"EVALUATION OF ALKALINE PHOSPHATASE AND OSTEOCALCIN IN GINGIVAL CREVICULAR FLUID AND SERUM IN PREMENOPAUSAL AND POSTMENOPAUSAL WOMEN WITH CHRONIC PERIODONTITIS: A BIOCHEMICAL STUDY"

Dissertation

Submitted to

BABU BANARASI DAS UNIVERSITY

LUCKNOW, UTTAR PRADESH.

In the partial fulfilment of the requirements for the degree

Of

MASTER OF DENTAL SURGERY

In

PERIODONTICS

By

DR. ASMITA JAISWAL

Under the guidance of

DR. VIVEK GOVILA

Principal

Professor and Head

Department of Periodontics

BABU BANARASI DAS COLLEGE OF DENTAL SCIENCES, LUCKNOW

Enrolment No. 1140328006

BATCH: 2014-2017

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BATCH: 2014-2017

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled **"EVALUATION OF ALKALINE PHOSPHATASE AND OSTEOCALCIN IN GINGIVAL CREVICULAR FLUID AND SERUM IN PREMENOPAUSAL AND POSTMENOPAUSAL WOMEN WITH CHRONIC PERIODONTITIS: A BIOCHEMICAL STUDY "** is a bonafide and genuine research work carried out by me under the guidance of **Dr. VIVEK GOVILA**, Principal, Professor and Head, Department of Periodontics, Babu Banarasi Das College Of Dental Sciences, Babu Banarasi Das University, Lucknow, Uttar Pradesh.

Date:

Place: LUCKNOW.

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Dr. ASMITA JAISWAL

CERTIFICATE BY THE GUIDE

This is to certify that the dissertation entitled **"EVALUATION OF ALKALINE PHOSPHATASE AND OSTEOCALCIN IN GINGIVAL CREVICULAR FLUID AND SERUM IN PREMENOPAUSAL AND POSTMENOPAUSAL WOMEN WITH CHRONIC PERIODONTITIS: A BIOCHEMICAL STUDY"** is a bonafide work done by **Dr. ASMITA JAISWAL,** under our direct supervision and guidance in partial fulfilment of the requirement for the degree of MDS in Periodontics.

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Dr. ASMITA JAISWAL

DEDICATED

TO

MY FAMILY

Acknowledgement

"Education is the manifestation of the perfection already in man."

-Swami Vivekananda

"Education is not the learing of the facts ,but the training of the mind to think"

-Albert Einstein

I owe my deepest gratitude to my guide ***Dr. Vivek Govila M.D.S, Head and Professor, Department of Periodontics, Babu Banarasi Das College of Dental Sciences, Lucknow,*** *who patiently provided the vision, advice and encouragement necessary for me to proceed through and complete my dissertation. His vast knowledge and ability to achieve excellence has proved to be very valuable throughout. I shall always remain greatly thankful for the scholarly guidance provided by him.*

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Dr. Asmita Jaiswal

Enrolment No.1140328006

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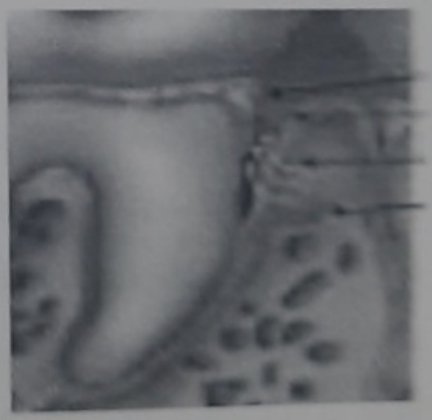
ABBREVIATIONS

GCF	Gingival Crevicular Fluid
OC	Osteocalcin
ANOVA	Analysis Of Variance
ALP	Alkaline Phosphatase
AST	Asparatate amimo Transferase
ACP	Acid Phosphatase
LDH	Lactate Dehydrogenase
PDLcs	Periodontal Ligament cells
ERs	Estrogen Receptors
RT-PCR	Reverse transcription- polymerase chain reaction
ES	Estrogen Supplement
ED	Estrogen Deficient
ALOSS	Attachment Loss
PGE_2	Prostaglandins
BMD	Bone Mineral Density
MCT	Metacarpel Cortical Thicknrss
CS	Chrondroitin Sulphate
PDA	Periodontal Disease Activity

RPMI-1640	Roswell ParkMemorial Institute
IOPA	Intra Oral Periapical Radiograph
CP	Chronic Periodontitis
ng/ml	Nano gram per milliletre
U/L	Units per litre



Abstract

Abstract

The interaction of microbial plaque and host defence mechanism is considered an important factor in the pathogenesis and onset of periodontal disease for that reason an indicator called a biomarker which is defined as a "Parameter that is objectively measured and evaluated as an indicator of normal biological or pathological processes, or pharmacological responses to a therapeutic intervention" (NIH 1998). Many diagnostic biomarkers have been found during bone formation and resorption such as alkaline phosphatase and osteocalcin in gingival crevicular fluid and serum.

As part of the aging process, women above 45 years of age enter into the phase of menopause that leads to silent physical changes including osteoporosis problems which are preventable to some extent. In the mean while, bones are constantly remodelled with old bone being replaced by new bone. However, the other main reason women experience rapid bone loss due to decline in estrogens during menopause. It is therefore, essential to identify these women at risk of developing osteoporosis.

The study aimed at comparing the levels of biochemical markers such as alkaline phosphatase and osteocalcin in gingival crevicular fluid and serum in pre and post menopausal women with chronic periodontitis. Study Design- Cross sectional, observational study. The study was carried out which comprised of 15 pre menopausal women aged between 30-40 years were selected as control (group A) whereas, 15 post menopausal women aged between 45-65 years were selected as case (group B)

It was observed that bone formation markers that is Serum ALP, Serum Osteocalcin levels were significantly increased in post menopausal when compared with premenopausal women .When post menopausal were compared to pre menopausal women ($p \leq 0.001$) also there was strong correlation between GCF Alkaline phosphatase and osteocalcin levels but not as much as serum Alkaline phosphatase and Osteocalcin.

In our study after comparing the biochemical parameter in pre and post menopausal women we conclude that both biomarkers Alkaline phosphatase and Osteocalcin are increased that is increased bone loss. Therefore gingival crevicular fluid (GCF) can be treated as a window for noninvasive analysis of periodontitis, taking into account indicators and markers of connective tissue and bone destruction.

Introduction

Review of Literature

Periodontitis is a chronic inflammatory disease characterised by gingival inflammation, periodontal destruction, and alveolar bone resorption. The interaction of microbial plaque and host defence mechanism is considered an important factor in the pathogenesis and onset of periodontal disease. Fossil evidence of alveolar bone loss surrounding the root surfaces of teeth demonstrates that it is an ancient disease and yet remains the most common cause of tooth loss around the world. Periodontal diseases are heterogeneous and include a variety of infections and inflammatory lesions. More recently Champagne et al has realized that host related factors might be the keys to understanding the disease processes in periodontitis.[1] Traditional clinical measurements probing pocket depth, bleeding on probing, clinical attachment loss, plaque index, radiographs used for periodontal diagnosis are often of limited usefulness in that they are indicators of previous periodontal diseases rather than present diseases activity. There is a need for development of new diagnostic tests that can detect the presence of active diseases, predict future disease progression and evaluate the response of periodontal therapy, thereby improving the clinical management of periodontal patients. Advances in oral and periodontal diagnostic research are moving towards identifying and quantifying by different objective measures.

'Biomarkers' refer to biologic substances that can be measured and evaluated to serve as indicators of biological health, pathogenic processes ,environmental exposure and pharmacological responses to a therapeutic intervention .It is an inflammatory exduate from gingival microcirculation that crosses inflamed periodontal tissue and enroute collects molecules of potential interest from the local inflammatory reaction. The constituents of the fluid are derived from a variety of

sources, a large collection of serum proteins, inflammatory mediators host cell degradation products and microbial metabolites. A variety of enzymes that degrade proteins, proteoglycans, lipid and carbohydrates have been detected in GCF[2]. GCF enzymes in a search for novel indicators that would guide early detection of periodontal disease and for monitoring tissue health following therapy [3]

Alkaline phosphatase (ALP), which is an important indicator of bone formation and is a phenotypic marker for osteoblast cell and associated with bone metabolism and is produced by various cells such as polymorphoneuclear leukocytes (PMNLs), osteoblasts, macrophages, and fibroblasts within the area of periodontium and GCF.[4]The level of ALP is positively correlated with the severity of the periodontal disease.[5]

On the other hand, numerous studies have demonstrated an increase in gingival inflammation during puberty, menstrual cycle and pregnancy concomitant with increased secretion of sex steroid hormones.[6] Lower osteocalcin levels have been postulated as a marker of inhibition of bone formation. The deficiency of osteocalcin in women at menopause is contributing factor to osteoporosis and considered one of the risk factors for periodontal disease.[7] It has been hypothesized that osteoporosis decreases alveolar bone density and in turn increases its susceptibility to resorption due to periodontal inflammation [8].

Accelerated bone loss in menopause is related to increased bone turnover. Alteration in GCF and serum Alkaline phosphatase and osteocalcin levels might be expected as an indication of periodontal disease activity. Thus the current study aimed at determining the effect of menopause in the levels of Alkaline phosphatase and osteocalcin in pre menopausal and post menopausal women with chronic periodontitis.

Aims & Objectives

The *Aim* of the present study was:

Evaluation of Alkaline Phosphatase(ALP) and Osteocalcin(OC) levels in Gingival Crevicular Fluid (GCF) and Serum in Premenopausal and Postmenopausal women with Chronic Periodontitis

The ***Objectives*** of the present study were:-

1) To compare the ALP and Osteocalcin levels in gingival crevicular fluid and Serum between Premenopausal and Postmenopausal women with chronic priodontitis.

2) To assess ALP levels in Gingival Crevicular Fluid and Serum as a predictor for increased alveolar bone loss in postmenopausal women.

Ishikawa and Cimasoni G (1970)[9] had conducted a study on periodontal inflammation in fluid of gingival sulcus, and the activity of ALP is significantly higher in gingival fluid than in serum, and we have attempted to investigate the correlation between its level of activity and various clinical parameters. 21 patients, aged 18-65 years and showing extensive gingival inflammation and periodontitis. Results stated that alkaline phosphatase activity indicated a mean of 99 ~0 p moles p-nitrophenol liberated l/min with a SD of 53 *6 in gingival fluid, against a mean of 33 * 7 f 9 * 6 S.D. in serum.

Elders PJ, Habets LL, Netelenbos JC, Van der Linden LW (1992)[10] investigated to evaluate relationship between intra-oral examination and measured lumbar bone mineral density (lumbar BMD) and metacarpal cortical thickness (MCT) in 286 female volunteers between 46 and 55 years of age,the alveolar bone height was measured on bite wing radiographs of the dentate subjects, no significant correlation was observed between the clinical parameters of periodontitis . Result suggested that systemic bone mass is not an important factor in the pathogenesis of periodontitis.

Kunimatsu K. et al (1993)[11] study was conducted to determine the levels of OC , a bone specific matrix protein, in GCF from periodontal disease patients and to investigate the relationship between GCF OC levels and clinical parameters. Nineteen initial visit patients, 5 patients with gingivitis and 14 patients with adult periodontitis, participated in this study, OC adsorbed on a strip was extracted in a plastic tube containing 150µ 1 of 10 mm sodium phosphate buffer (ph 6.5). Results strongly suggest the presence of GCF OC and may reflect the degree of the periodontal inflammation at the sampled sites.

Nakashima K, RoehrichN, Cimasoni G (1994)[12] study was conducted to examine if OC was present in GCF and to assess the relationships of OC, PGE_2, and ALP in GCF to periodontal conditions. GCF samples were collected with durapore strips from 34 healthy, 12 gingivitis and 118 periodontitis sites in 17 subjects. ALP was measured spectrophotometrically by using p-nitrophenyl phosphate as substrate. Results showed OC amounts in GCF, as well as PGE_2, and ALP levels, can be considered as potential markers of periodontitis, in order to better define the capacity of such mediators for diagnosis, additional longitudinal studies are needed.

McCulloch CA et al (1994)[13] studied on enzymes in GCF may lead to insights into pathogenesis and may provide a rational basis for the development of novel diagnostic tests. The enzyme of interest should be readily measured over a broad range of disease severity and in varied clinical settings. In the final `analysis, the utility of host enzymes as diagnostic indicators will need to be examined in randomized controlled trials .

Mohammad AR , Brunsvold M, Bauer R (1996)[14] examined the strength of association between systemic osteoporosis and periodontal status in postmenopausal non-Hispanic white women. Twenty subjects with low bone density and a spine bone density of 0.753 +/- 0.039 dual-energy x-ray absorptiometry units (g/cm2) and 22 subjects with high bone density and a spine bone density of 1.032 +/- 0.028 dual-energy x-ray absorptiometry units (g/cm2) were randomly selected from a cohort of 565 women. This study suggests that systemic osteoporosis may contribute to periodontal attachment loss in the form of gingival recession.

Nakashima K, Giannopoulou C, Andersen E, Roehrich N, Brochut P, Dubrez B, Cimasoni G (1996)[15] investigated to examine the relationship of

possible crevicular biochemical parameters to ALOSS, 330 sites from 8 untreated adult patients were monitored longitudinally at 3-month intervals, for up to 1 year. Attachment levels were measured with a force-sensing probe and an acrylic stent in duplicates at each study point. The result suggested that the combination of several biochemical parameters in crevicular fluid could give more information to predict future clinical ALOSS.

Morishta M et al (1999)[16] investigated the effects of estradiol on mineralized nodule formation by human PDLcs. The formation of mineralized nodules was assessed by staining the PDLcs with alizarin red and counting the number of mineralized nodule. Estradiol 20 ng/ml significantly enhanced the ALP activity and the mineralized nodule formation, compared to the control. Results suggested that estrogen status may modify the regenerative activity of periodontal tissue.

Marjorie K Jeffcoat, Cora Elizabeth Lewis, Michael S.Reddy, Redford CY M (2000)[17] investigated about bone loss in women occurs most rapidly in the years immediately following menopause when natural levels of estrogen are greatly reduced. Hormone replacement therapy is designed to replace estrogen after menopause since this immediate post-menopausal period is a time of rapid loss of bone mineral density Relationship between osteoporosis and oral bone loss has long been postulated, the existing studies have been preliminary in nature.

Kang-Moon Kim et al (2002)[18] study was done on 43 postmenopausal patients with no systemic disease, were grouped in to 3 groups by their periodontal conditions; 12 mild periodontitis, 11 moderate periodontitis, 20 advanced periodontitis. Blood ALP and OC were measured. Results revealed that the blood ALP and OC levels were similar among the groups with different periodontal condition.

Bullon P, Chandler L , Egea JJS, Cano R P (2004)[19] investigated to assess plasma, saliva and GCF levels of OC and correlate them with periodontal treatment outcome in postmenopausal women. Thirty-nine postmenopausal women (57.8 ±8.5 years old) were recruited for the study. Mean PD and mean CAL decreased significantly at second appointment in the group with serum OC concentration. Mean PD decreased significantly at second appointment in the groups with saliva OC concentration .

Bullon P et al (2005)[20] conducted a study to assess serum, saliva and GCF levels of OC and correlate there periodontal status and bone mineral density in postmenopausal women. 73 postmenopausal women (above 35 years were recruited for the study. Mean PD correlated significantly with GCF OC concentration. Concept of OC correlates with periodontal but not osteoporosis.

Daltaban O et al (2006)[21] study was conducted on 36 postmenopausal women on ES and 37 ED postmenopausal women , were divided in to two sub groups - chronic periodontitis and clinically healthy controls after clinical and radiographic examination . Results revealed that, periodontitis groups demonstrated significant increase in GCF ALP levels compared to control groups. GCF total ALP levels of ED periodontitis group were significantly higher than ES periodontitis group. These data suggested that the presence of ALP in GCF is not simply a reflection of local inflammation state but the estrogen status of the patient may possibly influence local ALP levels in GCF.

Totah A et al (2006)[22] investigated salivary a AST and ALP in patients presenting with probing depth ≥ 5mm, bleeding on probing and alveolar bone loss ≥ 40%. Results showed significant increase in salivary AST and ALP activity in

patients with periodontal disease compared to control. Study concluded that the salivary AST could be a useful marker for monitoring periodontal disease, increase in salivary ALP activity in periodontitis could be associated with alveolar bone loss. Salivary analysis for biochemical markers of periodontal disease can offer a cost effective approach for monitoring the disease.

Totan A, Greabu M, Totan C, Spinu T (2006)[23] study was done to investigate components of saliva proposed as disease markers, subjects, presented a probing depth >5 mm, bleeding on probing and alveolar bone loss >40%. Salivary AST, ALT and ALP activities were measured .Salivary AST activity in patients with periodontal disease was significantly increased ($p<0.01$) (median 81.75+/-23 U/L) compared with controls (15.25+/-10.5 U/L). Our results showed a significant ($p<0.01$) increase in salivary ALP activity (34.38+/-1.5 U/L) in patients with periodontal disease compared with controls (6.6+/-4.2 U/L).

Cao et al (2007)[24] evaluated the effects of estrogen deficiency on alveolar bone in ovariectomized rats by histometric measurement of attachment level in vivo. Using RT-PCR and Western-blot procedure, mRNA and protein products of ERs are detected. The effects of estrogen on bone forming capability is estimated by monitoring ALP activity and osteocalcin production in cultured human PDLcs. Results demonstrated that both ER-alpha and beta were expressed in PDLcs. When exposed to 17-ß estradiol , PDLcs exhibited positive modulation on ALP activity and OC production. Study suggested that estrogen and ERs may play an important role in periodontal diseases .

Kinney JS, Ramseier CA, Giannobile WV (2007)[25] a study was conducted on oral fluids to find possible biological samples for objective measures of current

disease state, treatment monitoring, and prognostic indicators have boosted saliva and other oral-based fluids to the forefront of technology. Although most biomarkers in oral fluids represent inflammatory mediators, several specific collagen degradation and bone turnover-related molecules have emerged as possible measures of periodontal disease activity. Timely detection and diagnosis of disease may significantly affect the clinical management of periodontal patients by offering earlier, less invasive, and more cost-effective treatment therapies.

Bullon P , Chandler L , Egea JJS , Cano RP, Sahuquillo AM (2007)[26] investigated a study aiming to assess plasma, saliva and GCF levels of OC and correlate them with periodontal treatment outcome in postmenopausal women. Thirty-nine postmenopausal women (57.8 ±8.5 years old) were recruited all women was carried out and plaque, bleeding on probing, PD, and CAL were recorded. Serum, saliva and GCF OC were measured. Six months after the first appointment a second periodontal examination was carried out. Results showed mean PD and mean CAL decreased significantly at second appointment in the group with serum OC concentration < 10 ng/ml (15.8 ±15.8% and 15.3 ± 21.2% respectively; $p < 0.05$). Mean PD decreased significantly at second appointment in the groups with salivary OC concentration < 3 ng/ml (17.1 ± 15.9%; $p < 0.05$) and 3 – 7 ng/ml (16.2 ± 18.1%; $p < 0.05$).

Desai S, Shinde H, Mudda J , Patil V (2008)[27] investigated ALP levels in saliva of patients with chronic periodontitis. Forty patients were assigned to each one of three groups C0, C3 and C4, based on their largest CPITN code, totaling 120 participants. Unstimulated saliva was collected and analyzed with autoanalyzer (RA-XT Tecnicon® Auto Analyzer, USA) .Results showed significant differences between

levels of ALP from groups C0, C3 and C4 and a significant positive correlation between clinical parameters and ALP concentration in saliva in each group.

Kalburgi V, Jenifer HD ,Wara SD, Bhola S, Chaudhari HL (2010)[28] investigated a study to evaluate the salivary ALP as a marker of periodontal disease activity in diabetics and non-diabetics before and after scaling and root planing therapy .16 systemically healthy and 16 type–II diabetic mellitus patients with chronic periodontitis were enrolled in this study. Measurements of clinical parameters including GI, PD, CAL and collection of unstimulated whole saliva were performed at baseline and 4 weeks after scaling. Salivary ALP levels were analyzed results showed significant difference in the mean GI score at baseline between the two groups and also significant correlation between the clinical parameters and salivary ALP levels after scaling and root planning therapy among diabetic patients.

Zia A, Khan S, Bey A, Gupta ND, Mukktar-Un-Nisar (2011)[29] reviewed the treatment and prevention of periodontal disease will be founded on diagnostic tests based on aetiopathogenic factors rather than just clinical experience. Clinical measurements used in diagnosis of periodontal diseases are often of limited usefulness in that case biochemical mediators in oral fluids like saliva and GCF are highly beneficial in the determination of current periodontal status. This review highlighted recent advances in the use of salivary and GCF biomarker-based disease diagnostics that focus on the identification of active periodontal disease.

Reddy S , Kau S, Prasad M.G.S, Agnihotri J, Asutkar H, Bhowmik N (2011)[30] conducted a review on the ability to monitor health status, disease onset and progression, and treatment outcome through non-invasive means is a most desirable goal in health-care promotion and delivery. There are certain ground rules for this

goal to be realized: specific biomarkers associated with a health or disease state, a non-invasive approach to detect and monitor the biomarkers, and the technologies to discriminate between and among the biomarkers. We in this present literature have tried to assess a pathway to achieve these goals using oral fluids as the diagnostic medium to analyse the health and/or disease status of individuals. As the "mirror of body", oral fluid is a perfect medium to explore regarding health and disease regulation.

Dabra S , Singh P (2012)[31] study was done to determine the salivary level ALP and ACP activities in patients with periodontal disease and to evaluate the use of these enzymes as biochemical markers for periodontal tissue damage , experimental groups consisted of 20 gingivitis , periodontitis and the control group had healthy subjects . The stimulated saliva of the patient was collected in a sterile test tube and analyzed using Hitachi's Diagnostic Automatic Analyser. Results that were obtained showed statistically significant increased activities of ALP and ACP in saliva from patients with periodontal disease in relation to control group. A significant reduction in the enzyme levels was seen after conventional periodontal therapy.

Kunjappu JJ , Mathew V B, Hegde S, Kashyap R, HosadurgaR (2012)[32] investigated a study for the assessment of ALP in GCF a biomarker in chronic periodontitis a longitudinal study was done GCF samples were collected and all the clinical parameters were checked. Patient recalled after 7,30,60 days. Results showed a sustained statically significant decrease GCF ALP values. Positive correlation with probing depth not with plaque index.

Jain BK, Patne SS, Bindra M (2013)[33] this study was conducted to evaluate serum OC as marker for osteoporosis in 45 newly diagnosed postmenopausal

osteoporotic women and in 56 age matched control group, who are not suffering from osteoporosis. the observed significantly higher levels of serum OC in study population 21.4 +3.8 ng/ml when compared to control group 18.9 + 4.6 ng/ml ($P<0.01$).Concluded by observing OC as best marker for bone turnover in osteoporosis, hence also can be used as marker for diagnostic and prognosis marker in PMO.

Kapur S , Singh RPM , Verma R , Khehra L S, Kapur G (2013)[34] a study was done aiming to assess total activity of ALP in the GCF collected from healthy sites, sites with gingivitis and chronic adult periodontitis. A total of 18 patients were equally divided into three groups viz. healthy sites (I), gingivitis (II), chronic periodontitis(III). The ALP level in GCF of all three groups was determined by spectrophotometric analysis. Result stated enzyme activity of ALP was significantly higher in periodontitis as compared to healthy and gingivitis sites.

Ramesh A , Bhandary R , Thomas B , D' Souza SR, Kumari S (2013)[35] investigated deficiency of estrogen in women at menopause is contributing factor to osteoporosis and considered one of the risk factors for periodontal disease. The study included 40 subjects, 20 in each group in the age group of 50-60 years. Group 1 comprised of 20 Postmenopausal women without chronic periodontitis. Group 2 comprised of 20 Postmenopausal women with chronic periodontitis. Each saliva sample was estimated for ALP levels. Result showed significant increase in ALP in postmenopausal women with periodontitis (Group 2) with p value.

Ngo LH, Darb IB, Veith, PD, Locke A G, Reynolds EC (2013)[36] study was done to observe mass spectrometric analysis of GCF may allow for the site-specific prediction of periodontal disease progression. Forty-one periodontal maintenance

subjects were followed over 12months, with clinical measurements taken at baseline and every 3 months thereafter. GCF was collected from subjects at each visit and analysed using MALDI-TOF mass spectrometry. A genetic algorithm was used to create a model based on pattern analysis to predict sites undergoing attachment loss. The clinical indices of PD, MGI, plaque levels and bleeding served as poor discriminators of GCF mass spectra. Models generated from the GCF mass spectra could predict attachment loss at a site with a high specificity (97% recognition capability and 67% cross validation).

Kumar A, Devi SG, Sharma S (2013)[37] conducted a study on to assess bone turnover using bone marker in pre and post menopausal women comparing serum bone ALP and serum N-terminal telopeptide.A total of 160 pre- and 95 post menopausal women .Results showed negative correlation of serum alkaline phosphatise and serum N-terminal telopeptide these results suggested that bone resorption in north Indian women with low BMD remains high after menopause.

Kurş unlu SF ,Mehmet VO, Baş Z (2013)[38] investigated a study gingival crevicular fluid (GCF) osteocalcin levels in chronic periodontitits (CP) and periodontal healthy in elderly subjects, 10 patients with CP alone and 10 healthy elderly patients were enrolled. Probing depth, clinical attachment level, plaque index, and papillary bleeding index were recorded. GCF osteocalcin levels were analyzed by enzyme-linked immunosorbent assay. Parametric tests were used for statistical analysis Result showed CP had higher GCF osteocalcin levels compared to healthy groups ($P < 0.05$). Fluctuating GCF levels of osteocalcin might point out to the abnormal bone turnover in periodontitis.

Beg M et al (2014)[39] Study was conducted aimed to evaluate significance of serum osteocalcin and prevelance of hypovitaminosis D in postmenopausal females to determine the effect on serum osteocalcin in postmemopausal females and concluded with a promising result that serum osteocalcin is a prominent marker for bone turnover.

Ali MR , Zaidan TF , Gorial FI (2014)[40] study conducted to assess validity of OC and ALP biomarkers in postmenopausal . Resulted in OC and ALP were valid biomarkers to diagnose postmenopausal women with low BMD. Biomarker score>1 had high accuracy and sensitivity to diagnose low BMD. This may suggest a new promising measure to early diagnose patients at high risk of low BMD and subsequently giving early appropriate treatment.

Shenoy BS. Shenoy P ,Talwar A, Thomas B, Sharath K S, ShettyKS (2014)[41] a study was done to compare the levels of LDH and ALP in post-menopausal women with and without periodontitis A cross-sectional pilot study with 50 postmenopausal women were recruited and categorized into two groups based on their periodontal status. LDH and ALP estimation in the laboratory .The activity of LDH and ALP were significantly higher in the post-menopausal women with periodontitis than those without periodontitis.

Khongkhunthian S. et al (2014)[42] study conducted to compare two biochemical markers, which have been previously used to determine the degrees of alveolar bone destruction, in evaluating periodontal disease severity. The WF6 epitope of CS and ALP levels were determined GCF samples collected from patients with various degrees of disease severity, including patients with gingivitis (50 gingivitis sites) and 33 patients with chronic periodontitis (including gingivitis, slight,

moderate and severe periodontitis sites; n = 50 each), as well as from ten healthy volunteers (50 healthy sites) by Periopaper strips. The levels of CS and ALP were measured by an ELISA and a fluorometric assay, respectively. The results demonstrated low levels of CS and ALP in non-destructive and slightly destructive periodontitis sites, whereas significantly high levels of these two biomolecules were shown in moderately and severely destructive sites ($p < 0.05$). Although a significant difference in CS levels was found between moderate and severe periodontitis sites, no difference in ALP levels was found.

Probal S, et al (2015)[43] study was done to determine the enzyme and levels tested in blood samples. And concluded with this that patients with chronic periodontitis have significantly elevated ALP and can considered as a biomarker for periodontal disease.

Ram VS, Parthiban, Sudhakar U, Mithradas N, PrabhakarR (2015)[44] conducted a study in which the increasing prevelance of periodontal disease was studied which paved way to the development of new diagnostic tests that could detect the presence of active disease ,the course of the disease and its response to treatment. Bone is a metabolically active tissue and undergo continuous remodelling, a process that largely relies on the activity of osteoclasts to remove bone and of osteoblasts to form bone. This review highlighted, the recent advances in the use of biomarkers of bone remodeling ,that could facilitate the screening, diagnosis and management of periodontal diseases.

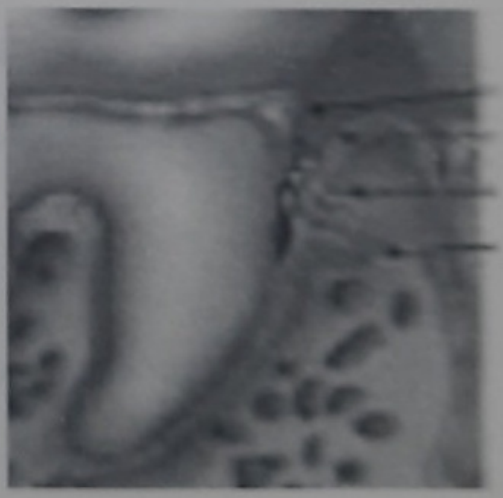
Singh S, Kumar D, Lal AK (2015)[45] investigated to detect serum OC as a diagnostic biomarker for primary osteoporosis in Women a case-control study, 82 post-menopausal females, between 40-70 years, were subjected to measurements of

bone mineral density and serum osteocalcin levels. Based on the results of DEXA scan they were divided into two. Significant association of age and years since menopause (YSM) was found with serum OC levels and BMD. Statistically significant difference between values of serum osteocalcin in postmenopausal non-osteoporotic women, post-menopausal women with low bone mass (osteopenia) and post-menopausal women with osteoporosis were seen.

Kurdukar PA, Kurdukar AA, Mahale SA, Beldar AM (2015)[46] a review of study was done in which conventional clinical and radiographical methods of periodontal diagnosis are only capable of retrospective diagnosis of attachment and bone loss. These are unable to either detect or predict PDA. For these reasons a large proportion of recent periodontal research has been concerned with finding and testing potential markers of PDA. This article reviews the various biomarkers in GCF used for assessing periodontal disease activity.

Thomas SS, Badade ZG, Sheikh AZ (2015)[47] conducted a study comparing the levels of biochemical markers such as bone formation and bone resorption in pre and post menopause women. The study comprised of 50 pre-menopause women aged between 40-40 years were selected as control (group 1) with regular menses and no complications related to menstruation, whereas, 50 post-menopause women aged between 41-60 years were selected as (group2). Result showed a significant decrease in Serum Total Calcium and Phosphorous in postmenopausal women ($p \leq 0.001$). Similarly it was observed that bone formation markers i.e. Serum ALP, Serum OC levels and bone resorption markers ACP and Urinary excretion of Hydroxyproline were significantly increased in postmenopausal women compared to premenopausal women ($p \leq 0.001$) and there was strong correlation between ALP and urinary hydroxyproline ($p \leq 0.001$).

Hassan AO, Hussein SM, Sahib WW(2016)[48] investigated salivary biomarkers so that difference between health and disease can be detected this is study conducted on a total 80 subjects; 29 (12 female &17 male) gingivitis patients, 27 (11 female & 16 male) chronic periodontitis patients and 24 (15 female &9 male) as a control subjects. Unstimulated whole saliva samples were collected to determine the levels of PGE2, MMP-8 and ALP. Clinical periodontal parameters were recorded at four sites per tooth. The study founded the prevalence of gingivitis and chronic periodontitis higher in males than females.



Materials & Methods

A comparative controlled clinical and in-vivo study for the correlation and evaluation of the levels of ALP and osteocalcin in gingival crevicular fluid (GCF) and serum in pre menopausal and post menopausal women with chronic periodontitis, study was conducted in the Department of Periodontics, Babu Banarasi Das College of Dental Sciences, Lucknow, Uttar Pradesh in collaboration with at Charak Diagnostic centre near KGMC, Lucknow.

A total of 30 volunteers irrespective of cast, creed visiting the O.P.D of the college were selected. They were divided into two groups, Group A - 15 subjects pre menopausal women suffering from chronic periodontitis age 30-40yrs, Group B -15 subjects post menopausal women suffering from chronic periodontitis age 45-55 yrs. The current study was executed after receiving an appropriate clearance from the Institutional Ethics Committee. A duly signed written consent was obtained from all the volunteers after explaining the study protocol and procedure.

SUBJECT SELECTION

At the time of screening medical and dental history was obtained. The selection of volunteers was based upon the following inclusion and exclusion criteria:

❖ INCLUSION CRITERIA:

1. Subjects within 5 years of menopause and not on hormonal replacement therapy.
2. Subjects who have not attained menupause and not on any OCP or hormonal therapy.

3. Subjects with chronic periodontitis with >=4mm of clinical attachment loss and >=30% of alveolar bone loss, involving at least 4 teeth in each arch.
4. Subjects with good systemic health and not received any periodontal therapy in the past 6 months.

❖ **EXCLUSION CRITERIA:**

1. Smokers, tobacco and/ or pan masala chewers, alcoholics, drug addicts.
2. Pregnant and lactating women and women in their menstrual phase.
3. Persons having systemic diseases or conditions that influence the progression and/or clinical characteristics of periodontal disease.
4. Mental capacity limited to the extent that the subject could not provide written informed consent.
5. Persons having taken antibiotics , anti-inflammatory drugs, steroids or hormonal therapy within the preceding 3 months or currently taking medications.
6. Periodontal treatment within last 6 months.

The study protocol was explained to all the volunteers and those patients were enrolled and grouped into the following categories:

Group A: 15 Premenopausal women with chronic periodontitis, in the age group of 30-40 years.

Group B: 15 Premenopausal women with chronic periodontitis, in the age group of 45-55 year

PRESURGICAL PROCEDURES

Each individual was subjected to a full diagnostic workup including

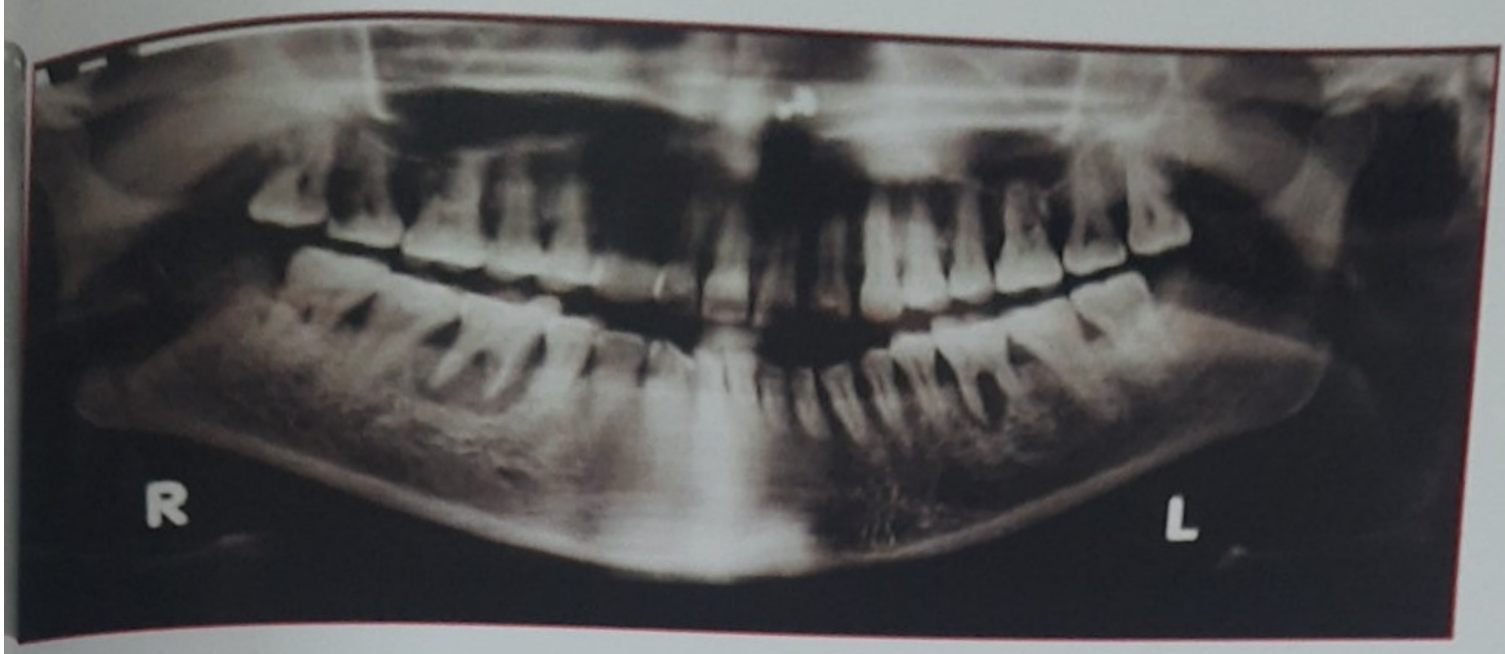
1. A detailed case history record **(*Appendix- I*)**
2. A duly informed written consent of each patient was taken for the purpose of the study ***(Appendix-II)***.
3. Orthopantomogram and Intra-oral periapical (IOPA) X-rays.

Following the diagnosis, Gingival Crevicular Fluid and Serum samples were collected from the volunteers to quantify the levels of alkaline phosphatase and osteocalcin. Before sample collection measurement of pocket depth at least at 6 sites ,which should be more than >= 4 . Then supragingival scaling was done just to remove plaque so that no hindrance during sample collection .**(PLATE I)**

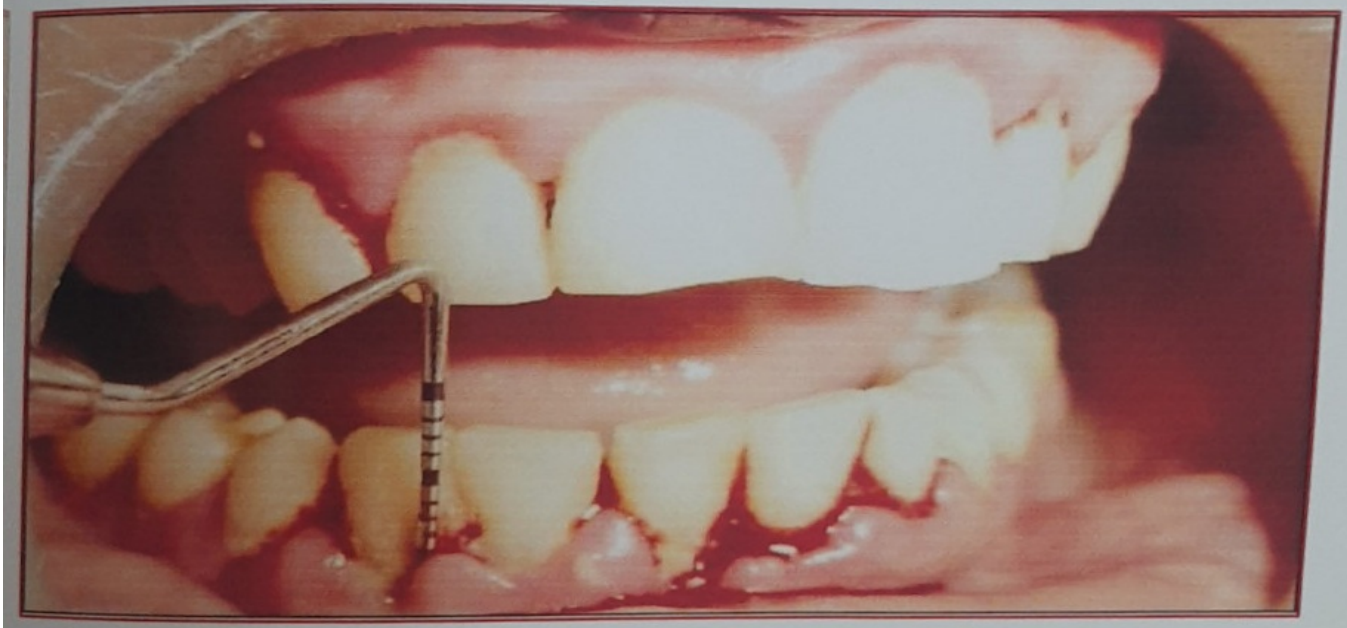
GINGIVAL CREVICULAR FLUID (GCF) COLLECTION

GCF samples were collected from 4 sites with deepest periodontal pockets from each volunteer. The supra - gingival plaque was removed after isolating the site with cotton rolls to prevent contamination with saliva. GCF sample was collected by gently inserting two absorbent paper point number 40 into the orifice of the periodontal pocket until a slight resistance was felt and then left there for 30 seconds and collected in Eppendorf tubes with 250 µ l of RPMI-1640 transport media.

Following collection the samples were kept in minicooler, maintaining a temperature of -20^{0}C and immediately transported to Charak Diagnostic Centre near KGMC Lucknow . **(PLATE II, III ,IV)**

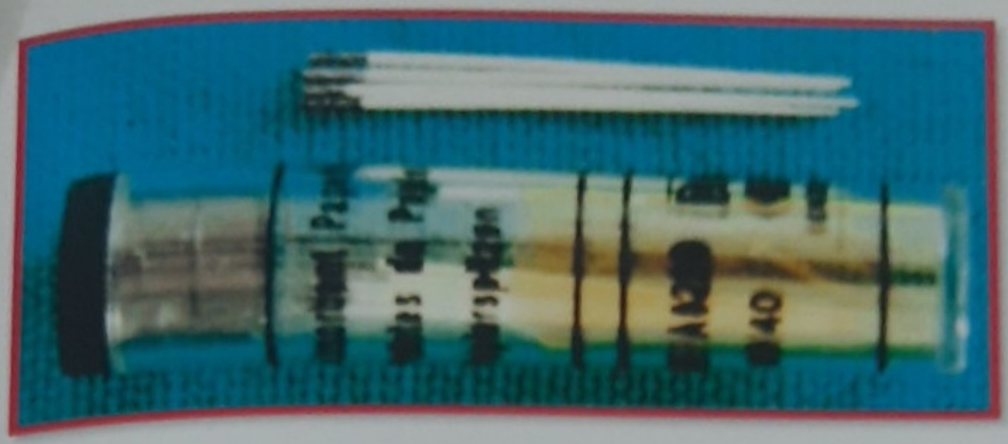


REPRESENTATIVE PHOTOGRAPH OF A CHRONIC PERIODONTITIS PATIENT

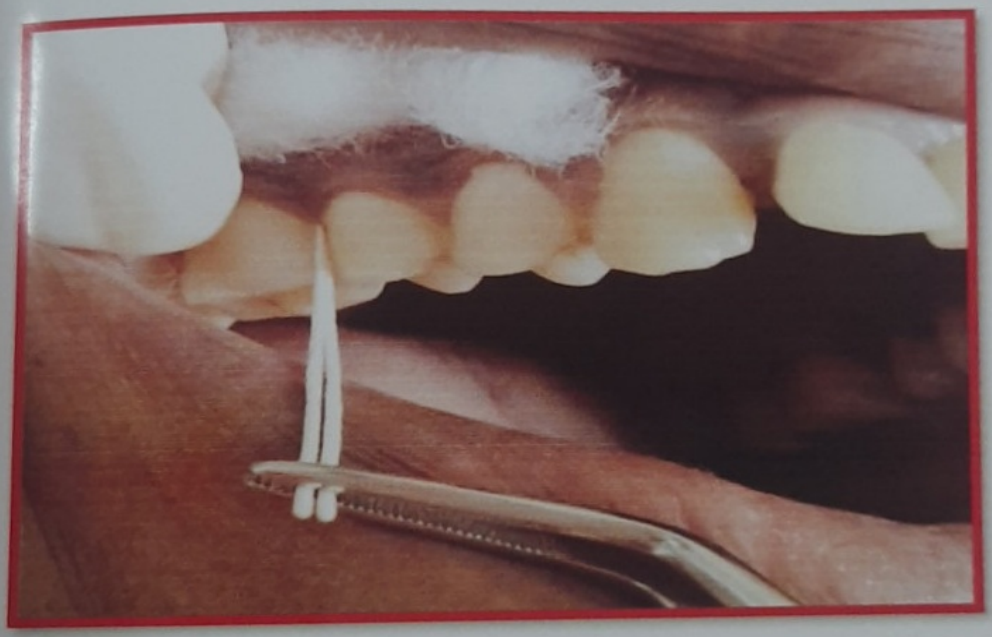


PRE OPERATIVE Probing Depth Measurement

PLATE NO. I

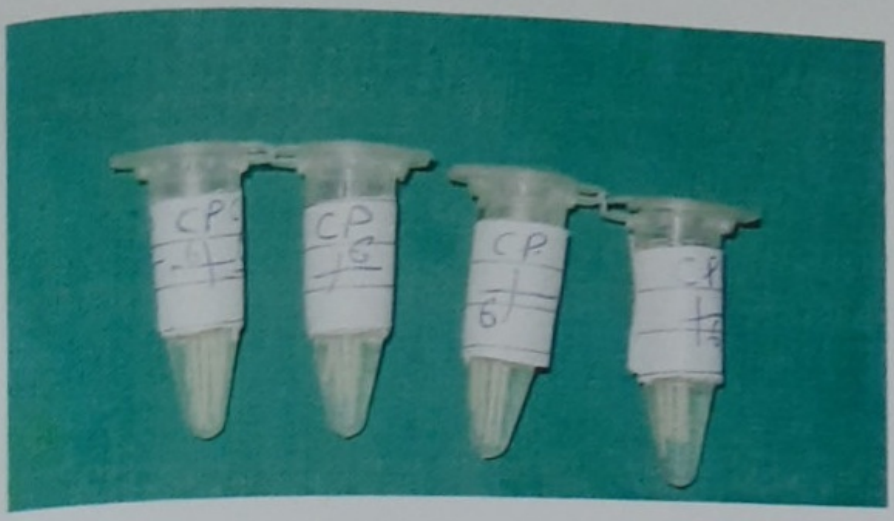


ABSORBENT PAPER POINT NO. 40
(For GCF collection)

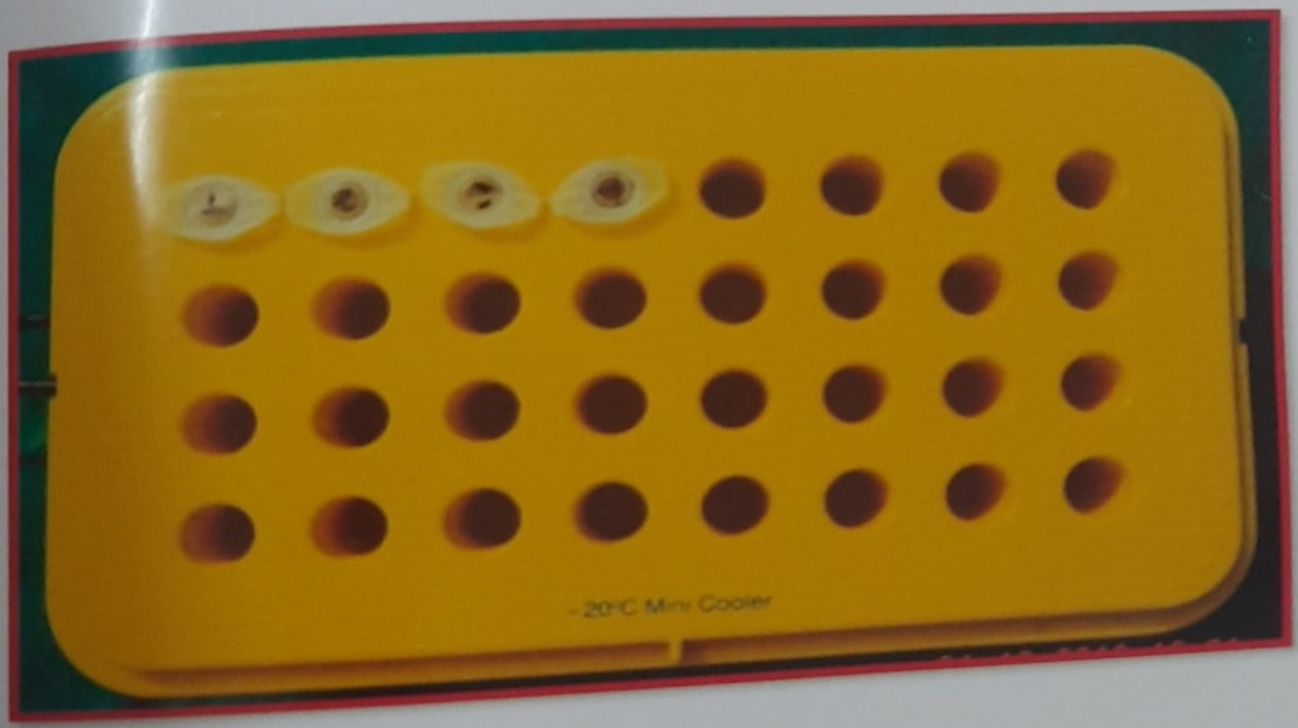


GCF COLLECTION

PLATE NO. II



PAPER POINTS AFTER GCF COLLECTION
(kept in transport media)



MINI COOLAR
Transport of GCF samples

PLATE NO. III

AUTOANALYSER
(Evaluation of GCF samples)

PLATE NO. IV

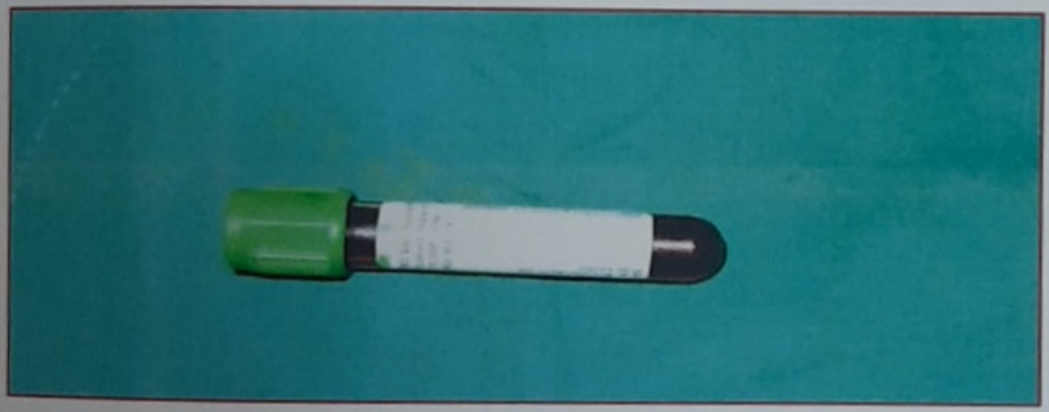
SERUM COLLECTION

- 10 ml intravenous blood was collected from the anticubital vein of each using the tourniquet and 24 gauge syringe.
- Following collection, the samples were kept on ice, maintaining the temperature 0-4^0 C and immediately transported to Charak Diagnostic Centre near KGMC, Lucknow, where they were stored at - 4°C and **(PLATE V,VI,VII)**
- The collected 10 ml blood sample was divided as per the following:

Separation of Serum

1. A 10 ml tube of whole blood will be collected following standard procedures using a 10 ml blood in BD Vacutainer rapid serum tube (RST). (Sterilized BD Vacutainer RST tube, 4ml) by BD Franklin Lakes NJ, USA, from each patient.
2. Allow samples to clot for one hour at room temperature
3. Centrifuge for 10 minutes at approximately 1000g
4. Using clean pipette technique Aliquot 210ul of serum into labeled cryovials.
5. Immediately freeze vials of serum at –4 degree freezer
6. Serum kept in two vials for detection of alkaline phosphatase and osteocalcin separately.

PERIPHERAL BLOOD COLLECTION



VACUTAINER WITH SODIUM HEPARIN
(For blood collection)

PLATE NO. V

BLOOD SAMPLES KEPT IN ICE
(for transportation)

PLATE NO. V I

ESTIMATION OF ALP IN SERUM

Serum which was extracted detected by Alkaline Phosphatase Assay Kit (Colorimetric)(ab83369) was used.

REQUIRED REAGENTS/SUPPLIES/EQUIPMENT

1. Spectrophotometer
2. Spectrophotometer cuvettes
3. Distilled deionized water
4. Physiological (0.9% NaCl) saline
5. Pipettes
6. Alkaline phosphatase reagent obtained from Pointe Scientific, Inc. Reconstitute reagent with 15 mL distilled water. Swirl gently to dissolve. When reconstituted as described, the reagent contains p-Nitrophenylphosphate 10.0 mM, Magnesium ions 1.0 mM, Buffer (pH 10) , activator and binder. The unreconstituted reagent is stored at 2-8°C. Once reconstituted, the reagent is stable for 48 hours at 25°C and for 30 days at 2-8°C.
7. Paraffin squares
8. Heating block or water bath 37°C
9. Timer

PROCEDURE

1. Turn on the spectrophotometer and let warm up for at least 15 minutes.
2. Set the wavelength to 405 nm.
3. Label cuvettes.
4. Add 1.0 mL of distilled deionized water to cuvette 1.
5. Add 1.0 mL of Alkaline phosphatase reagent to each control and patient cuvettes.
6. Incubate all cuvettes at 37°C for 5 minutes.
7. Add 25 uL of each control and patient sample to their respective cuvettes.
8. Mix each by inversion using the paraffin squares to prevent spillage.
9. Incubate control and patient cuvettes at 37°C for 1 minute.
10. After one minute, place cuvette 1 in the spectrophotometer and set the absorbance to read 0.000.
11. Read and record the absorbance for each of the patient cuvettes.
12. Return the patient cuvettes to 37°C and repeat readings for each cuvette every minute for the next two minutes.
13. Calculate the average absorbance difference per minute (ΔAbs/min).
14. Calculating the amount of enzyme using the following calculation:

$$\text{ALP (IU/L)} = \frac{\Delta\text{Abs/min} \times \text{TV} \times 1000}{18.75 \times \text{LP} \times \text{SV}}$$

ESTIMATION OF OSTEOCALCIN IN SERUM

Thermo Fisher Scientific kit was used The Human Osteocalcin ELISA is a solid phase enzyme-amplified sensitivity immunoassay (EASIA) performed on a microtiter plate.

1. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of human osteocalcin. Standards and samples react with the capture monoclonal antibody (MAb 1) coated on the microtiter well and with a monoclonal antibody (MAb 2) labeled with horseradish peroxidase (HRP).
2. After an incubation period allowing the formation of a sandwich (coated MAb 1 - human osteocalcin - MAb 2 – HRP), the microtiter plate is washed to remove unbound enzyme-labeled antibody.
3. Bound enzyme-labeled antibody is measured through a chromogenic reaction. Chromogenic solution (TMB, ready for use) is added and incubated.
4. The reaction is stopped with the addition of Stop Solution and the microtiter plate is then read at the appropriate wavelength 405-410nm.
5. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which is proportional to the human osteocalcin concentration.
6. A standard curve is plotted and human osteocalcin concentration in a sample is determined by interpolation from the standard curve.Values are expressed in ng\ml.

ESTIMATION OF OSTEOCALCIN IN GCF

Bender Med System, Vienna, Austria commercially available ELISA kit

PROCEDURE:

1. 96-well plates were coated with coating buffer (100µ L/well) and incubated overnight at 4^0C.

2. Following incubation, the wells were washed three times with wash buffer and the plates were dried by inverting them onto the absorbent paper. Blocking was done by 1X assay diluent and incubated for 1 hour at room temperature. Plates were re-washed as earlier.

3. After blocking, calcitonin standard (100µ L/well) or test samples (100µ L/well) was added to the wells and incubated at room temperature for 2 h. After incubation, the contents of the wells were aspirated, followed by

4. 3 washings as detailed earlier.

5. Detection antibody (100µ L/well) was then added and the plates were reincubated for 1 hour at room temperature. The contents were aspirated again and washing was done.

6. Detection enzyme pre-titrated avidin-HRP was added (100µ L/well) to each well and incubated for 30 minutes at room temperature. It was then aspirated and the wells were washed 6-7 times with wash buffer.

7. Finally, a substrate solution (100µ L/well) was added to each well and incubated for 15 min at room temperature.

8. Following the incubation, stop solution (50 µ L/well) was added to the each well and the plates were read at 570 and 450 nm wavelength and the OD values at 570 nm were subtracted from the OD values at 450 nm and the data

was analyzed with the help of standard curve plotted for this purpose to calculate quantity of calcitonin (pg/mL).

ESTIMATION OF ALP IN GCF

1. The GCF fluid so collected was diluted to 100µL with Sorensens media containinig 0.05% bovine serum albumin in phosphate-buffered saline pH 7.0 in a plastic cuvette.

2. The working reagent solution was prepared by dissolving 1 substrate tablet in 3.2mL buffer solution.

3. One mL of this working reagent solution was added to 20µL of the GCF sample solution in a plastic cuvette and the ALP activity was assayed with a spectrophotometer at 30^0C at 405nm.

4. The Alkaline Phosphatase kit used was of (DEA), (pNPP Kinetic method) , from Coral Clinical Systems, Volmolenheide, Belgium and the composition of it was : DEA Buffer 1M pH 10.3, magnesium chloride 0.5mM and pNPP 10mM.

5. ALP hydrolyses p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate. The rate of increase in absorbance at 405nm was monitored as the p-nitrophenol formed.

6. The absorbance was converted into enzyme activity units (1U=1mmol of p-nitrophenol released per minute at 30^0 C).

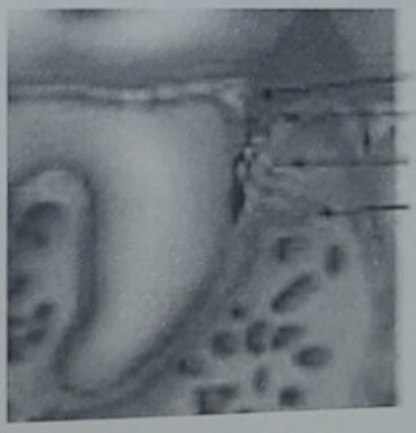
7. Readings were noted immediately after initiation of the reaction (A1), 1 minute later (A2), 2 minutes later (A3) and 3 minutes later (A4). The

summation of the changes over the 3 minutes period starting from A1 to A4 [(A2-A1) + (A3-A2) + (A4-A3)], was then calibrated and the change in absorbance was noted and designated as delta A.

8. The mean change in absorbance per minute was calculated (delta/min).

   Total alkaline phosphatase activity in U/L was calculated using the formula: Delta A/min x 2754.

9. According to the readings obtained in the spectrophotometer a master chart was prepared for the enzyme activity. The mean level of alkaline phosphatase activity was calculated and the standard deviation of the mean values of the enzyme activity was recorded.



Observations & Results

The present study included 30 subjects, 15 premenopausal women with chronic periodontitis 30-40 yrs and 15 postmenopausal women with chronic periodontitis 45-55yrs. The study protocol was explained to all subjects and they were grouped into following categories.

- **Group A: 15 Premenopausal women with chronic periodontitis 30-40 yrs.**
- **Group B: 15 Postmenopausal women with chronic periodontitis 45-55yrs.**

Changes in the level of alkaline phosphatase and osteocalcin in Gingival Crevicular Fluid (GCF) and serum were assessed in premenopausal and postmenopausal women with chronic periodontitis.

STATISTICAL ANALYSIS

The results were subjected to statistical analysis. Data were summarized as Mean±SD and percentage. The unpaired t- test was used to compare categorical variables. The variables between Group A and Group B were compared by paired test. The p-value less than 0.05 were considered as significant. All the analysis were carried using SPSS (16.0 version).

AGE

The premenopausal and postmenopausal age of both the groups are summarized in **Table 1** and also shown graphically in **Graph 1**.

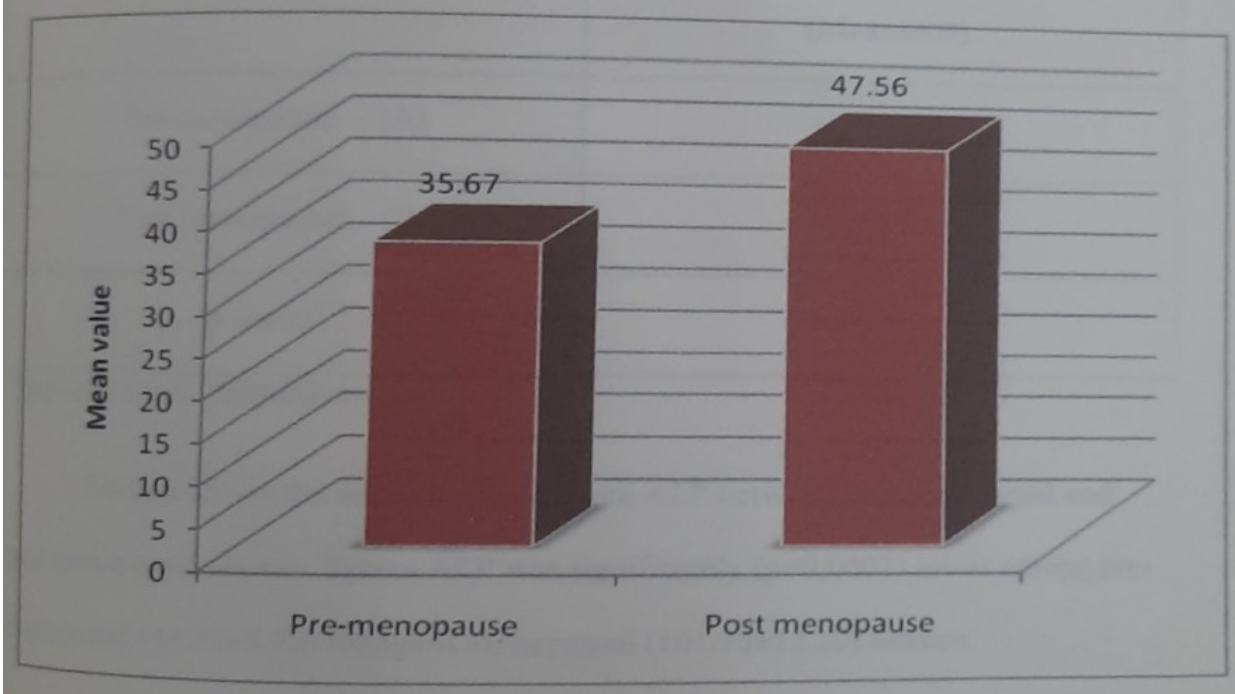
Table-1: Distribution of age between pre-menopausal and post-menopausal women

Groups	Age in years (Mean±SD)	Range
Pre-menopausal (A)	35.67±3.45	30-40
Post- menopausal(B)	47.56±7.67	45-55
p-value[1]	0.0001*	

[1]Unpaired t-test, *Significant

Table-1 shows the distribution of age between pre-menopausal and post menopausal women. The mean age of pre-menopausal women was 35.67±3.45 years and post menopausal was 47.56±7.67 years. There was significant (p=0.0001) difference in the age between pre-menopausal and post menopausal women.

Graph 1: Distribution of age between pre-menopausal and post-menopausal women



LEVELS OF SERUM ALP

Comparision of serum ALP between premenopausal and postmenopausal women of both the groups are summarized in **Table -2** and has shown graphically in **Graph -2**

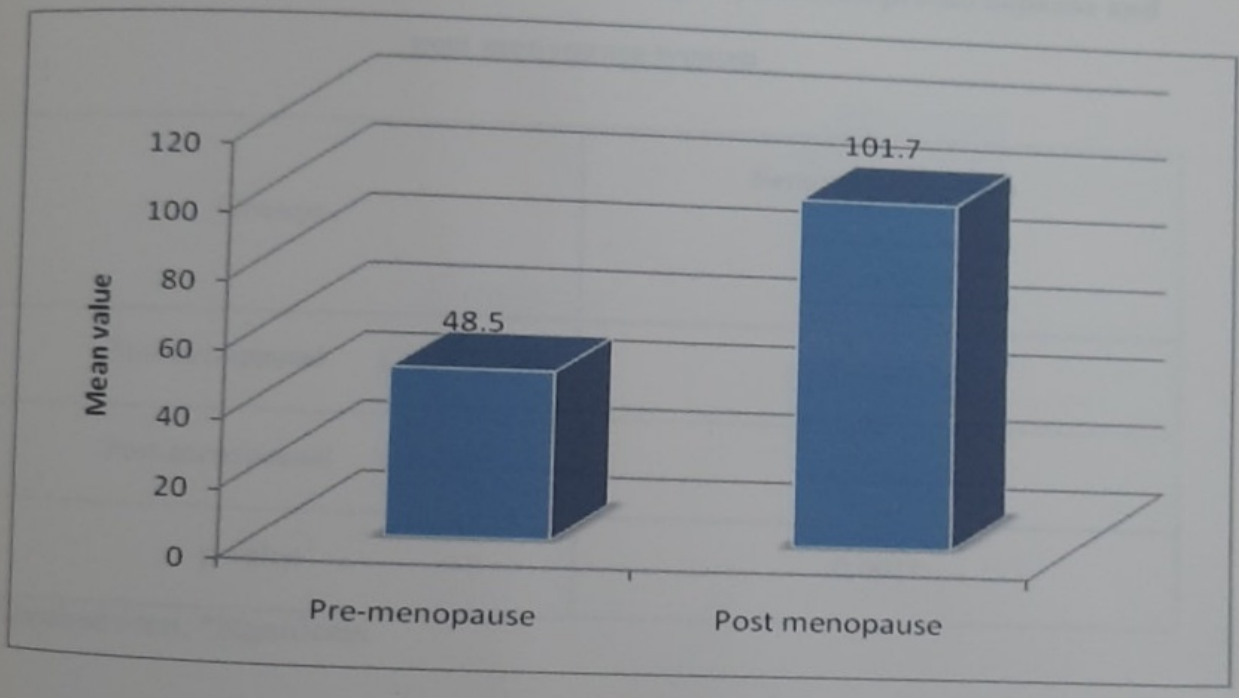
Table-2: Comparison of serum ALP(U/L) between pre-menopausal and post menopausal women

Groups	Serum ALP (U/L) (Mean±SD)
Pre-menopausal (A)	48.50±4.32
Post menopausal (B)	101.70±12.30
p-value[1]	0.0001*

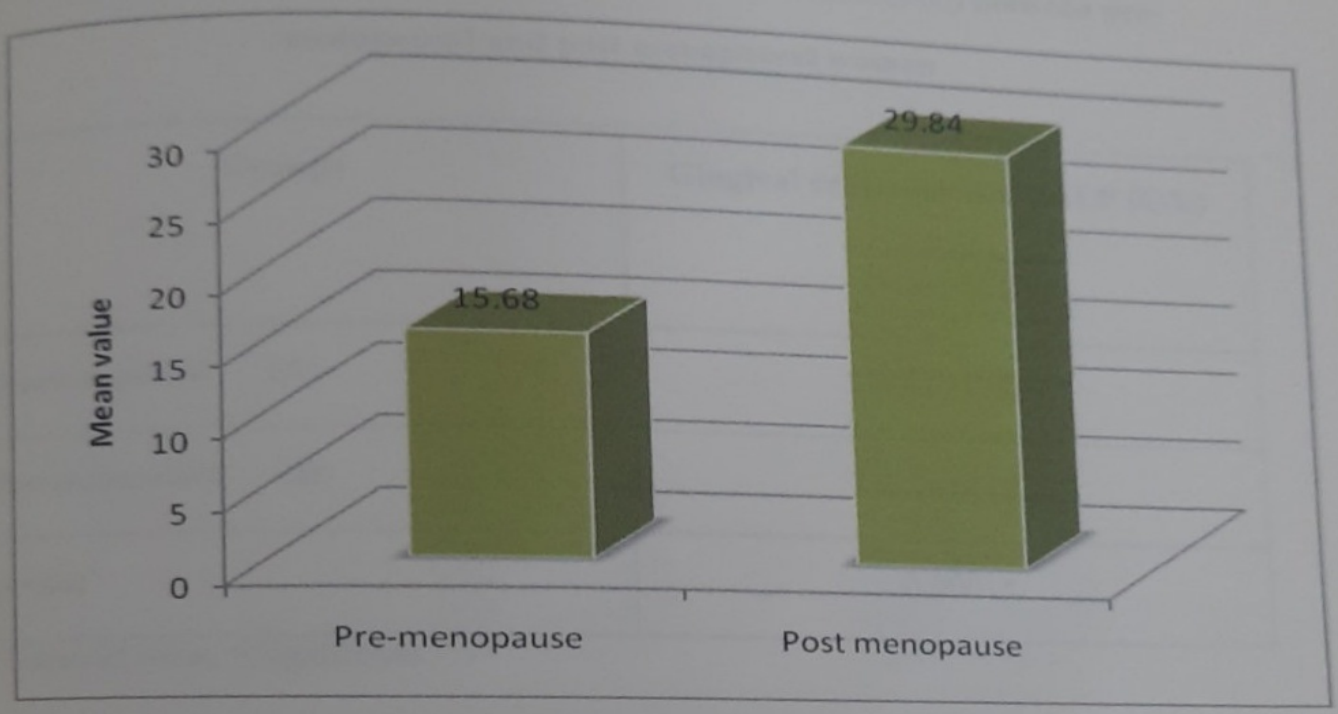
[1]Unpaired t-test, *Significant

Table-2 shows the comparison of serum ALP between pre-menopausal and post menopausal women. Serum ALP was significantly (p=0.0001) lower among pre-menopausal (48.50±4.32) than post menopausal (101.70±12.30) women.

Graph 2: Comparison of serum ALP between pre-menopausal and post menopausal women



Graph 3: Comparison of serum Osteocalcin between pre-menopausal and post menopausal women



Level of Gingival Crevicular Fluid ALP

Comparision of ALP level in Gingival crevicular fluid between premenopausal and postmenopausal women of both the groups are summarized in **Table-4** and has shown graphically in **Graph -4**

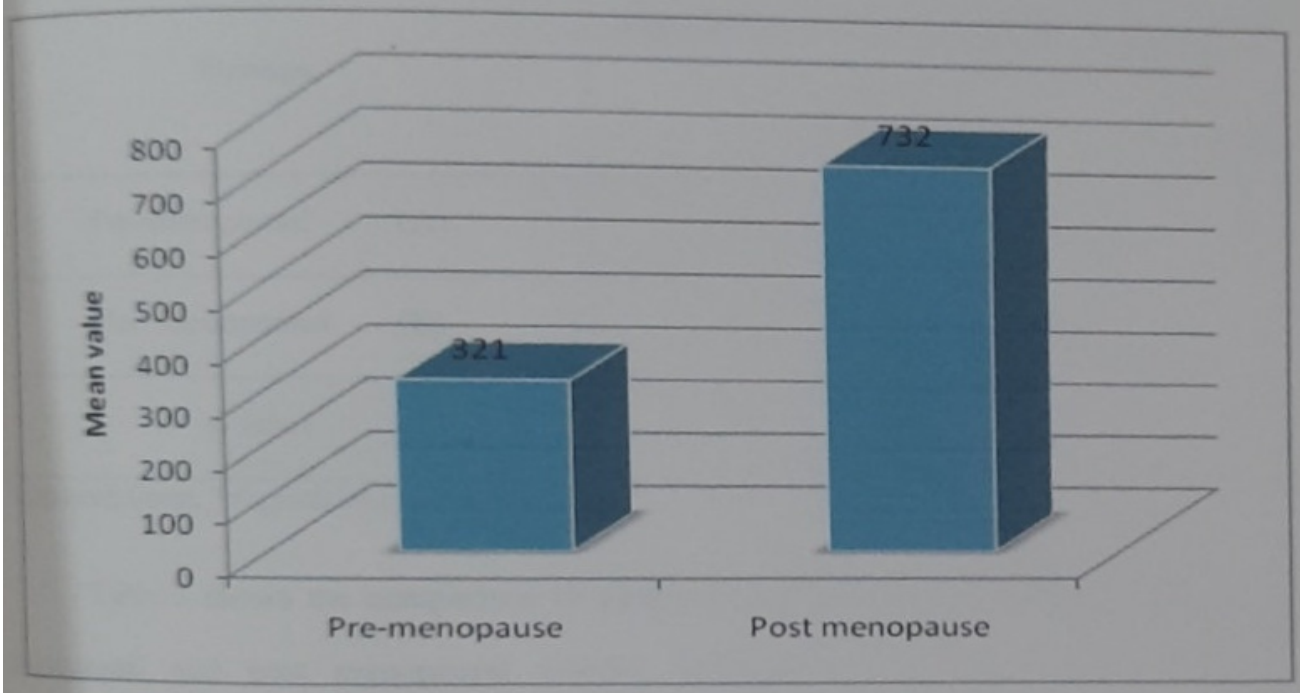
Table-4: Comparison of gingival crevicular fluid ALP(U/L) between pre-menopausal and post menopausal women

Groups	Gingival crevicular fluid ALP (U/L) (Mean±SD)
Pre-menopausal (A)	321.00±139.60
Post menopausal (B)	732.00±414.93
p-value[1]	0.0001*

[1]Unpaired t-test, *Significant

Table-4 shows the comparison of gingival crevicular fluid ALP between pre-menopause and post-menopausal women. Gingival crevicular fluid ALP was significantly (p=0.0001) lower among pre-menopausal (321.00±139.60) than post menopausal (732.00±414.93) women.

Graph 4: Comparison of gingival crevicular fluid ALP between pre-menopausal and post menopausal women



Level of Gingival Crevicular Fluid Osteocalcin

Comparision of Osteocalcin levels in Gingival crevicular fluid between the premenopausal and postmenopausal women of both the groups are summarized in Table-5 and has shown graphically in **Graph -5**

Table-5: Comparison of gingival crevicular fluid OC between pre-menopausal and post menopausal women

Groups	Gingival crevicular fluid OC (ng/ml) (Mean±SD)
Pre-menopausal (A)	5.20±3.20
Post menopausal (B)	18.30±9.80
p-value[1]	0.001*

[1]Unpaired t-test, *Significant

Table-5 shows the comparison of gingival crevicular fluid OC between pre-menopausal and post menopausal women. Gingival crevicular fluid OC was significantly (p=0.0001) higher among pre-menopausal (18.30±9.80) than post menopausal (5.20±3.20) women.

Graph 5: Comparison of gingival crevicular fluid Osteocalcin between pre-menopausal and post menopausal women

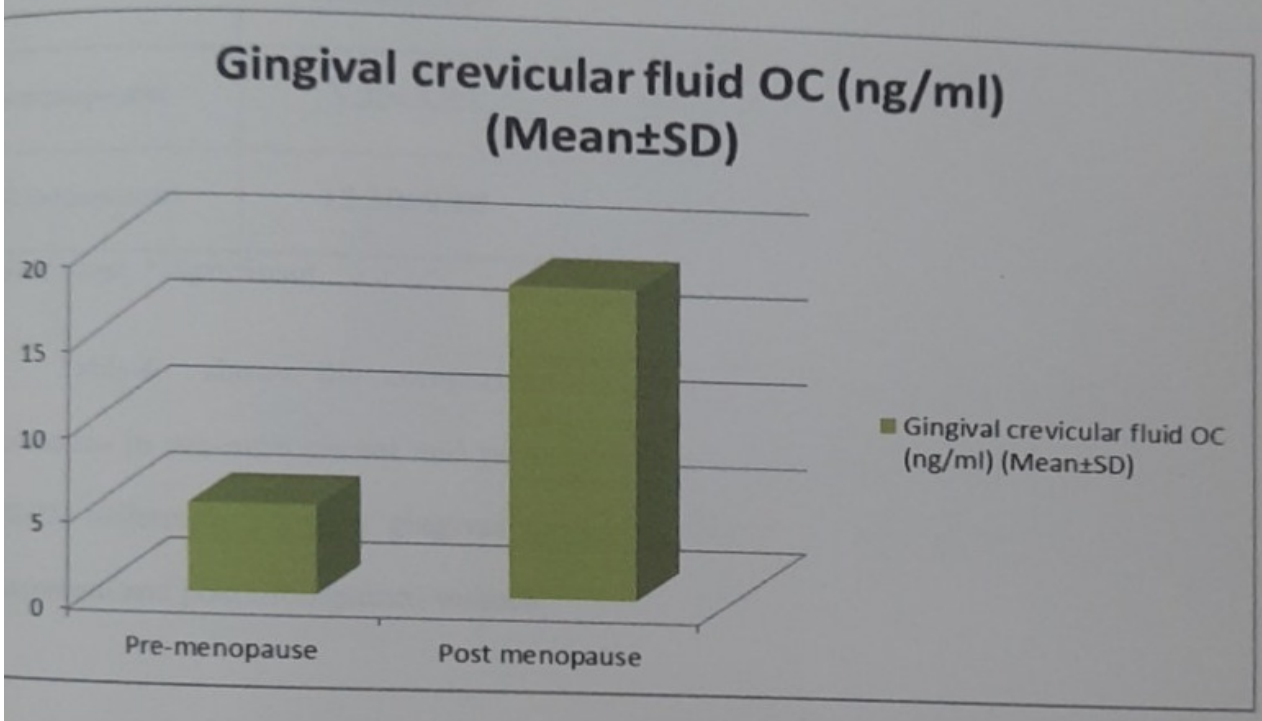


Table-6: **Comparison of gingival crevicular fluid and serum Osteocalcin in pre-menopausal and post menopausal women**

Groups	Gingival crevicular fluid OC (ng/ml) (Mean±SD)	Serum OC (ng/ml) (Mean±SD)	p-value[1]
Pre-menopausal	5.20±3.20	15.68±3.33	0.01*
Post menopausal	18.30±9.80	29.84±2.98	0.0001*

[1]Paired t-test, *Significant

Table-6 shows the comparison of gingival crevicular fluid and serum Osteocalcin in pre-menopausal and post menopausal women. There was significant ($p<0.05$) difference between gingival crevicular fluid OC and serum OC in pre-menopausal and post menopausal women.

Graph 6: Comparison of gingival crevicular fluid and serum Osteocalcin in pre-menopausal and post menopausal women

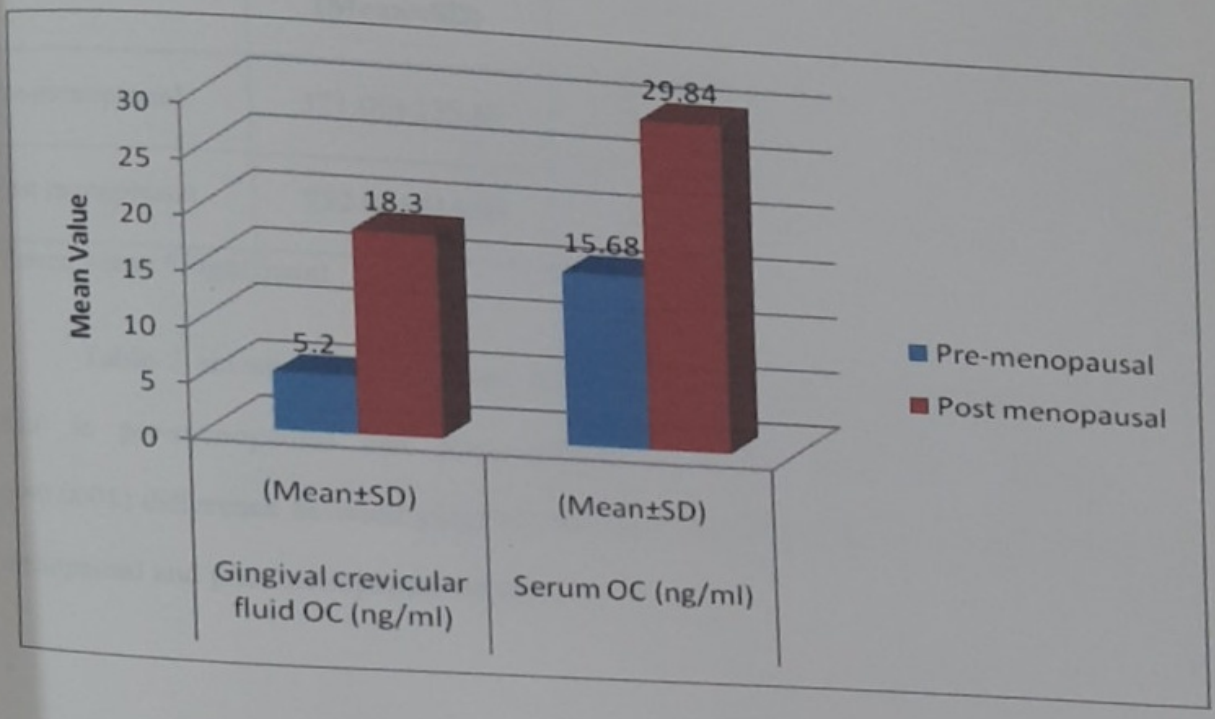


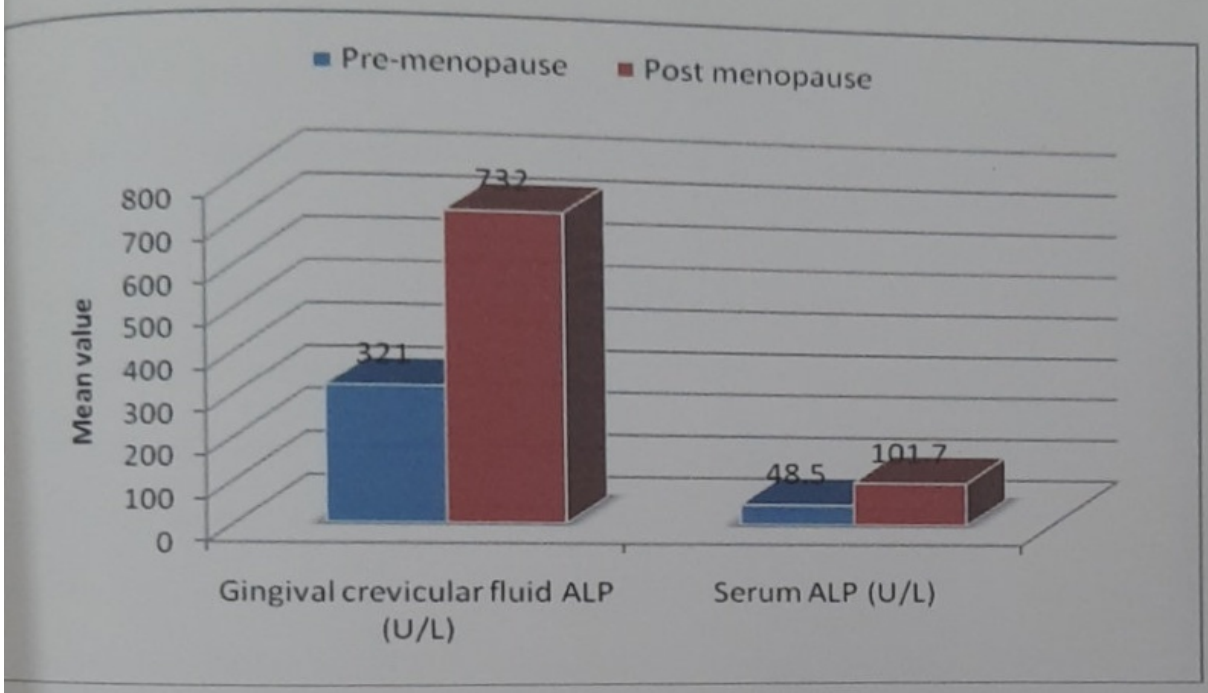
Table-7: **Comparison of gingival crevicular fluid ALP and serum ALP in pre-menopausal and post menopausal women**

Groups	Gingival crevicular fluid ALP (U/L) (Mean±SD)	Serum ALP (U/L) (Mean±SD)	p-value[1]
Pre-menopausal	321.00±139.60	48.50±4.32	0.0001*
Post menopausal	732.00±414.93	101.70±12.30	0.0001*

[1]Paired t-test, *Significant

Table-7 shows the comparison of gingival crevicular fluid ALP and serum ALP in pre-menopausal and post-menopausal women. There was significant (p=0.0001) difference between gingival crevicular fluid ALP and serum ALP in pre-menopausal and post-menopausal women.

Graph 7 : Comparison of gingival crevicular fluid ALP and serum ALP in premenopausal and post menopausal women



Discussion

Chronic periodontitis has been defined as "an infectious disease resulting in inflammation with the supporting tissues of the teeth, progressive attachment loss and bone loss." In periodontitis one of the mechanism of collagen loss is fibroblast phagocytize collagen fibres which contribute to the total Alkaline Phosphatase (ALP) level, its amount in the blood is calculated and represented as the total amount of Alkaline Phosphatase (ALP) released from the tissues. Among the host enzymes, the first one identified was Alkaline Phosphatase. Its the most effective in alkaline environment. The optimum pH for the activity is 8-8.5 depending on the source.

Gingival Crevicular Fluid is now widely used as the primary collection source for components of host and bacterial cell metabolism that may prove to be reliable indicators of disease activity or potential activity. Collection of Gingival Crevicular Fluid has been undertaken in the past using variety of methods like gingival washing, capillary tubes or micropipettes were used, as extremely small volumes of fluid collectable from a single site, necessitate highly sensitive analytical techniques to allow accurate quantification of fluid components and volume is influenced by many factors such as flow rate, gingival trauma and repeat sampling.[49-51] In the present study absorbent paper points was used for better analysis [52].

Alkaline Phosphatase (ALP) is enriched in the membranes of mineralizing tissue cells (e.g.osteoblasts) and is also present in PMN granules, plaque bacteria, fibroblasts and osteoclasts. It was suggested that host-derived Alkaline Phosphatase contributed to >80% of the enzyme in Gingival Crevicular Fluid inhibition in the studies conducted on suspensions of washed plaque.[53]

In the periodontium, ALP is very important enzyme as it is part of normal turnover of periodontal ligament, root cementum and maintenance, and bone

homeostasis. The deficiency of estrogen in women at menopause is contributing factor to osteoporosis and considered one of the risk factors for periodontal disease.It has been hypothesized that osteoporosis decreases alveolar bone density and in turn increases its susceptibility to resorption due to periodontal inflammation. Accelerated bone loss in menopause is related to increased bone turnover.

Osteocalcin, also called bone Gla-protein is a small (5. 4kDa) calcium-binding protein of bone, and is the most abundant noncollagenous protein of mineralized tissues [11]. It is a protein with a molecular mass of approximately 6kd, containing 49 amino acids. Osteocalcin(OC) is predominantly synthesized by osteoblasts, odontoblasts and hypertrophic chondrocytes and it has an important role in both bone resorption and mineralization.[54]

There are studies documented in literature that have investigated the changes in Alkaline Phosphate levels in Gingival Crevicular Fluid when pre menopausal were compared with post menopausal also certain studies have compared the Alkaline phosphate in serum of chronic periodontitis patients . However there are only few studies evaluating the levels of osteocalcin produced in serum and GCF of chronic periodontitis patients. Thus this study was conducted for evaluating the level of alkaline phosphatase (ALP) and osteocalcin(OC) in gingival crevicular fluid and serum in pre menopausal women compared with post menopausal women.

A total of 30 subjects were enrolled in the study and were divided into two groups as follows: 15 pre menopausal women(30-40 yrs) with chronic periodontitis (Group A) and 15 post menopausal women(45-55 yrs) with chronic periodontitis (Group B) for the assessment of the level of alkaline phosphatase and osteocalcin in GCF and serum.

CLINICAL PARAMETERS:

SERUM ALP LEVELS

When levels of ALP in serum was assessed in Group-A, serum ALP was significantly (p=0.0001) lower among pre menopausal (48.50±4.32) when compared with Group-B postmenopausal (101.70±12.30) women, this can be attributed to bone tissue remodelling slackens with decreasing bone density which reflect changes in the levels of biochemical bone remodelling markers that is ALP.

The values found in Group A was lower but within normal range of the age group among premenopausal women.

Due to Estrogen deficiency at the menopause which results in high turnover bone loss can be recognized receptors on the osteoblasts which do not function optimally due to the lack of hormones. This is reflected by a significant increase in the mean value of markers of resorption and formation in the serum from pre menopausal to post menopausal.[55]

It was a similar to a study done by Bhattarai et al [2014][56] measured serum ALP in 50 post menopausal women (experimental group) and 50 pre menopausal (control group). Pre menopausal women (control group). They found that serum ALP level was significantly increased in post menopausal women compared to controls.

There was in an agreement with study done by Nakumura M and Slots J et al who found increase in serum ALP activity in periodontitis which could be associated with alveolar bone loss, a key feature of periodontitis.[57]

Similarly a research done by Tutan A et al who observed a significant increase in the level of this enzyme ranged from 44.18 to 52.82 U/ml for pre-menopausal

women and from 89.4 to114.2 U/ml for post-menopausal women.($p<0.0001$)[58]. Similar study showed the tendency of linear increase in the level of ALP activity in serum reflects the advancing periodontal tissue injury and damage.[59]

Akshay et al[60] and Deepa S. et al[61] conducted study in which the total ALP levels were considerably raised in the postmenopausal women (226.44±44.36) 45-78yrs as compared to those in the premenopausal women (211.16±37.35) 24-47 yrs

SERUM OSTEOCALCIN LEVELS

When levels of osteocalcin in serum was assessed in GROUP-A, serum Osteocalcin was significantly ($p=0.0001$) lower within the normal age group among pre menopausal (15.68±3.33) than GROUP-B post menopausal (29.84±2.98) TABLE-3 andGRAPH-3.

The value found in Group A was lower but within normal range of the age group among pre menopausal women.

This observation was in agreement with a previous study performed by Vanita R et al 2011 where they found significantly higher ($p<0.0001$). which can be due to reduction in skeletal mass caused by an imbalance between bone resorption (osteoclastic activity) and bone formation (osteoblastic activity) due to lack of estrogen. It is therefore, essential to identify these women at risk of developing osteoporosis.[61]

In post menopausal women deficiency of calcium may lead to lowering of formation of hydroxyapatite crystals, with the decreased rate of bone mineralization, free osteocalcin may be available for circulation in the blood. Related findings were in accordance with the studies of other investigation Storm et al 1998 which explains

increased concentration of osteocalcin in the serum of postmenopausal women (53.52+/-5.30 yrs).[62]

Similar study observed high levels of serum osteocalcin in postmenopausal when compared to premenopausal women. The same increase in serum osteocalcin in postmenopause was also observed by Lori J Sokoll et al .[63] Estrogen deficiency may induce calcium loss due to decreased intestinal calcium absorption and decreased renal calcium conservation .[64] The same high levels in postmenopausal was also observed by Lie T. Merijanti Susanto .[65]

Plantalech et al.[66] reported that total OC serum levels were significantly higher in postmenopausal women than in premenopausal women, as was observed in our study. We considered that while the bone turnover rate is steady in premenopausal women, it was induced during the postmenopausal period

Bullon et al 2005[67] Serum osteocalcin is presently considered a valid marker of bone turnover when resorption and formation are coupled and a specific marker of bone formation when formation and resorption are uncoupled.

On the contrary, serum osteocalcin concentration correlated significantly with the periodontal treatment outcome. The authors conclude that high serum osteocalcin levels are associated with rapid bone loss, but the individual value of this marker is limited .

Osteoblasts produce osteocalcin and incorporate it into the bone matrix. Osteocalcin is released into the circulation from the matrix during bone resorption and, therefore, is considered a marker of bone turnover rather than a specific marker of bone formation. The production of osteocalcin is stimulated by 1,25 dihydroxy

vitamin D and depends on vitamin K. Vitamin K increases the carboxylation of osteocalcin, but it does not increase its overall rate of synthesis. Although its function is not completely understood, osteocalcin may exist as a deposition site for hydroxyapatite crystals; it may also affect energy metabolism via the production and action of insulin.

ALP LEVELS IN GCF

When the levels of GCF in alkaline phosphatase was assessed GROUP-A gingival crevicular fluid ALP was significantly ($p=0.0001$) lower among premenopausal (321.00±139.60) than GROUP-B in postmenopausal (732.00±414.93) women.Table4 Graph4

It is in accordance with the study done by Ishikawa and Cimasoni on ALP which showed similar results along with other clinical parameters in which the levels of enzymes ranged from 239+/- 187 for pre menopausal and 569+/-321 for post menopausal.

Very few studies are done on comparative evaluation of GCF ALP levels in pre and postmenopausal women the reason for this rise might be due to estrogen deficiency which is an associated factor with menopause as it induce increased bone resorption.

Comparable study done by Binder et al demonstrated that ALP concentration in GCF showed a positive relationship of bone loss with aging in postmenopausal women he stated that ALP level may also be useful as a potential bone turnover marker to establish the diagnosis and prognosis of periodontal disease.[69] Gibert

predicted ALP as an indicator for future loss of periodontium in postmenopausal women.70

The increase in ALP levels in GCF of post menopausal women's can be due to neutrophil predominance in the pocket epithelium and pocket itself which is the major source of ALP during inflammation. Polymorphoneuclear leukocytes (PMNLs) are produced within the area of periodontium and GCF which is associated with bone metabolism.[71] PMNs causes phagocytosis of bacteria causes cleavage of complement C5 to C5a along with cleavage of cytokineIL8 making it more active in PMN which causes increased PMNs at the site of infection.

In periodontal disease, the cellular inflammatory infiltrate of T cells, B cells, macrophages, and neutrophils within gingival connective tissue is increased, with a concurrent increase in the secretion of inflammatory mediators . These inflammatory cells also interact with stromal cells, such as osteoblasts, periodontal ligament, and gingival fibroblasts. RANKL-mediated osteoclastogenesis plays a pivotal role in inflammatory bone resorption, and its expression is increased in periodontitis

While PMNS produce RANKL, they might not be involved in bone resorption under physiological conditions. However, in inflammatory pathological resorptive states, activated T lymphocytes may mediate bone resorption through excessive production of sRANKL, and findings suggest that activated T and B lymphocytes are one of the major RANKL-expressing sources in diseased periodontal tissue .

GCF OSTEOCALCIN LEVEL

Levels in Gingival crevicular fluid of Osteocalcin of GROUP-A pre menopausal (5.20±3.20) was lower when compared with GROUP-B postmenopausal (18.30±9.80) which was significantly (p=0.001) higher in.Table5 Graph5

Similar study conducted by Nakashima K. in which OC was present in GCF from both healthy and diseased sites with mean concentrations more than ten times greater than normal serum levels. These data suggest that a significant amount of OC present in GCF is produced locally by periodontal tissues.[72]

However it was in concurrent with a study done by Kunimatsu K et al who did not find osteocalcin in GCF of patients with gingivitis while periodontitis GCF osteocalcin was positively correlated with aging.[73]

Also it was not in accordance in a cross-sectional study carried out by Lee et al[74] where no difference in GCF osteocalcin levels were observed between sites with and without periodontal pockets in the same patient.

Recently, Wilson et al. [75] in a study including 14 patients without periodontal treatment, did not detect osteocalcin in GCF. Most of these studies have been carried out in periodontal patients and did not compare healthy and diseased subjects, but osteocalcin levels in healthy and periodontally affected sites in periodontal patients.[76]

Osteocalcin levels found to be significantly associated with periodontal disease, since as during active resorption OC and its fragments are released from the extracellular matrix into GCF (Bullon et al. 2005).[77]

PayneJ.B in his study stated that estrogen deficiency become significant in postmenopausal and due to this alveolar bone loss occurs.[78]

INTRAGROUP COMPARISION

GCF and Serum ALP levels

Comparison of gingival crevicular fluid ALP and serum ALP in pre menopausal and post menopausal women. There was significant ($p=0.0001$) difference between gingival crevicular fluid ALP and serum ALP in pre menopausal and postmenopausal women.TABLE -7 AND GRAPH -7

The aforementioned observations could be explained owing to similar findings of McCauley&Nohutcu,2002 in which Alkaline phosphatase levels in gingival crevicular fluid are higher than in serum. In serum, the enzyme are associated with systemic bone disease,[79] and its elevation in gingival crevicular fluid could well reflect changes of alveolar bone which can be due to neutrophil predominance in the pocket epithelium and pocket itself which is the major source of ALP raise in GCF compared to serum.

GCF and Serum osteocalcin levels

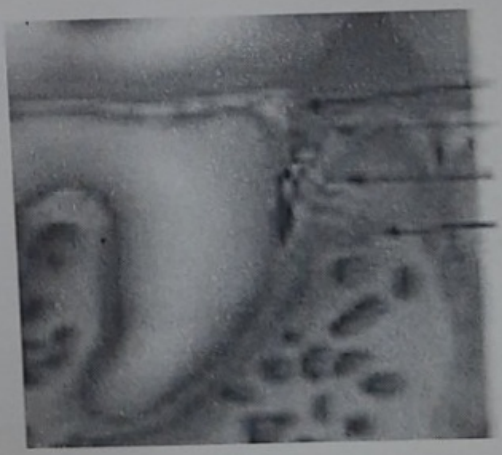
Comparisons was also done in which gingival crevicular fluid and serum Osteocalcin in pre menopausal and post menopausal women were compared. There was significant ($p<0.05$) difference between gingival crevicular fluid OC and serum OC in premenopausal and post menopausal women.TABLE-6 and GRAPH-6

Vanita R et al 2011 on the contrary, serum osteocalcin concentration correlated significantly than GCF OC with the periodontal treatment outcome.[80] The use of serum osteocalcin as osteoporosis marker has been studied, without treatment with estrogens.

The authors[81] conclude that high serum osteocalcin levels are associated with fast bone loss, but the individual value of this marker is limited. Similar study done by showed possible to verify that serum osteocalcin levels are determinant of the bone formation[82-84]

We can conclude after comparing the biochemical parameter in pre and post menopausal women both the biomarkers Alkaline phosphatase and Osteocalcin are increased with the increase in bone loss. It was observed that bone formation markers that is Serum ALP, Serum Osteocalcin levels were significantly increased in post menopausal when compared with pre menopausal women .When post menopausal were compared to pre menopausal women ($p \leq 0.001$).

There was a statistically significant difference between GCF Alkaline phosphatase and Osteocalcin levels. However, the results need to be confirmed and supported through further long term studies in larger and wider population groups.



Conclusion

Based on the analysis and clinical observations, the following conclusions can be drawn in the present study:

- Significant reduction in the levels of serum alkaline phosphatase(ALP) and Osteocalcin in Group A(pre menopausal) when compared with Group B (post menopausal)

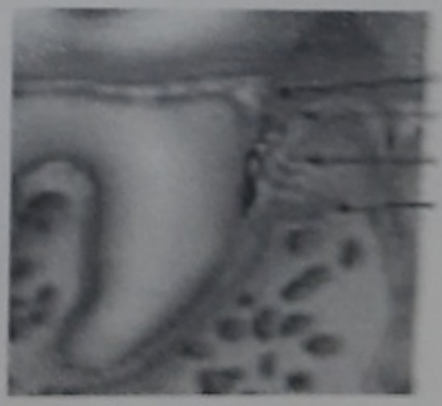
- Levels of alkaline phosphatase and osteocalcin in Gingival Crevicular Fluid was significantly higher in Group B (postmenopausal) than in Group A(pre menopausal) .

- A significant difference between Gingival Crevicular Fluid and Serum Osteocalcin levels were seen, with a significant difference in APL levels of Gingival Crevicular Fluid and Osteocalcin.

Osteocalcin (OC) and Alkaline Phosphatase (ALP) were valid biomarkers. Since GCF has the chance of being closely approximated to the periodontal tissues where periodontal disease begins, it seems to provide more information than markers in saliva. The molecules in saliva can be also originated from salivary glands which cellular and biochemical mediators in saliva may reflect the diseases and metabolic status of glands instead of periodontal diseases. Because Gingival Crevicular Fluid (GCF) constituents reflects the host response to the periodontopathogenic bacterial antigens and the disease progression is essentially dependent upon the host response, evaluation of the markers in Gingival Crevicular Fluid(GCF) is considered to be a reliable method in the determination of an individual risk for periodontal disease.

Detection and identification of biomarkers in the GCF and serum could be an important adjunct in early disease diagnosis for Chronic Detection and identification

of inflammatory mediators in the GCF could be an important adjunct in early disease diagnosis for Chronic Periodontitis as well as it may provide insight into disease mechanisms. However, the results need to be confirmed and supported through further long term studies in larger and wider population groups.



Summary

Chronic periodontitis has been defined as "an infectious disease resulting in inflammation with in supporting tissues of the teeth, progressive attachment loss and bone loss". Bone is constantly undergoing the process of remodelling. In bone remodelling, the bone is constantly resorbed on a particular bony surface, followed by a phase of bone formation. A biomarker is a substance used to indicate a biologic state. It can be defined as, - a substance that is measured objectively and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

The aim of the present study was to evaluate the levels of Alkaline Phosphatase(ALP) and Osteocalcin(OC) in Gingival Crevicular Fluid(GCF) and Serum of Chronic Periodontitis pre menopausal and post menopausal women and to find there correlation with each other.

There are studies documented in literature that have investigated the changes in levels of serum alkaline phosphatase and osteocalcin in pre menopausal and post menopausal women but very few comparing the levels of Alkaline Phosphatase and Osteocalcin levels in GCF. Therefore, now there is a whole new approach for the future use of oral fluids, especially in the field of diagnostics .Study Design- Cross sectional, observational study. The study was carried out which comprised of 15 pre-menopausal women aged between 30-40 years were selected as control (group A) whereas, 15 post-menopausal women aged between 45-55 years were selected as case (group B)

The material and methodology is described in brief. Following the diagnosis, Gingival Crevicular Fluid and Peripheral Blood samples were collected from the volunteers of both the groups Samples were analysed at the diagnostic centre.

Significant clinical outcome was there in Serum Alkaline Phosphatase and Osteocalcin levels that were significantly increased in postmenopausal when compared with premenopausal women .When postmenopausal were compared to premenopausal women ($p \leq 0.001$) also there was strong correlation between GCF Alkaline phosphatase and osteocalcin levels but not as much as serum Alkaline phosphatase and Osteocalcin levels . In contrary levels of gingival crevicular fluid osteocalcin in postmenopausal were higher in comparison to premenopausal the two major causes of bone loss are estrogen deficiency after the menopause and age-related process.

When the intargroup comparison was done significant ($p<0.05$) difference between gingival crevicular fluid and serum osteocalcin levels in Group A and Group B.

It is also clear that no single marker has been able to fulfil all the criteria necessary for assessment of the clinical state of the periodontium, and future research should be directed possibly at the production of "marker packages" As of now various efforts are on to develop an ideal test, but actual use as a chairside diagnostic is still illusive. Many more studies with larger population are required for further advancement of the invasive technique.

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Appendices

APPENDIX – I

ETHICAL COMMITTEE APPROVAL FORM

Babu Banarasi Das College of Dental Sciences
(A Faculty of Babu Banarasi Das University)
BBD City, Faizabad Road, Lucknow – 227105 (INDIA)

Dr. Lakshmi Bala
Professor and Head Biochemistry and
Member-Secretary, Institutional Ethics Committee

Communication of the Decision of the I[st] Institutional Ethics Sub - Committee

IEC Code: 22 **BBDCODS/22/2015**

Title of the Project: Evaluation of Alkaline Phosphatase and Osteocalcin levels in Gingival Crevicular Fluid and Serum in Premenopausal and Postmenupausal Woman with Chronic Periodontitis.

Principal Investigator: Dr. Asmita Jaiswal **Department:** Periodontology

Name and Address of the Institution: BBD College of Dental Sciences Lucknow
Type of Submission: New, MDS Protocol
Dear Dr. Asmita Jaiswal

The Institutional Ethics Sub-Committee meeting comprising following four members was held on 09-01-2015

1.	Dr. Amrit Tandan Member	Prof. and Head Deptt. of Prosthodontics, BBDCODS, Lucknow
2.	Dr. Jiji George Member	Prof., Deptt. of Oral Pathology, BBDCODS, Lucknow
3.	Dr. Asish Saini Member	Reader, Deptt. of Periodontics, BBDCODS, Lucknow
4	Dr. Lakshmi Bala Member Secretary	Prof. and Head, Deptt. of Biochemistry, BBDCODS, Lucknow

The committee reviewed and discussed your submitted documents of the research study in the meeting. The proposal was reviewed and thoroughly revised.

Decisions: The committee approved the above proposal from ethics point of view.

Forwarded by

(Dr. Lakshmi Bala)
Member-Secretary IEC

(Dr. Vivek Govila)
Dean, BBDCODS

PRINCIPAL
Babu Banarasi Das College of Dental Sciences
(Babu Banarasi Das University)
BBD City, Faizabad Road, Lucknow-227105

APPENDIX – II

CASE SHEET

BABU BANARASI DAS COLLEGE OF DENTAL SCIENCES, LUCKNOW

"The levels of alkaline phosphate and osteocalcin in gingival crevicular fluid and osteocalcin in premenopausal and postmenopausal women with chronic periodontitis: a biochemical study"

CLINICAL EVALUATION:

NAME:

AGE: **SEX:**

OPD No.:

DATE:

ADDRESS:

Chief complaint:

History of present illness:

GROUPS:

GROUP A	Premenopausal women with chronic periodontitis
GROUP B	Postmenopausal women with chronic periodontitis

Past dental history:

Past medical history:

History of medication:

CLINICAL EVALUATION:

I. Gingiva

Color

Consistency

Size

Position

Bleeding

Suppuration

II. Examination of teeth:

Number of teeth present

Mobility

CLINICAL PERIODONTAL PARAMETERS

PROBING POCKET DEPTH

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

RADIOGRAPHS

DIAGNOSIS

PROGNOSIS

TREATMENT PLAN

TREATMENT DONE

II. Examination of teeth:

Number of teeth present

Mobility

CLINICAL PERIODONTAL PARAMETERS

PROBING POCKET DEPTH

8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8

RADIOGRAPHS

DIAGNOSIS

PROGNOSIS

TREATMENT PLAN

TREATMENT DONE

MAINTENANCE PHASE

BIOLOGICAL PARAMETERS

1. Level of ALP and Osteocalcin in Gingival Crevicular Fluid and Serum in Group A
2. Level of ALP and Osteocalcin in Gingival Crevicular Fluid and Serum in Group B

APPENDIX - III

Informed Consent form

Study Title:

Subject's Initials: _____ Subject's Name: _____

Date of Birth/Age: _____

(Subject)

		Please initial box
(i)	I confirm that I have read and understood the information sheet dated __ __ for the above study and have had the opportunity to ask questions.	[ ]
(ii)	I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	[ ]
(iii)	I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s)	[ ]
(i)	I agree to take part in the above study.	[ ]

_____

Subject Name (Print)

_____ _____

Subject Signature **Date**

I certify tha provided was giv ... the subject.

_____ _____

Name of Person Obtaining Informed Consent

(Print)

_____ _____

Signature of Person Obtaining Informed Consent **Date**

Signature & Date of Principal Investigator: _____

(if other than the Person Explaining Consent)

(* If a patient has limited ability to read and write, and impartial witness should be present during the entire informed consent discussion and his/her legally acceptable representative should sign on patient's behalf). In these instances the patient places his/her left thumb impression in the place of the signature.

Is Subject illiterate?

☐ Yes

☐ No

Subject's Initials _____

APPENDIX-IV

STATISTICAL TOOLS EMPLOYED

The following Statistical formulas were used:

1. The Arithmetic Mean

The most widely used measure of central tendency is arithmetic mean, usually referred to simply as the mean, calculated as

$$\overline{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

2. The Standard Deviation

The standard deviation (SD) is the positive square root of the variance, calculated as

$$SD = \sqrt{\frac{\sum X_i^2 - \frac{(\Sigma X_i)^2}{n}}{n-1}}$$

where n= no. of observations

3. Unpaired t test

$$s_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$

$$SE(\bar{x}_1 - \bar{x}_2) = s_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

4.Paired test

$$SE(\bar{d}) = \frac{s_d}{\sqrt{n}}$$

$$T = \frac{\bar{d}}{SE(\bar{d})}.$$

5. Level of significance: "p" is level of significance

$p > 0.05$	=	Not significant
$p < 0.05$	=	Significant
$p < 0.01$	=	Highly significant
$p < 0.0001$	=	Very highly significant