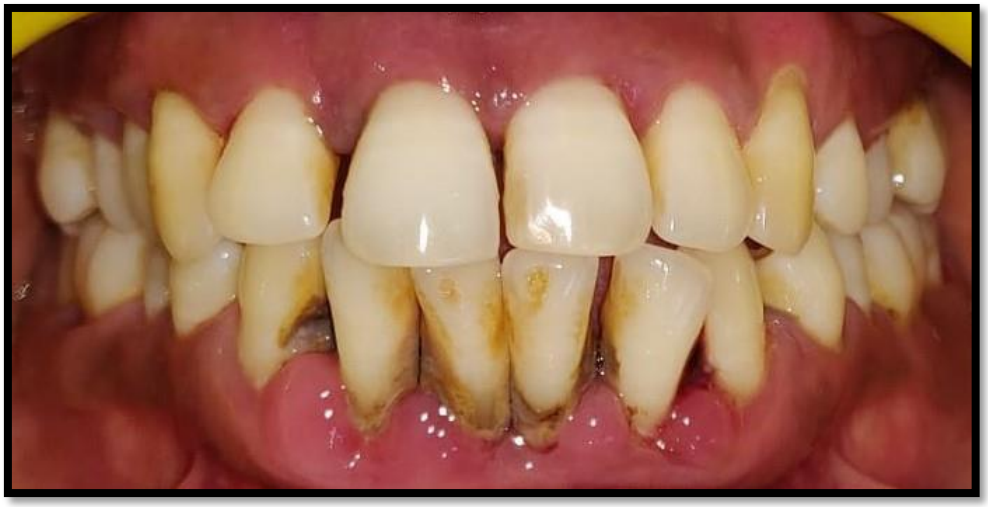
COLOR PLATE II

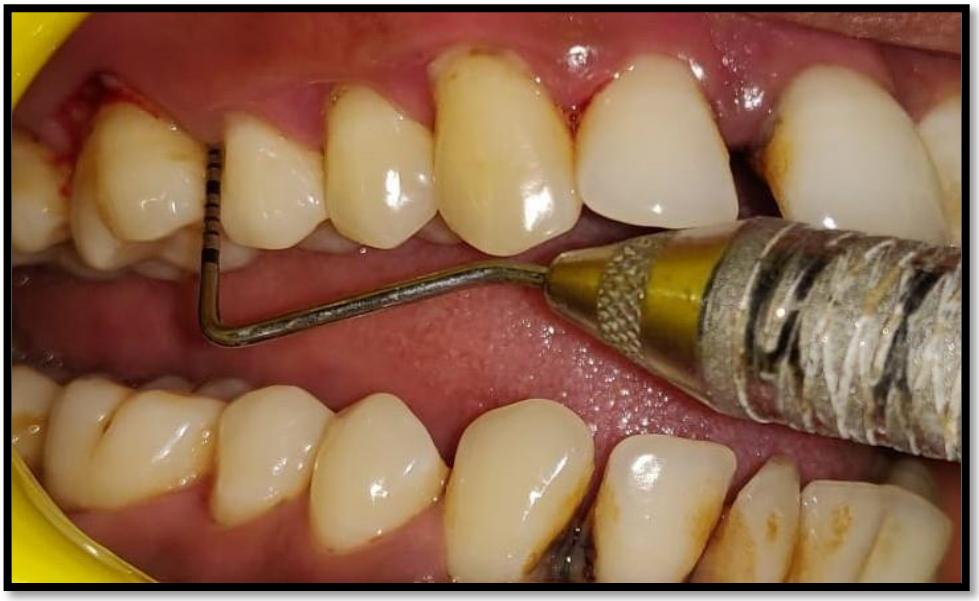
Group II (Gingivitis patients)



COLOR PLATE III

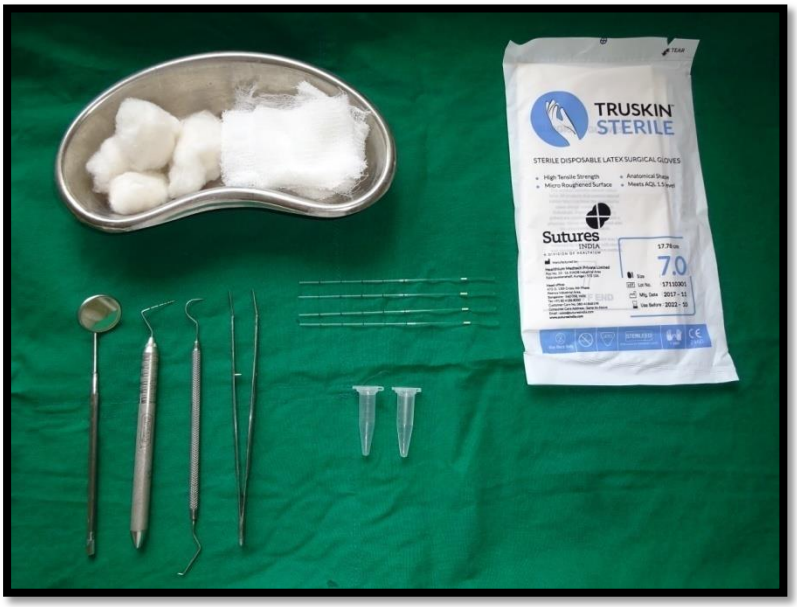
Group III (Stage III Periodontitis)



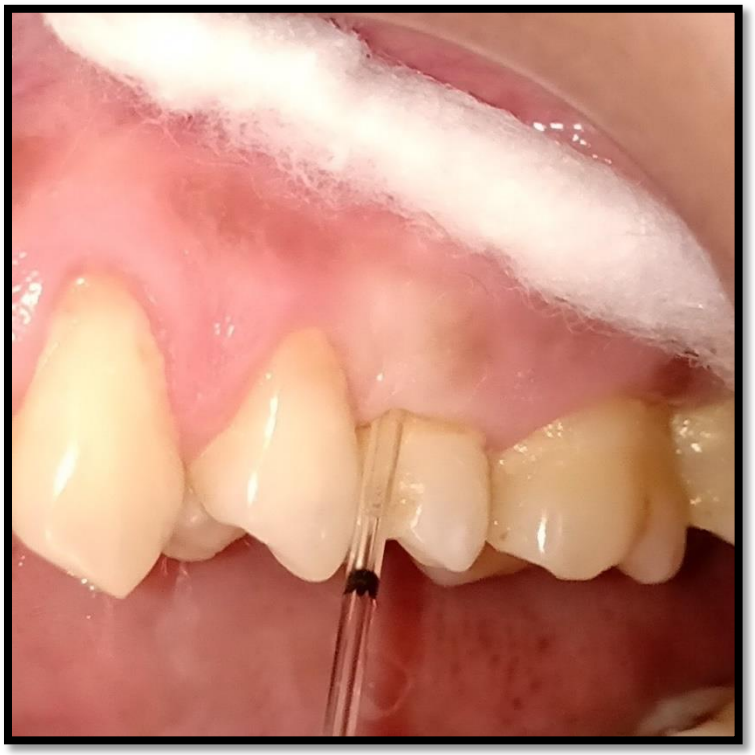


Probing depth ≥ 5mm

COLOR PLATE IV



Armamentarium for clinical examination and GCF collection

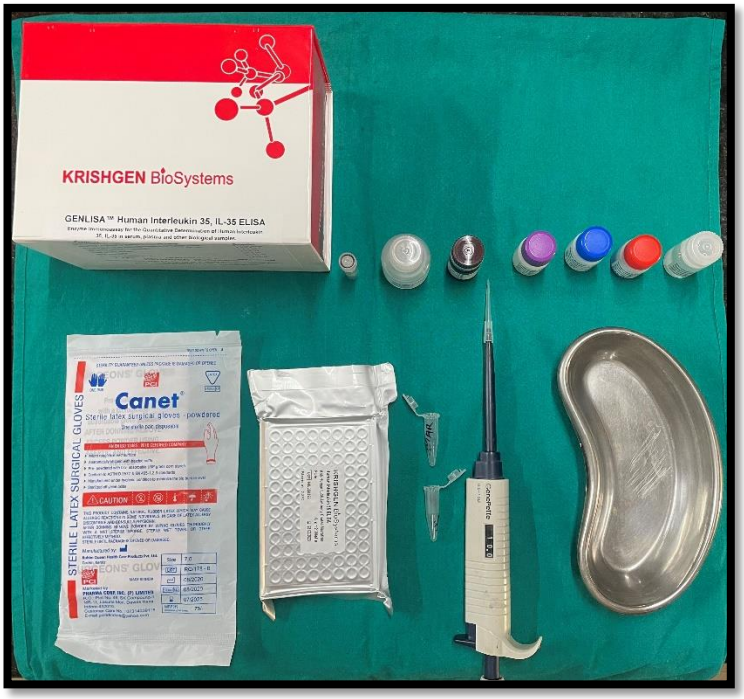


Collection of GCF from gingival sulcus

COLOR PLATE V

Deep Freezer

COLOR PLATE VI

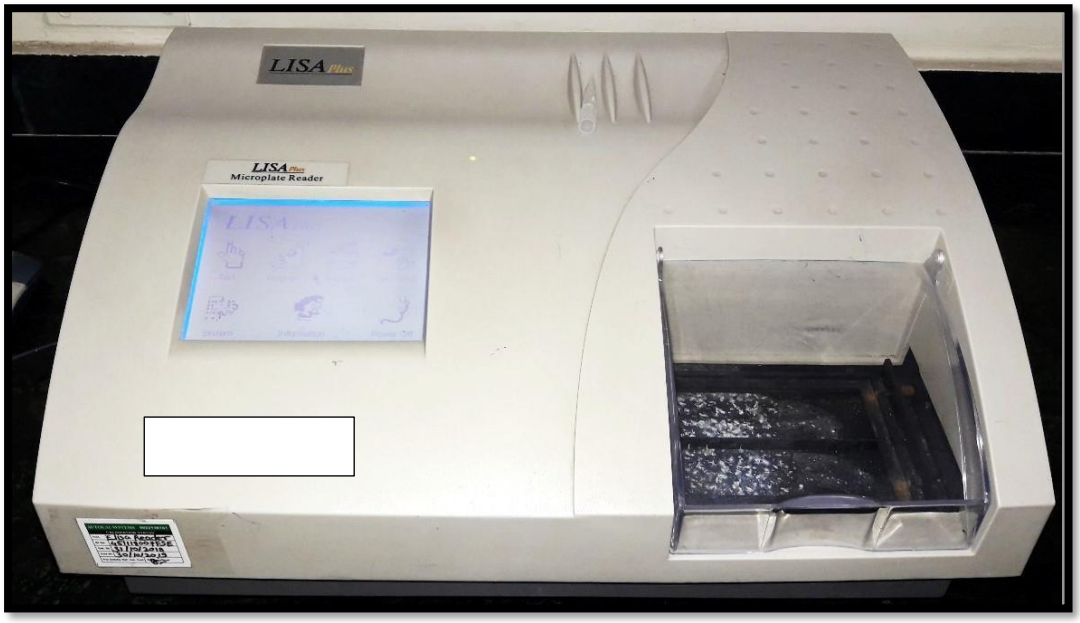


Interleukin-35 ELISA Kits

Vortex Mixer

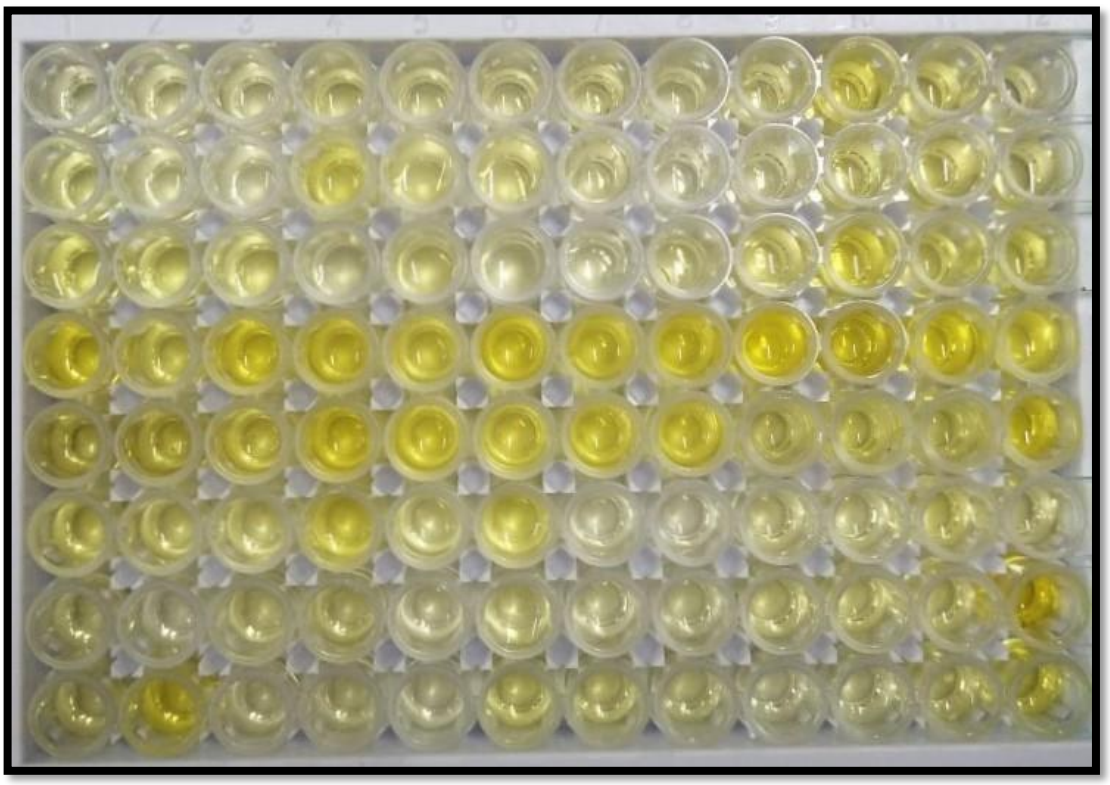
COLOR PLATE VII

ELISA Washer



ELISA Reader

COLOR PLATE VIII



ELISA plate after adding stop solution

RESULTS

The present study compared Interleukin-35 levels in Gingival crevicular fluid of periodontally healthy, gingivitis and Stage III Periodontitis group before and after Non-surgical periodontal therapy. The healthy samples were collected at baseline line and used for for comparison with gingivitis and Stage III periodontitis group both before and after NSPT. 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 at 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 individual group; females were 11 (55%) and males were 9 (45%). In gingivitis group, females were 10 (50%) and males were10 (50%). In Stage III Periodontitis group, females were 9 (45%) and males were 11 (55%).

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 healthy individuals and gingivitis group. In the age group of 18-22 years, there were 9 (45%) patients in healthy individuals group and 8 (40%) patients in Gingivitis group. Likewise, 23-27 years had 8 (40%) with healthy individuals and 8 (40%) with Gingivitis group. 28-32 years age group had 3 (15%) healthy individuals and 3(15%) with Gingivitis group, 33-37 years age group had no individuals in healthy group and 1 (5%) in Gingivitis group. The table also represents distribution of patients with respect to age in Stage III Periodontitis group. In the age group, in 43-47years age group, there were 8 (40%) patients, in 48-52 years, there were 6 (30%) patients, in 53-57 years, there were 5 (25%) patients and in the age group of 58-62 years, there was 1 (5%) patient.

Graph 2

The bar diagram represents distribution of patients with respect to age in healthy individuals and gingivitis 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.

Graph 3

The bar diagram represents distribution of patients with respect to age in Stage III Periodontitis group. The x-axis represents group and the y-axis represents percentage of patients in each age group. Patients were more in the age group from 43-47 years.

Table 3.

The table represents difference in healthy individuals, gingivitis and Stage III Periodontitis groups for GI, PI, PBI, PPD and CAL parameters at baseline. A significant difference was present between the groups for GI with $F=97.70$ and $p<0.0001$. A significant difference was present between the groups for PI with $F=233.05$ and $p<0.0001$. A significant difference was present between the groups for PBI with $F=129.53$ and $p<0.0001$. A significant difference was present between the groups for PPD with $F=160.49$ and $p<0.0001$. A significant difference was present between the groups for CAL with $F=630.71$ and $p<0.0001$. GI was more in Stage III Periodontitis group followed by gingivitis group and least in healthy individuals. Similarly, PI, PBI and PPD scores were also more in Stage III Periodontitis group followed by gingivitis group and least in healthy individuals group. The CAL scores in healthy and gingivitis patients groups were same i.e., zero while they were highest in the Stage III Periodontitis group.

Graph 4

The bar diagram represents the variation in the GI, PI, PBI, PPD and CAL parameters between healthy individuals groups, gingivitis group and Stage III Periodontitis group. The x-axis represents parameters; GI, PI, PBI, PPD and CAL and the y-axis represents the mean values of these parameters at baseline.

Table 4

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, PBI, PPD and CAL. The difference for GI ranged between 0.82 to 1.87 with $p<0.0001$. For PI, the difference between the groups ranged between 1.07 to 2.62 with $p<0.005$. For PBI, the mean difference ranged between 0.831 to 2.06 with $p<0.0001$. For PPD, the mean difference ranged between 1.10 to 4.65 with $p<0.005$. For CAL, the mean difference healthy/ gingivitis group with that of Stage III Periodontitis was 7.60 with $p<0.0001$.

Table 5

The table represents difference in the intervention groups for GI, PI, PBI, PPD and CAL parameters at 3 months. A significant difference was present between the groups for GI with mean difference of 0.98 and $p<0.0001$. A significant difference was present between the groups for PI with mean difference of 0.82 and $p<0.0001$. A significant difference was present between the groups for PBI with mean difference of 0.80 and $p<0.0001$. A significant difference was present between the groups for PPD with mean difference of 2.55 and $p<0.0001$. A significant difference was present between the groups for CAL with mean difference of 6.30 and $p<0.0001$. Parameters, GI, PI, PBI, PPD and CAL were significantly more in Stage III Periodontitis group compared to gingivitis group.

Graph 5

The bar diagram represents the variation in the GI, PI, PBI, PPD and CAL parameters between gingivitis group and Stage III Periodontitis group at 3 months. The x-axis represents parameters; GI, PI, PBI, PPD and CAL and the y-axis represents the mean values of these parameters 3 months. The values were higher in the Stage III Periodontitis group followed by gingivitis group.

Table 6.

The table represents mean IL-35 level in healthy individuals group. The mean IL-35 was 70.17 ± 3.10 with the values ranging between 64.10 to 78.20.

Graph 6

The bar diagram represents mean and standard deviation of IL-35 level in healthy individuals group. The x-axis represents the parameter and the y-axis represents the values of the parameter IL-35.

Table 7

The table represents mean IL-35 level in gingivitis group at baseline and 3 months. The mean IL-35 at baseline was 35.73 ± 3.36 and at 3 months was 64.84 ± 3.07. The table also represents mean IL-35 level in Stage III Periodontitis group at Site 1, Site 2 and Site 3 at baseline and 3 months. The mean IL-35 at Site-1 at baseline was 55.47 ± 2.64 and at 3 months was 62.69 ± 2.80. The mean IL-35 at Site-2 at baseline was 31.68 ± 2.60 and at 3 months was 47.05 ± 2.75. The mean IL-35 at Site-3 at baseline was 21.02 ± 3.36 and at 3 months was 30.41 ± 2.92. The IL-35 level was more at 3 months compared to baseline at all the three sites.

Graph 7

The bar diagram represents mean Il-35 in gingivitis group at baseline and 3 months. The x-axis represents time period and y-axis represents the values of the parameter IL-35. The IL-35 level increased from baseline to 3 months. The bar diagram also represents mean IL-35 level in Stage III Periodontitis group at Site1, Site 2 and Site-3 from baseline to 3 months. The x-axis represents time period and the y-axis represents the IL-35 level. The IL-35 level increased from baseline to 3 months at all the three sites.

Table 8

The table represents difference in the IL-35 level from baseline to 3 months in gingivitis group. IL-35 level significantly increased from baseline to 3 months with a mean difference of 29.66, t=20.69 and p<0.0001.

Table 9, 10, 11

The table represents difference in the IL-35 level from baseline to 3 months in Stage III Periodontitis group at Site-1, Site-2 and Site-3. At Site-1, IL-35 level significantly increased from baseline to 3 months with a mean difference of 7.22, t=8.37 and p<0.0001 (table 9.). At, Site-2, IL-35 level significantly increased from baseline to 3 months with a mean difference of 15.37, t=18.16 and p<0.0001 (table 10.). At, Site-3, IL-35 level significantly increased from baseline to 3 months with a mean difference of 9.38, t=9.41 and p<0.0001 (table 11.).

Table 12

The table represents difference in the IL-35 level between the three sites in Stage III Periodontitis group at baseline. There was a significant difference in IL-35 level with F=745.21and p<0.0001. The IL-35 was more at Site-1 followed by Site-2 and least at Site-3.

Graph 8

The bar diagram represents mean IL-35 level in Stage III Periodontitis group at Site-1, Site-2 and Site-3 at baseline. The x-axis represents sites and the y-axis represents the IL-35 level. The IL-35 levels at baseline were higher at Site-1 followed by Site-2 and Site 3.

Table 13

The table represents difference in the IL-35 level between the three sites in Stage III Periodontitis group at baseline using pot hoc Tukey test. The mean difference between the

sites at baseline for IL-35 level ranged from 10.65 to 34.44 with $p<0.0001$. A statistical significant difference was present between the sites for IL35 level.

Table 14

The table represents difference in IL-35 level at 3 months between the three sites in Stage III Periodontitis group. There was a statistical significant difference in IL-35 level between Site-1, Site-2 and Site-3 at 3 months with $F=650.72$ and $p<0.0001$. The IL-35 level was more at Site-1 followed by Site-2 and least at Site-3.

Graph .9

The bar diagram represents mean IL-35 level in Stage III Periodontitis group at Site-1, Site-2 and Site-3 at 3 months. The x-axis represents sites and the y-axis represents the IL-35 level. The IL-35 levels at 3 months were higher at Site-1 followed by Site-2 and Site 3.

Table 15

The table represents difference in the IL-35 level between the three sites in Stage III Periodontitis group at 3 months using pot hoc Tukey test. The mean difference between the sites at 3 months for IL-35 level ranged from 15.64 to 32.28 with $p<0.0001$. A statistical significant difference was present between the sites for IL35 level.

Table .16

The table represents difference in the IL-35 level at baseline between the three groups. There was a statistical significant difference in IL-35 level between healthy individuals, gingivitis group and Stage III Periodontitis groups at baseline with $F=1183.00$ and $p<0.0001$. IL-35 level was more in Healthy individuals group followed by gingivitis group and least in Stage III Periodontitis group.

Graph .10

The bar diagram represent mean IL-35 level in healthy individuals, gingivitis and Stage III Periodontitis group at baseline. The x-axis represents groups and the y-axis represents the IL-35 level. The IL-35 levels at baseline were higher in healthy patients group followed by gingivitis group and Stage III Periodontitis group.

Table .17

The table represents difference in IL-35 level at baseline between the groups using post hoc Tukey test. The mean difference between the groups at baseline for IL-35 level ranged from 14.70 to 49.14 with $p<0.0001$. A statistical significant difference was present between the groups for IL-35 level.

Table 18

The table represents difference in the IL-35 level at 3 months between the three groups. There was a statistical significant difference in IL-35 level between healthy patients, gingivitis patients and Stage III Periodontitis patients groups at 3 months with $F=1008$ and $p<0.0001$. IL-35 level was more in Healthy individuals group followed by gingivitis group and least in Stage III Periodontitis group.

Graph 11

The bar diagram represent mean IL-35 level in healthy individuals, gingivitis and Stage III Periodontitis group at 3 months. The x-axis represents groups and the y-axis represents the IL-35 level. The IL-35 levels at 3 months were higher in healthy individuals group followed by gingivitis group and Stage III Periodontitis group.

Table 19

The table represents difference in IL-35 level at 3 months between the groups using post hoc Tukey test. The mean difference between the groups at 3 months for IL-35 level ranged from 5.33 to 39.75 with $p<0.0001$. A statistical significant difference was present between the groups for IL-35 level.

Overall, with respect to the parameters like GI, PI, PBI, PPD and CAL; the scores significantly reduced from baseline to 3 months in both gingivitis group and Stage III Periodontitis group. The difference between the parameters at baseline was significant with higher scores among Stage III Periodontitis group followed by gingivitis group and the least among the healthy individuals group. Similarly, at 3 months, the difference in the GI, PI, PBI, PPD and CAL was significant between the gingivitis and Stage III Periodontitis group with higher scores among Stage III Periodontitis group followed by gingivitis group.

With respect to the IL-35 level in Stage III Periodontitis group at Site,1 Site-2 and Site-3; the levels significantly increased from baseline to 3 months at all the sites. There was a significant difference in IL-35 level at all the site at baseline as well as at 3 months with higher levels at Site-1 followed by Site-2 and Site-3. With respect to IL-35 level, the level significantly increased from baseline to 3 months among gingivitis group. At baseline the difference among the three groups was significant. Similarly, at 3 months there was a significant difference in IL-35 level with higher level among healthy individuals followed by gingivitis group and Stage III Periodontitis group.

DISCUSSION

The present study was conducted to measure GCF IL-35 levels in the periodontitis and gingivitis patients to evaluate the levels in different disease state and correlate it with the severity of the disease and also to evaluate the effect of NSPT on the levels of IL-35 in periodontitis and gingivitis patients. Total of 60 patients with age group ranging from 18-60 were selected and divided into three groups i.e Group I (healthy Individuals group), Group II (gingivitis group), Group III (Stage III Periodontitis group) each group consists of 20 subjects. A total of 180 GCF samples were collected, 20 samples from healthy Individuals, 40 samples from gingivitis group 20 before NSPT and 20 after NSPT i.e after 3 months, 120 samples from Stage III Periodontitis group i.e 1 sample from each site namely healthy site, 1 sample from sites showing signs of gingivitis and 1 sample from sites showing signs of periodontitis before and after NSPT. All 180 GCF samples were analyzed for assessing IL-35 levels using ELISA test.

Mean full mouth plaque index (PI) score, mean full mouth gingival index score (GI) and papillary bleeding index (PBI) score in Gingivitis group were significantly higher compared

to healthy individuals before NSPT. In Stage III Periodontitis group the mean full mouth plaque index (PI) score, mean full mouth gingival index score (GI) and papillary bleeding index (PBI) score showed significant greater values as compared to Gingivitis and Healthy groups before NSPT.

Comparison of probing pocket depth (PPD) and clinical attachment levels (CAL) between the groups showed significantly more PPD and CAL, both full mouth assessment and at selected sites, indicating that division of groups as well as sites into healthy gingiva group, gingivitis group and Stage III Periodontitis group as well as sites before NSPT. These clinical parameters showed improvement after NSPT in the Gingivitis group and Stage III Periodontitis group except for the PPD and CAL in the gingivitis group as it was considered as a fixed value according to the inclusion criteria. The parameters also showed a significant difference between the 3 groups after NSPT that the GI, PI and PBI was higher in the gingivitis and Stage III Periodontitis group.

Periodontal diseases are considered to be multifactorial diseases, where putative periodontal pathogens trigger inflammatory and immune responses. It is characterized by irreversible loss of alveolar bone and connective tissue attachment in the periodontium which ultimately results in the loss of teeth. These pathogens are known to produce proteolytic enzymes as well as elicit signals in resident gingival cells or in immune cells infiltrating the gingival tissues that result in immune responses; these responses lead to either the successful removal of the pathogens or to host mediated destruction of the periodontal tissue.[60]

Gingival inflammation is regulated by reciprocal interactions between various cell types including, leukocytes, epithelial cells, fibroblasts and endothelial cells. Among the many immune and inflammatory mediators known cytokines have attracted special attention. Pro-inflammatory and anti-inflammatory cytokines play a key role in the pathogenesis of

periodontal disease there by influencing destruction, remodeling and repair of periodontal tissues.[61]

The search for potential markers associated with the severity, as well as the susceptibility of periodontal disease, has recently been receiving considerable attention.[62] Although roles of many other cytokines have been evaluated in the pathogenesis of periodontal diseases, the exact role of IL-35 has not yet been studied. We compared GCF levels of IL-35 with the severity of periodontal disease process among the 3 groups and also the effect of the NSPT on the IL-35 levels of GCF to obtain an insight into the probable role of IL-35 in immune-pathogenesis of periodontal disease, as well as to assess future periodontal disease site activity.

In the present study mean GCF IL-35 levels at healthy sites in healthy individuals was 70.17 pg/µl, while at it was significantly reduced to 35.7345 pg/µl in gingivitis group further reduced to 21.0275 pg/µl in the periodontitis sites in the Stage III Periodontitis group at both time points i.e before NSPT and after NSPT. This results were correlative with the results obtained by Koseglu et al. 2015[7] who reported mean GCF concentration of 63.19 pg/µl in periodontally healthy subjects which was significantly higher than that of the chronic gingivitis and chronic periodontitis group. There is always an interaction between host and bacteria even in clinically healthy gingival crevices. This subclinical inflammation may cause IL-35 expression from micro vascular endothelial and epithelial cells. [63]

In the present study, GCF IL-35 levels in gingivitis group was 35.7345 pg/µl which were significantly improved to that of 64.84 pg/µl after NSPT but when compared to the healthy individuals this levels were significant lower. GCF IL-35 levels of periodontitis sites in the Stage III Periodontitis group at baseline 21.0275 pg/µl and 3 months 30.4150 pg/µl when compared to gingivitis group showed the gingivitis group showed significant higher values

both at baseline and 3 months. This results are in accordance with the study done by Kaustubh et al. [25] in which the levels of GCF IL-35 in gingivitis patients was 36.28 pg/µl and chronic periodontitis patients was 19.93 pg/µl indicating that the levels of IL-35 decreases with increase in the inflammatory status hence playing a role in suppressing gingival inflammation and maintaining periodontal health. [25]

In the present study the levels of GCF IL-35 were significantly higher in the Healthy sites 55.47 pg/µl (site1) in Stage III Periodontitis group which significantly reduced in the sites with Gingivitis 31.68 pg/µl (site 2) further-more it was significantly reduced in the Periodontitis sites 21.02 pg/µl (sites 3), and same changes were seen in the Stage III Periodontitis group after NSPT. The GCF IL-35 levels were significantly increased in the Stage III Periodontitis group in all the sites after NSPT. In general GCF IL-35 levels was progressively and significantly reduced from periodontally healthy sites, gingivitis sites and periodontitis sites. Similar findings have been reported by Koseglu et al.[7] In present study the GCF IL-35 levels in healthy sites in healthy individuals were 70.17 pg/µl and in healthy sites in periodontitis patients were 55.47 pg/µl. Can be considered as a GCF IL-35 concentration at which the disease stage might be initiated. So this, level can be considered as a probable point of conversion of healthy gingiva to diseased state. Similarly, GCF IL-35 concentration in gingivitis patients in sites with gingivitis was 35.7345 pg/µl and the gingivitis sites in periodontitis patients was 31.68 pg/µl. So, the GCF IL-35 levels of 31.68 pg/µl can be considered as a level at which progress from gingivitis to periodontitis starts, however further studies are required in this aspect.

A study by Raj et al. (2018)[23] showed that the GCF IL-35 values were higher in the chronic periodontitis group i.e 70.26 pg/µl than the Gingivitis group i.e 62.36 pg/µl and further lower in the healthy group 54.81 pg/µl, in this study the GCF IL-35 levels were also checked after NSPT but only in the chronic periodontitis group after which the levels were decreased to

55.72 pg/µl.[23] In a study by Mitani et al. (2015)[28] the GCF concentration of IL-35 was significantly higher in the chronic periodontitis group than the Gingivitis and healthy groups. In another study by Jin Y et al. (2017)[12] showed that the relative expression of IL-35 subunit mRNAs in the affected tissues of patients with chronic periodontitis was significantly higher compared with that in the samples from healthy controls.[12]The mean concentration of IL-35 protein in the GCF and sera of patients with periodontitis was also significantly higher compared with that in samples from healthy controls. These results are not in agreement with our results in terms of GCF concentration of IL-35. This difference could be because of the fact that chronic periodontitis has two period of activities characterized by short periods of activity and long periods of quiescence.[64] During those active periods, there is periodontal breakdown. In the quiescent episode, tissue repair occurs, and there is reduced inflammatory response with little or no alveolar bone loss. One of the possible explanations in their study is that GCF samples might be taken from quiescent sites rather than active sites in patients with CP, and some GC sample volumes in patients with CP may be lower than GCF sample volumes in healthy individuals.

In a study by Li et al. 2012[65] on the IL-35 and new categorization of anti-inflammatory cytokines. Their new system in categorizing anti-inflammatory cytokines and new working model provide considerable insight into the following issue: how anti-inflammatory cytokines share their functions. The housekeeping cytokines, for example TGF-b, prevent the formation of inflammation, whereas the responsive cytokines, including new cytokine IL-35, repress full blown inflammation. The other studies about IL-35 expression in the literature support their results.[66,67,68] IL-35 was induced by bacterial lipo-polysaccharides and stimulations of different proinflammatory cytokines in Treg cells,[14] smooth muscle cells,[69] microvascular endothelial cells,[70] and monocytes[71]. Furthermore, IL-35 was also expressed in epithelial cells by IL-1b, tumour necrosis factor (TNF)-α and interferon-g.[72] "Housekeeping" anti-

inflammatory cytokines, such as TGF-b, are expressed in homeostatic tissues to prevent formation of inflammation. After the inflammation, proinflammatory agents can induce tissues to express "responsive" anti-inflammatory cytokines, such as IL-35, by specific transcription factors to countervail the inflammation response.

The loss of IL-35 has also been shown to be associated with the development and exacerbation of disease, including many inflammatory diseases such as encephalomyelitis and inflammatory bowel disease. In multiple models of encephalomyelitis, wild type tregs can prevent the onset and severity of disease.[14] However, animals that lack functional IL-35 were shown to have enhanced inflammatory immune responses and increased disease. Similar observations been shown in inflammatory bowel disease, liver fibrosis, and models of lethal autoimmune disease.[14] Conversely, given that the loss of IL-35 is associated with increased incidence and severity of inflammatory diseases, the induction of IL-35 expression has been shown to alleviate a variety of disease symptoms. In models of inflammatory bowel disease, IL-35 gene therapy and the adoptive transfer of IL-35 expressing tregs have been shown to cure colitis symptoms.[73] In contrast to the inflammatory diseases, tumor models have shown that IL-35 can act to suppress tumor infiltrating lymphocytes that may have anti tumor activity, as well as potentially supporting the proliferation of tumor cells by promoting angiogenesis.[74] Therefore, in the present study the reduced levels of GCF IL-35 could be due to increase in the periodontal inflammation with increase in the severity of periodontal disease and hence the after NSPT in both gingivitis and periodontitis group there was increase in the anti-inflammatory cytokine i.e IL-35 with the reduction in the inflammation and the convergence of the diseased tissue in more healthy one.

Along with the human studies a study done by Cafferata et al. (2020) on mice looking at the therapeutic effect of IL-35 in which the local as well as systemic administration of IL-35 was done in the mice who were affected by periodontitis. The findings of the study showed the

inhibitory effect of alveolar bone resorption in periodontitis mice.[36] In the present study the emphasis was given on using GCF biomarkers for prediction of future disease progression. The use of panels of GCF biomarkers for disease diagnosis may hold promise.[75] GCF biomarkers, specifically IL-35 present in high concentrations were able to predict stability for 100 % of subjects who were clinically stable. GCF IL-35 levels within specific group showed that those undergoing clinical disease progression also had low concentrations of GCF IL-35. Offenbacher et al.[76] proposed a diagnostic periodontal disease classification scheme called the "biologic systems model". This model is based on medical and dental findings and contributory biologic phenotypes. Underlying "biologic phenotypes" consider the biofilm and the host inflammatory and immune response to be at the biofilm-gingival interface. As a whole, the biologic system model is built on a framework of components, starting with the recognition of subject-level exposures interacting with genetic and epigenetic factors, and including cellular and molecular processes and inflammatory biomarkers to define difference clinical phenotypes of periodontal disease detection and prediction. The present investigation supports the use of GCF IL-35 biomarker as indicators for periodontal disease progression. The use of GCF IL-35 biomarker offers potential for the prediction of periodontal disease progression or stability to potentially determine susceptible sites for future destruction in larger patient populations. In this study also the NSPT was used as the treatment modality or Gingivitis and periodontitis and the increase in the GCF IL-35 after NSPT shows that it has a significant effect on treatment of the periodontal diseases.

SUMMARY AND CONCLUSION

The purpose of the study was to evaluate the levels of GCF IL-35 for assessment of periodontal diseases. With the objective of determining and comparing the levels of IL-35 in GCF of healthy subjects, gingivitis patients and Stage III Periodontitis patients before and after NSPT.

From the analysis of the results and within the limitations of the present study following conclusions were drawn:

The clinical parameters like GI, PI, PBI, PPD, CAL were significantly higher in the Stage III Periodontitis group compared to the gingivitis group followed by healthy individuals both before and after NSPT.

The mean GCF IL-35 levels were significantly higher in the healthy individuals as compared to gingivitis group and Stage III Periodontitis group, also the mean GCF IL-35 levels in gingivitis group were significantly higher in gingivitis group when compared to Stage III

Periodontitis patients before NSPT suggesting with the increase in inflammatory status there is decrease in the levels of GCF IL-35.

The mean GCF IL-35 levels were significantly higher in the healthy individuals as compared to gingivitis group and Stage III Periodontitis group, also the mean GCF IL-35 levels in gingivitis group were significantly higher in gingivitis group when compared to Stage III Periodontitis group after NSPT suggesting with the NSPT there is an decrease in inflammation and hence there is increase the levels of GCF IL-35.

The mean GCF IL-35 were higher in healthy individuals, gingivitis group and periodontitis group after NSPT when compared to the GCF IL-35 levels before NSPT in same groups. This might be probably due to IL-35 being a responsive cytokine, it plays a role in suppressing inflammation.

The mean GCF IL-35 levels were higher in healthy sites as compared to gingivitis sites and periodontitis sites, also the IL-35 levels were significantly higher in gingivitis sites when compared to periodontitis sites in the Stage III Periodontitis group before NSPT and after NSPT.

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TABLES

Table no.1- Distribution of patients with respect to gender in the groups

Gender	Healthy individuals group		Gingivitis group		Stage III Periodontitis group	
	Frequency	Percentage	Frequency	Percentage	Frequency	Percentage
Females	11	55.0	10	50.0	9	45.0
Males	9	45.0	10	50.0	11	55.0
Total	20	100.0	20	100.0	20	100.0

Table no.2- Distribution of patients with respect to age in Healthy individuals, gingivitis group and Stage III Periodontitis group

Age	Healthy individuals		Gingivitis group		Age	Stage III Periodontitis group	
	Frequency	Percentage	Frequency	Percentage		**Frequency**	**Percentage**
18-22 years	9	45	8	40	43-47 years	8	40
23-27 years	8	40	8	40	48-52 years	6	30
28-32 years	3	15	3	15	53-57 years	5	25
33-37 years	0	0	1	5	58-62 years	1	5

Table no.3-Difference in the parameters at baseline

Parameters	Groups	Mean	F	Significance (p)
GI	Healthy individuals	.2290	97.70	**<0.0001***
	Gingivitis group	1.2833		
	Stage III periodontitis group	2.1085		
PI	Healthy individuals	.2695	233.05	**<0.0001***
	Gingivitis group	1.3460		
	Stage III Periodontitis group	2.8929		
PBI	Healthy individuals	.0490	129.53	**<0.0001***
	Gingivitis group	1.2870		
	Stage III Periodontitis group	2.1170		
PPD	Healthy individuals	1.2000	160.49	**<0.0001***
	Gingivitis group	2.3000		
	Stage III Periodontitis group	5.8500		
CAL	Healthy individuals	.0000	630.71	**<0.0001***
	Gingivitis group	.0000		
	Stage III Periodontitis group	7.6000		

Table no.4- Post hoc test for difference in parameters between the groups at baseline

Parameters	Groups		Mean Difference	Significance (p)
GI	Healthy individuals	Gingivitis patients	1.05425*	**<.0001***
		Stage III Periodontitis group	1.87950*	**<.0001***
	Gingivitis group	Healthy individuals	1.05425*	**<.0001***
		Stage III Periodontitis group	.82525*	**<.0001***
	Stage III Periodontitis group	Healthy individuals	1.87950*	**<.0001***
		Gingivitis patients	.82525*	**<.0001***
PI	Healthy individuals	Gingivitis patients	1.07650*	**<.0001***
		Stage III Periodontitis group	2.62340*	**<.0001***
	Gingivitis group	Healthy individuals	1.07650*	**<.0001***
		Stage III Periodontitis group	1.54690*	**<.0001***
	Stage III Periodontitis group	Healthy individuals	2.62340*	**<.0001***
		Gingivitis patients	1.54690*	**<.0001***
PBI	Healthy individuals	Gingivitis patients	1.23800*	**<.0001***
		Stage III Periodontitis group	2.06800*	**<.0001***
	Gingivitis group	Healthy individuals	1.23800*	**<.0001***
		Stage III Periodontitis group	.83000*	**<.0001***
	Stage III Periodontitis group	Healthy individuals	2.06800*	**<.0001***
		Gingivitis patients	.83000*	**<.0001***
PPD	Healthy individuals	Gingivitis patients	1.10000*	**<.0001***
		Stage III Periodontitis group	4.65000*	**<.0001***
	Gingivitis group	Healthy individuals	1.10000*	**<.0001***
		Stage III Periodontitis group	3.55000*	**<.0001***
	Stage III Periodontitis group	Healthy individuals	4.65000*	**<.0001***
		Gingivitis patients	3.55000*	**<.0001***
CAL	Healthy individuals	Gingivitis patients	.00000	1.000
		Stage III Periodontitis group	7.60000*	**<.0001***
	Gingivitis group	Healthy individuals	.00000	1.000
		Stage III Periodontitis group	7.60000*	**<.0001***
	Stage III Periodontitis group	Healthy individuals	7.60000*	**<.0001***
		Gingivitis patients	7.60000*	**<.0001***

Table no.5- Difference in parameters between the groups at 3 months

Parameters	Groups	Mean	Mean difference	t-value	Significance (p)
GI	Gingivitis group	.3620	0.98	7.01	**<0.0001***
	Stage III Periodontitis group	1.3505			
PI	Gingivitis group	.4535	0.82	6.62	**<0.0001***
	Stage III Periodontitis group	1.2800			
PBI	Gingivitis group	.5560	0.80	5.51	**<0.0001***
	Stage III Periodontitis group	1.3565			
PPD	Gingivitis group	2.2000	2.55	8.40	**<0.0001***
	Stage III Periodontitis group	4.7500			
CAL	Gingivitis group	.0000	6.30	21.64	**<0.0001***
	Stage III Periodontitis group	6.3000			

*Significant difference is present the groups for GI, PI, PBI, PPD and CAL with higher values of parameters in Stage III Periodontitis group compared to gingivitis group

Table no.6- Mean IL-35 values in Healthy individuals group

Parameter	N	Minimum	Maximum	Mean	Std. Deviation
IL-35	20	64.10	78.20	70.1700	3.10909

Table no.7- Mean IL-35 values in patients with gingivitis and Stage III Periodontitis group

IL-35	N	Minimum	Maximum	Mean	Std. Deviation
Gingivitis Baseline	20	30.10	43.10	35.7345	3.36236
Gingivitis 3months	20	58.70	69.00	64.8400	3.07458
Stage III Periodontitis at Site 1 Baseline	20	50.42	58.90	55.4725	2.64265
Stage III Periodontitis at Site 1 3months	20	58.30	68.20	62.6960	2.80789
Stage III Periodontitis at Site 2 Baseline	20	28.30	36.60	31.6840	2.60014
Stage III Periodontitis at Site 2 3months	20	41.90	51.30	47.0545	2.75024
Stage III Periodontitis at Site 3 Baseline	20	15.75	27.25	21.0275	3.36069
Stage III Periodontitis at Site 3 3months	20	27.20	36.10	30.4150	2.92921

Table no.8- Difference in the IL-35 from baseline to 3 months in gingivitis group

Time period	Mean	Mena difference	t-test	Significance (p)
Baseline	35.7345	29.66	20.692	**<0.0001***
3months	64.8400			

*Significance at $p<0.05$

A statistical significant difference is present from baseline to 3 months in IL-35 level in gingivitis group. IL-35 levels were significantly more at 3 months compared to baseline.

Table no.9- Difference in the IL-35 from baseline to 3 months in patients with Stage III Periodontitis group at site1

Time period	Mean	Mena difference	t-test	Significance (p)
Baseline	55.4725	7.22	8.378	**<0.0001***
3months	62.6960			

*Significance at p<0.05

A statistical significant difference is present from baseline to 3 months in IL-35 level in Stage III Periodontitis group at site1. IL-35 levels were significantly more at 3 months compared to baseline.

Table no.10- Difference in the IL-35 from baseline to 3 months in patients with Stage III Periodontitis group at site2

Time period	Mean	Mena difference	t-test	Significance (p)
Baseline	31.6840	15.37	18.162	**<0.0001***
3months	47.0545			

*Significance at p<0.05

A statistical significant difference is present from baseline to 3 months in IL-35 level in Stage III Periodontitis group at site2. IL-35 levels were significantly more at 3 months compared to baseline.

Table no. 11-Difference in the IL-35 from baseline to 3 months in patients with Stage III Periodontitis group at site3

Time period	Mean	Mena difference	t-test	Significance (p)
Baseline	21.0275	9.38	9.417	**<0.0001***
3months	30.4150			

*Significance at p<0.05

A statistical significant difference is present from baseline to 3 months in IL-35 level in Stage III Periodontitis group at site3. IL-35 levels were significantly more at 3 months compared to baseline.

Table no.12- Difference in IL-35 level between Site1, 2 and 3 at baseline in Stage III Periodontitis group

Site	Mean	F	Significance (p)
Site 1	55.4725	745.21	**<0.0001***
Site 2	31.6840		
Site 3	21.0275		

*Significance at p<0.05

A statistical significant difference is present between the sites for IL-35

Table no.13-Post hoc test for difference in IL-35 between the sites at baseline

Groups		Mean Difference	Significance (p)
Site 1	Site 2	23.78850*	**<0.0001***
	Site 3	34.44500*	**<0.0001***
Site 2	Site 1	23.78850*	**<0.0001***
	Site 3	10.65650*	**<0.0001***
Site 3	Site 1	34.44500*	**<0.0001***
	Site 2	10.65650*	**<0.0001***

*Significance at p<0.05

A statistical significant difference is present between the sites for IL-35 at baseline

Table no.14- Difference in IL-35 level between Site1, 2 and 3 at 3 months in Stage III Periodontitis group.

Sites	Mean	F	Significance (p)
Site 1	62.6960	650.72	**<0.0001***
Site 2	47.0545		
Site 3	30.4150		

*Significance at p<0.05

A statistical significant difference is present between the sites for IL-35 at 3 months

Table no.15- Post hoc test for difference in IL-35 between the sites at 3 months

Groups		Mean Difference	Significance (p)
Site 1	Site 2	15.64150*	**<0.0001***
	Site 3	32.28100*	**<0.0001***
Site 2	Site 1	-15.64150*	**<0.0001***
	Site 3	16.63950*	**<0.0001***
Site 3	Site 1	-32.28100*	**<0.0001***
	Site 2	-16.63950*	**<0.0001***

*Significance at p<0.05

A statistical significant difference is present between the sites for IL-35 at 3 months

Table no.16- Difference in the IL-35 at baseline between Healthy individuals, gingivitis group and Stage III Periodontitis group

Groups	Mean	F	Significance (p)
Healthy individuals	70.1700	1183.00	**<0.0001***
Gingivitis group	35.7345		
Stage III Periodontitis group	21.0275		

*Significance at p<0.05

A significant difference is present in IL-35 between groups at baseline.

Table no.17- Post hoc test for IL-35 between groups at baseline

Groups		Mean Difference	Significance (p)
Healthy individuals	Gingivitis group	34.43550*	**<0.0001***
	Stage III Periodontitis group	49.14250*	**<0.0001***
Gingivitis group	Healthy individuals	34.43550*	**<0.0001***
	Stage III Periodontitis group	14.70700*	**<0.0001***
Stage III Periodontitis group	Healthy individuals	49.14250*	**<0.0001***
	Gingivitis group	14.70700*	**<0.0001***

*Significance at p<0.05

A significant difference is present in IL-35 between groups at baseline.

Table no.18- Difference in the IL-35 level between Healthy individuals, gingivitis group and Stage III Periodontitis group at 3 months

Groups	Mean	F	Significance (p)
Healthy individuals	70.1700	1008.16	**<0.0001***
Gingivitis group	64.8400		
Stage III Periodontitis group	30.4150		

*Significance at p<0.05

A statistical significant difference is present in IL-35 level between Healthy individuals, gingivitis group and Stage III Periodontitis group at 3 months.

Table no.19- Post hoc Tukey test for IL-35 level between Healthy individuals, gingivitis group and Stage III Periodontitis group

Groups		Mean Difference	Significance (p)
Healthy individuals group	Gingivitis group	5.33000	**<.0001***
	Stage III Periodontitis group	39.75500	**<.0001***
Gingivitis group	Healthy individuals group	5.33000	**<.0001***
	Stage III Periodontitis group	34.42500	**<.0001***
Stage III Periodontitis group	Healthy individuals group	39.75500	**<.0001***
	Gingivitis group	34.42500	**<.0001***

*Significance at $p<0.05$

A statistical significant difference is present in IL-35 level between:

Healthy individuals & gingivitis group

Healthy individuals & Stage III Periodontitis group

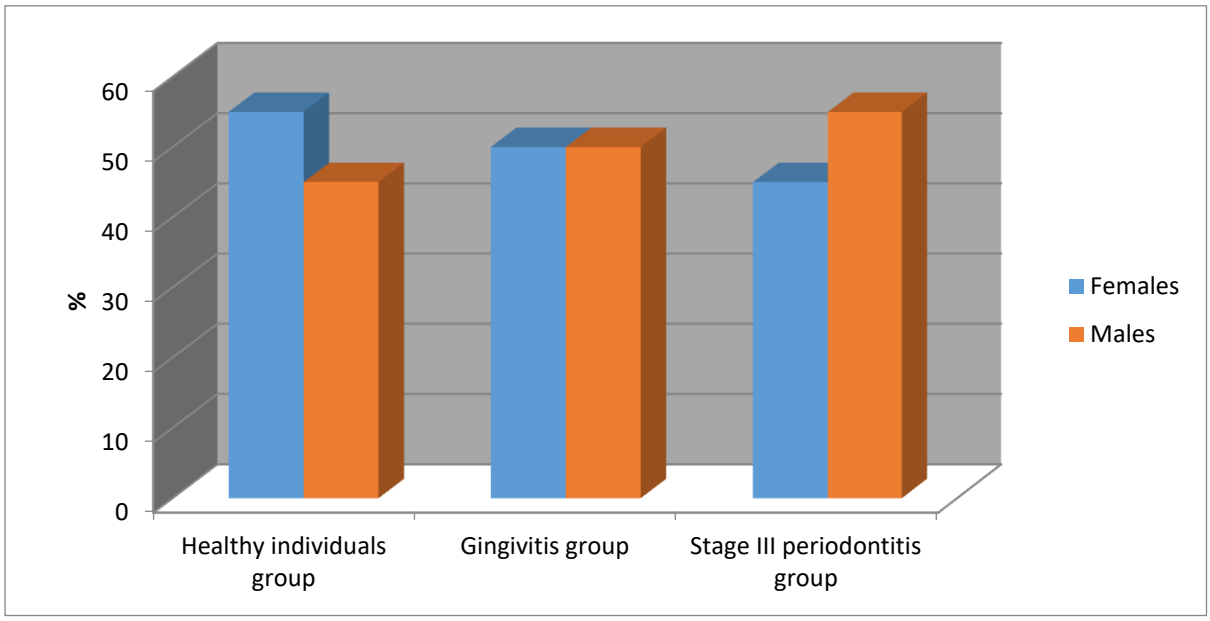
Gingivitis patients & Stage III Periodontitis group

IL-35 levels were:

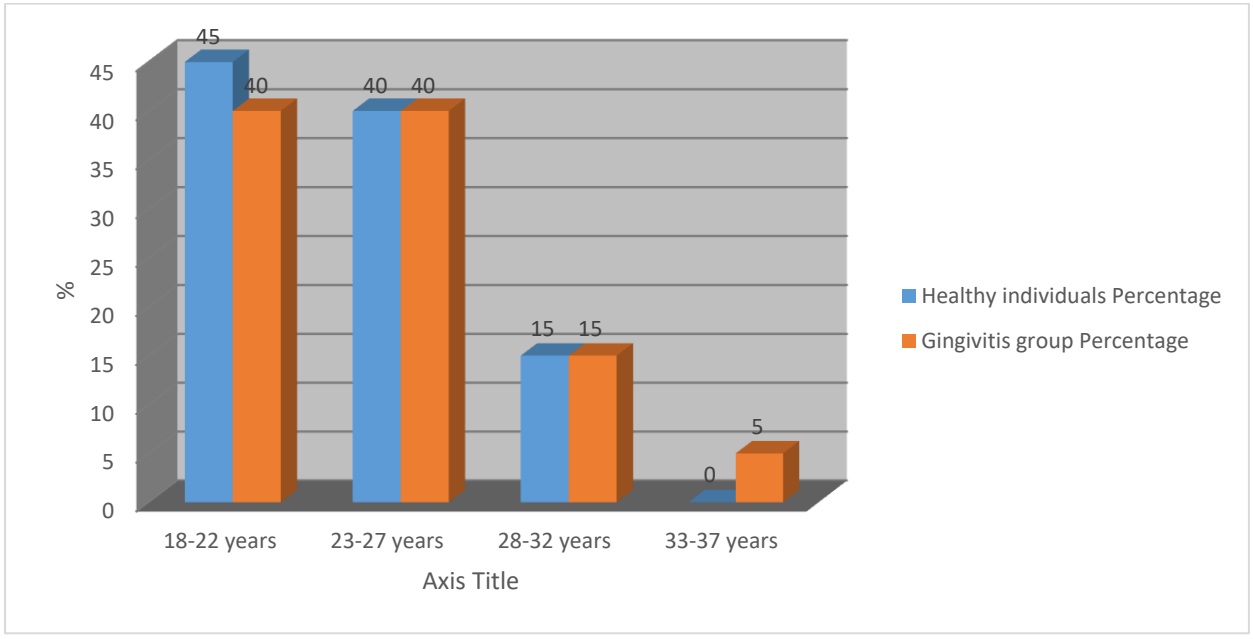
Healthy individuals > Gingivitis group > Stage III Periodontitis group

GRAPHS

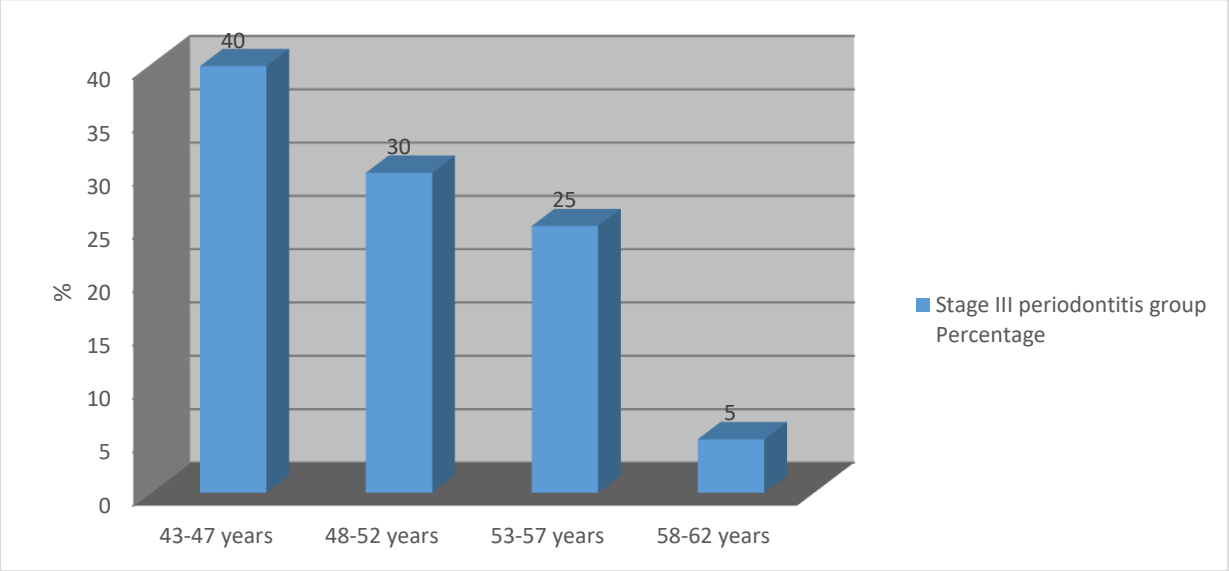
Graph no.1- Bar diagram representing distribution of patients with respect to gender



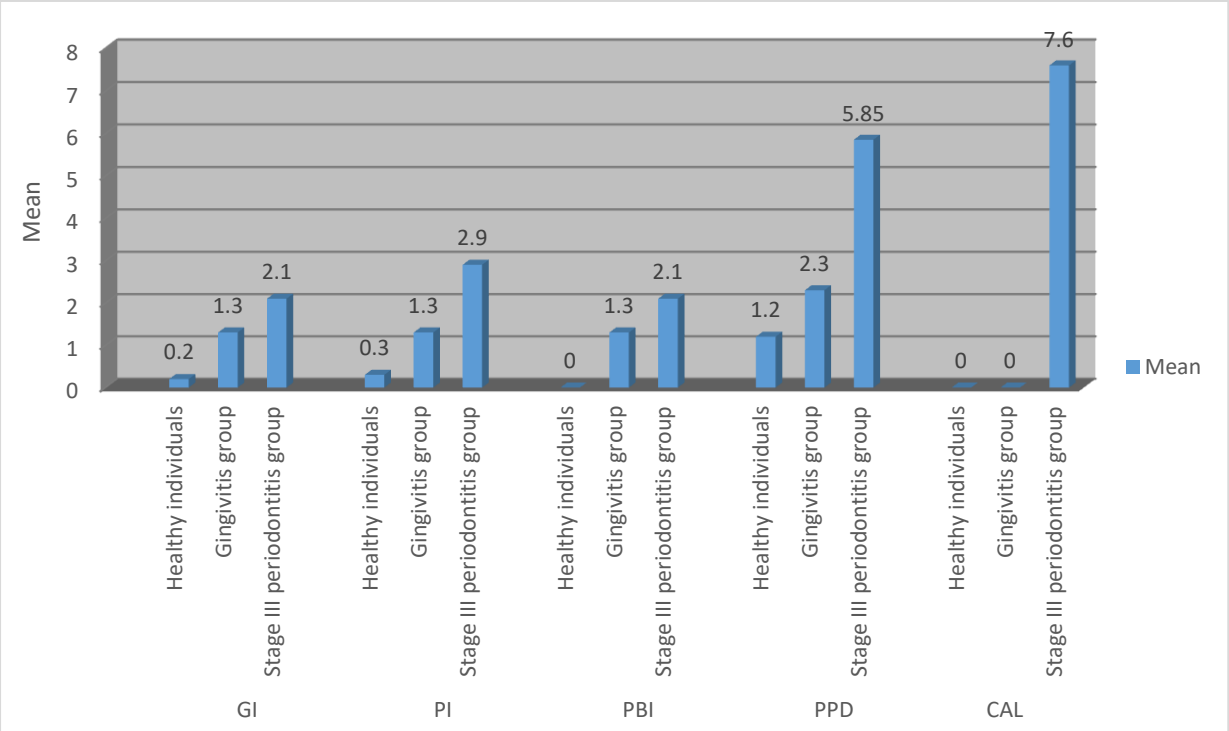
Graph no.2- Bar diagram representing distribution of patients with respect to age in healthy individuals and gingivitis patients



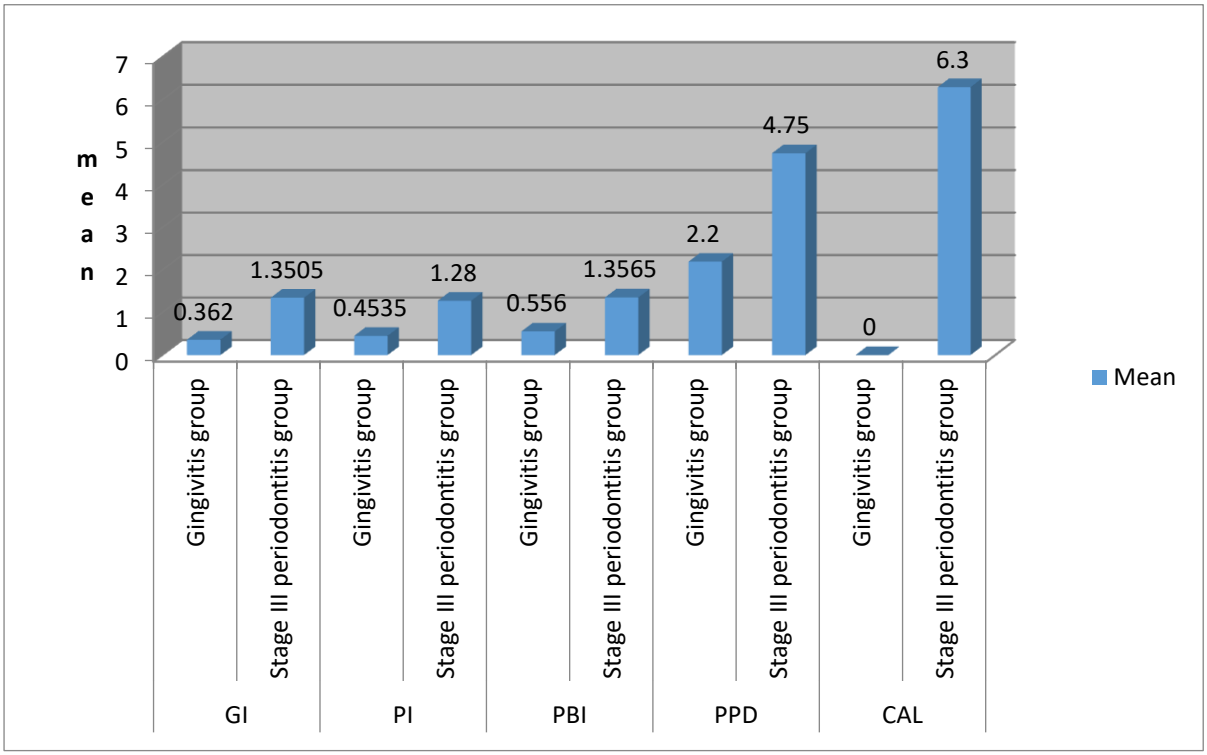
Graph no.3- Bar diagram representing distribution of patients with respect to age in Stage III Periodontitis group



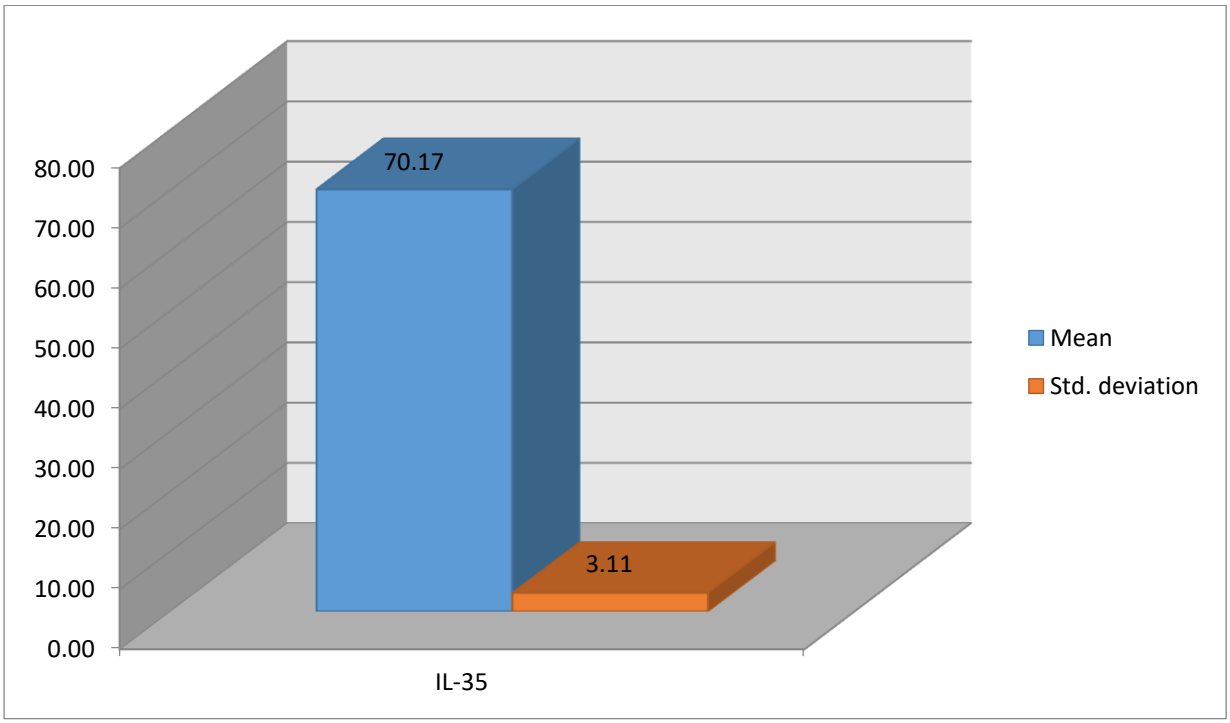
Graph no.4- Bar diagram representing parameters in three groups at baseline



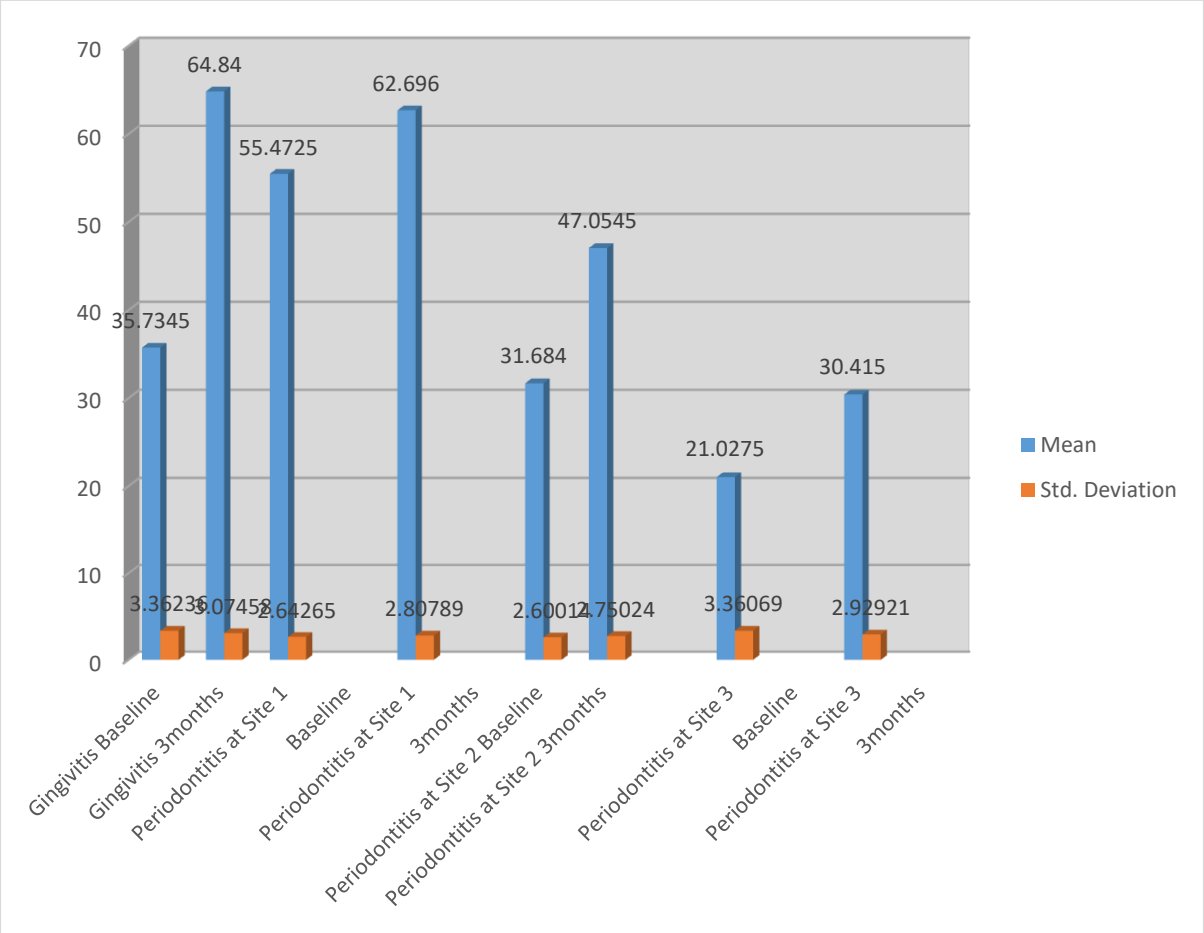
Graph no.5- Bar diagram representing parameters in the groups at 3 months



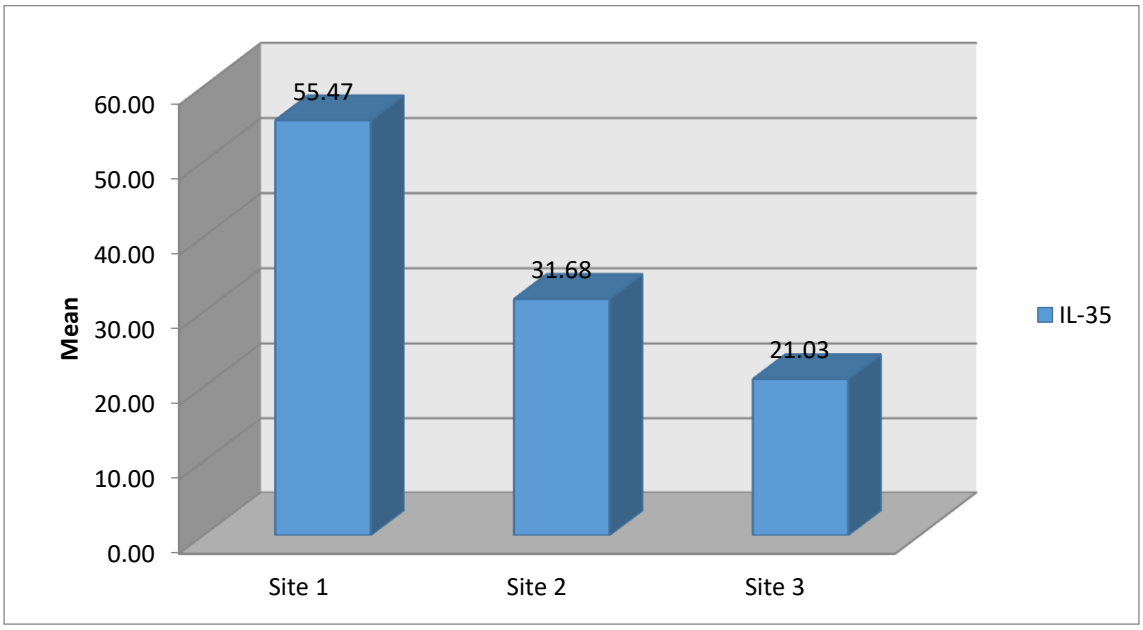
Graph no.6- Bar diagram representing IL-35 in Healthy individuals group



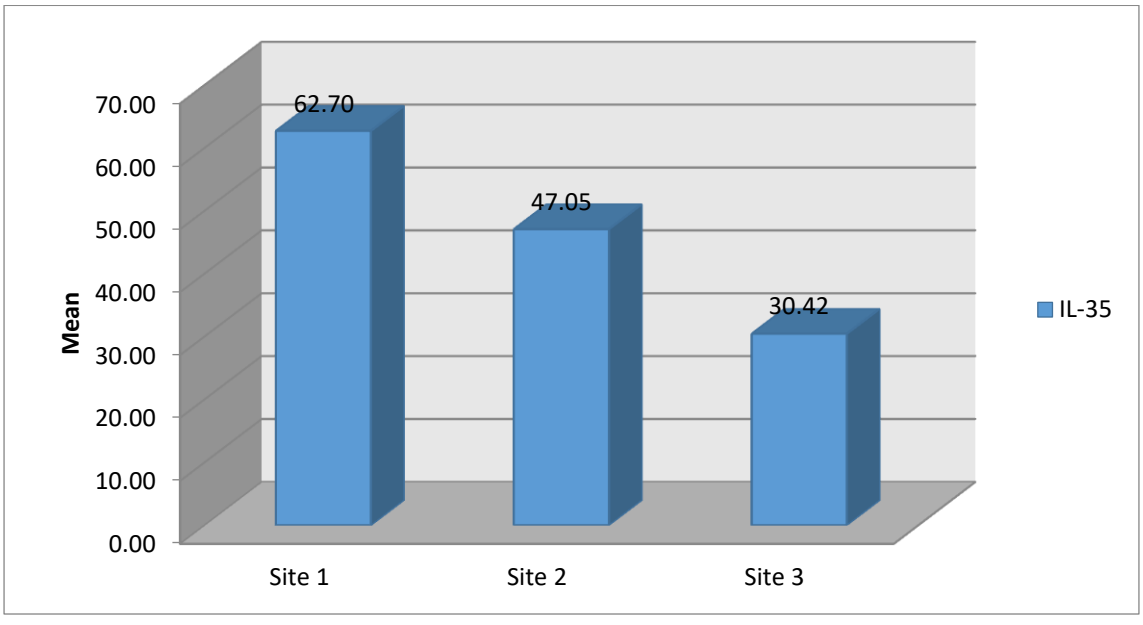
Graph no.7- Bar diagram representing IL-35 levels in gingivitis group and Stage III Periodontitis group all sites at baseline and 3 months



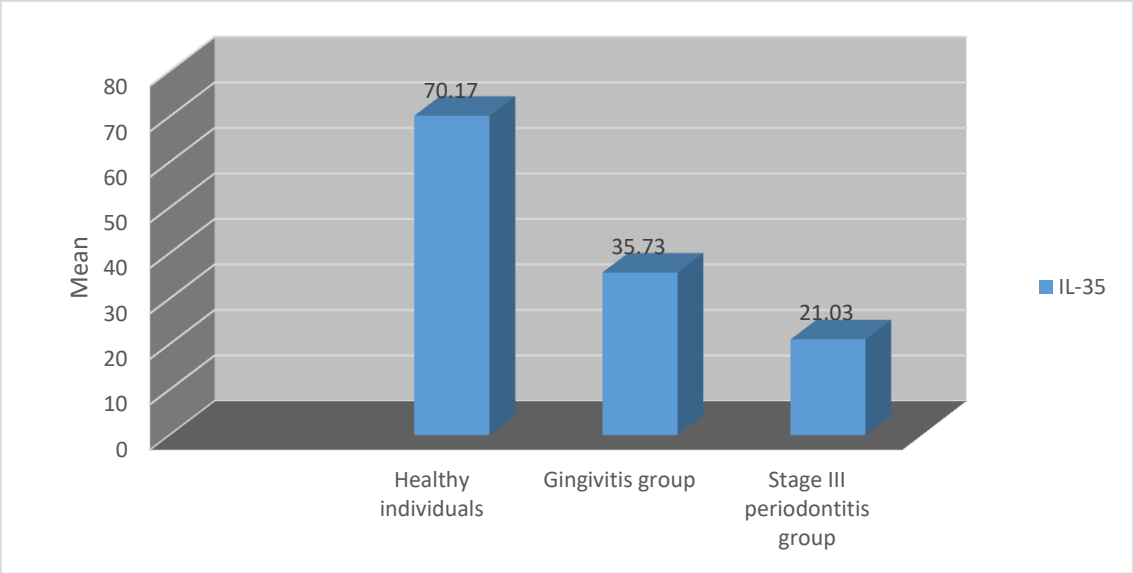
Graph no.8- Bar diagram representing IL-35 levels at baseline for site 1, 2 and 3 in Stage III Periodontitis group



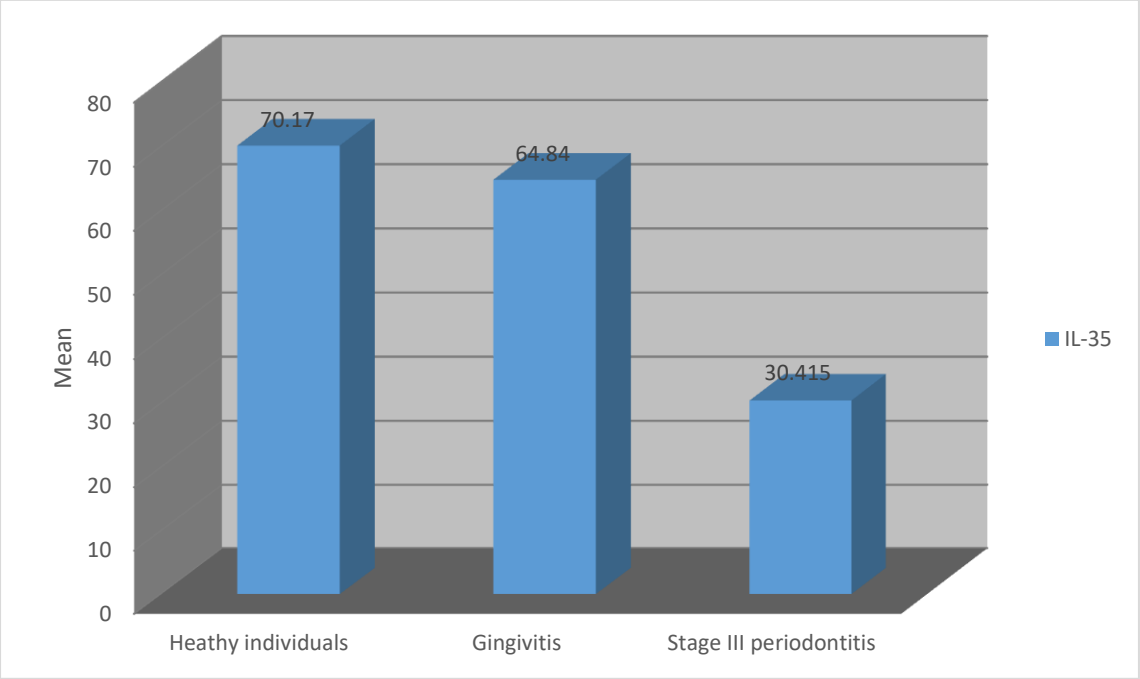
Graph no.9- Bar diagram representing IL-35 levels at 3 months for site 1, 2 and 3 in Stage III Periodontitis group



Graph no.10-Bar diagram representing IL-35 levels in three groups at baseline



Graph no.11- Bar diagram representing IL-35 levels in three groups at 3 months



MASTERCHART

Periodontal clinical and Biochemical parameters

HEALTHY INDIVIDUALS (GROUP I)

Sr. No	Age	Gender	HEALTHY INDIVIDUALS (GROUP I)					
			GI	PI	PBI	PPD	CAL	IL-35
1	18	F	0.8	0.08	0.01	1	0	70.1
2	20	M	0.12	0.25	0.01	1	0	68
3	30	F	0.12	0.7	0.01	1	0	67.1
4	26	F	0.25	0.42	0.06	1	0	72.1
5	25	F	0.25	0.45	0.03	1	0	72.9
6	24	M	0.25	0.16	0.04	1	0	71
7	26	F	0.08	0.16	0.07	1	0	69.1
8	19	M	0.15	0.2	0.01	2	0	78.2
9	20	F	0.08	0.25	0.01	2	0	64.1
10	27	M	0.25	0.25	0.05	1	0	69.1
11	25	M	0.25	0.16	0.07	1	0	75.1
12	23	F	0.25	0.29	0.17	2	0	71.3
13	21	M	0.25	0.3	0.12	1	0	70.9
14	29	F	0.08	0.25	0.01	1	0	69.1
15	26	M	0.3	0.37	0.07	1	0	69.7
16	30	F	0.08	0.25	0.02	2	0	71.1
17	21	F	0.45	0.16	0.07	1	0	66.1
18	19	F	0.25	0.24	0.07	1	0	67.3
19	20	M	0.16	0.29	0.07	1	0	70
20	18	M	0.16	0.16	0.01	1	0	71.1
			0.229	0.2695	0.049	1.2	0	70.17

Periodontal clinical and Biochemical parameters

GINGIVITIS GROUP (GROUP II)

Sr. no	Age	Gender	GINGIVITIS GROUP (GROUP II)											
			GI		PI		PBI		PPD		CAL		IL-35	
			Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months
1	23	F	0.7	0.06	1.04	0.48	1.06	0.48	3	2	0	0	39.4	60.1
2	25	M	1.5	0.49	1.5	0.62	0.8	0.08	2	2	0	0	35.9	67.7
3	18	M	1.8	0.99	2.2	0.78	2.2	1.08	3	3	0	0	43.1	64.7
4	30	F	1.3	0.49	1.2	0.08	1.5	0.78	2	2	0	0	30.1	65.4
5	26	M	1.75	0.54	1.6	0.18	1.48	0.38	2	2	0	0	34.9	63.9
6	21	F	1.04	0.43	1.08	0.46	1.03	0.28	2	2	0	0	38.79	67
7	27	F	1.41	0.5	1.08	0.36	1.2	0.58	2	2	0	0	33.1	69
8	19	M	1.9	0.99	1.7	0.78	2.07	1.35	3	2	0	0	34.9	68.5
9	18	F	1.9	0.89	1.7	0.78	1.38	0.46	2	2	0	0	35.2	67
10	20	M	0.8	0.01	1	0.08	0.8	0.08	2	2	0	0	37.8	65.7
11	25	F	1.7	0.19	1.5	0.58	1.34	0.62	2	2	0	0	33.1	63.2
12	35	M	1.6	0.09	1.6	0.77	0.9	1	3	3	0	0	30.9	62
13	31	M	0.32	0.04	0.5	0.79	0.5	0.18	2	2	0	0	33.1	67.7
14	19	F	1.3	0.12	1.5	0.58	1.68	0.78	3	3	0	0	38.9	68
15	20	F	0.75	-0.01	0.66	-0.16	0.53	0.08	2	2	0	0	39.4	59.5
16	22	M	0.45	0.2	0.66	-0.12	0.67	0.08	1	1	0	0	37.9	66.4
17	27	F	0.875	-0.01	1	0.09	1.1	0.28	2	2	0	0	36.3	61.9
18	25	F	1.58	0.69	2.2	1.28	2.1	1.19	3	3	0	0	30.9	58.7
19	24	M	1.79	0.19	2.1	-0.01	2.2	0.78	3	3	0	0	36.9	65.7
20	30	M	1.2	0.31	1.1	0.09	1.2	0.58	2	2	0	0	34.1	64.7
			1.28325	0.36	1.346	0.4245	1.287	0.556	2.3	2.2	0	0	35.7345	64.84

Periodontal clinical and Biochemical parameters

STAGE III PERIODONTITIS GROUP(GROUP III)

Sr. No	Age	Sex	STAGE III PERIODONTITIS GROUP(GROUP III)															
			GI		PI		PBI		PPD		CAL		IL-35					
			Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months	Site 1 (Baseline)	Site 1 (3 months)	Site 2 (Baseline)	Site 2 (3 Months)	Site 3 (Baseline)	Site 3(3 months)
1	43	F	2.04	1.5	2.9	1.7	2.1	1	7	6	8	7	58.3	59.34	34.8	42.7	17.51	28.2
2	48	M	2.14	1.7	2.7	1.3	1.3	0.43	4	3	7	5	54	62.32	33	50.9	15.75	36.1
3	47	F	1.64	0.5	2.9	1.32	2.5	1.9	8	7	9	8	54.9	62.3	31	48.9	18.35	35.2
4	45	M	2.04	1.5	2.98	1.68	2.18	1.9	5	4	6	5	58.1	61.2	36.6	44.5	27.25	30.2
5	45	F	1.64	1.2	2.4	1	2.23	2	6	6	8	8	50.42	68.2	30	47.9	17.75	31
6	60	F	2.04	1.4	2.98	0.88	1.9	1.3	5	4	7	6	57.1	58.3	35.3	43.2	20.36	29.1
7	56	F	2.94	1.8	3.8	1.7	2.6	2.02	6	4	7	5	57.09	58.4	30.4	48.3	17.65	27.2
8	53	M	2.93	2.1	3.19	1.7	2.6	1.5	7	5	8	7	56.1	64.4	30	47.9	23.35	27.2
9	53	M	2.04	0.9	2.9	1.6	1.7	1.03	5	4	7	6	51.09	63.8	34	41.9	25.35	32.2
10	49	F	2.24	1.91	2.9	1.42	2.4	1.3	5	5	7	7	52.2	64.7	32.2	50.1	23.55	29.2
11	46	F	2.18	1.5	2.7	0.6	2.3	1.9	4	3	6	5	53.3	61.9	31.2	49.1	23.55	33.2
12	47	F	1.93	0.79	3.4	1.3	2.03	1.1	7	6	8	7	58.2	60.9	33.4	51.3	16.55	29.4
13	57	M	1.44	1	2.4	1	1.6	1.1	4	3	6	5	52.3	60.17	36.3	44.2	25.75	29
14	53	M	3.44	2.3	3.6	2	3.2	2.1	5	4	7	5	53.1	61.35	29.33	43.89	19.75	27.2
15	52	M	2.44	1.8	3.108	1.8	2.4	1.3	5	4	7	5	58.9	66.67	28.3	47.7	20.23	27.8
16	52	M	1.59	1.1	2.3	0.7	1.5	0.82	5	4	6	5	56.9	64.8	28.9	46.8	17.65	29.2

Cont…….

Periodontal clinical and Biochemical parameters
STAGE III PERIODONTITIS GROUP (GROUP III)

Sr. No	Age	Sex	STAGE III PERIODONTITIS GROUP(GROUP III)															
			GI		PI		PBI		PPD		CAL		IL-35					
			Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months
17	51	M	1.64	0.7	2.4	0.8	2.1	1.9	7	5	8	7	57.03	67.5	30.3	48.2	21.25	34.2
18	50	M	2.34	1.72	3.2	1.6	1.6	0.5	7	6	10	8	58.16	60.97	28.9	46.8	22.75	35.4
19	45	F	2.04	1.29	2.9	0.8	2.3	0.93	7	5	9	6	57.14	62.8	29.2	47.9	23.95	28.1
20	43	M	1.44	0.3	2.2	0.7	1.8	1.1	8	7	11	9	55.12	63.9	30.55	48.9	22.25	29.2

CASE HISTORY PROFORMA

CASE HISTORY PROFORMA

NAME: **OPD NO:**

AGE/SEX: **DATE:**

ADDRESS: **PHONE NO:**

OCCUPATION:

CHIEF COMPLAINT:

PAST DENTAL HISTORY:

PAST MEDICAL HISTORY:

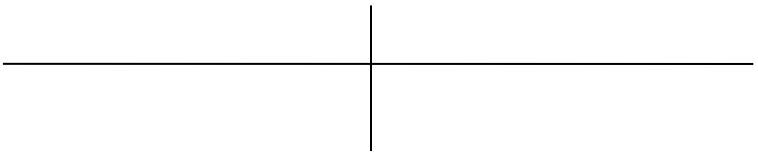
DRUG HISTORY:

PERSONAL HISTORY:

ORAL HYGIENE HABIT:

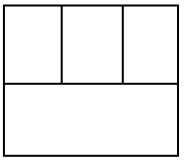
PHYSICAL EXAMINATION:

TEETH PRESENT:



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

16



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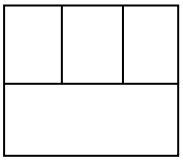

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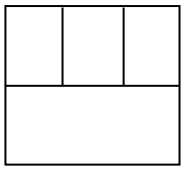

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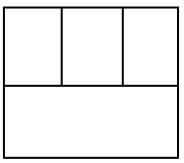

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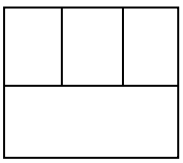
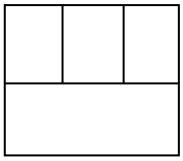


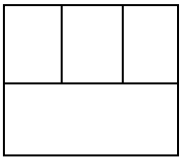
36

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

GINGIVAL INDEX *(Loe&Sillness1963*) (3months)



16



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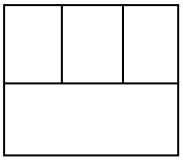

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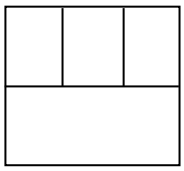

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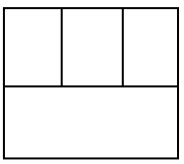

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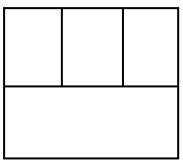
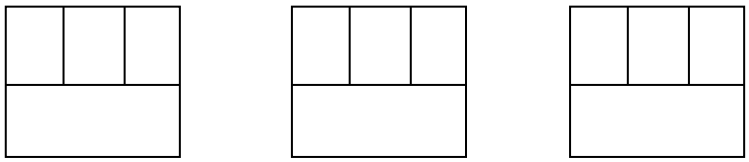


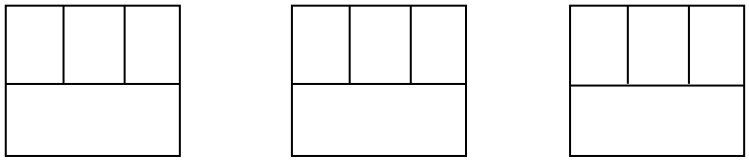
36

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

PLAQUE INDEX (*Sillness & Loe1964) (Baseline)*



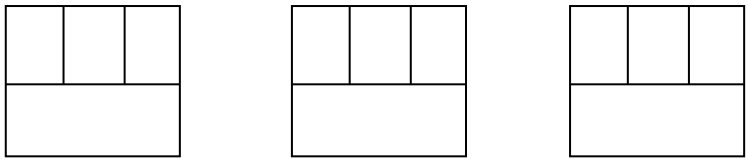
16 12 24



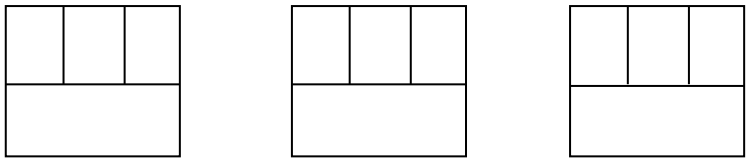
44 32 36

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

PLAQUE INDEX (*Sillness & Loe1964)(3 months)*



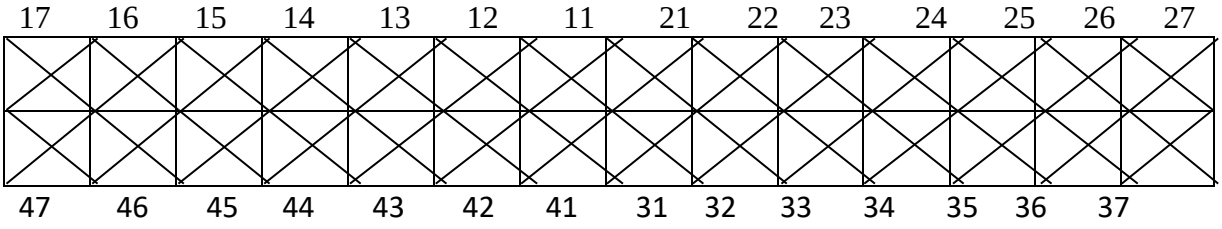
16 12 24



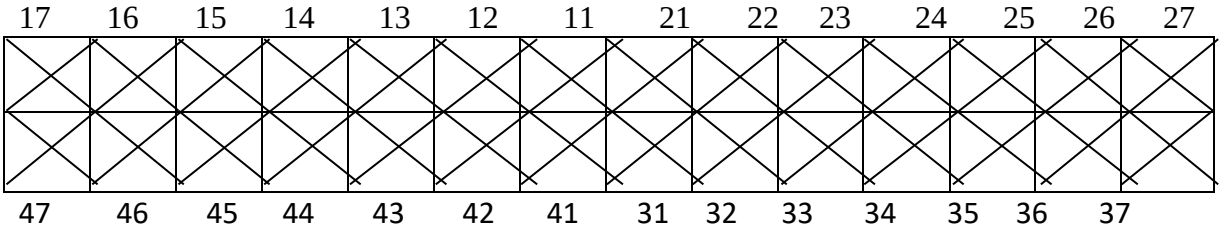
44 32 36

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

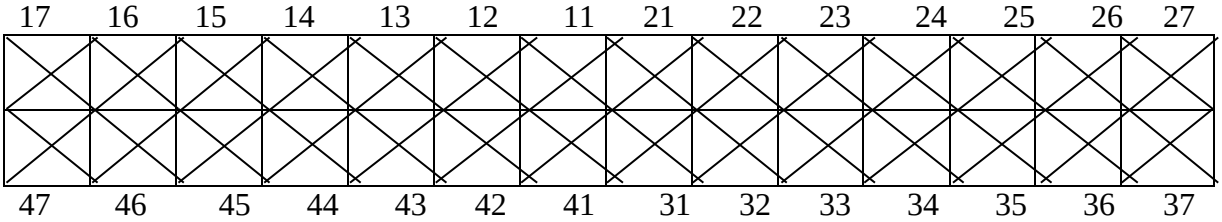
PROBING POCKET DEPTH (mm): (Baseline)



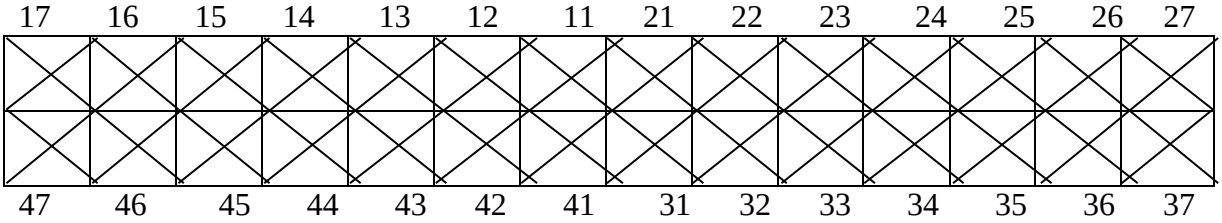
PROBING POCKET DEPTH (mm) (3 months)



CLINICAL ATTACHMENT LEVELS (mm): (Baseline)



CLINICAL ATTACHMENT LEVELS (mm): (3 months)



PAPILLARY BLEEDING INDEX (PBI) *(*Muhlemann H.R. 1977) (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) (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:

For healthy individuals- Biochemical Parameters	**Gingival crevicular fluid**
Interleukin 35 (ng/ml)	

For gingivitis Group- Biochemical Parameters	**Gingival crevicular fluid at baseline.**	**Gingival crevicular fluid at 3months.**
Interleukin 35 (ng/ml)		

For periodontitis Group- Biochemica-l Parameters	**Gingival crevicular fluid at baseline**			**Gingival crevicular fluid at 3months.**		
Interleukin 35 (ng/ml)	**Healthy sites**	**Gingiviti s sites**	**Periodont-itis sites**	**Healty sites**	**Gingivits sites**	**Periodontit i-s sites**

Informed Consent Form

Evaluation and comparison of Interleukin-35 levels in Gingival crevicular fluid of Periodontally healthy, Gingivitis and Severe periodontitis patients before and after Non-surgical periodontal therapy: A Clinical controlled trial

Mr./Master/Mrs./Miss._____

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.

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 GCF 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	Signature	Date	Time