"ESTIMATION AND CORRELATION OF HUMAN SALIVARY CLUSTERIN LEVELS IN PERIODONTAL HEALTH, GINGIVITIS AND GENERALISED CHRONIC PERIODONTITIS - A CROSS SECTIONAL STUDY"

By

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LIST OF ABBREVIATIONS

GCF	Gingival Crevicular Fluid
CLU	Clusterin
MMP-9	Matrixmetalloproteinase-9
MIP-1β	Macrophage Inflammatory Protein-1β
MCP-1	Monocyte Chemotactic Protein-1
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
LPS	Lipopolysaccharides
LDLR	Low Density Lipoprotein Receptor
GCSF	Granulocyte Colony Stimulating Factor
KC	Keratinocyte Chemoattractant
TNF-α	Tumour Necrosis Factor-alpha
IL-1ra	Interleukin-1 receptor antagonist
M-CSF	Macrophage colony-stimulating factor
IL-27	Interleukin- 27
ROS	Reactive Oxygen Species

ABSTRACT

INTRODUCTION: Gingivitis and periodontitis are chronic inflammatory conditions that may affect as much as 80% of the adult population, making them one of the most prevalent diseases in humankind. The disease process initiates due to the accumulation of bacteria and exposure to bacterial products triggers inflammation in the tissue. The absence of pain in periodontal diseases is usually one of the main reasons why patients do not seek professional care in their early or even more advanced stages. The traditional clinical criteria are often insufficient for determining sites of active disease, monitoring the response to therapy, or measuring the degree of susceptibility to future disease progression. To overcome the challenge, various molecular disease biomarkers are being researched for identifying the hidden lethal threat before the disease becomes complicated.

Saliva contains an abundance of proteins and genetic molecules and is readily accessible *via* a totally noninvasive approach. A novel protein clusterin is expressed in almost all mammalian tissue including plasma, serum, milk, saliva, urine, cerebrospinal fluid, and semen. It is believed to perform some important functions in the body that are prime to maintaining homeostasis. It is postulated that the protein might also play important role in the occurrence or progression of periodontitis.

Hence, this study was designed to estimate and correlate the clusterin levels in saliva in health, gingivitis, and periodontitis.

AIM: To estimate and correlate human salivary clusterin levels in periodontal health, gingivitis, and generalized chronic periodontitis.

MATERIALS AND METHODS: This was a cross-sectional study. A total of 75

subjects were included in this study. Depending on the clinical examination, they were divided into three groups: Healthy/ control group, gingivitis group, and chronic periodontitis group. Saliva samples were collected from all the subjects and clusterin values were evaluated using ELISA. The data was entered in Excel and analyzed statistically using the SPSS software version. Intergroup comparisons were done by One way ANOVA. A pairwise Comparison of groups was also carried out using post hoc test. All statistical tests were performed at a significance level of 5% ($p<0.05$).

RESULTS AND CONCLUSION: In the healthy group, the mean clusterin value was 222.9± 85.978, whereas, for gingivitis group, the value was 136.56 ± 37.720. For periodontitis group, the mean value was 105.91 ± 23.82. There was a statistically significant difference observed between the three groups with mean values. The mean SBI scores, PPD, and CAL scores of all three groups also showed significant difference. The clusterin value was observed to be decreased in patients with chronic periodontitis as compared to periodontally healthy and patients with gingivitis. The study concluded that salivary clusterin levels are lowered in patients with chronic periodontitis and can play an important role in creating a proinflammatory condition in the disease process.

KEYWORDS: Biomarkers, Clusterin, periodontitis, saliva, salivary biomarkers

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INTRODUCTION

Oral cavity is the gateway to the human body. According to World Health Organisation (WHO), examination of oral is very vital because of the incidence and prevalence of infections occurring in the cavity. Although dental caries is one of the leading causes of pain and discomfort for which patients seek professional dental care, diseases of gums namely gingivitis and periodontitis affect oral health even more.[1] Hence, early detection of such infections is beneficial in order to maintain the delicate equilibrium within a highly dynamic oral environment. Over the years naturally occurring molecules known as biomarkers have played and continue to play a crucial role in the early detection, prediction of the severity of existing periodontal destruction, or identification of a future risk for periodontal disease.[2]

Many biomarkers have been discovered to date, most of which are present in gingival crevicular fluid (GCF) as well as saliva. GCF is a long accepted diagnostic marker of periodontal disease progression as well as resolution. It however poses grave challenges in terms of the method of collection, quantity collected as well as cross contamination. Over the years saliva has also been utilized for investigation of various biomarkers. Although the majority of saliva constitutes of water, it also consists of many other molecules such as glycoproteins, phosphate, calcium ions, enzymes, and other organic-inorganic substances.[2] Selecting saliva as a "Point of care" has many benefits such as a convenient collection of samples, time efficiency, relatively cheaper, presence of factors and molecules from GCF as well. It also allows convenient usage in screening large populations.[3] New salivary biomarkers have come to light recently and many more are in the pipeline. A heterodimeric

glycoprotein namely Clusterin is one among the recent biomarkers that have been found in this regard.[4]

Clusterin, also called apolipoprotein J (ApoJ), SPG2, TRPM-2, or CLU was first discovered and isolated in 1979 in rat testis. As the name suggests it has the ability to aggregate blood cells *in vitro.*[5] Earlier, it was very hard to believe that this apolipoprotein performs so many important roles and created a challenge for scientists. It is a multifunctional, disulfide-linked, heterodimeric glycoprotein found almost in body fluids like plasma, serum, milk, saliva, urine, cerebrospinal fluid, and semen, and in the intracellular matrix at some sites.[6] There are mainly two isoforms of this protein: a small nuclear isoform (nCLU) of around ~49kD size is present in the cytosol which can translocate to nucleus and is usually responsible for apoptotic processes, whereas mitochondria contain a ~53 kDa sized isoform- Secretory form (sCLU) which is unglycosylated, uncleaved. The sCLU falls into a process of reactions that produce a mature form of two ~40 kDa subunits linked with disulfude bonds- a mature sCLU form of size ~75-80kDa and is usually present in the endoplasmic reticulum, golgi apparatus as an α-β heterodimer.[6] The secretory form regulates majority of the functions that clusterin performs and mainly functions to prevent cell death. Secretion takes place via secretory vesicles and other non-regulated routes. Uptake and degradation are believed to be mediated by the endocytic receptor gp330/megalin, a part of the Low-Density Lipoprotein Receptor (LDLR) gene family.[6,7]

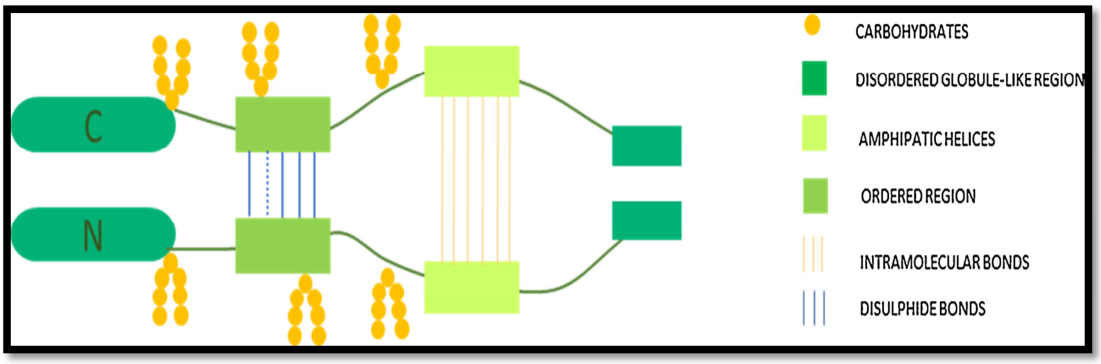


FIG 1. Schematic presentation of the structure of Clusterin

There are plentiful functions that clusterin performs and some of them are even dualistic leading to outcomes that are opposite because of which this protein is even more perplexing. It performs functions like clearance of cellular debris, cytoprotection at fluid–tissue interface, cell membrane recycling during the development and in case of injury, regulation of complement-mediated membrane attack, and scavenging.[8] In addition to this, clusterin can either advance or forestall cell death depending upon the nature of molecular species. Expression of clusterin is seen to surge in diseases where either abnormal cell death or proliferation is occurring. There is upregulation of the protein in diseases such as Alzheimer's, retinitis pigmentosa, glomerulonephritis, atherosclerosis, myocardial infarction whereas cancers including gliomas and testicular, prostate, breast, liver and lung cancer have shown a marked decrease.[5,7,9,10,11]

Clusterin was first detected in GCF via proteomic analysis in the year 2012.[13] As saliva consists of contents from GCF also, the possibility of clusterin existing in saliva was assumed. In the year 2018, proteomic analysis was done to detect clusterin in saliva for the first time.[4] These studies have shown that clusterin levels in saliva, as well as GCF, are low in chronic periodontitis as compared to periodontally healthy

sites. Downregulation of the clusterin leads to activation of p53 gene.[14] This activation affects the gingival fibroblasts through increased ROS production and mitochondrial dysfunction. The damage occurs even more when the fibroblasts are affected by lipopolysaccharides of periopathogens such as *Porphyromonas gingivalis (Pg)* leading to LPS induced inflammation. The cascade of p-53 and LPS eventually commence the apoptotic activity and causes cell death.[15] On the contrary low levels of the protein can also lead to the lack of apoptotic induction of macrophages allowing them to remain in the tissues and consequently maintain a proinflammatory situation.[16] Furthermore, it participates in translocation of NF- KB which leads to κBα- degradation followed by increased expression of Matrixmetalloproteoinase-9 (MMP-9), a gelatinase responsible for periodontal destruction. It is also found that the clusterin promotes Tumor Necrosis factor- α (TNF-α) secretion.[17,18] As already established, TNF-α acts directly as a chemoattractant for macrophages or monocytes and a mediator of inflammation. The synthesis of chemokines like Monocyte Chemotactic Protein-1 (MCP-1), Macrophage Inflammatory Protein-1β (MIP-1β), and RANTES in macrophages is also boosted because of clusterin along with the promotion of chemotactic migration of macrophages.[17] Clusterin also functions to counter-balance the damaging consequences of oxidative stress.[19] Keeping this aspect in mind , it can be theorized that its reduced levels in the periodontal environment can lead to compromise in the oxidative balance and cause more periodontal tissue damage.

Hence, this study was designed to estimate the levels of salivary clusterin in periodontally healthy patients, patients with gingivitis, and patients with chronic periodontitis. Further we also decided to explore the correlation of clusterin levels in all three groups and to the severity of periodontal disease.

AIM AND OBJECTIVES

AIM OF THE STUDY: To estimate and correlate human salivary clusterin levels in periodontal health, gingivitis and generalized chronic periodontitis.

OBJECTIVES:

1) To assess salivary human clusterin levels in periodontal health, gingivitis and generalized chronic periodontitis.

2) To intercompare salivary human clusterin levels in periodontal health, gingivitis and generalized chronic periodontitis.

REVIEW OF LITERATURE

Clusterin is a heterodimeric glycoprotein detected in nearly all fluids of mammals like plasma, serum, milk, saliva, urine, cerebrospinal fluid and semen, and the intracellular tissue at some sites. It is related to many biologic functions as well as pathologic conditions. Changes in the clusterin levels are appreciated in aging which is a physiologic condition. Levels are found to fluctuate in pathologies like Alzheimer's disease, glomerulonephritis, retinitis, atherosclerosis, cancer. In the recent past, the presence of clusterin has been detected in both GCF and saliva. Research has continued to find out the possible correlation between periodontal disease and clusterin levels. Its downregulation causes increased redox imbalance through p53 gene and mitochondrial dysfunction through Bcl-2 gene. Also, there is a rise in the levels of cytokines like IL- 1β, TNF- α, increased translocation of NF KB leading to increased expression of MMP-9. All these factors make clusterin one of the important factors that can affect periodontal destruction.

Jia Liu et al (2018)[15] conducted a study to check the participance of p53 gene in the inflammation process induced by lipopolysaccharides (LPS) in human gingival fibroblasts (HGF). In this study, HGFs were cultured in a medium consisting of LPS extracted from *Porphyromonas gingivalis* (Pg). The p53 gene expression was assessed using reverse transcription polymerase chain reaction (qRT-PCR) and Western blot analysis. Production of ROS, cellular respiration and cytokine secretion was also assessed. This study also mentioned the previously proven fact that increase in p53 activity leads to an increase in ROS production and distorts the balance between proapotic and antiapoptic activity of Bcl-2 family members, resulting in an increased redox imbalance and mitochondrial dysfunction respectively. The study

showed that cell damage was predominantly noted in the presence of lipopolysaccharides. There was also increase in expression of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α eliciting an inflammatory response. All these changes were transient in nature and could be reversed by the inhibition of p53. As stated earlier the reduced levels of clusterin activates the p53 gene and this study showed the harmful effects of p53 activation in presence of lipopolysaccharides.

Bostanci N et al (2018)[4] performed a quantitative proteomic analysis" to identify new salivary protein biomarkers in periodontal health, gingivitis and periodontitis. Periodontal examination was carried out for all the subjects followed by saliva sample collection. Proteomic analysis was then conducted for all the samples. This led to the discovery of 119 new proteins. Increased levels of clusterin were observed in healthy patients as compared to periodontitis. The protein- protein interaction showed that it was strongly related to Ras-related protein (RAP1A) which performs functions like regulation of inflammatory reactions, osteoclastic functions and bone resorption and MMP-9, which have a well-established role in periodontal destruction. The study also explained the protective nature of clusterin against cellular oxidative stress and postulated that its reduced expression in periodontitis can impair the redox balance which in turn is one of the factors responsible for periodontal destruction.

Won JC et al (2014)[20] carried out a study where they inspected anthropometric and clinical factors affecting the plasma levels of clusterin. This study was done in Healthy Korean population. Serum clusterin levels were estimated in 111 males and 93 females using ELISA kit. The results showed that plasma clusterin levels were higher in overweight and obese subjects as compared to lean subjects. The levels were seen to be correlated positively with Body Mass Index (BMI), Waist- hip ratio. It was

also seen to be associated with inflammatory markers like high sensitivity C-reactive protein, uric acid, ferritin and retinol binding protein- 4. This study concluded that plasma clusterin levels could be a reliable and sensitive marker for systemic inflammation and oxidative stress.

Young-Nam Lee et al (2012)[19] conducted a study in flies that is in Drosophila melanogaster strain (fruit flies). The purpose was to check whether clusterin protects against aging or its expression is a consequence of aging. Authors recognized genetically modified Drosophila allelomorphs carrying the secretory form of human clusterin using a system to induce the ectopic overexpression of human clusterin in fruit flies. To understand the relation between overexpression of clusterin and aging, lifespan of clusterin overexpressing flies and their isogenic controls was evaluated. Results showed that there was an increase in the lifespan of transgenic fruit flies as compared to their controls. Antioxidant activity of clusterin and ROS production was also determined. It was seen that overexpression of clusterin provided protection against heat shock, starvation and oxidative stress. Also, there was less production of ROS in transgenic fruit flies than that of isogenic controls. It was concluded that clusterin functions to reduce reactive oxygen species through cysteine sulfhydryl groups which eventually leads to increased tolerance to oxidative stress. This increased resistance was considered to be responsible for increased lifespan of Drosophila those were overexpressing the clusterin. Hence, we can consider that when there is downregulation or underexpression, the resistance against oxidative stress will be impaired and there will be more production of ROS leading to redox imbalance.

Baliban RC et al (2012)[13] carried out a proteomic analysis in which they identified new biomarkers in periodontally healthy and disease. The study included patients who presented with chronic periodontitis and periodontally healthy patients. 12 patients were included in each group. GCF samples were collected from all the patients. Clinical parameters such as pocket probing depth, gingival recession and bleeding on probing were recorded. This was followed by collection of GCF samples and liquid chromatography, mass spectrometry and proteomic analysis of the samples. Results showed identification of total 432 new proteins. These proteins were then broadly categorized to assess the relationship of relevant ones with chronic periodontitis. Among 432 protein biomarkers, 123 proteins were related to only chronic periodontitis and 230 were detected in both healthy as well as chronic periodontitis samples. Clusterin was detected in GCF for the first time. Its levels were observed to be more in periodontally healthy patients as compared to the patients with chronic periodontitis. Other biomarkers which were suggested to be reliable indicators of periodontal health and disease were angiotensinogen, thymidine phosphorylase, carbonic anhydrase-1 & 6, elongation factor 1-gamma.

Shim YJ et al (2012)[17] carried out a study to determine the role of clusterin in secretion of TNF- α and chemotactic migration of macrophages. This was an in vitro study. First, macrophages were grown in vitro on a suitable media and then stimulated by clusterins. Western blot analysis, cytokine array, RT-PCR and transwell filter assay was carried out for the macrophages. Test results showed that the expression of cytokines in macrophages was upregulated by clusterin. The secretions of Granulocyte Colony Stimulating Factor (GCSF), Keratinocyte Chemoattractant (KC), TNF-α and Monocyte Chemotactic Protein-1 (MCP-1) were markedly elevated as compared to the controls when seen in cytokine array analysis. Also, Macrophage

Inflammatory Protein-1β (MIP- 1β), Interleukin-1 receptor antagonist (IL-1ra), and RANTES levels were discreetly increased and Macrophage colony-stimulating factor (M-CSF), IL-27 were marginally increased. The primary inference was that clusterin stimulated secretion of TNF-α by macrophages and enhanced chemotactic migration of macrophages and monocytes. Other cytokines and clusterin itself also showed chemotactic activity helping in the migration of the cells. All of these factors can be considered as an important part of any inflammatory process.

Shim YJ et al (2011)[18] from an in vitro study showed, for the first time, that clusterin induces expression of MMP-9 through extracellular signal- regulated kinase (ERK) and Nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) pathways. This was examined in murine monocytes and macrophages. Cells were cultured in vitro using a suitable media. Western blot analysis, RT-PCR and MMP-9 gene promoter assay were then carried out. Clusterin was seen to increase the enzymatic activity of the cells along with increased secretion of MMP-9. There was increased proteolytic activity. It was seen that clusterin performed a part in the nuclear translocation of NF-κB p65 gene along with κBα- degradation and phosphorylation, this step is vital for the expression of MMP-9. This study also demonstrated that the glycosylated form of clusterin was able to induce the MMP-9 expression and that the carbohydrate component of its structure is important for this activity. NF-κB performs central role in regulation of inflammation. Hence it was inferred that clusterin might also have an important function in regulating the inflammation indirectly.

An animal study was conducted by **Park HK et al (2008)[21]** where the expression of clusterin was observed under restraint stress condition in the submandibular salivary glands. Dawley rats were utilized for this purpose. There were two groups viz normal

and restraint stress group. The rats in the study group were exposed to stress by means of stress cages. Rats were sacrificed at day 0, day 3 and some at day 5. This was followed by excision of submandibular gland immediately. Immunohistochemistry and Northern blot analyses were used for measuring the levels of clusterin and mRNA in the tissues, respectively. The results showed that clusterin was expressed in the glands as an early response to the stress in order to protect the glandular cells and was detected only after immediate restraint stress application. On the contrary, if the stress was strong and acting for a longer duration, there was under expression of clusterin and the cells eventually underwent apoptosis. Thus, it was concluded that clusterin poses as a reliable marker of stress.

Ioannis P. Trougakos et al (2002)[22] conducted a study to analyse the potential relationship of clusterin with in aging in patients, its potential relationship with development of atherosclerosis and similar effect in developing atherosclerosis in type II diabetes. Sandwich ELISA assay and immunoblotting analysis were used to examine the relationship. There was a substantial surge in serum clusterin levels with age in males. Levels also increased significantly in type II Diabetes patients, and patients who either suffered from developing coronary heart disease, or myocardial infarction. The study concluded that accumulation of high amounts of clusterin is related to generalized stress initiation mechanism and it is the potential indicator of vascular injury.

Sensibar et al (1995)[23] examined the role of clusterin in apoptosis of cells. This was an in vitro study which used LNCap cell line. This is a human cell line is commonly used in the oncology field. The cells were stimulated with TNF-α. The immunocytochemical staining was done to understand the activity of clusterin.

Initially there was a weak activity; but it peaked at the end of 2 hours and then depleted after 6 hours. After the levels of clusterin were reduced a phase of cell death was observed. Based on these observations authors concluded that the distinct rise in the levels of clusterin was a protective mechanism for preventing the cell death and hence there was overexpression as a resistance to cytotoxicity of TNF- α. Hence, author concluded that clusterin provides protection against TNF- α induced cell death.

MATERIALS AND METHODS

SOURCE OF DATA:

The study was a descriptive cross-sectional study. It was conducted in the Outpatient Department of Periodontics, KAHER's KLE V.K. Institute of Dental Sciences, Belagavi. The laboratory procedures were carried out in the Department of Clinical Biochemistry (High-Tech Laboratory), KLE's Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi. An ethical clearance was obtained from the Ethical Committee, KAHER's KLE V.K. Institute of Dental Sciences, Belagavi before conducting the study.

CRITERIA FOR GROUP SELECTION:

The study population was decided to be divided into three groups:

A. Control/ Periodontally healthy group
B. Gingivitis group
C. Chronic generalized periodontitis group

A detailed clinical examination was done. Diagnosis of periodontal disease was given on the basis of the 1999 International Workshop for Classification of Periodontal Disease and Conditions.[24] Before starting, the purpose and format of the study were described to the patients in a language that they understand and a written consent was obtained from them. After recording the demographic and clinical data, collection of saliva sample was carried out.

INCLUSION CRITERIA:

Group A- Control/ Periodontally healthy group ***(Photo 1)***:

1. A minimum complement of 20 teeth to be present at the time of evaluation.
2. Age- 17 to 65 years of either sex.
3. No history of dental treatment in the preceesing 6 months including oral prophylaxis.
4. A bleeding index score of zero.

Group B- Gingivitis group ***(Photo 2)***:

1. A minimum complement of 20 teeth to be present at the time of evaluation.
2. Age- 17 to 65 years of either sex.
3. No history of dental treatment in the preceding 6 months including oral prophylaxis.
4. A bleeding index score of ≥ 1.

Group C- Generalized Chronic Periodontitis group ***(Photo 3***):

1. A minimum complement of 20 teeth to be present at the time of evaluation.
2. Age- 17 to 65 years of either sex.
3. No history of dental treatment in the preceding 6 months including oral prophylaxis.
4. A bleeding index score of ≥ 2.
5. Pocket probing depth of ≥ 5.
6. Clinical attachment loss of ≥ 2.

EXCLUSION CRITERIA:

1. Patients with a history of any systemic disease.
2. Smokers and patients consuming tobacco in any other form.
3. Patients who are lactating mothers and pregnant women.
4. Subjects who have undergone any periodontal therapy 6 months prior to the study.
5. Patients who have taken any anti-inflammatory drugs, antibiotics or mouth rinses over the past 3 months.

SAMPLE SIZE ESTIMATION:

$S = \frac{S_1 + S_2}{2}$

Z_α= 1.96 at 5% α error.

Z_β= 0.84 at 20% β error.

S1= 18.29

S2= 20.87

d= -15.5

$$n = \frac{2S^2 (Z_\alpha + Z_\beta)^2}{d^2} = 25 \text{ (In each group)}$$

CLINICAL ARMAMENTARIUM *(Photo 4)*:

1. Mouth mirror
2. Explorer
3. Straight probes
4. Tweezers
5. Kidney tray
6. Cotton roll and gauze
7. William's graduated periodontal probe
8. Mouth mask

9. Disposable latex gloves
10. 15 ml falcon tube
11. Ice pack with container for transportation to the laboratory

LABORATORY ARMAMENTARIUM ***(Photo 6-11)***:

1. -20°C Refrigerator for storage
2. ELISA microplate reader (absorbance at 450 nm)
3. Micropipette
4. Centrifuge machine
5. Laminar air flow
6. Ependdroff tubes of 2 ml

CLINICAL PARAMTERES:

Following clinical parameters were recorded:

1. Sulcus bleeding index (Muhlemann and Son- 1971)
2. Pocket Probing depth
3. Clinical attachment loss

Sulcus Bleeding Index (Muhlemann H. R and Son S. 1971)[25]:

The Sulcus Bleeding Index (SBI) is an index for assessment of gingival bleeding. The rationale of the index is to locate areas of bleeding from gingival sulcus upon probing with optimum pressure and thus identifying and recording the presence of early inflammatory gingival disease.

Procedure:

The SBI is based on the evaluation of bleeding from gingiva on probing which is first sign to occur, gingival contour and gingival colour changes. Four gingival units were scored for each tooth: The labial and lingual marginal gingiva (M units) and the mesial, distal papillary gingiva (P units). The probing of four areas was carried out under proper illumination. The probe was hold and walked parallel to the long axis of the tooth for M units and directed towards the col area for P units. After probing was done, 30 seconds time was allowed to pass before scoring gingival units. The gingiva was dried gently to observe the colour changes clearly.

Scoring criteria:

Score	Criteria
0	Healthy appearance of O and M, no bleeding upon sulcus probing, gingiva of normal texture and colour
1	Apparently healthy P and M units showing no colour or contour changes and no swelling; but bleeding from sulcus on probing.
2	Bleeding on probing and colour changes caused by inflammation (reddening). No swelling or macroscopic edema.
3	Bleeding on probing, change in colour, slight edematous swelling.
4	Bleeding on probing and/ or colour change OR obvious swelling.
5	Spontaneous bleeding on probing, colour change, marked swelling with or without ulceration.

Pocket Probing Depth[26]:

Pocket probing depth was measured from margin of the free gingiva to the base of the pocket using William's graduated periodontal probe. The probe was walked through the gingival sulcus along the circumference of the tooth.

Three measurements were done the buccal aspect (Mesio-facial, facial, disto-facial) and three measurements on the lingual aspect (Mesio-lingual, lingual, disto-lingual) of each tooth.

Clinical Attachment Loss[27]:

Clinical attachment levels were measured from the cementoenamel junction (CEJ) to the pocket base using William's graduated periodontal probe.

A. When the free gingival margin was located coronal to the CEJ, the loss of attachment was determined by subtracting the distance between the free gingival margin and the CEJ from the pocket probing depth.
B. When the free gingival margin was located on the CEJ, the loss of attachment was as same as pocket probing depth.
C. When the free gingival margin was located apical to the CEJ, the loss of attachment was calculated by adding the distance between the free gingival margin and the CEJ to the pocket probing depth.

Three measurements were done the buccal aspect (Mesio-facial, facial, disto-facial) and three measurements on the lingual aspect (Mesio-lingual, lingual, disto-lingual) of each tooth.

Collection of Saliva Sample *(Photo 5)*:

The format of the study was explained to all patients in the language they understand. Patients were advised not to perform any oral hygiene measures or consume any liquid or solid food substances 1 hour prior to saliva collection.[28] At first, patients rinsed their mouth thoroughly with water to remove food debris. Subjects were then asked to spit out the saliva that was collected in initial 30 seconds. After this whole saliva was collected into 15 ml Falcon tube using spitting method.[29] A final volume of 3 to 5 ml whole saliva was obtained for each patient. Each saliva sample was immediately placed on icepacks for transportation to the laboratory. Samples were stocked at -20° C till the time of assay. At the time of assay, samples were first centrifuged at 10000 x g at 4°C for 10 minutes. The estimation was done using Enzyme- linked Immunosorbent assay (ELISA).

ASSAY PROCEDURE[30]:

Levels of Clusterin were determined by using Picokine Human Clusterin (CLU) ELISA kit, Bosterbio, USA.

REAGENTS:

1. Anti-Human CLU Pre-coated 96-well Strip Microplate: 12 strips of 8 wells each- 1 unit.
2. Human CLU Standard: 50 ng/tube- 2 units.
3. Human CLU Biotinylated Antibody (100x): 100 µl- 1 unit.
4. Avidin-Biotin-Peroxidase Complex (100x): 100 µl- 1 unit.
5. Sample Diluent: 30 ml- 1 unit.
6. Antibody Diluent:12 ml- 1 unit.
7. Avidin-Biotin-Peroxidase Diluent: 12 ml- 1 unit.

8. Colour Developing Reagent (TMB): 10 ml- 1unit.
9. Stop Solution: 10 ml- 1 unit.
10. Wash Buffer (25x): 20 ml- 1 unit.
11. Plate Sealers- 4 units.

ADDITIONAL MATERALS REQUIRED:

1. Microplate reader (Absorbance at 450 nm)
2. Microipettes capable of precisely dispensing 0.5 µl through 1 ml volumes of aqueous solutions.
3. Distilled water.
4. 500 ml graduated cylinders.
5. Test tubes for dilution.
6. Log graph paper standard or sample dilutions.

PREPARATION OF REAGENTS:

REAGENT	PREPARATION
All reagents were brought to room temperature (18-25°C) before the assay. Assay was done at 37°C.	
Wash buffer	500 ml was prepared by diluting the supplied 20 ml of Wash Buffer (25 x) with 480 ml of deionized or distilled water. If crystals formation occurred in the concentrate, solution was warmed to room temperature and mixed gently until crystals had completely dissolved
Biotinylated Anti-Human CLU antibody	Reagent was prepared immediately prior to use by diluting the antibody with Antibody Diluent. 100 µl was prepared by adding 1 µl of Biotinylated antibody (100x) to 99 µl of Antibody Diluent for each well.

Avidin-Biotin-Peroxidase Complex	Same as above except using Avidin-Biotin-Peroxidase Complex as a reagent.
Human CLU Standard	Prepared 2 hours prior to performing the experiment. One 50 ng of lyophilized Human CLU standard was used. The vial was gently spun prior to use. The standard to a stock concentration of 50 ng/ml was reconstituted using 1 ml of sample diluent.
Samples	Samples were diluted so that the expected range of concentrations fell within the detection range of this kit.

DILUTION OF HUMAN CLU STANDARD:

1. Test tubes were numbered from 1 to 8.
2. For standard #1, 1000 µl of undiluted standard stock solution was added to tube 1.
3. 300 µl of sample diluent was added to all tubes from 2-7.
4. To generate standard # 2, 300 µl of standard # 1 was added from tube 1 to tube 2 for a final volume of 600 µl. It was mixed thoroughly.
5. To generate standard # 3, 300 µl of standard # 2 from tube 2 was added to tube 3 for a final volume of 600 µl. It was mixed thoroughly.
6. The serial dilution was continued in this manner for tube 4-7.

Final Concentrations to be Tube 1 –50 ng/ml

Tube 2 –25 ng/ml

Tube 3 – 12.5 ng/ml

Tube 4 – 6.25 ng/ml

Tube 5 – 3.125 ng/ml

Tube 6 – 1.562 ng/ml

Tube 7 – 0.781ng/ml

Tube 8 – Sample Diluent served as the standard (0.0 ng/ml)

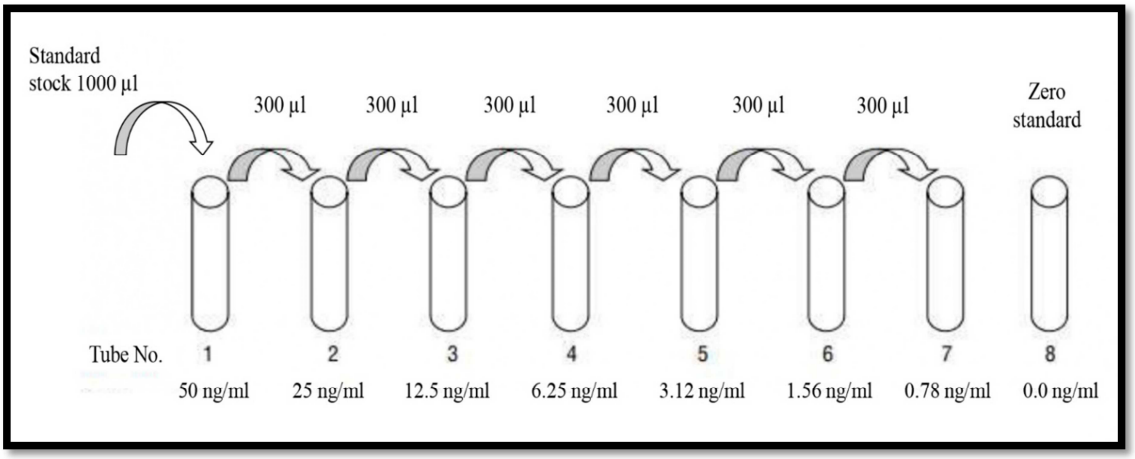


FIG 2: Dilution series

ASSAY PROTOCOL:

All reagents and materials were equilibrized to room temperature (18-25°C)

1. All reagents and working standards were prepared as directed previously.

2. 100 µl of the standard, samples, or control were added per well.

3. 100 µl of the Sample Diluent was added into the zero well.

4. The plate was covered with the plate sealer followed by incubation for 120 minutes at room temperature.

5. The cover was removed and the liquid in the wells was discarded into a waste receptacle. The plate was inverted on the benchtop onto a paper towel and tapped gently to remove the remaining liquid; but wells were not completely dry at any time.

6. 100 µl of the prepared 1x Biotinylated Anti-Human CLU antibody was added to each well.

7. A plate sealer was covered and incubated for 90 minutes at room temperature.

8. The plate was washed 3 times with the 1x wash buffer:

A. The liquid in the wells was discarded into a waste receptacle. Then, the plate was inverted on the benchtop onto a paper towel and tapped gently to remove the remaining liquid.

B. 300 µl of the 1x wash buffer was added to each assay well.

C. Steps A and B were repeated 2 additional times.

D. The wash buffer was discarded into a waste receptacle. Then, the plate was inverted on the benchtop onto a paper towel and tapped gently to blot any remaining liquid.

9. 100 µl of the prepared 1x Avidin-Biotin-Peroxidase Complex was added into each well. The plate was covered with the sealer provided and incubated for 40 minutes at room temperature.

10. The plate was washed 5 times with the 1x wash buffer:

A. The liquid in the wells was discarded into a waste receptacle. Then, the plate was inverted on the platform on a paper towel and tapped gently to remove remaining liquid.

B. 300 µl of the 1x wash buffer was added to each assay well.

C. Steps A and B were repeated 4 additional times.

D. The wash buffer was discarded into an appropriate waste receptacle. Then, the plate was inverted on the benchtop onto a paper towel and tapped gently to remove remaining liquid.

11. 90 µl of Colour Developing Reagent was added to each well. The plate was covered with the plate sealer followed by incubation in the dark for 30 minutes at room temperature.

12. 100 µl of Stop Solution was added to each well. The colour immediately changed to yellow.

13. Within 30 minutes of stopping the reaction, the O.D. absorbance was read with a microplate reader at 450nm.

STATISTICAL ANALYSIS:

- The Statistical Package for Social Science software (SPSS) version 20.0 was used to perform analyses.
- In all three groups, Mean and Standard deviation (SD) were calculated for all the parameters.
- Distribution of age and gender was assessed in all three groups. Percentage distribution of gender was done for all groups.
- Correlation between gender distribution and clusterin values was assessed using Chi square test.
- The mean values of the measured clinical parameters (SBI, PPD, CAL) and levels of salivary Clusterin levels for all the study groups were assessed by Analysis of variance (ANOVA) and Tukey's multiple post hoc test.
- The correlation of significance between the salivary Clusterin values with PPD, CAL and SBI were done using Karl Pearson's Correlation ratio.
- Level of significance was fixed at p= 0.05. Any value less than or equal to 0.05 was considered as statistically significant.

Photo 1: Control/ Periodontally healthy group

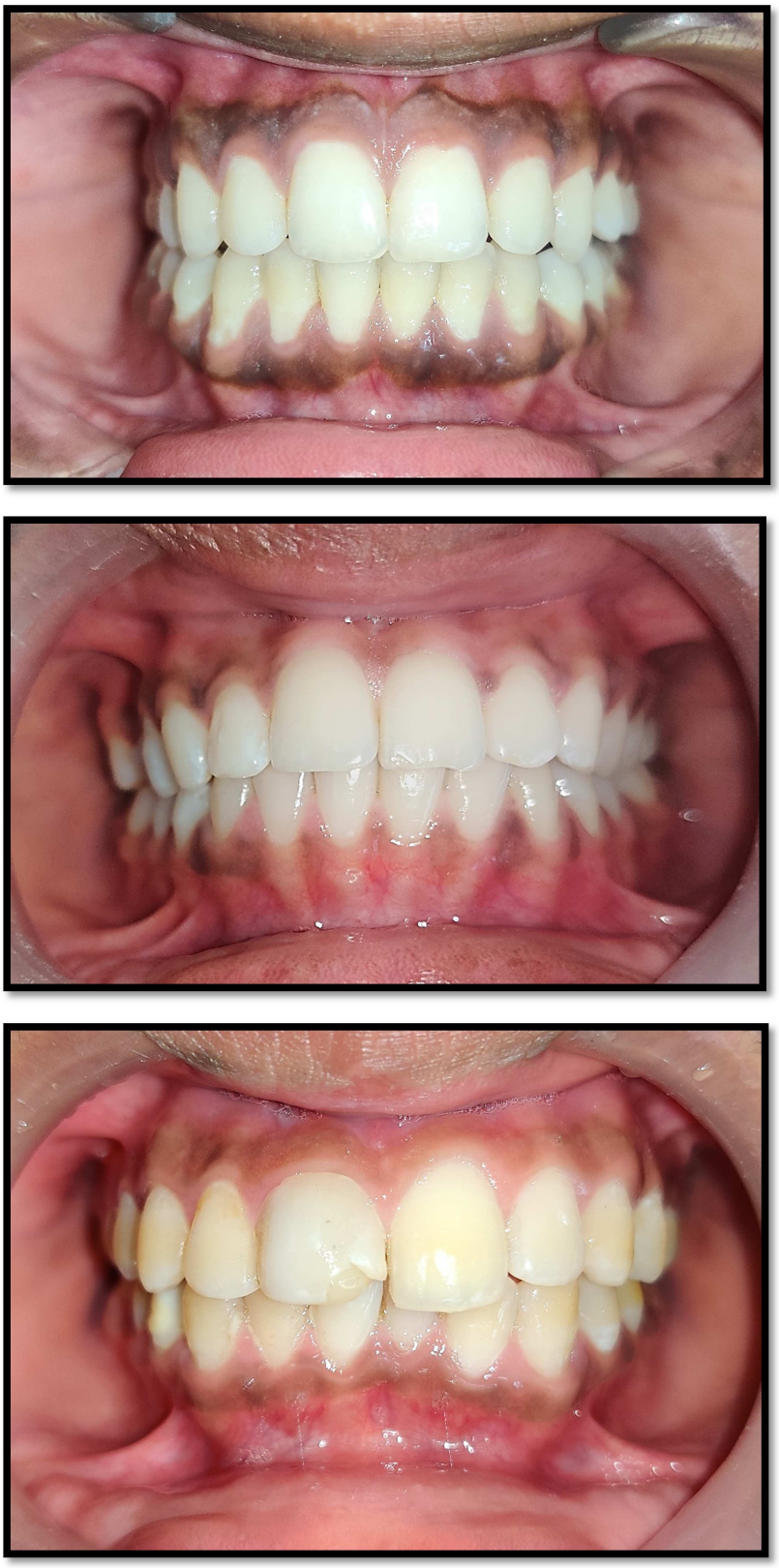


Photo 2: Gingivitis group

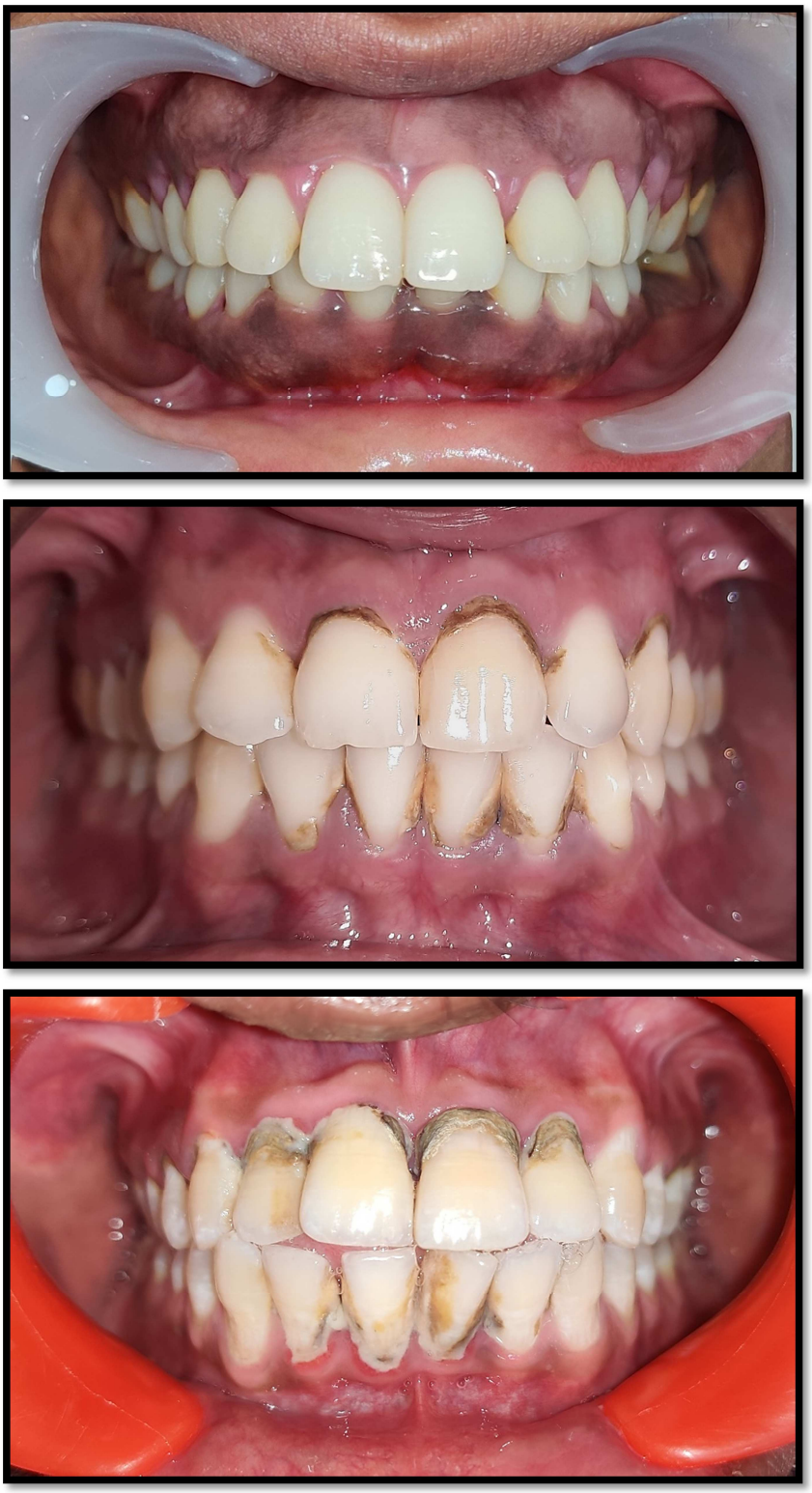


Photo 3: Chronic periodontitis group

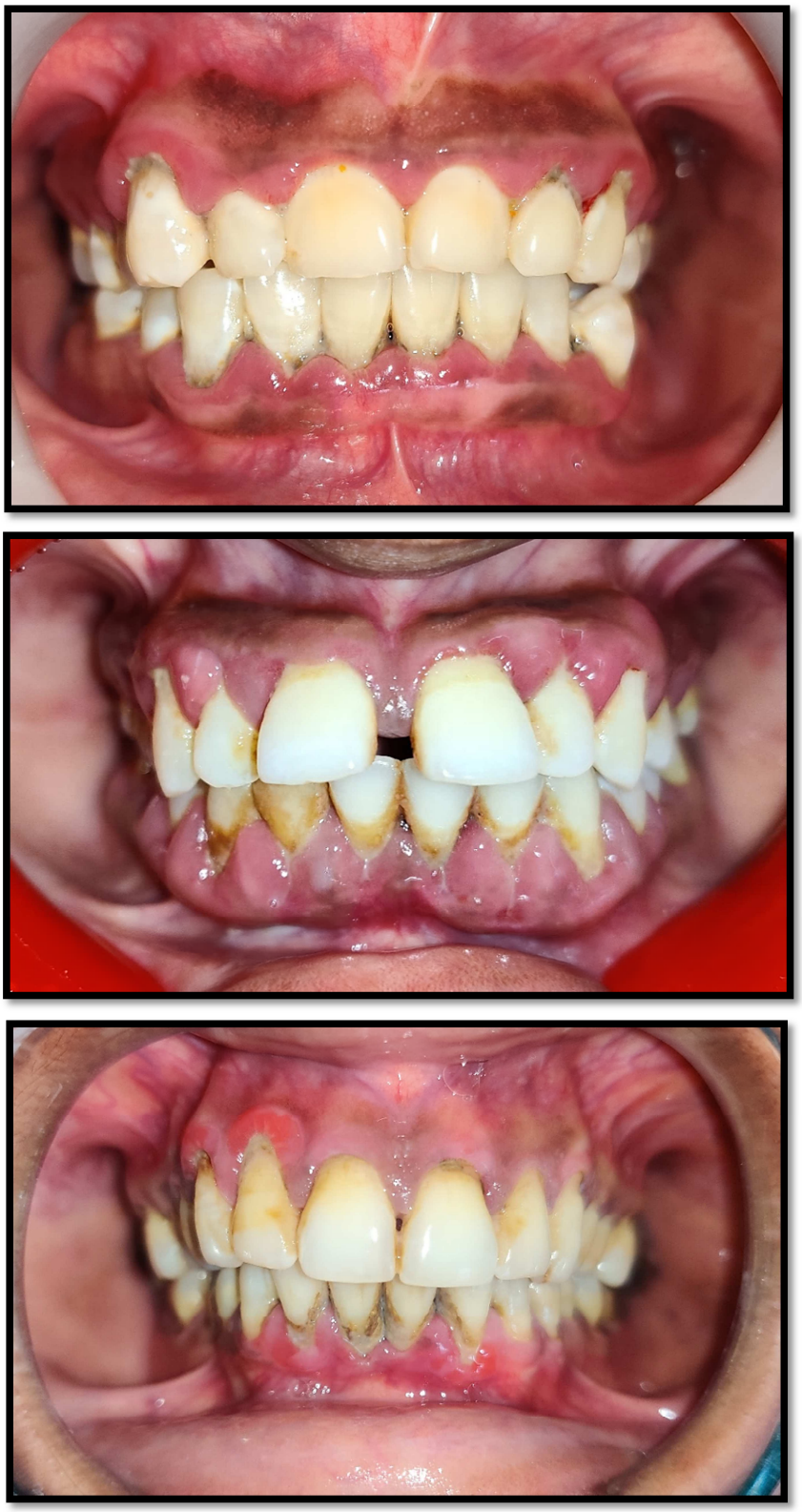


Photo 4: Clinical Armamentarium

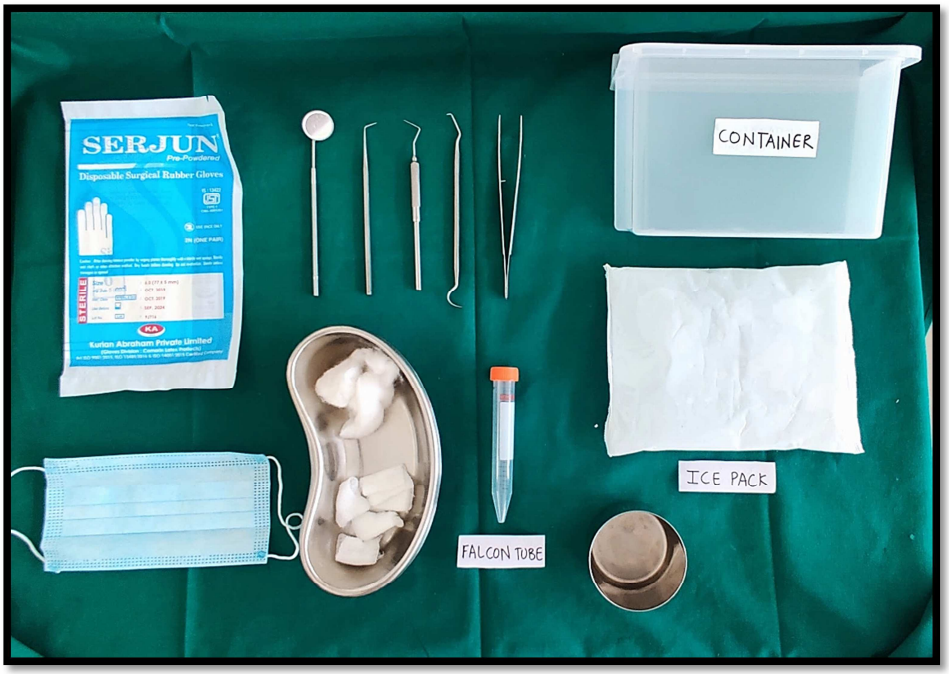


Photo 5: Collection of saliva

Photo 6: -20° C Refrigerator

Photo 7: Laminar Air Flow

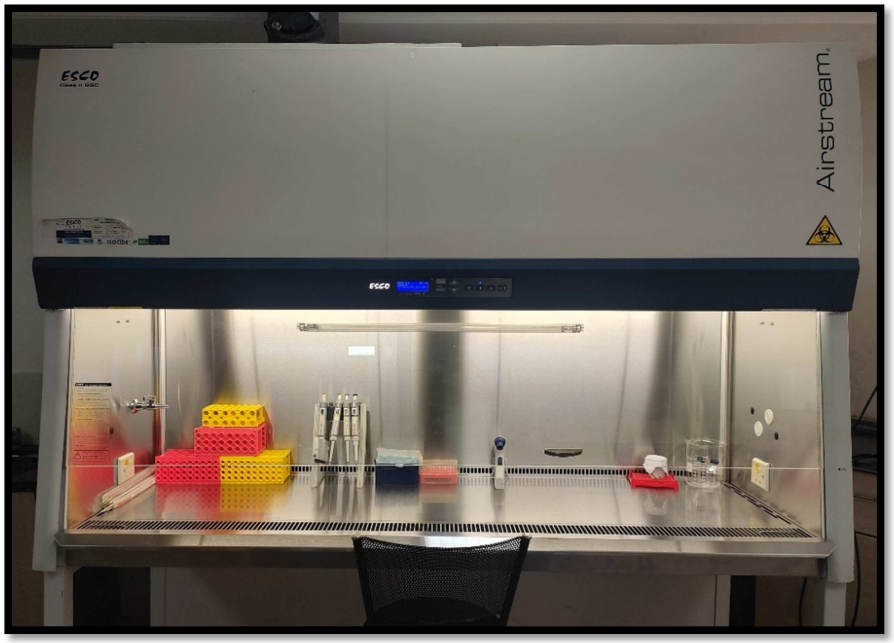


Photo 8: Micropipette

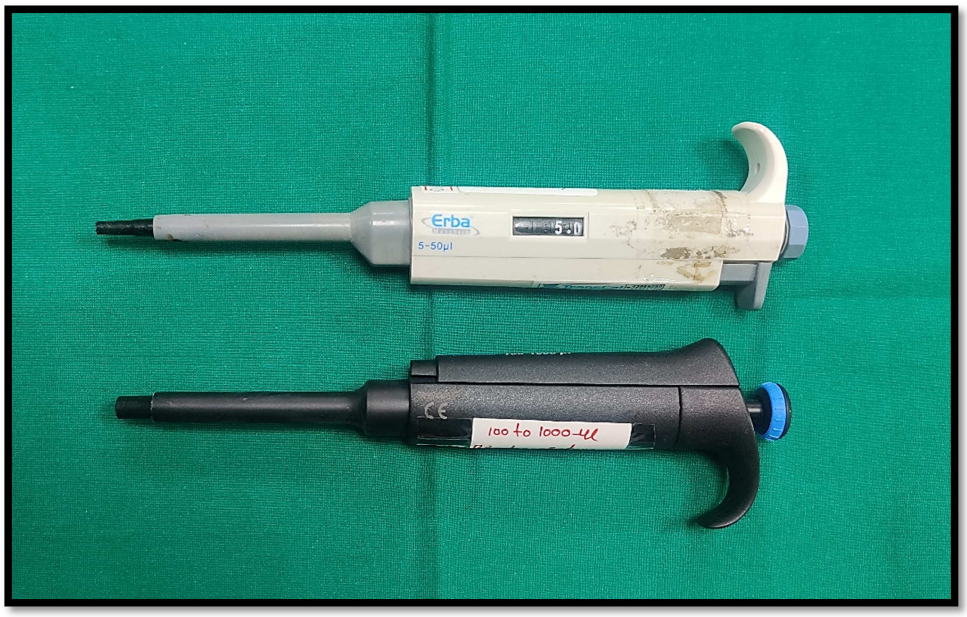


Photo 9: Centrifuge machine

Photo 10: ELISA kit contents

Photo 11: Vortex machine

Photo 12: ELISA microplate reader

RESULTS

Table 1: Age distribution of patients in three groups

Variable (n= 25)	Minimum	Maximum	Mean	Std. Deviation
Healthy	20.00	57.00	36.92	10.32
Gingivitis	18.00	52.00	32.32	8.78
Periodontitis	27.00	58.00	40.92	8.87

Observations:

In healthy group, age of the patients ranged between 20 to 57 with mean 36.92± 10.31. In case of gingivitis group, the range was from 18 to 53 with the mean age of 32.32 ± 8.77. Similarly, in periodontitis group, age of the patients was between 27 and 58, the mean age was 40.92 ± 8.86.

Table 2: Frequency table for gender of the patients and Correlation of gender with clusterin levels among health, gingivitis and periodontitis groups

Groups	**Healthy**	**Gingivitis**	**Periodontitis**	**Chi square p value**
Male	9 (36%)	11 (44%)	13 (52%)	**0.11**
Female	16 (64%)	14 (56%)	12 (48%)	
Total	25	25	25	

Observations:

Frequency distribution was done. In healthy group, among 25 subjects, 9 were males and 16 were females. The frequency percentage of males was 36% and females was 64%. Gingivitis group included 11 males and 14 females with frequency of 44% and 56% respectively. The periodontitis group comprised of total 25 patients among which 13 were males with frequency percentage of 52% and 12 were males with frequency percentage of 48%.

The Chi square test correlating both the genders and values of clusterin level showed no significance ($p > 0.05$) suggesting that the increase or decrease in clusterin levels was not associated with any gender.

Table 3: Comparison of three groups (Healthy group, gingivitis group and chronic periodontitis group) with respect to Sulcus Bleeding Index (SBI) scores by ANOVA

Groups	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval	
					Lower Bound	Upper Bound
Healthy	25	0.00	0.00	0.00	0.00	0.00
Gingivitis	25	1.29	0.41	0.08	1.12	1.46
Periodontitis	25	2.92	0.61	0.12	2.67	3.17
Total	75	1.40	1.27	0.14	1.11	1.69
F value	295.06					
P value	0.0001*					

*$p < 0.05$

Observations:

The mean value of sulcus bleeding index scores in healthy, gingivitis and periodontitis groups were 0.0, 1.29± 0.41, 2.92 ± 0.61 respectively. Significant difference was observed between three groups with mean SBI scores (F value- 295.06, p= 0.001)

Table 4: Comparison of three groups (Healthy group, gingivitis group and chronic periodontitis group) with respect to pocket probing depth (PPD) scores by ANOVA

Groups	N	Mean	Std Deviation	Std Error	95% Confidence interval	
					Lower Bound	Upper Bound
Healthy	25	1.68	0.41	0.08	1.51	1.85
Gingivitis	25	2.40	0.73	0.14	2.10	2.70
Periodontitis	25	5.79	0.60	0.12	5.54	6.03
Total	75	3.29	1.89	0.21	2.85	3.73
F value	333.62					
P value	0.0001*					

*P < 0.05

Observations:

The mean value of pocket probing depth healthy, gingivitis and periodontitis groups were 1.68± 0.41, 2.4± 0.73 and 5.79± 0.6 (in mm) respectively. Significant difference was observed between three groups with respect to mean pocket probing depth. (F value-333.62, P= 0.0001)

Table 5: Comparison of three groups (Healthy group, gingivitis group and chronic periodontitis group) with respect to Clinical Attachment Loss (CAL) scores by ANOVA

Groups	N	Mean	Std Deviation	Std Error	95% Confidence interval	
					Upper Bound	Lower Bound
Healthy	25	0.00	0.00	0.00	0.00	0.00
Gingivitis	25	0.00	0.00	0.00	0.00	0.00
Periodontitis	25	3.21	0.52	0.10	2.99	3.43
Total	75	1.07	1.55	0.17	0.71	1.42
F value	922.06					
P value	0.0001*					

*P < 0.05

Observations:

The mean value of clinical attachment level scores in healthy, gingivitis and periodontitis groups were 0.0, 0.0 3.21± 0.52 (in mm) respectively. Significant difference was observed between three groups with mean pocket probing depth. (F value- 922.064, P= 0.0001)

Table 6: Tukey's Multiple Post hoc tests for pairwise comparison of clinical parameters (SBI, PPD, CAL) between all three groups

Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
SBI	H	G	-1.29*	0.12	**0.000**	-1.58	-1.00
	H	P	-2.92*	0.12	**0.000**	-3.21	-2.63
	G	P	-1.62*	0.12	**0.000**	-1.91	-1.33
PPD	H	G	-0.71*	0.16	**0.000**	-1.12	-0.31
	H	P	-4.10*	0.16	**0.000**	-4.51	-3.69
	G	P	-3.38*	0.16	**0.000**	-3.79	-2.97
CAL	H	G	0.00	0.08	**1.000**	-0.20	0.20
	H	P	-3.21*	0.08	**0.000**	-3.41	-3.00
	G	P	-3.21*	0.08	**0.000**	-3.41	-3.00

* The mean difference is significant at the 0.05 level.

H- Healthy, G- Gingivitis, P- Chronic periodontitis

Observations:

In this pairwise comparison of clinical parameters viz sulcus bleeding index (SBI), pocket probing depth (PPD) and clinical attachment loss (CAL) between all the three groups showed a statistically significant difference between the values ($p < 0.05$) except the comparison of CAL between healthy and gingivitis group.

Table 7: Comparison of three groups (Healthy group, gingivitis group and chronic periodontitis group) with respect to Clusterin values by ANOVA

Groups	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval	
					Lower Bound	Upper Bound
Healthy	25	222.90	85.97	17.19	187.40	258.39
Gingivitis	25	136.56	37.72	7.54	120.99	152.13
Periodontitis	25	105.91	23.82	4.76	96.07	115.74
Total	75	155.12	74.36	8.58	138.01	172.23
F value	29.41					
P value	0.0001*					

*P < 0.05

Observations:

Mean clusterin value of healthy group was 222.9± 85.97, whereas for gingivitis group, the value was 136.56 ± 37.72. For periodontitis group, the mean value was 105.91 ± 23.82. Significant difference was observed between three groups with mean values of clusterin.

(F value- 29.41, P= 0.0001)

Table 8: Tukey's Multiple Post hoc tests for pairwise comparison of clusterin levels in all three groups

Variable		Mean Difference (I-J)	Std. Error	P value	95% Confidence Interval	
					Lower Bound	Upper Bound
H	G	86.33*	15.81	**0.0001**	54.80	117.86
	P	116.99 *	15.81		85.45	148.52
G	H	86.33*	15.81	**0.0001**	117.8	54.80
	P	30.65	15.81		25.87	62.18
P	H	116.99*	15.81	**0.0001**	148.52	85.45
	G	30.65	15.81		62.18	25.87

H- Healthy, G- Gingivitis, P- Periodontitis

*The mean difference is significant at the 0.05 level.

Observations:

The pairwise comparison of values of clusterin levels between all the three groups showed a statistically significant difference between the values ($p < 0.05$).

Table 9: Correlation of clusterin levels (All three groups) with increase in clinical parameters

		SBI	PPD	CAL
Pearson's ratio	**R- Value**	-0.48	-0.513	-0.477
Clusterin	**n**	75	75	75

Pearson's correlation test revealed a negative correlation between clusterin levels and all three clinical parameters viz Sulcus Bleeding Index, Pocket probing depth and Clinical attachment level. The strength of correlation was moderately strong as all R-values were between -0.3 and -0.5.

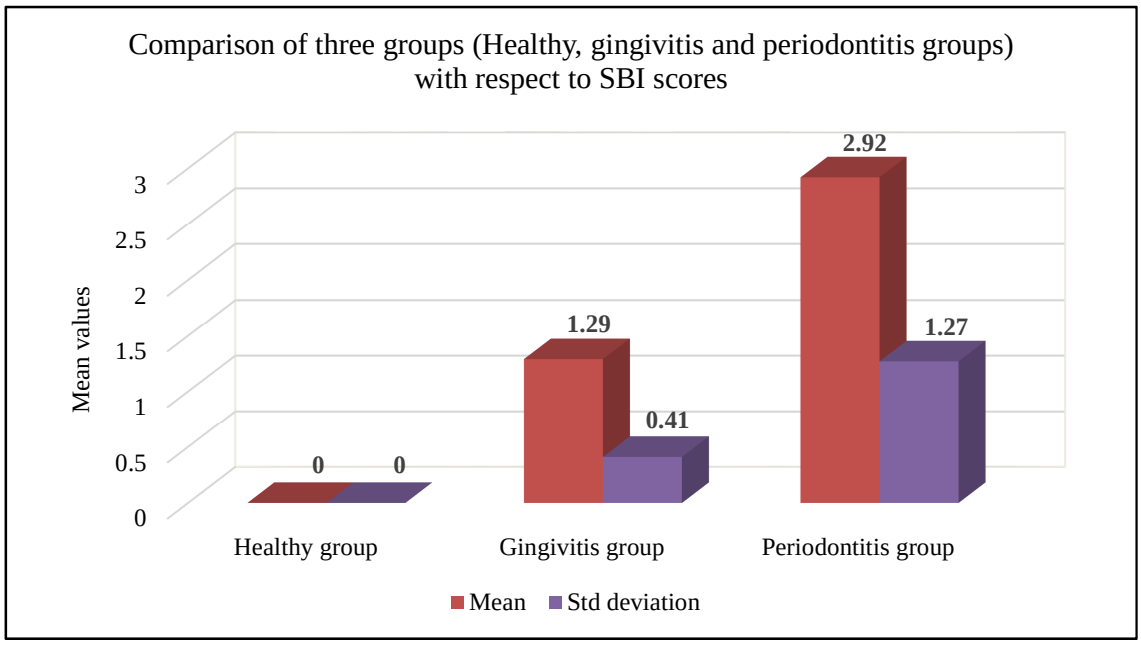
Table 10: Significance of corelation of Clusterin with clinical parameters

		SBI	**PPD**	**CAL**
Pearson's correlation ratio	Healthy (n= 25)	.	0.162	.
	Gingivitis (n= 25)	0.28	0.03	.
	Chronic periodontitis (n= 25)	**-0.52**	**-0.41**	**-0.43**

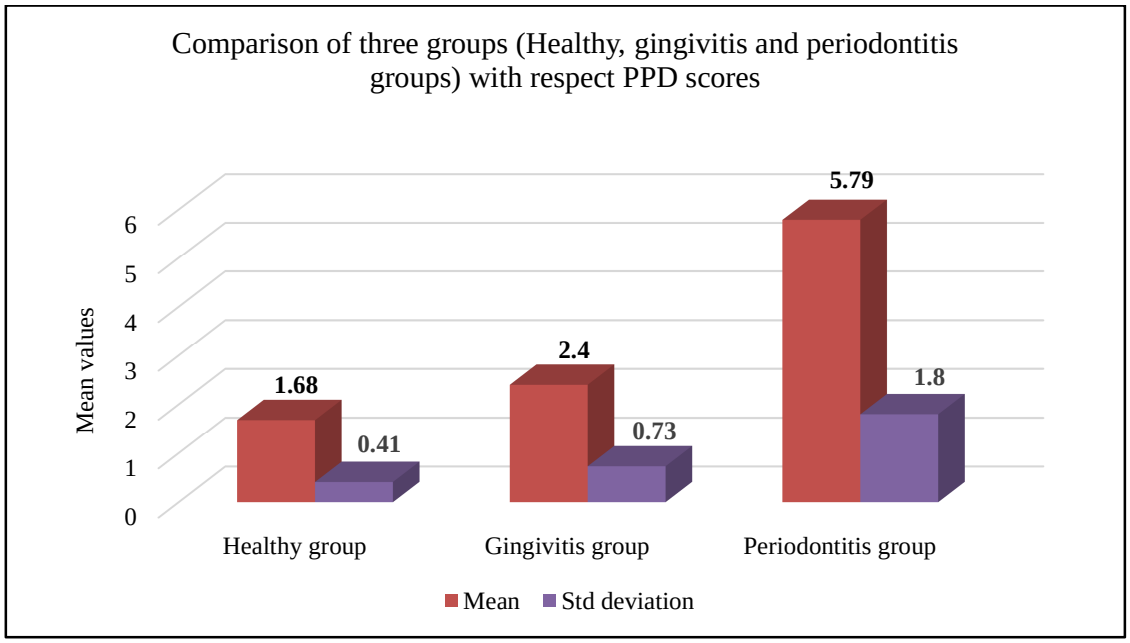
Person's correlation ratio revealed a weak positive correlation between clusterin values and pocket probing depth in healthy group as the R value lied between 0 and 0.3. Similar correlation was found between clusterin values and sulcus bleeding index, pocket probing depth in gingivitis group (R value between 0-0.3). Whereas, there was moderately strong negative correlation between clusterin values and all three parameters in chronic periodontitis group as the values were present between (-0.3 to -0.5).

GRAPHS

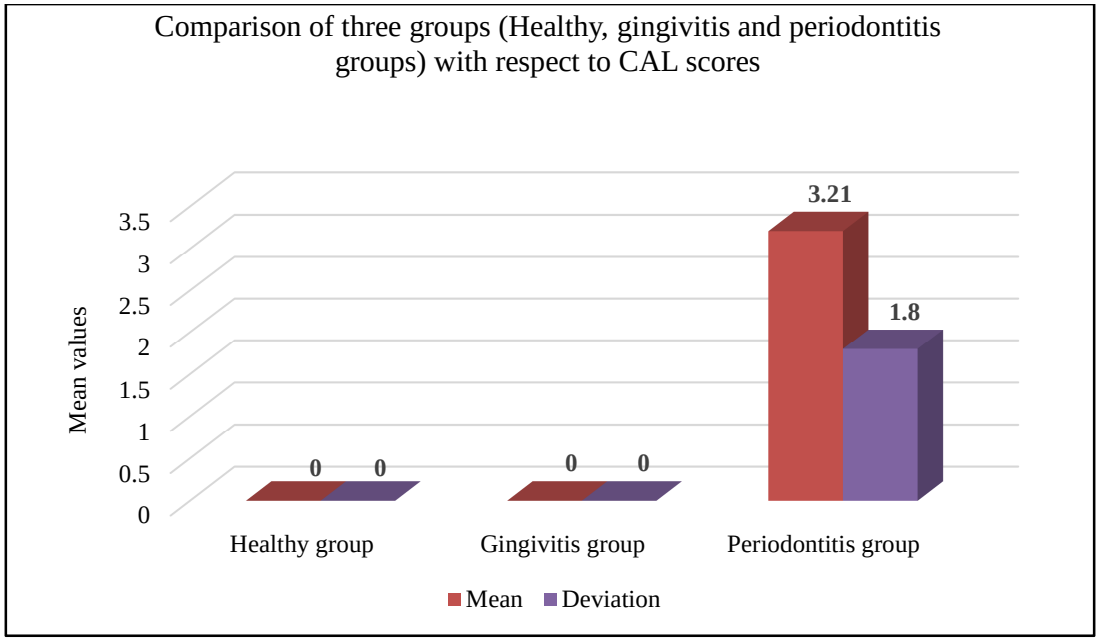
GRAPH 1: Comparison of Sulcus Bleeding Index scores between study groups



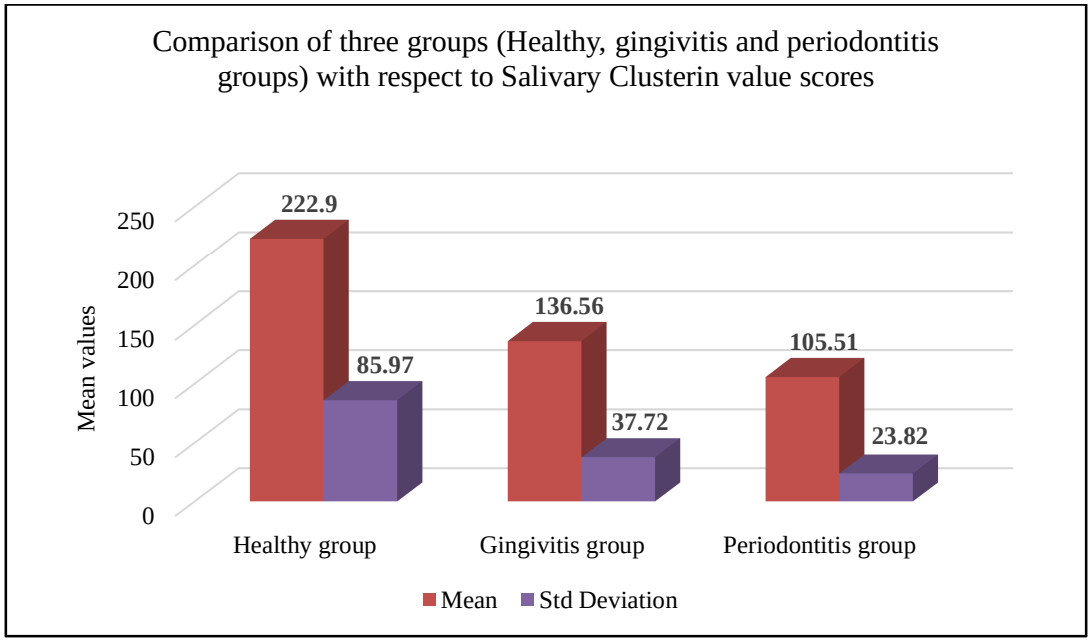
GRAPH 2: Comparison of Pocket Probing Depth scores between study groups



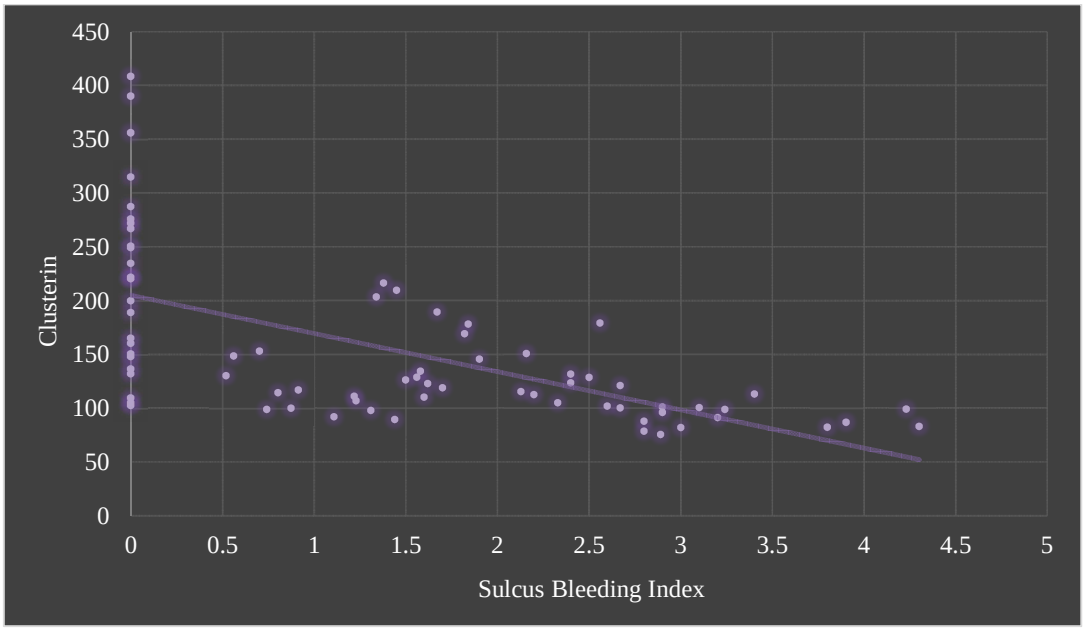
GRAPH 3: Comparison of Clinical Attachment Level scores between study groups



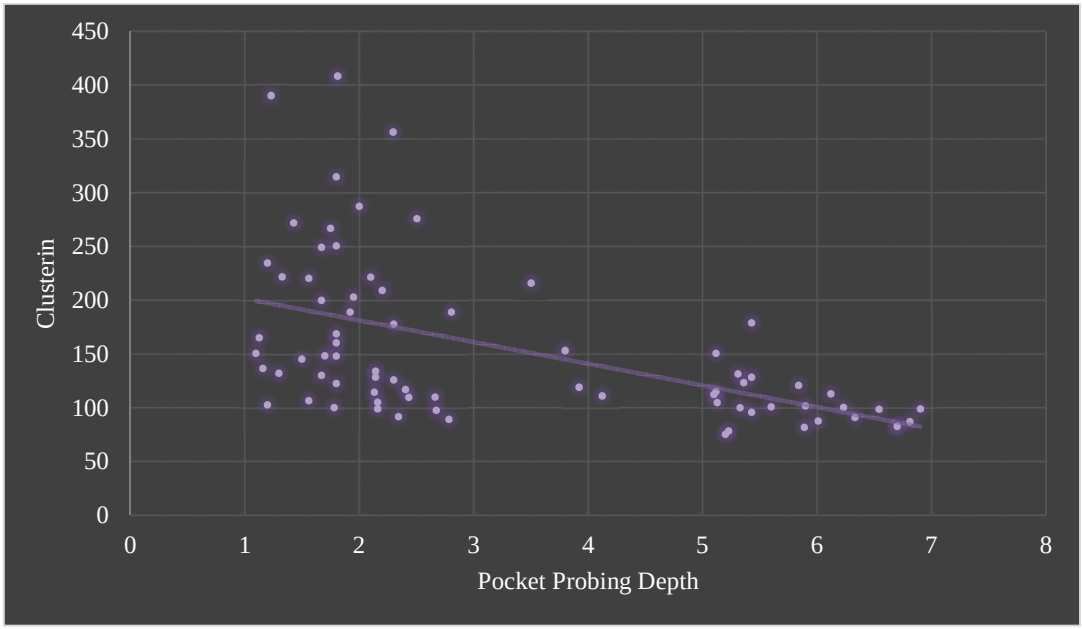
GRAPH 4: Comparison of Salivary Clusterin value scores between study groups



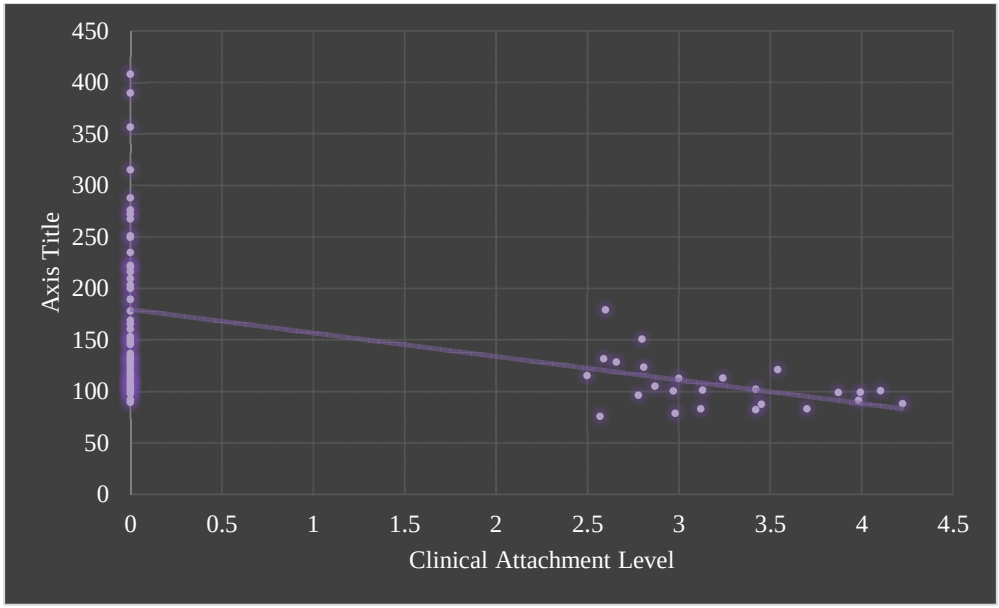
GRAPH 5: Correlation between SBI and Clusterin levels- Overall



GRAPH 6: Correlation between PPD and Clusterin levels- Overall



GRAPH 7: Correlation between CAL and Clusterin levels- Overall



DISCUSSION

Periodontal disease is multifactorial in origin and tends to be affected by numerous environmental and host factors, apart from its microbial etiology. During its occurrence and progression, a variety of molecules show quantitative changes that can possibly be correlated with the severity of periodontal destruction. Hence, there is a constant need to identify and understand newer molecular changes that can explain the disease progression along with a pathway to achieve better clinical outcomes.

Comment [MP1]: Why not viruses and fungi…suggesting word microbial etiology.

GCF is a long accepted diagnostic marker of periodontal disease progression as well as resolution. It however poses grave challenges in terms of the method of collection, quantity collected as well as cross contamination. Over the years saliva has also been utilized for investigation of various biomarkers. Factors derived from GCF & plaque are found in whole saliva also. Collection of saliva sample is non-invasive, uncomplicated, time-efficient, relatively cheaper and can be useful in screening large populations. Hence, it serves the purpose of investigation for periodontal disease as an alternative to GCF.

Clusterin performs an important role in the clearance of cellular debris, cytoprotection at fluid–tissue boundaries, membrane recycling during development and in response to injury, and regulation of complement-mediated membrane attack and scavenging. In addition to this, clusterin can either advance or forestall cell death depending upon the nature of molecular species. Clusterin has been extensively studied in the medical field because of its pivotal role in basic functions however its role in oral microbial infection i.e., periodontitis has not been extensively explored till date. We therefore undertook the study with this very aim to assess the relationship of clusterin in periodontal health and disease.

Comment [MP2]: Suggesting microbial infection.

Our samples panned across the age range of 25 to 58 years. The possibility of reduction of clusterin values with age changes was ruled out as the mean age for all three groups ranged between 36.92 and 40.92. Similarly, there was no association seen between the gender of the participants and the clusterin values **(Table 2).**

Further we observed that salivary clusterin levels significantly (p= 0.0001) reduced in the periodontitis group as compared to gingivitis and healthy control groups. **(Table 5, Graph 4)**. This result was reconfirmed further when pairwise comparison between all three groups was done **(Table 6)**. It was seen that the difference of clusterin levels between all three groups was statistically significant (p= 0.0001). This can be attributed to the fact that expression of clusterin by oral and gingival keratinocytes tends to reduce as there is a progression from periodontal health to gingivitis and further to periodontitis.

We also investigated the possible association and correlation of clusterin levels with the severity of periodontal disease by comparing the clinical parameters (SBI, PPD and CAL) with the clusterin values. The overall comparison showed a moderately strong negative correlation **(Table 10, Graphs 5, 6, 7)** whereas the individual group comparison showed a weak positive correlation in the periodontally healthy and gingivitis group and a moderately strong negative correlation in the chronic periodontitis group **(Table 11).**

Comment [MP3]: Long form kaun mention karega be

Comment [MP4]: Healthy?

A plausible biological reason for our observation could be that clusterin acts as a versatile molecule and performs various functions. Its downregulation can affect the tissue directly or indirectly. Whenever there is downregulation of clusterin, it leads to activation of the p53 gene.[14] This activation increases oxidative stress through increased production of ROS and induces damage to mitochondria of gingival

fibroblasts. This p53 induced redox imbalance and mitochondrial dysfunction is further enhanced by lipopolysaccharides of periopathogens such as *Porphyromonas gingivalis* affecting the normal activity of gingival fibroblasts. In this way, lower levels of clusterin exert a direct influence on the periodontium as well as indirectly through the p53- LPS cascade. Furthermore, activated p53 itself can decrease the expression of clusterin in turn increasing cellular pathways towards the apoptotic death of the cells and more tissue destruction.[15]

Another mechanism that can be implicated is the stimulation of macrophages to produce and secrete TNF-α, which is one of the popular mediators involved in the occurrence and progression of the inflammation. Reduced levels of clusterin also elevate the secretion of a vast array of cytokines like GCSF, KC, MCP-1, MIP- 1b, IL-1ra, and RANTES coupled with a slight increase in the expression of M-CSF, IL-27. It is well known that increased expression of all these cytokines will help in maintaining a persistent inflammation, which in turn is the basis of chronic periodontal disease.[17]

Comment [MP5]: Expression of? reframe.

Comment [MP6]: Either write the details or give reference. Don't do both.

NF-κB performs central role in regulation of inflammation. Clusterin regulates this NF-κB pathway and hence, in a way assists in regulation of the inflammation too. When the levels of clusterin drops, it accelerates nuclear translocation of NF-κB p65 along with κBα- degradation and phosphorylation. This step is crucial for the expression of MMP-9, a gelatinase that is considered pivotal in periodontal destruction. Thus, it is seen that downregulation of clusterin enhances the expression of MMP-9 indirectly and thereby upregulates periodontal connective tissue destruction.[18]

A delay in apoptotic induction is caused by a secretory isoform of clusterin as mentioned earlier, clusterin can either induce or prevent the cell death. Though, it is said that low levels activate the proapoptotic cascade, studies have shown that lower levels in the tissue might delay the clearance of debris, forestall the apoptosis of monocytes and macrophages. Continued presence of these cells in the tissue will help to maintain the proinflammatory environment.[4] Further, they will secrete TNF-α and other chemokines. TNF-α will act as a chemoattractant and will have a direct effect on migration of macrophages furthering recruitment of chronic inflammatory cells in the tissue will eventually increase connective tissue destruction.[12] Clusterin exerts a protective influence on intracellular oxidative stress a phenomenon that can hasten cellular destruction. This effect is lost when clusterin levels are reduced in periodontal disease and an increase in periodontal cellular destruction is concomitantly seen.[19,20]

Bearing all these biological correlations in mind, it can be postulated that downregulation of clusterin participates in upholding an inflammatory status in periodontal tissues. Our study showed a significant decrease in clusterin levels in the chronic periodontitis group as compared to the periodontally healthy group. Only two studies carried out by Baliban RC et al (2012) and Bostanci et al (2018) have found similar observations[4,13]. They also mentioned the quantitative analysis of clusterin for both periodontal health and periodontitis, but did not mention the possible mechanisms for the difference in levels in periodontal health and disease.

Based on the inferences drawn from our study it can be safely concluded that clusterin levels and functions affect the periodontium via multiple mechanisms. Further immunohistochemical and microbial studies may be able to endorse the clinical and biochemical observations of our study.

SUMMARY AND CONCLUSION

Periodontal diseases are inflammatory diseases that can cause tooth supporting tissue destruction and eventually tooth loss. Though pathogenic bacteria are considered to be the prime etiological factor, host immune response is also an equally important factor that decides the progression as well as the disease prognosis. Clusterin is a heterodimer glycoprotein, present in almost all the mammalian fluids. They are involved in many important functions like clearance of cellular debris, cytoprotection at fluid–tissue boundaries, membrane recycling during development and in response to injury, and regulation of complement-mediated membrane attack, scavenging. In addition to this, clusterin can either advance or promote cell death depending upon the nature of molecular species. Though this protein was detected in saliva a few years ago, its exact role as a diagnostic marker or its correlation with disease progression has never been investigated in patients suffering from chronic periodontitis.

Hence, this study was carried out to check whether there was any correlation between the destruction caused in periodontal tissue as seen in chronic periodontitis and the levels of clusterin present in saliva. The study was conducted in Outpatient Department of Periodontics, KAHER's KLE V.K. Institute of Dental Sciences, Belagavi.

A total of 75 subjects were included in the study. Written informed consent was obtained from all the subjects. Detailed medical history and thorough dental examination which included Sulcus Bleeding Index, Pocket probing depth, Clinical attachment loss. This was followed by division of the subjects into three groups of 25 subjects each. The three groups were as follows: Group I: Periodontally healthy/

Control group, Group II: Gingivitis group, Group III: Chronic periodontitis group. All the data was obtained with the help of proforma.

On the day of saliva sample collection, participants were asked to refrain from brushing, drinking or consuming food 1 hour before the collection. Each participant was instructed to rinse the mouth thoroughly with water and then expectorate whole saliva into a 15 ml falcon tube and a final volume of 3 to 5 ml was obtained. Each sample was immediately placed on ice for transportation to the laboratory. Measurement of salivary clusterin concentrations was performed using Enzyme Linked Immunosorbent Assay (ELISA) kit, Bosterbio, USA.

Data obtained from the participants was statistically analysed using ANOVA, Tukey's multiple post hoc test and Pearson's correlation ratio.

In light of the observations seen in the present study the following conclusions can be drawn:

1. Salivary clusterin levels were decreased in subjects with chronic periodontitis as compared to healthy controls.
2. A significant difference was observed in the mean values of clusterin of all three groups.
3. A pairwise comparison of three groups with each other also showed a statistically significant difference with respect to mean values of clusterin levels.
4. Salivary levels of clusterin correlated negatively with clinical parameters of Sulcus Bleeding Index, Pocket Probing Depth and Clinical Attachment Level.

Hence, the study revealed that decreased production of clusterin could be an additive factor in progression of periodontal disease. It can be deemed as a biomarker which will be able to indicate the severity and progression the disease. Further, site specific (Gingival tissue, GCF and saliva) clusterin levels and their value as a risk indicator for periodontal disease needs to explored to extrapolate the observations found in our study.

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ANNEXURES- I: ETHICAL CLEARANCE CERTIFICATE

Research and Ethics Committee
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KLE University
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Sl. No. : 1484

CERTIFICATE

This is to Certify that the synopsis titled

Estimation and correlation of human salivary clusterin (CLU) levels in periodontal health, Gingivitis and Generalised chronic periodontitis – A cross sectional study.

Submitted by

Dr. _____ *P. G. Student /*

Staff, Guided by *from Department of*

Periodontics. *has been critically evaluated by committee members and granted ethical clearance to conduct the above mentioned study*

Date : 5/5/21

Member Secretary
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

Chairman
Research and Ethical Committee
KLEVK Institute of Dental Sciences
Belagavi

ANNEXURE II- PLAGIARISM CERTIFICATE

Scientific Correspondence and Review Committee

KLE VK Institute of Dental Sciences

A Constituent Unit of KLE Academy of Higher Education and Research
(Deemed-to-be-University u/s 3 of the UGC Act, 1956)
Nehru Nagar, Belagavi - 590 010, Karnataka State

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Date : 24.12.22 Serial No. : 133

PLAGIARISM CHECK REPORT

Name of the Applicant :
UG / PG / Ph.D / Staff : POST GRADUATE STUDENT
Batch & Year : 2020-23
Department : PERIODONTICS

The soft copy of Research Work / Manuscript by ..... entitled
"ESTIMATION AND CORRELATION OF HUMAN SALIVARY CLUSTERIN LEVELS IN PERIODONTAL HEALTH, GINGIVITIS AND CHRONIC PERIODONTITIS - A CROSSSECTIONAL STUDY....."
under the guidance of .....has been submitted for Anti-Plagiarism check to the Scientific Correspondence & Review Committee of KLE VK Institute of Dental Sciences using "Turn-it-in" software.

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Scientific Correspondence and Review Committee
KLEVK Institute of Dental Sciences
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ANNEXURE- III: BIOSTATISTICIAN CERTIFICATE

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Biostatistics Clearance Certificate

This is to certify that the Biostatistics aspect of the Dissertation of **Post Graduate Student**, under the guidance of **Department of Periodontics**, entitled "Estimation and correlation of human salivary clusterin (CLU) levels in periodontal health, gingivitis and generalised chronic periodontitis - a cross sectional study" has been done under my guidance and considered satisfactory.

Dr. J. B. Prasad

Place: Belagavi
Date: 19/12/22

Name & Signature of Biostatistician

DEPARTMENT OF EPIDEMIOLOGY & BIOSTATISTICS • KAHER'S BELAGAVI

ANNEXURE- IV- CONSENT FORM

DEPARTMENT OF PERIODONTICS
KLE V.K. INSTITUE OF DENTAL SCIENCES, BELAGAVI

ESTIMATION AND CORRELATION OF HUMAN SALIVARY CLUSTERIN (CLU) LEVELS IN PERIODONTAL HEALTH, GINGIVITIS AND GENERALISED CHRONIC PERIODONTITIS - A CROSS SECTIONAL STUDY

I _____, aged _____ years have been informed about my involvement in the study.

I agree to give my personal details like Name, Age, Gender, Residential Address, past and present dental history and any other details if required for the study to the best of my knowledge.
I will co-operate with the dentist.
I will follow the instructions given by the dentist during study.

I permit the dentist to utilize the information given and results obtained from this study for presentation and publication without disclosing my identity.
I have understood the nature of the study and permit the dentist to carry out the procedure of collection of saliva sample.
If for any reason I am unable to participate in the study, for reasons unknown, I can withdraw from the study at any given point of time.
In my full consciousness and presence of mind, after understanding all the procedures and related complications if any, in my vernacular language, I am willing and give my consent to participate in this study.

Date:
Name of the Patient:
Signature:
Address
Phone No:

CONSENT FORM

DEPARTMENT OF PERIODONTICS

KLE V.K. INSTITUE OF DENTAL SCIENCES

BELAGAVI

मी,_____, वय_____ वर्षे, मला ह्या अभ्यासाबद्दल पूर्ण कल्पना देण्यात आली आहे.

मी माझी वैयक्तिक माहिती जसे की, नाव, वय, लिंग, पत्ता, आधी झालेल्या आणि आता सुरू असलेल्या दंत उपचाराची माहिती देण्यास सहमत आहे.

मी दंतचिकित्सकांना त्यांच्या अभ्यासात सहकार्य करीन.

मी दंतचिकित्सकांनी दिलेल्या सर्व सूचना पाळीन.

मी दंतचिकित्सकांनी सांगितलेल्या वेळेस व तारखेस त्यांच्या अभ्यासासाठी हजर राहीन.

मी दंतचिकित्सकांना दिलेली माहिती आणि संशोधनात मिळालेले परिणाम माझी ओळख उघड न करता वापर करण्याची परवानगी देतो/देते.

मला दंतचिकित्सकांचा अभ्यास समजला आहे आणि त्यासाठी दंतचिकित्सकांना माझी परवानगी आहे.

मी कोणत्याही कारणास्तव अभ्यासात भाग घेऊ शकत नसेन, तर मी ह्या अभ्यासातून केव्हाही बाहेर पडू शकतो/शकते .
मी पूर्ण शुद्धीत आणि मनाच्या उपस्थिती, माझ्या मातृभाषेत सर्व प्रक्रिया आणि संबंधित समस्या समजून माझ्या मातृभाषेत ह्या अभ्यासात सहभागी होण्यास संमती देतो/देते.

तारीख :- स्वाक्षरी :-
पत्ता:
दूरध्वनी क्र:

CONSENT FORM

DEPARTMENT OF PERIODONTICS

KLE V.K. INSTITUE OF DENTAL SCIENCES, BELAGAVI

Principal Investigator:

ನಾನು _____ ವಯಸ್ಸಿನ _____.

ಅಧ್ಯಯನದಲ್ಲಿ ನನ್ನ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆಯ ಬಗ್ಗೆ ನನಗೆ ತಿಳಿಸಲಾಗಿದೆ.

ನಾನು ಹೆಸರು, ವಯಸ್ಸು, ಲಿಂಗ, ವಸತಿ ವಿಳಾಸ, ಹಿಂದಿನ ಮತ್ತು ಪ್ರಸೆಂಟ್ ಹಲ್ಲಿನಬಾಗ್ಗೆ ಮತ್ತು ನನ್ನ ಜ್ಞಾನದ ಅತ್ಯುತ್ತಮ ಅಧ್ಯಯನಕ್ಕೆ ಬೇಕಾಗುವ ಯಾವುದೇ ಇತರ ವಿವರಗಳು ಹಾಗೆ ನನ್ನ ವೈಯಕ್ತಿಕ ವಿವರಗಳನ್ನು ನೀಡಲು ಒಪ್ಪುತ್ತೀರಿ.

ನಾನು ಅಧ್ಯಯನದ ಸಮಯದಲ್ಲಿ ದಂತವೈದ್ಯ ನೀಡಿದ ಸೂಚನೆಗಳನ್ನು ನುಸರಿಸಿ.

ನಾನು ನನ್ನ ಹೆಸರನ್ನು ಬಹಿರಂಗಪಡಿಸದೇ ನೀಡಿದ ಮಾಹಿತಿ ಈ ಅಧ್ಯಯನದಿಂದ ಪಡೆದ ಫಲಿತಾಂಶಗಳನ್ನು ಬಳಸಿಕೊಳ್ಳಲು ದಂತವೈದ್ಯರಿಗೆ ಅನುಮತಿ ನೀಡುತ್ತೇನೆ.

ನಾನು ಅಧ್ಯಯನ ಸಲುವಾಗಿ ಲಾಲಾರಸ ಕೊಡಲು ಒಪುತೇನೆ.

ಯಾವುದೇ ಕಾರಣಕ್ಕಾಗಿ ನನಗೆ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಸಾಧ್ಯವಾಗದಿದ್ದರೆ, ಅಜ್ಞಾತ ಕಾರಣಗಳಿಗಾಗಿ, ನಾನು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆ ಸರಿಯಬಹುದು.

ನನ್ನ ಪೂರ್ಣ ಪ್ರಜ್ಞೆ ಮತ್ತು ಮನಸ್ಸಿನ ಉಪಸ್ಥಿತಿಯಲ್ಲಿ, ಎಲ್ಲಾ ಕಾರ್ಯವಿಧಾನಗಳು ಮತ್ತು ಸಂಬಂಧಿತ ತೊಡಕುಗಳು ಯಾವುದಾದರೂ ಇದ್ದರೆ, ನನ್ನ ಸ್ಥಳೀಯ ಭಾಷೆಯಲ್ಲಿ ಅರ್ಥಮಾಡಿಕೊಂಡ ನಂತರ, ನಾನು ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಸಿದ್ಧನಿದ್ದೇನೆ ಮತ್ತು ನನ್ನ ಒಪ್ಪಿಗೆಯನ್ನು ನೀಡುತ್ತೇನೆ.

ದಿನಾಂಕ: ಸಹಿ:

ವಿಳಾಸ ಮತ್ತು

ದೂರವಾಣಿ ಸಂಖ್ಯೆ.:

ANNEXURE V- PROFORMA

DEPARTMENT OF PERIODONTICS
KAHER'S KLE's V.K. INSTITUTE OF DENTAL SCIENCES
BELAGAVI.

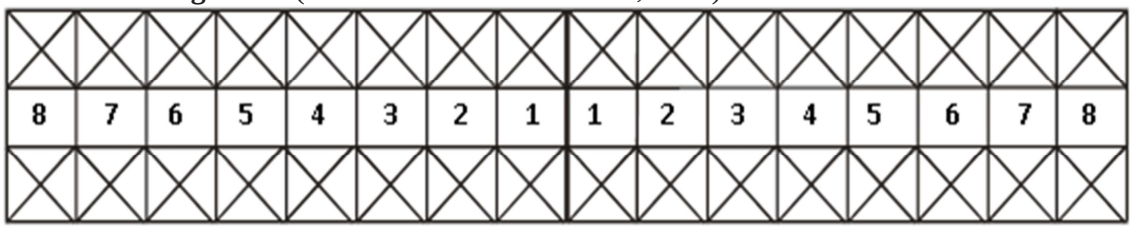
ESTIMATION AND CORRELATION OF HUMAN SALIVARY CLUSTERIN (CLU) LEVELS IN PERIODONTAL HEALTH, GINGIVITIS AND GENERALISED CHRONIC PERIODONTITIS -A CROSS SECTIONAL STUDY

Case No: OPD No:
Name:
Age: Sex: Occupation:
Address:
Chief Complaint:

Medical History:

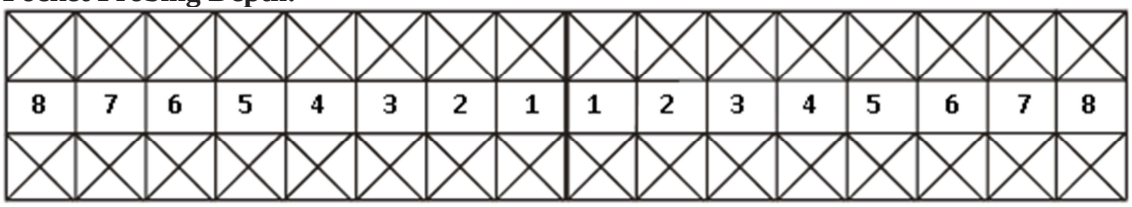
Dental history:

DENTAL PARAMETERS
Number of teeth present:-
Sulcus Bleeding Index (Muhlemann H.R and Son S, 1971):



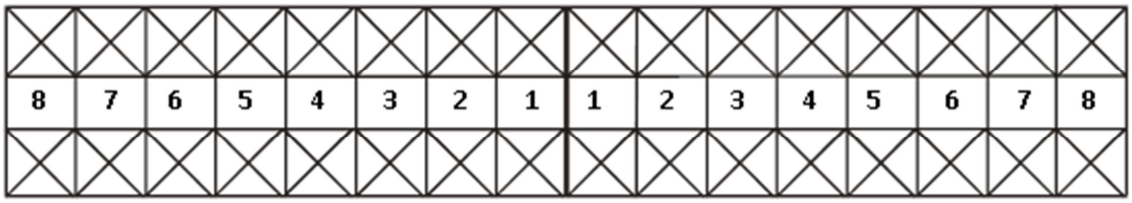
SBI Score-

Pocket Probing Depth:-



PPD Score:

Clinical Attachment Level:-



CAL Score:

Biochemical Parameter	Reading of Clusterin (ng/ml)
Saliva Sample	

Signature of the Student

Signature of the Patient

ANNEXURE VI- Group I (Periodontal Health/ Control)

SL No.	AGE (Years)	SEX	SBI	PPD	CAL	CLUSTERIN (ng/ ml)
1	20	F	0	1.2	0	103
2	24	F	0	1.43	0	272
3	38	M	0	1.8	0	148.3
4	34	F	0	2.1	0	221.5
5	42	F	0	1.8	0	250.8
6	44	M	0	2.3	0	356.2
7	48	F	0	1.33	0	222
8	23	M	0	1.8	0	160.6
9	36	F	0	1.67	0	249.2
10	41	F	0	2.16	0	105.6
11	39	F	0	2.5	0	275.8
12	25	M	0	1.1	0	151
13	26	F	0	1.56	0	220.6
14	49	F	0	1.23	0	390.1
15	33	F	0	1.8	0	314.6
16	54	M	0	2.43	0	110
17	29	M	0	1.16	0	136.9
18	35	F	0	1.2	0	234.7
19	35	M	0	1.67	0	200
20	46	F	0	1.81	0	408.1
21	52	F	0	2	0	287.4
22	27	M	0	1.3	0	132.3
23	28	F	0	1.75	0	267
24	57	F	0	1.92	0	189.3
25	38	M	0	1.13	0	165.5

Group II (Gingivitis)

SL No	AGE (Years)	GENDER	SBI	PPD	CAL	CLUSTERIN (ng/ ml)
1	30	F	1.23	1.56	0	107
2	25	F	1.5	2.3	0	126.2
3	28	M	1.82	1.8	0	169
4	32	F	0.91	2.4	0	117.4
5	38	M	0.8	2.14	0	114.7
6	28	M	1.62	1.8	0	122.9
7	39	F	1.31	2.67	0	98
8	26	M	0.7	3.8	0	153.1
9	44	F	1.38	3.5	0	216
10	39	M	1.6	2.66	0	110.2
11	32	F	1.67	2.8	0	189.2
12	25	F	0.52	1.67	0	130.5
13	28	M	1.11	2.34	0	92.3
14	37	F	1.9	1.5	0	145.5
15	22	M	1.34	1.95	0	203.1
16	24	M	1.58	2.14	0	134.4
17	31	F	0.87	1.78	0	100.5
18	36	F	1.44	2.78	0	89.6
19	42	M	0.74	2.16	0	99.4
20	38	F	1.22	4.12	0	111.3
21	19	F	1.56	2.14	0	128.9
22	27	F	0.56	1.7	0	148.6
23	48	M	1.45	2.2	0	209.2
24	52	M	1.7	3.92	0	119
25	18	F	1.84	2.3	0	178.1

Group III (Chronic Periodontitis)

SL No.	AGE (Years)	GENDER	SBI	PPD	CAL	CLUSTERIN (ng/ ml)
1	32	M	2.67	5.84	3.54	121
2	42	M	2.4	5.36	2.81	123.4
3	45	F	2.13	5.12	2.5	115.3
4	38	M	3.8	6.7	3.12	83
5	27	F	3.24	6.54	3.87	98.8
6	39	M	2.89	5.2	2.57	75.7
7	51	F	2.8	6.01	4.22	88.1
8	35	F	2.33	5.13	2.87	105
9	28	M	3.1	6.23	4.1	100.5
10	48	F	2.5	5.43	2.66	128.5
11	43	M	3	5.89	3.42	82.1
12	42	F	2.56	5.43	2.6	178.9
13	50	M	2.2	5.1	3	112.6
14	53	F	2.4	5.31	2.59	131.5
15	48	M	2.9	5.43	2.78	96.2
16	37	F	3.2	6.33	3.98	91.4
17	28	F	2.8	5.23	2.98	78.7
18	35	F	2.6	5.9	3.42	102
19	39	M	3.4	6.12	3.24	113
20	46	M	4.3	6.7	3.7	83.1
21	58	M	2.9	5.6	3.13	101.2
22	55	M	4.23	6.9	3.99	99.2
23	40	M	3.9	6.81	3.45	87.5
24	37	F	2.67	5.33	2.97	100.4
25	27	M	2.16	5.12	2.8	150.6