EVALUATION OF OSTEOCALCIN AND OSTEONECTIN LEVELS FROM PERI IMPLANT SULCULAR FLUID AROUND ENDOSSEOUS IMPLANTS

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY

In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

BRANCH II

PERIODONTOLOGY

MAY 2019

THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY
CHENNAI

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"EVALUATION OF OSTEOCALCIN AND OSTEONECTIN LEVELS FROM PERI IMPLANT SULCULAR FLUID AROUND ENDOSSEOUS IMPLANTS"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. G. SIVARAM, M.D.S.,** Professor, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

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CERTIFICATE

This is to certify that this dissertation titled "**EVALUATION OF OSTEOCALCIN AND OSTEONECTIN LEVELS FROM PERI IMPLANT SULCULAR FLUID AROUND ENDOSSEOUS IMPLANTS**" is a bonafide record of work done by **Dr. ALI FIROOZI** under my guidance during the study period 2016-2019.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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Acknowledgement

ACKNOWLEDGEMENT

*First of all, I would like to thank the Almighty, "**Allah**" for making me achieve my dreams, he was the light when I felt like I was lost.*

I would like to express my gratitude to all the people who supported me in the completion of this thesis.

*I take this opportunity to sincerely thank **Dr. N.S. Azhagarasan, MDS, Principal,** Ragas Dental College and Hospital, for his support during my postgraduate course at Ragas Dental College.*

*I would like to express my gratitude and appreciation to my respected professor **Dr.K.V.Arun**, **MDS, Professor,** Head of the Department of Periodontics, Ragas Dental College Chennai, for his inspiring advice and guidance. I thank him for his hands-on involvement when it came to helping me in my course and also the motivation, he provided making my love for the subject grow.*

*I would like to express my gratitude to my guide, **Dr. G. Sivaram, MDS,** Professor who has constantly inspired me and has unceasingly guided me throughout my study process with his constant support. He taught me a lot of techniques and personally taught me the subject when I was unable to comprehend certain difficult topics**.***

*I would like to thank **Dr.Shivakumar, MDS, ,** Professor who has generously guided with the valuable advice and constant encouragement which helped me throughout the post-graduation course.*

I would like to extend my deepest gratitude to ***Dr. Shreemogana****,* ***MDS****, senior lecturer, for her overwhelming help and valuable advice without which I would have never accomplished this particular research.*

I would like to express my profound sense of gratitude to ***Dr. Ramya Arun, MDS,*** *Reader,* ***Dr. Swarna Alamelu, MDS,*** *Reader and* ***Dr. Archana Meenakshi, MDS,*** *Reader for their constant support and encouragement throughout my tenure.*

I would like to thank ***Dr. Deepavalli, MDS,*** *Senior Lecturer and* ***Dr. A.R. Akbar, MDS,*** *Senior Lecturer for their continuous support and guidance. I would also like to thank* ***Dr. R.S. Pavithra, MDS,*** *Senior Lecturer and* ***Dr. J. Velkumar, MDS,*** *Senior Lecturer for their constant support.*

Here, I would like to say a special thanks to ***Dr. Gayathri,*** *for her extensive help and constant support which has been a vital part for the completion of this dissertation.*

I thank my batchmates ***Dr. Asha, Dr. Cynthia Johns, Dr. Kavipriya****,* ***Dr. Krupa*** *and* ***Dr. Santhosh*** *for their support and encouragement.*

I further extend my thanks to my juniors ***Dr. Indhumathi, Dr. Meenakshi, Dr. Ragamalika, Dr. Davis, Dr. Gayathri, Dr.Ashwathy, Dr.Lakshmi, Dr.Shalini*** *and* ***Dr.Shanmugapriya.***

I extend my thanks to the non-teaching staffs ***Mrs.Parvathi, Mr.Chellapan,*** *and* ***Mrs.Rosamma*** *for their timely help during the tenure.*

Finally, to my father ***Mr. MORTEZA*** *and my mother* ***Mrs. AMENEH,*** *my* ***brothers*** *and* ***sister*** *to whom I dedicate this work as an honour for their love, understanding, support and all the sacrifices they have made to see me succeed in my life.*

LIST OF ABBREVIATIONS

BSP	-	Bone Sialoprotein
CAL	-	Clinical Attachment Level
Cbfa1	-	Core Binding Factor Alpha 1
ECM	-	Extracellular Matrix
ELISA	-	Enzyme-Linked Immunosorbent Assay
GCF	-	Gingival Crevicular Fluid
HRP	-	Horse Radish Peroxidase
MMP	-	Matrix Metalloproteinases
NCP	-	Non-Collagenous Proteins
OD	-	Optical Density
ON	-	Osteonectin
OPN	-	Osteopontin
PISF	-	Peri Implant Sulcular Fluid
PD	-	Probing Depth
PDL	-	Periodontal Ligament
RANKL	-	Receptor Activator of Nuclear factor kappa B Ligand
RGD	-	Arginine glycine and aspartate sequence
SPARC	-	Secreted protein acidic and rich in cysteine
TRAP	-	Tartrate-Resistant Acid Phosphatase

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Introduction

INTRODUCTION

Management of edentulous ridge with implant treatment has been proved to be a successful and predictable treatment for tooth loss over the past years[49]. Mombelli. et al, stated that the two form of peri implant inflammation are peri-mucositis and peri implantitis [87]. The early and reliable detection of any adverse peri implant tissue reaction is a pre-requisite for treatment planning in patients treated with endosseous implant and also to avoid future failure of implant. (Oh et al) [98].

Alveolar bone is constantly undergoing remodelling by formation of new bone to compensate for the bone lost by resorption [120]. This is referred to as coupling. In both periodontitis and peri-implantitis there is failure to form an equivalent amount of new bone following resorption, this is the process of uncoupling which has an impact on the progression of periodontal disease or implant failure.

Albrektsson stated that, in dental implants the osseointegration occurring after the placement of the implant is a process in which the stable anchorage of an implant is obtained by direct bone-to-implant contact. It involves a series of cellular activities that closely resemble the development and growth of bone during embryonic and postnatal life [1].

Bone regeneration follows similar pathways: i.e. direct or primary healing, where a scaffold of woven bone, closely associated with an expanding vascular net, invades the granulation tissue and organizes the initially formed blood clot. In indirect or secondary healing, the connective tissue and/ or fibrocartilage differentiates within the fracture gaps and is replaced by bone as in endochondral ossification. Osseointegration belongs to the category of direct or primary healing. The final stage of osseointegration is the bone remodeling though this process continues throughout the life. Both the resorption and formation of bone around implant are coupled in a spatial and temporal manner.

Extra cellular matrix plays a crucial role in bone remodelling. Alveolar bone consists of osteoblasts, osteocytes, bone lining cells and osteoclasts that are embedded in the extra cellular matrix. The extra cellular matrix of alveolar bone is similar to the other bone tissues, which comprises of the collagenous and non – collagenous proteins. Non-collagenous proteins (NCP) play a vital role in different steps of differentiation and activity of bone cells and in the maturation and mineralisation of bone matrix. Thus, the non-collagenous proteins of the extracellular matrix function not only in maintaining the peri implant homeostasis but also involved in the process of osseointegration [120].

Among the non-collagenous proteins osteocalcin (OCN), osteonectin (ON), Bone sialoprotein (BSP), osteopontin(OPN) are considered to be the major components.

Osteocalcin comprises of less than 15% of the non-collagenous protein. It is produced by osteoblasts and is involved in the process of mineralisation rather than matrix production. Osteocalcin is known to be regulator of Hydroxyapatite crystal growth and may also recruit osteoclasts to sites of formed bone and is a potent marker in sites of bone turnover. Thus, osteocalcin is involved in both bone resorption and bone formation [(110)].

Ram et al stated that, Osteonectin is a calcium binding glycoprotein comprising of 25% of the non-collagenous protein. Osteonectin plays a role in collagen turnover and enhances mineralisation of collagen matrix. Studies have shown the presence of osteonectin is high during developmental phases and also during wound healing. Implant osseointegration is a process similar to bone healing and osteonectin is thus considered to be important for proper implant stability [(110)].

The combination of clinical and radiographic parameters, such as probing depth (PD), BOP, suppuration of implant, mobility and marginal bone loss, are the commonly used parameters for the diagnosis of patients with peri implantitis. However, these diagnostic processes might not be sensitive or specific enough to distinguish disease onset, development, and activity. Clinical measurements around implants as like natural teeth might be challenged by the force and direction of probing, implant geometry, prosthesis design and peri-implant soft tissue biotype. In addition, both peri-implant mucositis and peri-implantitis lesions can present with BOP and/or

suppuration, with PDs greater than 4 mm. Therefore, clinicians and researchers may often observe the early, and sometimes the late diagnosis of peri-implantitis.

Early detection of peri-implant destruction, as well as monitoring progression of bone loss is extremely important. Currently, blunt surrogate markers are being used such as radiographs and peri-implant probing. These tests have obvious limitations as they only tell us the past history of the disease and do not identify the present scenario or the future progression of the disease process. As main markers of peri-implantitis are bone destruction and inflammation biomarkers and enzymes in peri implant sulcus fluid (PISF), it is therefore essential in focusing on these entities for a precise conclusion. Such knowledge may potentially lead to new diagnostic strategies and candidate disease markers for peri-implant conditions.

A biomarker can be defined as an indicator of a biological state and can help to distinguish between normal and pathologic processes. One of the main advantages of evaluating biomarkers is the non-invasive nature of obtaining samples for analysis. Biomarkers can be measured in secretions such as saliva and gingival crevicular fluid (GCF), or in the case of implants which this particular thesis is based on, peri-implant sulcular fluid. Studies have been conducted to look at a vast array of biomarkers and enzymes around dental implants as an early sign of peri-implantitis.

We have chosen osteocalcin and osteonectin for biomarker analysis because osteocalcin was considered to be a marker for differentiated osteoblasts and osteonectin is considered to be associated in the mineralisation process and is expressed by osteoblasts during bone formation [110].

Hence, this study was done to evaluate the levels of osteocalcin and osteonectin in the PISF of healthy implant sites 6 months after loading and compare it to the GCF obtained from periodontally healthy sites.

Aims and Objectives

AIM AND OBJECTIVES

The aim and objective of the present study is:

1. To evaluate and compare the Osteocalcin levels in GCF and PISF of periodontally healthy tooth and implant sites.
2. To evaluate and compare the Osteonectin levels in GCF and PISF of periodontally healthy tooth and implant sites.

Review of Literature

REVIEW OF LITERATURE

In all multicellular organisms development is influenced by the interactions between cells and their extracellular matrix (ECM). ECM provides the cell with information about the positional and temporal clues, such as where it is, where it should migrate, when to differentiate, and in some instances, when it is time for apoptosis.

Mathew s et al[77] stated that ECM enhances cell recruitment through the cell surface receptors called integrins, which decides the cell to ECM interactions and stimulate the specific cellular functions such as adhesion, migration, proliferation and differentiation.ECM is also a pool of growth factors such as bone morphogenic proteins (BMPs) and transforming growth factor (TGF)-β1.

According to **ellis et al,** and **ly et al**[33], the other important feature of ECM is that it aids in the proteolytic degradation by proteases that in turn guides the tissue repair and remodelling. ECM mechanical properties are important to mediate and determine stem cell differentiation, besides its chemical composition.

The most important properties of ECM is its functional diversity. According to **Robert P et al**[80]**,** all have a highly specialized functions with a modular design with different roles.

ALVEOLAR BONE:

Specialized part of the mandibular and maxillary bones is the alveolar bone which forms the primary support structure for teeth. Alveolar bone is subjected to continual and rapid remodelling associated with tooth eruption and subsequently the functional demands of mastication in comparison to the other bone tissues in the body.

During embryonic development, osteoblasts arising from condensing mesenchyme in the facial region that creates bony alveoli that house the individual teeth of the developing dentition forms the intra membranous bone of the maxilla and mandible [(120)]. In three dimensions, these osteoblasts form an extensively perforated sheet of otherwise contiguous osteoblasts which, not only produces the alveolar bone matrix proper, in addition they embed continuously remodelling periodontal ligament fibers in a precise manner [(62)].

it is followed by the maturation and then the osteoblasts may undergo apoptosis or embed in the matrix as osteocytes or remain on the bone surface as bone lining cells.

Osteoblasts that transformed to osteocytes occupy spaces (lacunae) in bone and are termed as cells surrounded by bone matrix. Important factor is the ability of bone to respond to biological regulatory factors and functional forces is the capacity of the large, multinucleated osteoclasts to resorb bone[(120)]. An extensive homeostatic network of cells formed together with osteocytes, bone-lining cells and their connecting cell processes capable of

regulating plasma calcium concentration through mechanisms partly independent of those related to the bone remodelling system [79].

ALVEOLAR BONE ECM:

The osteoblasts synthesis bone matrix in two main steps: deposition of organic matrix and its subsequent mineralization. In the first step, the collagenous proteins, mainly type I collagen, non-collagen proteins (OCN, osteonectin, BSP II, and osteopontin), and proteoglycan including decorin and biglycan, which form the organic matrix are secreted by the osteoblasts. Then the mineralization of bone matrix occurs.

60% inorganic, and 40% organic compounds (40%) makes up the alveolar bone. The hydroxyapatite crystals are formed by the nucleation of the calcium and phosphate ions, which are represented by the chemical formula $Ca_{10}(PO_4)6(OH)_2$. Along with collagen, the non collagenous matrix proteins form a scaffold for hydroxyapatite deposition and such correlation is responsible for the typical stiffness and resistance of bone tissue [79].

Clarke[24] also stated that the organic phase is made up of 90% of collagen type I and10% of mineral binding non-collagenous proteins (NCP) such as glycoproteins, gamma carboxy-glutamic acids, proteoglycans, and osteogenic proteins such as BMPs and TGF.

Collagen is the most unique ECM protein and is designed in such a way to to provide structure and resiliency to tissues [81].

Morgan et al and Roach et al[90,116] said that non collagenous protein such as bone sialoproteins(BSP), osteocalcin(OC), osteonectin, osteopontin(

OPN) and proteoglycans such as decorin and biglycan containing a protein nucleus with side- chains of glycosaminoglycans including chondroitin sulfate(CS) and hyaluronic acid**,** controls bone mineralization and bone mass. Hydroxyapatite and collagen type I both binds with BSP and **Addison et al** [(4)] said that OPN mediates the growth of hydroxyapatite crystals, and promotes the osteoclasts attachment onto the inorganic structure of bone.

The association between the bone formation and the bone resorption maintains the bone mass .Formation of bone, involves the proliferation and differentiation of stromal stem cells along an osteogenic pathway that leads to the formation of osteoblasts. Osteogenic line of cells give rise to preosteoblasts, osteoblasts, osteocytes, and bone lining cells, which, in turn, derived from the primitive mesenchymal cells in bone marrow stroma and from pericytes adjacent to connective tissue blood vessels.

Differentiation of these osteogenic cells requires activation of the Osf2/Cbfa gene, which initiates the expression of osteocalcin, bone sialoprotein (BSP), osteopontin (OPN), and collagen synthesis, which is followed by stimulation from bone morphogenetic protein- (BMP-) 2 and transforming growth factor beta (TGF-β)[(112,116)].

According to **Glowacki et al**[(40)], the osteocalcin promotes the recruitment and attachment of osteoclast precursor to bone matrix and promotes them to differentiate to osteoclasts. **Venkatesh B et al**[(132)] said that, the absence of non-collagenous proteins, especially small integrin-binding

ligand N-linked glycoproteins (SIBLINs) prevents ossification in vertebrates, which highlights their significant role in bone formation.

Both structural and biological functions are served by bone ECM, as the tissue's mechanical properties are achieved by the mineralized matrix, while it also provides chemical cues that regulate bone cells and acts as a reservoir for ions. ECM– integrin bonds that enable the formation of adhesive structures and activate signalling pathways, which regulate cell spreading, survival, and differentiation are regulated by the bone ECM.

Thus, bone matrix comprises of a complex and organized framework that provides mechanical support and plays an important role in the bone homeostasis. Several molecules that interfere in the bone cells activity and, consequently, has a participation in the bone remodeling are secreted by the bone matrix [41].

Interactions between Bone Cells and Bone Matrix

As mentioned earlier, bone matrix not only provides support for bone cells, but also plays a significant role in regulating the activity of bone cells via several adhesion molecules [41,144]. The most common adhesion molecules involved in the interaction between bone cells and bone matrix are the integrins [142].

Integrins aid osteoblasts to interact with bone matrix, which recognize and bind to RGD and other sequences present in bone matrix proteins including osteopontin, fibronectin, collagen, osteopontin, and bone

sialoprotein [27,76]. $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha 5\beta 1$ are the common integrins present in the osteoblast [48]. During osteoid synthesis these proteins play a key role in osteoblast organization on the bone surface [142].

In addition, the interaction between osteoclasts and bone matrix is essential for osteoclast function, because the bone resorption occurs only when osteoclasts bind to mineralized bone surface [113]. Thus, $\alpha v\beta 3$ and $\alpha 2\beta 1$ integrins expressed by the osteoclast during bone resorption to interact with the extracellular matrix, in which the former bind to bone-enriched RGD-containing proteins, such as bone sialoprotein and osteopontin, whereas $\beta 1$ integrins bind to collagen fibrils [47,48].

Stimulation of the osteoclast activation occurs after binding of RANKL to its receptor RANK, present in the membrane of osteoclast precursors. Osteoclast becomes polarized by reorganizing its cytoskeleton; the ruffled border (RB) and clear zone (CZ) are membrane specializations observed in the portion of the osteoclast proximal to the bone resorption surface, Howship lacuna (HL) [35].

The osteoclast initiates acidification due to pumping of hydrogen ions (H+) to the HL. H+ ions and bicarbonate (HCO_3 −) derived from the cleavage of carbonic acid (H_2CO_3) under the action of carbonic anhydrase II (CAII) and causes the dissolution of the hydroxyapatite which present proximal to the ruffled border. Cathepsin, matrix metalloproteinase-9 (MMP-9), and tartrate-

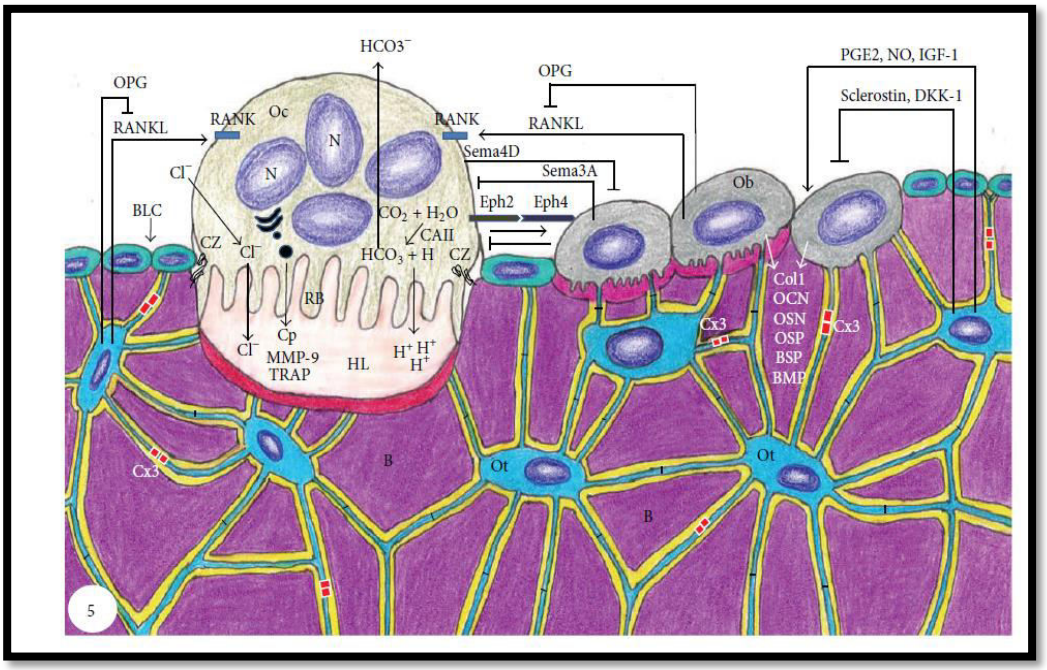
resistant acid phosphatase (TRAP) that degrade the organic matrix are released by the osteoclast, following the dissolution of hydroxyapatite.

Then the EphrinB2 (Eph2) present in osteoclast membrane binds to ephrinB4 (Eph4) in osteoblast (Ob) membrane, which mediates its differentiation, whereas the reverse signalling (ephrinB4/ephrinB2) inhibits osteoclastogenesis. Osteoblasts are inhibited by Sema4D, which is produced by osteoclasts, while Sema3A produced by osteoblasts inhibits osteoclasts. Osteoblasts (Ob) also forms receptor activator of nuclear factor KB (RANKL) and osteoprotegerin (OPG), which promotes and decrease osteoclastogenesis, respectively [35].

Thus the activated osteoblasts secrete collagenous (Col1) and noncollagenous proteins such as osteocalcin (OCN), osteopontin (OSP), osteonectin (OSN), bone sialoprotein (BSP), and bone morphogenetic proteins (BMP) are produced by these activated osteoblast.

Osteocytes are located within lacunae surrounded by mineralized bone matrix crosses canaliculi with its cytoplasmic process to make connection with other neighbouring osteocytes processes by gap junctions, mainly composed by connexin 43 (Cx3), as well as to cytoplasmic processes of osteoblasts and bone lining cells (BLC) on bone surface.

Osteoclastogenesis is stimulated by the RANKL secreted by osteocytes, while the osteoblastic activity is stimulated by the prostaglandin E2 (PGE2), nitric oxide (NO), and insulin-like growth factor (IGF). On the other hand, osteocytes secrete OPG that inhibits osteoclastogenesis; moreover, osteocytes produce sclerostin and dickkopfWNT signalling pathway inhibitor (DKK-1) that decrease osteoblast activity [35].



ALVEOLAR BONE HOMEOSTASIS:

Bone is markedly an active and dynamic tissue, which undergoes constant renewal in response to mechanical, nutritional, and hormonal influences. A prerequisite for the regeneration of tissues lost through

periodontal disease and for osseointegration of implants is the formation of the alveolar bone.

An equilibrium between the coupled processes of bone resorption by osteoclasts and bone formation by osteoblasts is required for a healthy periodontium [16,126,127]. Under physiologic conditions, the above said processes are very carefully controlled by systemic hormones and local factors and orchestrated by osteocytes and bone lining cells which fine-tune interstitial fluid and plasma calcium levels.

A strong similarity between the cellular and molecular events involved in bone remodelling and inflammation & repair is seen, and the relationships between matrix molecules, such as osteopontin, bone sialoprotein, SPARC and osteocalcin, and blood clotting and wound healing are clearly evident. The bone remodelling cycle operates continually as osteoclasts are constantly resorbing the mature bone, with new bone simultaneously formed by osteoblasts [128]. This multistep process occurs in four distinct phases of activation, resorption, reversal, and formation.

Initiating event that converts a resting bone surface into a remodelling surface is defined as activation [127,7]. This involves the recruitment of mononuclear osteoclast precursors to the bone surface, differentiate and fuse into functional osteoclasts [135]. Terminal differentiation and mononuclear cell fusion is controlled by cell-to-cell interactions between osteoclast progenitors and osteoblasts/stromal cells and by contact with the mineral phase, particularly with **osteocalcin** [4,131]**.**

Non mineralized osteoid encasing the mineralized bone matrix must be dissolved before the osteoclasts can attach to the mineralized matrix and initiate resorption [134]. Following this process, the activated osteoclasts attach to the bone matrix and reorganize the cytoskeleton; and uptake a polarized morphology, forming a sealed zone to isolate the resorption site and develop ruffled borders which secrete protease enzymes.

During the "resorption" phase, osteoclasts work in an environment which removes both mineral and organic components of the bone matrix. Howship's or resorption lacuna, which is the erosion of the scalloped appearance, is the hallmark of the resorbing surface.

There is a "reversal" phase, marking the transition from destruction to repair after most of the mineral and organic matrix has been resolved. Here, the coupling of resorption to formation takes place. The osteoclast can move along the bone surface and restart resorption or undergo apoptosis, after the completion of one resorption lacuna [128].

During resorption by the acidic environment created by osteoclasts the coupling factors are released from their binding proteins, and through the negative feedback they further inhibit resorption, by suppressing osteoclast formation and initiating osteoblastogenesis [39]. Thus, in a series of events which is locally controlled autoregulated cell activation, osteoclastic resorptive phase is usually follows a "**repair phase**" [39].

A cascade of differentiation events occurs including chemotaxis, cell attachment, mitosis, and differentiation of osteoblast precursors takes place, leading to new bone deposition, during repair [39].

Two theories generally clarify how calcification proceeds: the matrix vesicle theory and the nucleation theory; it is speculated that both theories work in parallel depending on the type of skeletal tissue involved [127]. After the mineralization process is initiated, the mineral content rapidly upregulates over the first few days to 75% of final mineral content, taking up to a year for the matrix to reach maximum mineral content. The important constituent of the mature mineral phase is hydroxyapatite [39].

Matrix mineralization requires, cellular adhesion, and regulation of cell activity for coupling of formation and resorption to occur and for this, the non-collagenous bone matrix proteins play a key role. **Osteocalcin**, one of the most abundant of these proteins, has a prominent role in mineralization, it acts as a chemoattractant, and may be crucial for osteoclast differentiation. Bone sialoprotein (BSP), a highly specific bone protein, has high calcium-binding potential, thus inhibiting mineral deposition.

It also initiates the adhesion of osteoclasts to bone matrix molecules through the key RGD (arginine-glycineaspartic acid) peptide sequence and controls the osteoclast formation. Osteopontin and **osteonectin** too are primary in osteogenic cell activity [39].

The osteoclast activation and differentiation are regulated by three members of the TNF ligand and receptor superfamilies: the osteoclastogenesis

inducers RANKL, RANK, and OPG. RANKL (receptor activator of nF-κB ligand) is a member of the TNF superfamily

Osteoprotegerin (OPG), its decoy receptor, is a circulating protein, produced by a variety of cell types including osteoblasts and marrow stromal cells, which inhibits osteoclast formation by binding mRANKL, thereby preventing the stimulatory cell-to-cell interaction with preosteoclasts and inhibiting RANKL/RANK interactions. Hence, these three proteins are important for osteoclast differentiation directed by osteoblasts, and the balance between RANKL and OPG in osteoblasts directs new osteoclast recruitment [93,100,52].

In periodontal disease, the RANKL is produced by the T cells present in the gingival tissues. Lytic enzymes like TRAP, are present abundantly in the fully matured osteoclast, these enzymes cause the demineralization of the mineralized tissues which leads to bone resorption and activation of the osteoinductive factors like BMP. The Cbfa1 and other genes which initiates osteoblasts activation and differentiation and lead to bone formation are activated by the BMP[51].

The presence of the periodontal pathogen leads to the production of proinflammatory cytokines, such as TNF-α and IL1β, which stimulate expression and activation of matrix metalloproteinases (MMPs) that degrade extracellular connective tissue matrix [51].

Osteoclastogenesis can be independently stimulated by the cytokines such as TNF-α while other cytokines stimulate RANKL expression that leads to formation of osteoclasts and osteoclast activity. The high levels of inflammation and bone resorption are led by the combined innate and adaptive immune responses. These proinflammatory cytokines are thought to produce an amplification loop that contributes to periodontal and peri implants disease progression [51].

BONE TO IMPLANT:

Endosseous dental implants are widely placed in maxillae and mandibles as an alternate for teeth in to restore or replace function in partially or completely edentulous patients. Following the functional loading of dental implants, the process of osseointegration occurs [13].

Various factors such as implant surface, anatomical site, surgical trauma, time of specimen observation plays a pivot role to the tissue response giving rise to osseointegration [131]. When inserting an implant into the ostectomy site a sequence of different biological events occurs at the bone-implant interface until the implant surface is fully covered with a newly formed bone.

In an orchestered manner cell types, tissues, growth factors and cytokines are involved during the inflammatory, formation and remodelling phases of bone healing. This indicates that osseointegration should be

regarded not as an exclusive reaction to a specific implant material but as the expression on the endogenous basic regenerative potential of bone [100].

The first clinical outcome of surgical procedures is the primary stability of the implant, which consists of a rigid fixation of the implant within the host bone cavity together with absence of micro-motion of the implant [39].

A fibrous membrane formation around the implant occurs if there is an excessive mobility present and cause displacement at the bone-implant interface inhibiting osseointegration [32].

A close approximation between implant and host bone may impede osteogenesis because in these areas there is no evidence of early bone formation. In contrast, space more than 500 mm predicts a reduction in the quality or quantity of the newly-formed bone and delay the rate of gap filling[42]. So a high quality bone seems to be important for the primary stability of implant.

MECHANISM OF OSSEOINTEGRATION:

The principle mechanism of the osseointegration around implants are very similar to those occurring during bone fracture repair and involve a series of various cellular and extracellular events [44]. The initial host response after implant placement is characterized by an inflammatory reaction elicited mainly by the inevitable surgical trauma and modified by the presence of the implant.

Immediately after the surgical procedure the bone walls are rapidly covered with blood, hence it is the first tissue coming into contact with the implant surface after the implant is positioned in the bone cavity. Inflammatory cells, primarily polymorphonuclear granulocytes, and later monocytes, emigrate from post-capillary venules and migrate into the tissue surrounding the implant.,

Proteins are adsorbed from blood and tissue fluids, after the blood comes into contact with the implant surface. Oxidation of the metallic implants occurs in both in vitro and in vivo [86]. Then the inflammatory cells respond after stimulation by secretion of proteins with effects on inflammation, bone healing and immune reactions evokes an response by several of the inflammatory cells detected at the interface. The structure and physiochemical properties of the implant surface are altered by these products.

STAGES OF OSSEOINTEGRATION:

Osseointegration follows a common, biologically determined program that is subdivided into **3** stages:

Incorporation by woven bone formation, followed by adaptation of bone mass to load (lamellar and parallel-fibered bone deposition); and then the adaptation of bone structure to load (bone remodelling).

Osteoblasts begin to deposit collagen matrix directly on the early formed cement line/lamina limitans layer described on the implant surface, after a few days of implantation [3]. In the peri-implant environment early bone formation

is the result of an appositional process on the cement line/lamina limitans holding onto the solid surface of the implant.

The osteoblasts cannot always migrate so rapidly to avoid being completely enveloped by the mineralizing front of calcifying matrix and thus they become clustered as osteocytes in bone lacunae. An appositional process on the cement line/lamina limitans holding onto the solid surface of the implant results in the early bone formation in the peri implant environment.

Following the early deposition of new calcified matrix on the implant surface, the arrangement of the woven bone and bone trabeculae developing in three dimensional directions and delimiting marrow spaces occurs [(84)].

This tissue consists of woven bone, cancellous bone or trabecular bone and is particularly suitable for the implant healing process as it shows a very active wide surface area, contiguous with marrow spaces including many vessels and mesenchymal cells [(84)].

The healing calcified tissue which can fill the gap between implant and bone more rapidly, and offer a mechanical resistance to loading thanks to its three-dimensional structure (a network of bone trabeculae arranged in arches) is defined as the trabercular bone. In two different directions peri-implant osteogenesis consists in woven bone and trabecular bone formation proceeds i.e., from the host bone towards the implant surface

(distance osteogenesis) and from the implant toward the healing bone (contact osteogenesis)[73].

Wound maturation around implants occurs like normal and physiological bone adaptation in the skeleton through the modelling and remodelling mechanisms, the two primary mechanisms by which bone at the interface can adapt to mechanical loading, are thought to be accountable for reshaping or consolidation of bone at the implant site. In cortical, as well as in cancellous bone, remodelling occurs in discrete units, often called a bone multicellular unit, as proposed by Frost.

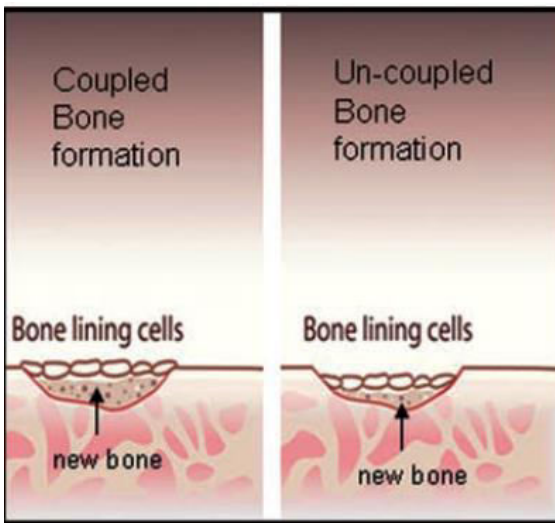
Remodelling starts with osteoclastic resorption, followed by lamellar bone deposition. Resorption and formation are coupled in space and time. This process usually initiates as an outcome of a fatigue damage to bone and involves four different processes: osteoclasts activation, bone resorption, osteoblasts activation, and finally mineralization of new bony tissue [15].

Bone remodelling is a multifaceted process involving not only interactions between cells of the osteoblastic lineage and bone matrix proteins, but also a numerous systemic and local regulatory factor are involved (Dempster, 1995). The cells of bone coordinate their proliferation and activities by the expression and response to hormones (PTH), and cytokines (IGFs, TGF-b1, FGF, BMP, EGF, PDGF, etc) [65].

Remodelling in the third stage of osseointegration contributes to an adaptation of bone structure to load in two ways:

1. It improves bone quality by replacing pre-existing, necrotic bone and/or initially formed, more primitive woven bone with mature, viable lamellar bone.
2. It leads to a functional adaptation of the bone structure to load by changing the dimension and orientation of the supporting elements.

It has been mentioned already that bone remodelling continues throughout life and thus becomes important for the longevity of implants.



BIOMARKERS:

A substance indicating a biologic state is termed as a biomarker. It is defined as, a substance that is measured objectively and evaluated as a measure of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

Detection of disease activity and treatment outcome non-invasively could be the most desirable goal in health cause promotion and delivery. Oral

fluid biomarkers that have been studied for periodontal diagnosis include proteins of host origin (e.g., enzymes and immunoglobulins), phenotypic markers, host cells (e.g., PMNs), hormones, bacteria and bacterial products, ions, and volatile compounds [60].

Because of the complex, complex nature of periodontal disease, it is undesirable that a single biomarker will prove to be a stand-alone measure for periodontal disease diagnosis. More probable the invention of an oral fluid-based diagnostic using a combination of host- and site-specific markers that accurately assess periodontal disease status [60].

There are no dependable tests to diagnose and predict progression of periodontitis, present currently. Although clinical diagnosis of periodontitis based on visual and radiographic assessment in addition to measurement of pocket depth, tissue attachment, and "bleeding on probing" (BOP) in different locations in multiple teeth is well established in dental practice, but they demand time from the periodontist [114].

There is a need for tests that tell the clinician more than the conventional diagnostic tools at his/her disposal, for example, visual changes, clinical assessment (e.g., BOP), and radiographs; these traditional diagnostic procedures give an indication of severity and, therefore, may reflect disease occurred but not current disease activity and they do not identify susceptible individuals who might be at risk of future periodontitis [103].

For effective clinical management of periodontitis, the application of scientific evidence and patient-specific information is now considered to be central [60]. Clinical mismanagement occurs due to lack of evidence-based knowledge of individual patients' disease, for example, failure to identify disease activity and inappropriate antimicrobial therapy.

The need for a dependable biomarker to distinguish progressive periodontitis from normal biological processes is considered essential to identify periodontitis at an earlier or even preclinical stage, to initiate preventative pretreatment, and also to conduct epidemiological studies [60].

For studying bone metabolism in population studies biochemical markers of bone turnover have proved to be a useful, non-invasive and relatively an inexpensive tool and it is also gradually becoming established in clinical practice. However, they are used mainly in monitoring response to treatment.

Increase in the knowledge of the pathophysiology of periodontitis can be obtained due to the continued development of new markers of bone turnover. The continued development of new markers of bone turn over will increase the knowledge of pathophysiology of periodontitis and metabolic bone diseases like osteoporosis. After further evaluation these markers may find a place in the clinical case of post-menopausal women.

For risk assessment and comprehensive screening of biomarkers, new diagnostic technologies such as microarray and microfluidics are available now. For the detection of clinical biomarkers electrochemical biosensors coupled to Magnetic Beads are used. A way for better treatment approaches to periodontal disease can be obtained from the recent advances in biomarkers

Parallel with better understanding of biochemical processes in bone and isolation and characterization of cellular components of skeletal matrix, the number of new potential biochemical markers of bone formation and resorption is found. Generally, markers are classified into the following groups [60].

a. Enzyme activity markers of bone formation (connected with osteoblast activity) and of bone resorption (connected with osteoclast activity);

b. Bone matrix proteins and resorption products of organic skeletal matrix, which are secreted into circulation during bone formation and resorption;

c. Inorganic skeletal matrix markers (calcium, phosphorus which, above all, reflect calcium-phosphorus homeostasis).

The continued development of new markers of bone turn over will enhance the knowledge of pathophysiology of periodontitis and other metabolic bone diseases like osteoporosis. After further assessment these markers may find a place in the clinical case of post-menopausal women

During the past decade a considerable attention have been gained on biomarkers of disease. These markers generally fall into three categories:

1. Indicators of current disease activity;
2. Predictors of future disease progression;
3. Predictors of future disease initiation at currently healthy sites.

The potential biomarkers in the GCF have been grouped into three general categories

- Host-derived enzymes
- Inflammatory mediators and products
- Tissue-breakdown products

For risk assessment and comprehensive screening of biomarkers new diagnostic technologies such as microarray and microfluidics are now currently available. For the detection of clinical biomarkers Electrochemical biosensors coupled to Magnetic Beads are used. These recent advances in biomarkers and diagnostic tools could prove a way for better treatment approaches to periodontal disease

CONNECTIVE TISSUE BREAKDOWN PRODUCTS AS BIOMARKERS

A prospect of determining the metabolic or structural state of the tissue is represented by the identification of tissue-specific structural molecules in oral fluids derived from periodontium.

Mature intermolecular cross links of collagen are pyridinoline and deoxypyridinoline, they are released upon osteoclastic bone resorption and collagen matrix degradation. It is specific to alveolar bone hence it is considered as a potential marker of bone turnover.

Kunimatsu K et al[60] reported that, osteocalcin was absent in the GCF of the gingivitis patient whereas they were predominantly present in the periodontitis patients, thus they were the indicators for the periodontal disease progression.

"On the assessment of the combination of the biochemical markers osteocalcin, collagenase, prostaglandin E2, α2-macro-globulin, elastase, and alkaline phosphatase, a significant diagnostic sensitivity and specificity values of 80% and 91%, respectively, were reported" by **Nakashima et al.** In 1996[94].

Zung P et al[145] postulated that in the presence of active osteoblast bone formation markers are increased though its actual functions are not clear.

Kuminastu et al (1993)[61] demonstrated a positive correlation between amino terminal peptide, GCF osteocalcin and clinical parameters in a

cross sectional study with gingivitis and periodontitis patients. Also, osteocalcin was not detected in patients with gingivitis [61].

Ng PY et al (2007)[95] showed that with bone-loss scores in patients with periodontal disease are inversely proportional to the salivary levels of osteocalcin and osteonectin.

Smith AJ et al (1997)[119] suggested a positive correlation have been made with glycosaminoglycan levels and periodontal pathogens such as *P. gingivalis.*

OSTEOCALCIN AND OSTEONECTIN

Osteocalcin:

Osteocalcin is a non – collagenous calcium binding protein which is present enormously in the calcified tissues.

Osteocalcin, the most abundant non-collagenous protein of mineralized tissues is also called as bone Gla-protein is composed of 15% of non-collagenous proteins. It is a small (5. 4kDa) calcium binding protein of bone and is a protein with a molecular mass of approximately 6kd, containing 49 aminoacids.

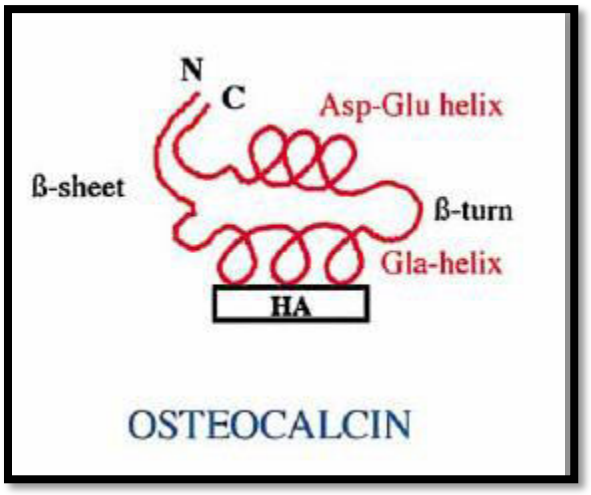
Osteocalcin is predominantly secreted by osteoblasts, odontoblasts and hypertrophic chondrocytes and it plays an important role in both bone mineralization and resorption.

Immunohistochemically demonstrated their presence in the alveolar bone, highly conserved protein that is 5.8-kDa characteristically modified by vitamin K–dependent carboxylating enzymes that convert two to three glutamic acids into g-carboxyglutamic acids (gla groups)

The promoter region of the osteocalcin gene has been extensively characterized for its transcriptional regulation and the tissue-specific expression in which the runt domain transcription factor and osteoblast-specific transcription factor cbfa1 are directly implicated. The gla groups formed on the pro-osteocalcin prior to secretion bind calcium ions strongly increase the affinity of osteocalcin for bone mineral. A predicted spacing of the gla groups in an α-helical conformation corresponds to the 0.545 nm spacing of the calcium ions in the 001 plane of the hydroxyapatite crystal.

It involves in recruiting osteoclasts to sites of newly formed bone and function as a regulator. During periods of rapid bone turnover serum osteocalcin levels are increased. A valid marker of bone turnover when resorption and formation are coupled is osteocalcin and also a specific marker of bone formation when formation and resorption are uncoupled.

Osteocalcin and osteocalcin fragments are likely to be released from the extracellular matrix into the GCF, during active bone resorption.



A pathology in bone metabolism can be determined by the changes in the measure of the bone protein and thus osteocalcin can be used as a potent clinical diagnostic marker **[Ram VS et al, 2015]** [110].

Kunimatsu K et al[60] reported that, osteocalcin was absent in the in the GCF of the gingivitis patient whereas they were significantly present in the periodontitis patients, thus indicates their role as predictors for the periodontal disease progression.

Studies have reported that the levels of osteocalcin are proportional to the disease progression. Several studies reveal about the role of osteocalcin in PCF of subjects with periodontitis [21–23]. Using a limited number of endosseous implants, **Murata et al.**[91] presented that osteocalcin in the GCF may reflect an enhanced bone turnover around oral implants, and PISF

osteocalcin levels in sulcular fluid of implants with gingivitis were significantly upregulated than the healthy ones.

Nakashima et al. In 1996[94] reported a "significant GCF osteocalcin levels from gingivitis and periodontitis patients." "On evaluation of a combination of the biochemical markers osteocalcin, collagenase, prostaglandin E2, α2-macro-globulin, elastase, and alkaline phosphatase, enhanced diagnostic sensitivity and specificity values of 80% and 91%.

Masashi Murata [91] stated that increased levels of GCF osteocalcin from periodontitis sites may be related to the sensitivity of alveolar bone resorption and/or repair. PISF osteocalcin levels showed a tremendous difference between peri-implant mucositis and healthy implants, although there were no significant differences between peri-implantitis and other sites.

Murata et al.[91] also reported that osteocalcin was a factor of osteoblast activity and bone formation as well as bone resorption, and serum osteocalcin levels achieved from peri implantitis sites, peri-implant gingivitis (mucositis) sites and peri-implant healthy sites did show any significant difference and all osteocalcin serum levels were in normal limits. Osteocalcin seemed to be a more consistent marker of oral implant bone destruction.

Onder gurluk[43] said that smoking seems to decrease salivary osteocalcin level but ICTP levels were not affected by smoking. This suppression in OC levels may be one mechanism of deteriorating effects of smoking on periodontal health. the serum concentration of OC could also be

used as a marker for bone formation (Price 1985; Hoffmann et al. 1996). This was also confirmed in this study, where serum concentrations of OC were higher in patients taking Pamidronate has alveolar remodelling where higher in such patients.

Cakal OT[17] said that Soft tissue inflammation around dental implants does not cause a variation in osteocalcin, osteopontin, and osteonectin levels in PISF. Also, peri-implantitis does not seem to give rise to an increase in PISF levels of osteocalcin, osteopontin, and osteonectin.

Through the results of various studies in the past it is determined that the alveolar bone extracellular matrix proteins ostenectin and osteocalcin are involved in bone remodelling and in osteogenesis.

Osteonectin:

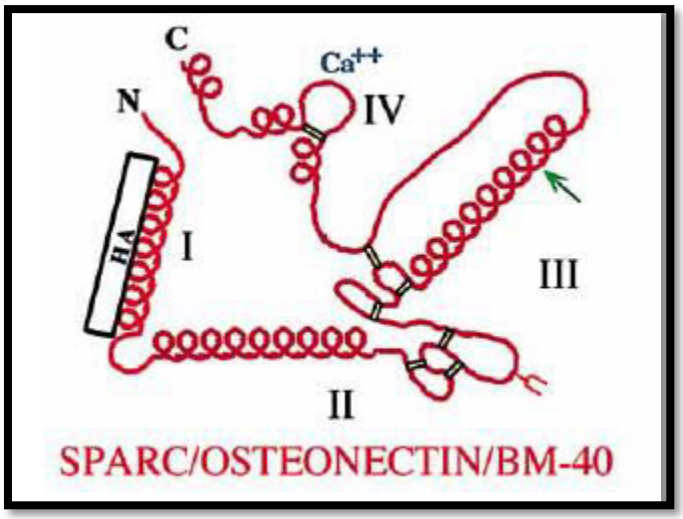
The principal extracellular matrix (ECM) protein in cementum, bone, and PDL is the type I collagen, an appreciation of cellular mechanisms that control collagen assembly and deposition in these tissues is important to improve treatments that enhance regeneration. For the formation of collagen fibrils and fibers collagen undergoes postsynthetic processing in the extracellular space [110].

For the collagen fibril synthesis, collagen fiber assembly and the deposition of collagen into the ECM collagen-binding proteins are essential. **Secreted Protein Acidic and Rich in Cysteine (SPARC)**/Osteonectin is a collagen binding protein with a profound effect on ECM structure and content.

It is also referred to as **basement membrane protein (BM-40).** Osteonectin is a single-chain polypeptide that binds strongly to hydroxyapatite and other extracellular matrix proteins including collagens. Osteonectin is composed of 25% of the non-collagen proteins.

The gene encoding SPARC/osteonectin is predicted to produce a 33-kDa protein which further undergoes post translational glycosylation so that secreted SPARC/ osteonectin protein is 43 kDa in most tissues. SPARC/ osteonectin is a matricellular protein, that binds to collagen through the Ca21 extracellular domain in the C terminal portion of the protein [(110)].

Osteonectin is a **de-adhesion matricellular protein** which aids the cell to move from a state of strong adherence to a state of weak adherence, which is also deemed necessary as this feature helps in the migration of the cells towards the implant, which in turn aids in the osseointegration and bone remodelling. Osteonectin is one such important matricellular protein that aids the cells to stay in spreaded morphology, antagonizing the pro-adhesive activity of other matrix proteins, which is very essential for cell proliferation occurring during any cellular injury [(93)].



During embryogenesis they are widely expressed, and in vitro studies have suggested their roles in the control of cell adhesion and proliferation, and in the modulation of cytokine activity. SPARC can bind to thrombospondin 1 and collagen, although the collagen-binding site is cryptic, lying between two a-helices and exposed by proteolysis [17].

Studies of primary fibroblasts reveals that the osteonectin in pericellular processing of procollagen, and a function in collagen turnover at the cell surface has been proposed. Both a collagen-binding domain and an hydroxyapatite binding region are resent in osteonectin.

Osteonectin has been implicated in the early phases of tissue mineralization because of its affinity for collagen and hydroxyapatite. In the osteoid, SPARC has been proposed to bind to the collagen and hydroxyapatite

crystals and they release calcium ions perhaps enhancing mineralization of the collagen matrix in bones [11].

In addition to collagen binding, SPARC have been attributed to several additional activities. For example, the extracellular domain was shown to regulate **anti proliferative and counter adhesive activities** in cells in culture (2;3; 4; 5). Interactions between the cell and ECM is modulated by the osteonectin and they also influence the efficacy of certain growth factors [6].

Expression of osteonectin is found to be more significant during the development of the tissues, and re-expression of SPARC/osteonectin is frequently associated with ECM remodelling events such as wound healing [7].

Studies have shown that the activity of osteoblasts and osteoclasts are regulated by osteonectin; the two primary cell types regulating bone homeostasis and repair. Osteoblasts undergoing active matrix deposit express osteonectin.

Levels of osteonectin protein are at the peak at times coinciding with initial stages of differentiation and subsequently they down regulate as cells mature and begin to express mature osteoblastic markers.

Ram VS et al, 2015[110] have reported that the osteonectin, a non-collagenous protein in the bone matrix, aids in the cell matrix interaction in the remodelling tissues and it is produced by the osteoblast, endothelial cells and fibroblast.

Zung P et al[145] postulated that it is a bone formation marker enhanced in the presence of active osteoblast though its actual functions are not clear.

In a cross-sectional study by **Bowers *et al***[14]. From patients with gingivitis, at moderate or severe periodontal disease states GCF's samples were analyzed. Using a dot blot assay, it was analyzed that both osteonectin and N-propeptide alpha I type I collagen were upregulated in patients with periodontal disease.

In addition, the sites where the probing depth measures were increased, there was an elevation in the protein concentration in the GCF [3] .At the final analysis of this study, in comparison with the N-propeptide alpha I type I collagen ,osteonectin appeared to be the more sensitive marker for detection of periodontal disease status.

GCF:

In 1899, Black was the first to report about the presence of a fluid in the periodontal sulcus. But, the exact nature of the fluid, its origin, and its composition has been controversial. **Brill and Egelberg s**uggested that due to an increase in the permeability of the vessels underlying the junctional and sulcular epithelium GCF was produced, which might occur due to inflammation, trauma, or mechanical stimulation.

Later, **Alfano**[5] suggested that the GCF as the initial transudate fluid produced due to the presence of highly osmotic substances in the gingival sulcus (bacterial products, plaque).

This hypothesis was supported by **Pashley**[102], who suggested that when the rate of capillary filtrate exceeds that of lymphatic uptake, fluid will accumulate as edema and/or leave the area as GCF. The filtration coefficients of the capillary endothelium and the osmotic pressure within the different compartments are the factors which alter the process.

GCF is a serum transudate in a healthy state, and in pathological condition, a more inflammatory exudate from the vessels of gingival plexus and it is the part of gingival defence system, rich in leucocytes polymorpho nuclear neutrophils which is chemotactic gradient of bacterial/host origin.

It also has host-derived molecules from blood, micro-organisms from bacterial plaque (**Ahlo-2007**)[63]**.** Ozkavaf et al stated that the GCF flow and volume increases during inflammation.

In healthy sites, GCF flow is 3µl/h and 20µl/h for intermediate pockets and 44µl/h for deep pockets [27].

Ng et al[95] analyzed salivary biomarkers related with alveolar bone loss. They found that several individual biomarkers were significantly related with bone loss score (IL-1β, IL-6, PGE2, osteonectin, and osteocalcin) but, multivariate analysis revealed that IL-1β and osteonectin were the only two biomarkers studied were increased in the bone loss.

GCF continuously flush the dentogingival crevice and secrete the antimicrobial components of serum such as antibodies and complement enzymes. In disease, the crevicular fluid flow increases by 30 times more than in health.

The GCF is a reliable tool in active phase of the destructive disease**.** The presence of alkaline phosphatise, α glucoronidase, interleukins and prostaglandins are specific indicators for periodontal disease progression. [Bang et al 1970] and some enzymes are detected much earlier, before the diseased is established clinically.

PISF

The long-term success of an osseointegrated implants depends on various factors which maintains the integrity of the peri-implant tissue**.** **[James and lozada 1989]**[134]**.** Anatomically, environmentally and functionally the peri implant sulcus is similar to the periodontal sulcus [**berglundh et al 1991**][8] and provides a medium for microbial colonisation. Peri-implant crevice is created surgically and the pocket depth is determined by various factors like abutment height, depth of fixture countersinking at stage-I surgery, the amount of tissue thinning during stage-II surgery. The reversible inflammation in response to the plaque accumulation is termed as periimplant mucositis, whereas the peri implantitis is irreversible

inflammatory reaction which involves the supporting bone around a functional implant. Thus, these peri implant diseases are recorded clinically by probing depth, gingival index, mobility and bleeding on probing and by radiographs**. [Albrektsson et al 1986]**[(1)]**.** But they do not explain either the disease progression or the current disease activity, they only infer the extent of the peri implant tissue lost**. [Mombelli and Lang 1994]** [(89)]**.**

Though the radiographs can represent the bone loss, but they are seen only in the later stages of demineralization, thus the initial stages of the disease could not be screened. While looking upon the clinical evaluation like the probing depth and bleeding score, they depend on the skill of the examiner and could not be relied. **[Errricson and Lindhe,1994]**[(9)]**.** Moreover, the nature of the peri implant tissue varies from the periodontal tissue which allows the entry of the probe apically easily. [**Mombelli et al 1997**] [(88)]. The bleeding on probing is not specific to the inflammation in the peri implant tissue**. [erricson and lindhe]**[(9)]**,** whereas the absence of the bleeding can be inferred has a clinical indicator for the peri-implant stability. Thus, simple and reliable clinical tests are required to detect the disease progression in the initial and reversible stages. **[Mombelli and Lang 1994]**[(94)]**.**

Curtis et al. stated that "markers of disease" might encompass three separate categories:

1. indicators of current disease activity;
2. predictors of future disease progression;
3. predictors of future disease initiation at currently healthy sites.

The Peri implant Sulcular Fluid (PISF) is an osmotically mediated inflammatory exudates, it is upregulated in volume during inflammation and capillary permeability. They also detect the osseointegration and bone resorption to the occlusal loading of the prosthesis [Last et al]. As they present with abundant components in them they can be used to detect the disease in the initial and reversible stage **[lang et al]**[28].

Thus, our study is to determine the biological changes occurring of bone biomarkers osteocalcin or ostenectin around healthy gingival tissue and peri implant tissue.

RATIONALE FOR GCF / PISF SAMPLING

Various cellular and biochemical molecules associated with disease activity are present in GCF. Sampling of crevicular fluid has advantages that they are equivalent to drawing of blood. Its advantages are the site specificity, non-invasiveness, and comparatively easy to obtain. GCF an inflammatory exudate which reflects the ongoing events in the periodontal environment that produce it.

GCF / PISF SAMPLING METHODS

Using a variety of methods the collection of GCF can be accomplished, each with distinct advantages and disadvantages. Based on the objective of the study, the method is usually selected. For the collection of GCF three techniques are commonly used.

- Use of absorbing paper strips / points
- Microcapillary pipettes
- Intra crevicular washing

Absorbing paper points / strips

Brill and Krasse(1958)[100]were the first to use filter paper strips for the collection of crevicular fluid. These paper strips are either placed within the sulcus (intrasulcular method) or at the entrance (extra sulcular method). By using filter paper strips volume of GCF collected can be determined by various methods -

- Linear measurement of fluid migration on strip
- Weighing the strip
- Use of stains
- Ninhydrin
- Fluroscein administration to patient 2 hours before the GCF/PISF collection and strips are examined under UV light
- Electronic measuring devices –Periotron, which allows quick and accurate measuring of GCF volume.

Microcapillary method

The use of fixed volume micropipette permits the collection of fluid by capillarity. As the internal diameter is known, the volume of GCF collected

can be accurately determined by measuring the distance which the fluid has migrated.

Gingival washing method

In this method, an isotonic solution such as Hank's balanced solution is perfused into the sulcus, usually a fixed volume or using a custom made acrylic stent which isolates gingival tissues from rest of the mouth. The fluid obtained represents the dilution of crevicular fluid. For harvesting cells from the gingival crevice this technique is more valuable.

The crevicular fluid is collected by the above methods, depending on the analyte under investigation. The filter paper is used commonly for enzymes assays and microcapillary pipette for analysis of connective tissue metabolites.

LIMITATIONS OF GCF SAMPLING

1. Contamination

Blood, saliva or plaque can contaminate the GCF, which in turn affect both volume and quality of the substance sort out for in the fluid.

2. Sampling time

While using filter paper strips it has to be left in place for 5 seconds. On the other hand, it is said that collecting a minimum volume has can take upto 20-30 min.

3. Volume determination:

The fluid from the filter paper evaporates, which is the primary problem, as the volume collected usually would be less than 1 µl.

More powerful diagnostic tools for clinicians to optimize their treatment predictability are made possible through the recent advances. Though there are many difficulties, the use of oral fluid based diagnostics appears to be a promising tool for diagnosing periodontal disease and to prognosticate periodontal treatment outcomes.

Materials and Methods

MATERIALS AND METHODS

24 patients recruited from pool of patients referred for periodontal treatment to the Department of Periodontics, Ragas Dental College, Chennai of which 12 patients who were followed 6 months post loading of endosseous implants were included in the study. Patients were explained regarding the study procedure and written informed consent was obtained from those agreed to participate in this study. The patients were informed that this research work was in no way directly related to the therapy or cure of the disease. The study was undertaken following approval from the institutional review board.

The patients were selected on the following criteria:

Selection criteria:

Group A: comprises of subjects exhibiting no signs of periodontal disease: absence of bleeding on probing; PPD ≤3mm and no clinical and radiographic evidence of attachment or bone loss.

Group B: comprises of subjects with implant placed and loaded before 6months exhibiting no signs of peri implant disease: absence of bleeding on probing and Peri implant pocket depth ≤4mm and no clinical and radiographic evidence of bone loss beyond the first thread of the implant.

INCLUSION CRITERIA

- Age group between 20-65years of both genders
- Systemically healthy individuals
- Patient who are highly compliant and willing to participate and follow up overtime period

EXCLUSION CRITERIA

- Presence of active periodontal disease and
- Patient with any systemic disease and patients who are under medication that interfere with the final treatment outcome
- Patient with known risk factor and risk modifiers which can influence the overall outcome of the treatment were excluded from the study
- Pregnant and lactating women
- Patient on antibiotic therapy for the past 6months
- Patient on radiotherapy

CLINICAL EVALUATION

Clinical evaluation was done using mouth mirror, William's periodontal probe and plastic probe with William's markings. PDs were measured at six locations per tooth (mesial-buccal, midbuccal, distal-buccal, mesial-lingual, mid-lingual, and distal-lingual) using a probe. The probing depth and bleeding on probing was evaluated for tooth and implant site.

GINGIVAL CREVICULAR FLUID COLLECTION

The supragingival plaque was removed from the selected site, using an ultrasonic scaler tip, without touching the marginal gingiva to avoid contamination and blocking of microcapillary pipette. The site was then air dried and isolated with cotton rolls. GCF was collected from sites using 1- 5µL calibrated volumetric microcapillary pipettes by placing the tip of the pipette extracrevicularly (unstimulated) for 5-20 min, a standardized volume of 5µL GCF was collected using the calibration on the micropipette [(11)]. The collected GCF was immediately transferred to eppendrof vials containing 200µL of elutionbuffer (50mM TrisHCl, 0.2M NaCl, 5mM CaCl2 & 0.01% Triton X-100 at pH 7.5) and stored at -70°C until the time of assay.

PERI IMPLANT SULCULAR FLUID COLLECTION

The implant sites were then air dried and isolated with cotton rolls. PISF was collected from sites using 1- 5µL calibrated volumetric microcapillary pipettes by placing the tip of the pipette extracrevicularly (unstimulated) for 5-20min, a standardized volume of 5µL PISF was collected using the calibration on the micropipette. The collected PISF was immediately transferred to eppendrof vials containing 200µL of elution buffer (50mM TrisHCl, 0.2M NaCl, 5mM $CaCl_2$& 0.01% Triton X-100 at pH 7.5)and stored at -70°C until the time of assay[**(90,108)**].

Armamentarium

- 5µl calibrated volumetric microcapillary pipettes (Sigma Aldrich Company)
- Autoclavable Eppendrof tubes (2mL)
- Cotton rolls
- Double sided cheek retractors
- Refrigerator
- Microtiter plate reader fitted with appropriate filters
- 10, 50 and 100 adjustable single channel micropipettes with disposable tips

REAGENTS:

Sandwich Elisa Kits

1. **Bioassay Technology Laboratory** Human Osteocalcin/Bone Gla Protein ELISA Kit
2. **Bioassay Technology Laboratory** Human Osteonectin ELISA Kit

Principle of the method

The Osteocalcin and the Osteonectin ELISA kit used in the study is a solid phase sandwich ELISA for the in-vitro qualitative and quantitative determination of proteins in oral or biological fluid. A capture Antibody highly specific for the protein to be detected has been coated to the wells of the microtiter strip plate provided during manufacture. Binding of protein samples and known standards to the capture antibodies and subsequent

binding of the biotinylated a secondary antibody to the analyte is completed during the incubation period. Any excess unbound analyte and secondary antibody is removed. The HRP conjugate solution is then added to every well including the zero wells, following incubation excess conjugate is removed by careful washing. A chromogen substrate is added to the wells resulting in the progressive development of a blue coloured complex with the conjugate. The colour development is then stopped by the addition of acid turning the resultant final product yellow. The intensity of the produced coloured complex is directly proportional to the concentration of the protein present in the samples and standards. The absorbance of the colour complex is then measured and the generated OD values for each standard are plotted against expected concentration forming a standard curve. This standard curve can then be used to accurately determine the concentration of protein in the sample tested.

ELISA KIT CONTENTS

Osteocalcin kit included 96 well microtiter strip plate, plastic plate covers, standard (100pg/ml), standard diluent, biotinylated human OT/BGP antibody, streptavidin-HRP, Wash buffer, substrate solution A, substrate solution B and stop reagent.

Osteonectin kit included 96 assay plates, standard solution, biotinylated human ON antibody, streptavidin-HRP, substrate solution A, substrate solution B, wash buffer and stop solution.

ASSAY preparation for Osteocalcin ELISA Kit- For GCF and PISF samples:

The number of microwell strips required to test the desired number of samples and appropriate number of wells needed for running zeros and standards were determined and the wash buffer and standard diluent buffer for IL-6 were prepared according to the instruction given by the manufacturer.

Preparation of the standard for Osteocalcin:

Standard vials must be reconstituted with the volume of standard diluent shown on the vial immediately prior to use. 120µl of the standard (160ng/ml) with 120µl of standard diluent should be reconstituted to generate a 80ng/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (80ng/ml) 1:2 with standard diluent to produce 40ng/ml, 20ng/ml, 10ng/ml and 5ng/ml solutions. Standard diluent serves as the zero standard. Any remaining solution should be frozen at -20 and used within one months

Preparation of wash water

Wash Buffer Dilute 20ml of Wash Buffer Concentrate 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

PREPARATION OF STREPTAVIDIN -HRP

5 µl of streptavidin is mixed with 0.5 mL of HRP diluent immediately before use. Further the HRP solution to volumes appropriate for the required number of wells is diluted in a clean glass vial.

METHOD

Assay procedure for osteocalcin

S.No	Assay steps	Details
1	Addition	Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use.
2	Addition	Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3	Addition	Add 50µl standard to standard well.
4	Addition	Add 40µl sample to sample wells and then add 10µl anti-OT/BGP antibody to sample wells, and then add 50µl streptavidin-HRP to sample wells and standard wells. (Not blank control well) .
5	Incubation	Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
6	Wash	Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
7	Addition	Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°Cin the dark.
8	Addition	Add 50µl Stop Solution to each well, the blue colour will change into yellow immediately.
9	Determination	Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 30 min after adding the stop solution.

Osteonectin reagent preparation

All reagents should be brought to room temperature before use.

Standard reconstitute the 120µl of the standard (24ng/ml) with 120µl of standard diluent to generate a 12µg/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (12µg/ml) 1:2 with standard diluent to produce 6µg/ml,0.75µg/ml solutions. Standard diluent serves as the zero standard. Any remaining solution should be frozen at -20°C and used within one month.

Preparation of wash water

Wash Buffer Dilute 20ml of Wash Buffer Concentrate 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

PREPARATION OF STREPTAVIDIN -HRP

5 µl of streptavidin is mixed with 0.5 mL of HRP diluent immediately before use. Further the HRP solution to volumes appropriate for the required number of wells is diluted in a clean glass vial.

METHOD

Assay procedure for Osteonectin:

S.No	Assay steps	Details
1	Addition	Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2	Addition	Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3	Addition	Add 50µl standard to standard well. Note: Don't add antibody to standard well because the standard solution contains biotinylated antibody.
4	Addition	Add 40µl sample to sample wells and then add 10µl anti-ON antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells. Not blank control well.
5	Wash	Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
6	Addition	Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°Cin the dark.
7	Addition	Add 50µl Stop Solution to each well, the blue colour will change into yellow immediately.
8	Determination	Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 30 min after adding the stop solution.

STATISTICAL ANALYSIS:

The Osteocalcin and Osteonectin results obtained were analysed statistically using the Mann-whitney U test and the independent t-test. P value ≤ 0.05 was considered to be statistically significant.

Photographs

Fig. 1: HEALTHY GINGIVA (GROUP A)

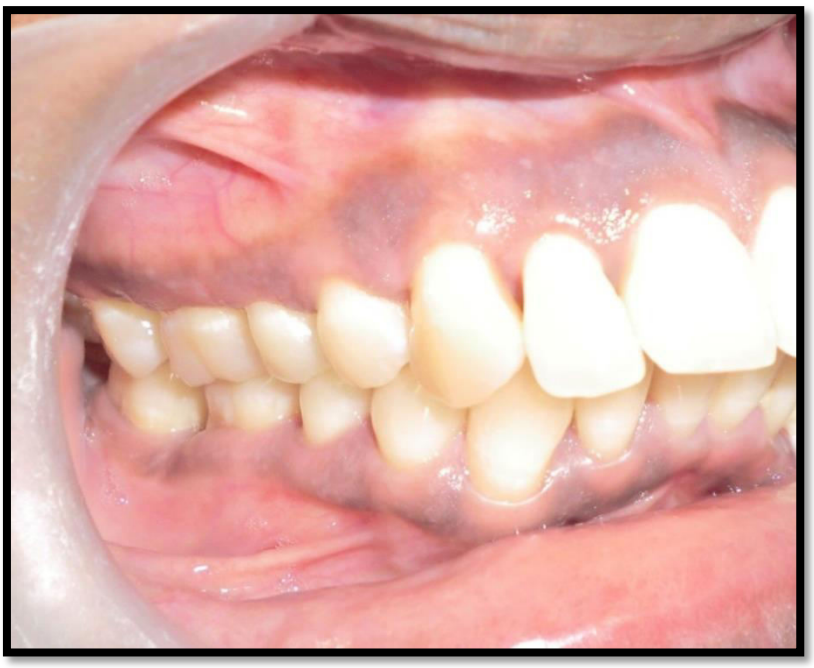


Fig. 2: HEALTY PERI-IMPLANT SITE (GROUP B)

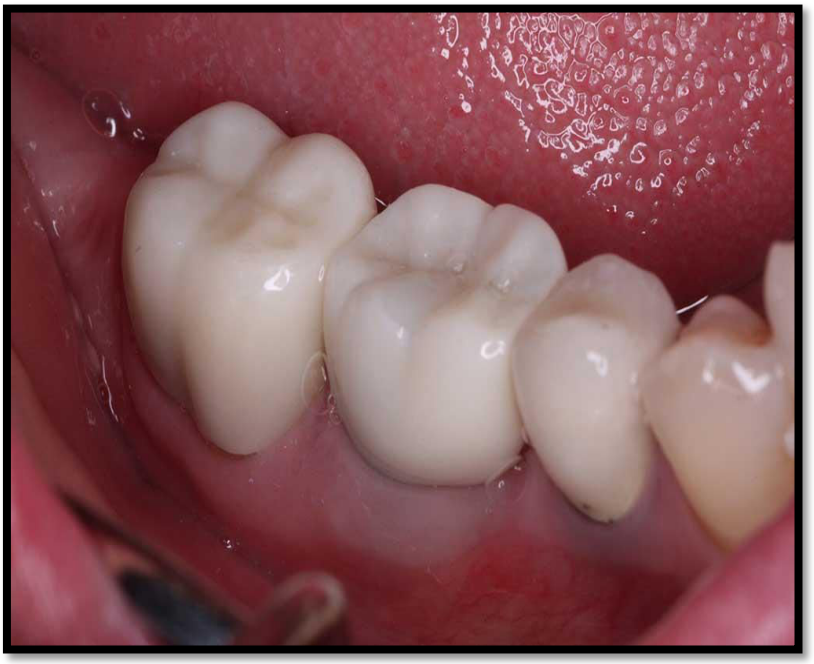


Fig. 3: ARMAMENTARIUM FOR GINGIVAL CREVICULAR FLUID COLLECTION

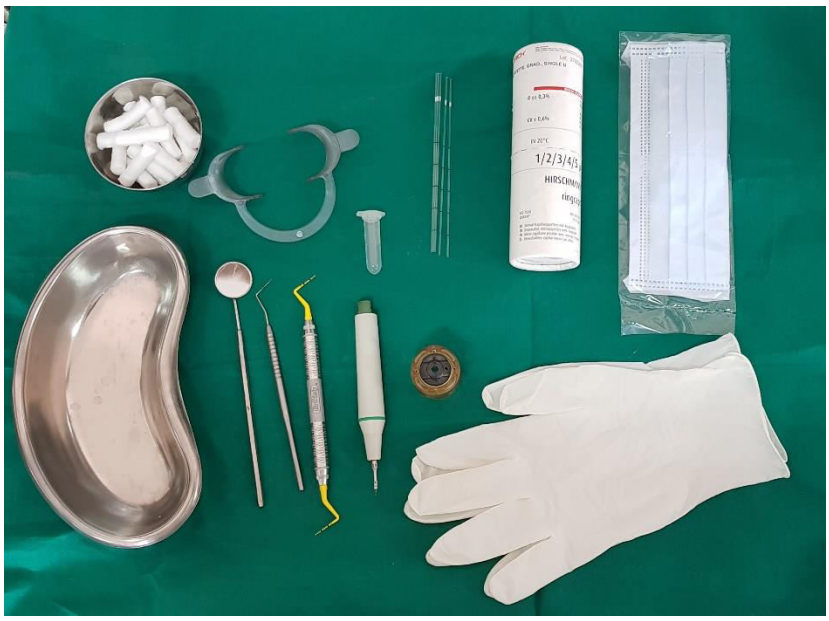


Fig. 4: MICROCAPILLARY PIPETTES

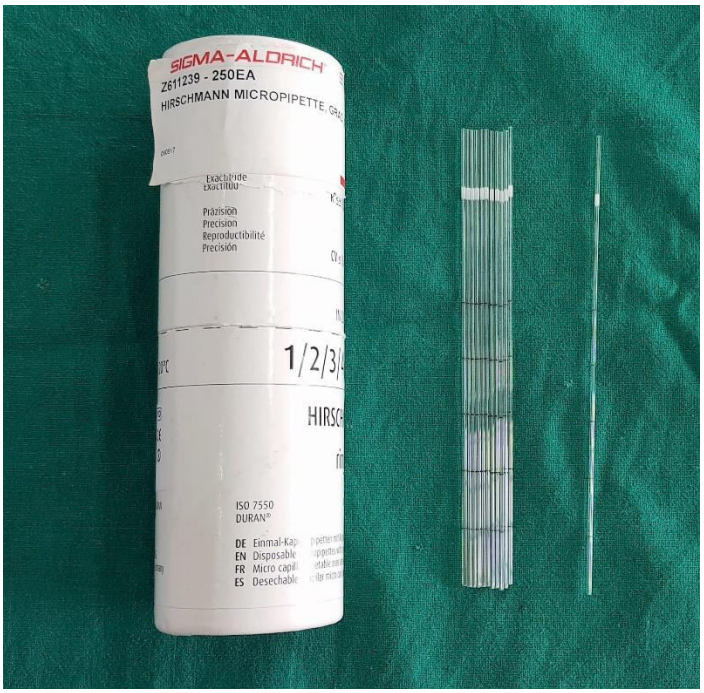


Fig. 5: GCF COLLECTION USING MICRO-PIPETTE (GROUP A)

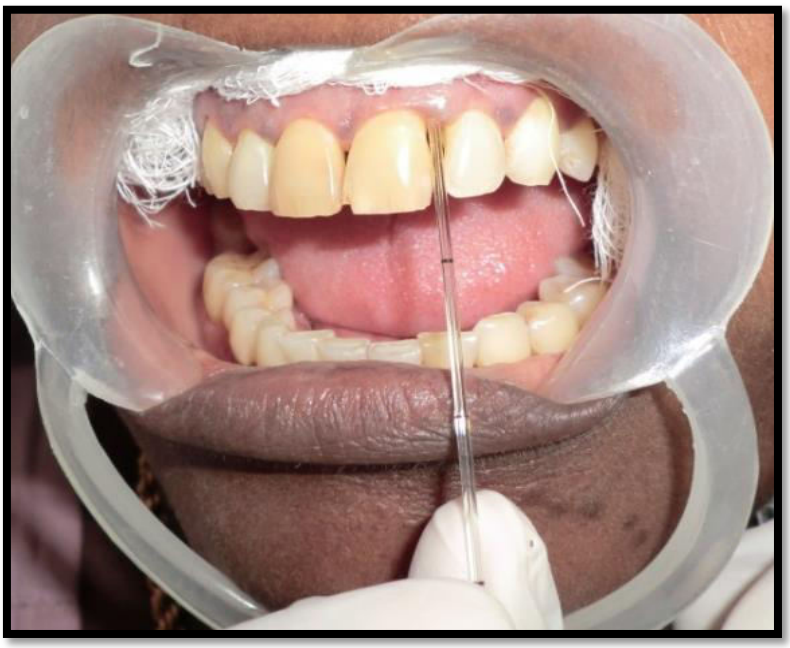


Fig. 6: PISF COLLECTION USING MICRO-PIPETTE (GROUP B)

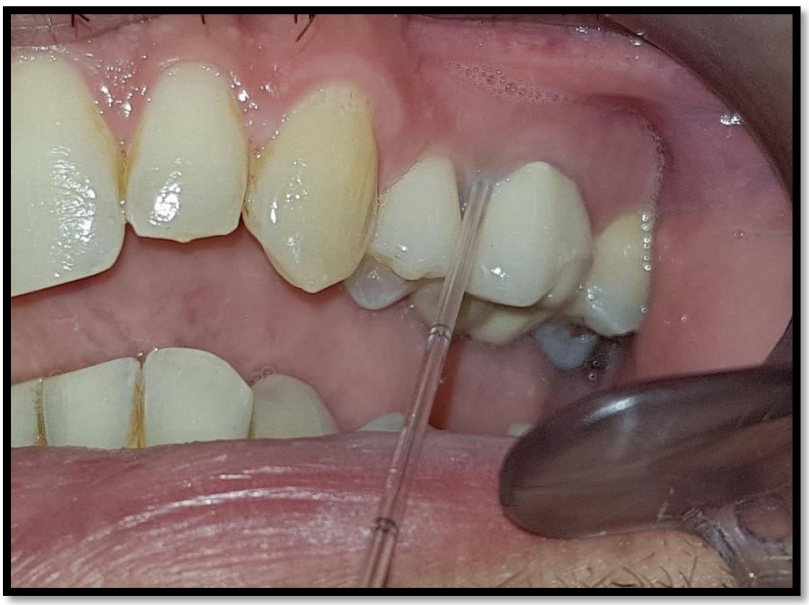


Fig. 7: ELISA KIT FOR OSTEOCALCIN

(Bioassay Technology Laboratory)

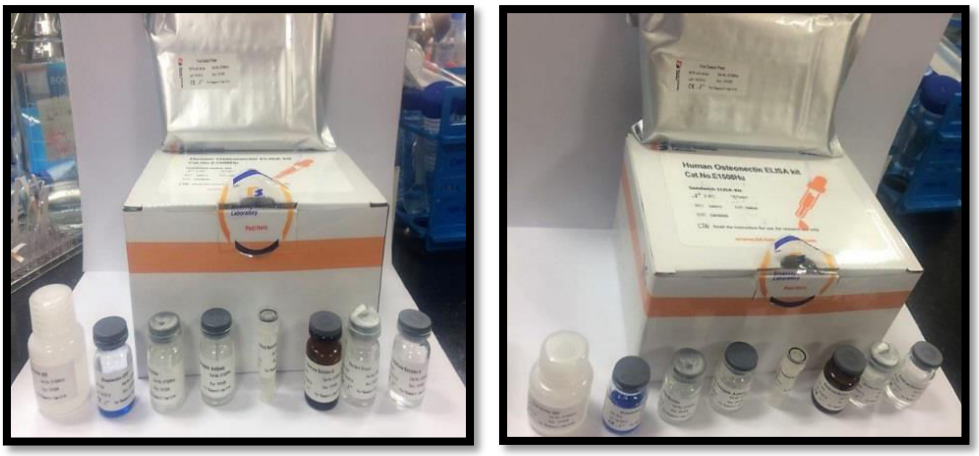


Fig. 8: ELISA KIT FOR OSTEONECTIN

(Bioassay Technology Laboratory)

Fig. 9: ARMAMENTARIUM FOR ELISA

Fig. 10: ELISA AUTOMATED WASHER

Fig. 11: ELISA READER

Fig. 12: COMPUTER

PROCEDURE

Fig. 13: MICRO PLATE WELL DURING PROCEDURE

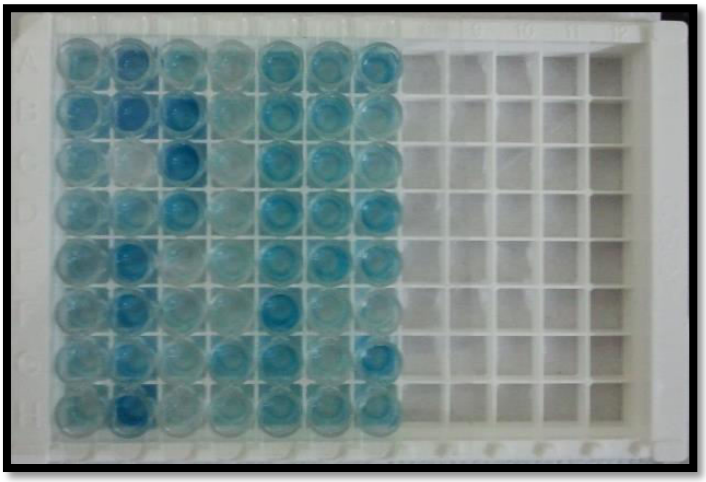
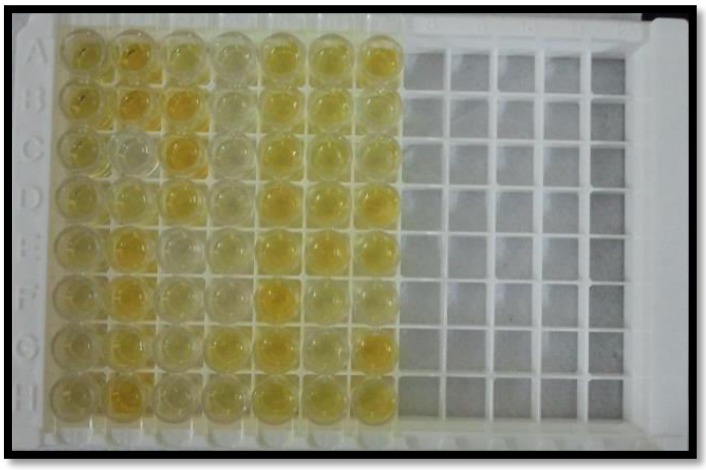


Fig. 14: MICRO PLATE WELL AFTER STOP REAGENT



Results

RESULTS

The present study was done to evaluate the levels of osteocalcin and osteonectin in GCF in patients with healthy periodontal tissues and in the peri implant sulcular fluid in implant sites 6 months after loading.

12 GCF samples and 12 PISF samples were collected from the patients. Osteocalcin and Osteonectin levels were assessed using sandwich ELISA technique. Absorbance was measured at 450nm as primary wavelength in terms of pg/ml, and the results were obtained.

Descriptive statistics included were the mean, standard deviation, minimum (min) and maximum(max).

Evaluation of GCF and PISF levels of Osteocalcin in periodontal health

The levels of osteocalcin in the GCF of clinically healthy periodontium and in the PISF in peri implant health six months after loading in healthy subjects were evaluated.

There was a statistically significant increase in the osteocalcin levels in peri-implant fluid when compared to health. (Table 1)(Graph 1)

Since the data did not follow the normal distribution, the mean Osteocalcin levels in GCF and PISF was assessed using Mann Whiteny U test (Non-Parametric Data).

The mean value of osteocalcin in 12 healthy subjects was observed to be 453.22 and the standard deviation was 205.19. In patients who underwent dental implants, the peri implant sites had a mean value of 2923.50 with a standard deviation of 2152.80. This gave a p value of 0.001 which was highly statistically significant.

Evaluation of GCF and PISF levels of Osteonectin in periodontal health

The levels of osteonectin in the GCF of clinically healthy gingiva on an intact periodontium and in the PISF in peri implant health six months after loading in healthy subjects were evaluated.

There was an increase in the osteonectin levels in peri-implant fluid when compared to health but there was no statistically significant difference. (Table 2) (Graph 2)

The mean Osteonectin levels in GCF and PISF was assessed using independent 't' test.

The mean value of osteonectin in 12 healthy subjects was observed to be 268.92 and the standard deviation was 81.40. In patients who underwent dental implants, the peri implant sites had a mean value of 369.67 with a standard deviation of 255.07. This gave a p value of 0.206 which was not statistically significant.

Tables and Graphs

Table 1.Mean Osteocalcin levels in the GCF and PISF in periodontal health using Mann Whiteny U test (Non-Parametric Data)

	GRP	N	Mean	Std. Deviation	P
OSTEOCALCIN	HEALTHY PERIODONTIUM	12	453.22	205.19	0.001
	PERI-IMPLANT HEALTH	12	2923.50	2152.80	

Graph 2: LEVELS OF OSTEOCALCIN

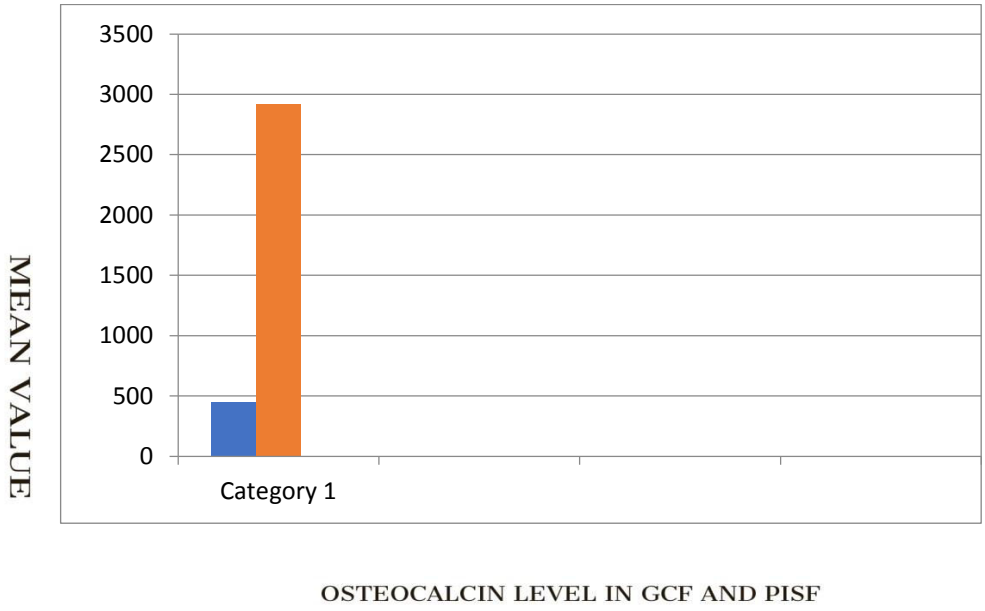
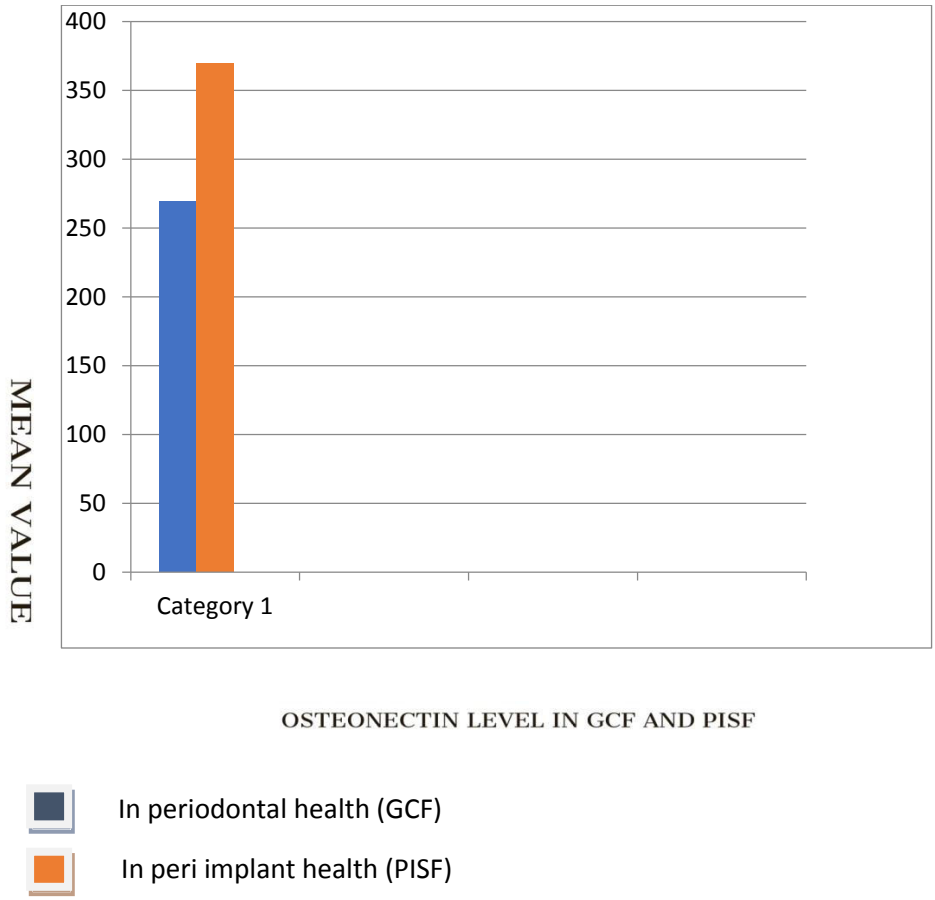


Table 2.Mean Osteonectin levels in the GCF and PISF in periodontal health using using independent T test

	GRP	N	Mean	Std. Deviation	P
OSTEONECTIN	HEALTHY PERIODONTIUM	12	268.92	81.40	0.206
	PERI-IMPLANT HEALTH	12	369.67	255.07	

Graph 1: LEVELS OF OSTEONECTIN



Discussion

DISCUSSION

Physiological bone remodeling is a continually occurring process that occurs in a highly coordinated manner. This cycle operates continually as osteoclasts are constantly removing mature bone, with new bone simultaneously formed by osteoblasts [129].

A balance between the coupled processes of bone resorption by osteoclasts and bone formation by osteoblasts is essential for maintaining periodontal homoeostasis [16,128,129].

The overall quality and quantity of bone will be affected by any factor that influence either of these processes or perturb this balance. The hallmark of periodontal disease is alveolar bone loss and this occurs as a result of imbalance in the coupling/uncoupling mechanisms[50]. Similarly, when an implant is placed, it involves of a series of events at the bone to implant interface for osseointegration to occur. Osseointegration is defined as a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant, it is critical for implant stability, and is considered a prerequisite for implant loading and long-term clinical success of end osseous dental implants[87].

Like periodontal health, the peri implant health around implants is also maintained in homeostasis via modeling and remodeling mechanisms[28]. These processes are the two primary mechanisms by which bone at the

interface can adapt to mechanical loading, and thus are responsible for reshaping or consolidation of bone at the implant site[28].

Healing around an implant can be divided into two phases; namely the early and late phases. Upon placement of an implant, there is a response to a foreign material, namely, protein adsorption, platelet activation, clotting and inflammation. Early healing produces immature woven bone that fills the gap between the implant and bone via contact and distance osteogenesis, the later stages of healing comprise of the conversion of woven into lamellar bone[73]. The remodeling of the peri implant bone continues for the life of the implant and is a complex process which involves the interactions between cells of alveolar bone and extracellular matrix components.

The diagnostic methods employed should provide the relevant information which aid to differentiate between different stages of periodontal disease, the degree of the destruction and the prognosis of the disease. The conventionally available clinical methods to evaluate the periodontal status like bleeding on probing, clinical attachment level and radiographs can only predict the destruction occurred previously but not the active disease progression[99]. Similarly, in case of implant sites they also pose challenges like the force and direction of probing which differs between the individuals performing, implant and tooth morphology and the biotype of the surrounding tissues. Thus, there is considerable interest in developing diagnostics using the GCF and PISF as medium.

A biomarker can be defined as an indicator of a biological state and can be used to distinguish between normal and pathologic processes [65]. One of the main advantages of evaluating biomarkers from biofluids is the repeatability and non-invasive nature of obtaining samples for analysis. GCF represents a transudate of gingival tissue interstitial fluid in health, but in the course of gingivitis and periodontitis, it is transformed into a true inflammatory exudate. Presence and functions of proteins, especially enzymes in GCF were first explored by **Sueda, Bang and Cimasoni**[121]. It was soon understood that enzymes released from inflamed periodontal tissue possessed an enormous potential for periodontal diagnosis. Similar expectations have been reported with the PISF obtained from the implant sites[31].

GCF and PISF from which the biomarkers are detected, which are the objective and measurable tools characteristic of the biological processes and are in close proximity to the inflamed sites [66]. They also seem to be influenced more by the local reactions than the systemic inflammatory processes and thus they serve as a more accurate diagnostic tool.

The present study was done to evaluate the levels of osteocalcin and osteonectin in the crevicular fluid of periodontally healthy tooth and in healthy peri implant site 6 months post loading. 24 sulcular fluid samples were collected i.e., the sulcular fluid was obtained from the gingival sulcus of the 12 periodontally healthy subjects and from 12 peri-implant site between 20-65 years of age, using micropipette. The samples collected were immediately

transferred to eppendroff vials which was stored at -70℃ and assessed with sandwhich ELISA.

Solid-phase sandwich ELISA was performed to evaluate the GCF and PISF levels of osteocalcin and osteonectin. ELISA is known to be a sensitive method for protein quantification and it can be performed without undue cost and technical barriers. Point of Care application depends mainly on economically feasible technological tools so that vast populations can be surveyed simultaneously.

ELISA was carried out according to the manufacturer's instructions and the results of the GCF and PISF levels of osteocalcin and osteonectin are represented in table 1 and 2.

Osteocalcin being a major non-collagenous matrix protein of bone, dentin, and cementum, is found in tight association with the calcium phosphate mineral phase of these tissues [(60)]. In the presence of calcium, the Gla residues promote osteocalcin binding to hydroxyapatite and further accumulation in bone matrix. Osteocalcin secreted by osteoblast, is a crystal growth regulator as they interact specifically with the plane of grown crystals and they subsequently inhibit the crystal growth [(111)].

The results of our study states that the Osteocalcin, the crystal growth regulators are significantly high around the implants after six months of implant loading when compared to periodontal health. In implant sites, the

process of contact osteogenesis is complete and is followed by the osseointegration by distant osteogenesis, where the remodeling is at its peak.

Osteonectin has a diverse function in wide range of biological processes, such as development, differentiation, mineralization and tissue remodeling. Osteonectin is a de-adhesion matricellular protein [92] which aids the cell to move from a state of strong adherence to a state of weak adherence, which is also deemed necessary as this feature helps in the migration of the cells towards the implant, which in turn aids in the osseointegration and bone remodeling. Osteonectin is one such important matricellular protein that aids the cells to stay in spreaded morphology, antagonizing the pro-adhesive activity of other matrix proteins, which is very essential for cell proliferation occurring during any cellular injury [93].

In our study there was an increase in the levels of osteonectin in the PSIF of implant, which are loaded before six months when compared to periodontal health. This supports its role as a bone turnover component and a deadhesion protein all signifying the process of distant osseointegration occurring around the implant.

The clinical relevance of this study indicates that there is a always a constant remodeling mechanism which is occurring around an implant, as a result of which bone related glycoproteins are presumably expressed in higher levels in PISF when compared to GCF in periodontally healthy sites. The increase in the values of these non-collagenous proteins must not be inferred

as a state of peri implant disease. Also, a baseline normality range has to be established which may differ for individual implants. Hence, only sequential measurements made from the time of implant placement through loading and follow ups may help at arriving at the diagnosis if these bone related proteins are to be used as biomarkers of peri implant diseases.

The results from our study suggests that the mean Osteocalcin was 453.22 ± 205.19. Standard deviation and the mean osteonectin was 268.92 ± 81.40 (Standard deviation) of GCF in periodontal health.

The large Standard deviation observed suggests that these is a considerable inter individual variability within the healthy group. The reason for this inter individual variation could be the differences in the masticatory load and stomatognathic system which in turn led to a considerable difference in the bone turnover among individuals.

In case of subjects with implants placed, the mean osteocalcin was 2923.50 ±, 2152.80 (Standard deviation) and the mean osteonectin was 369.67 ± 255.07 (Standard deviation) in PISF in peri implant health. This also exhibited a large standard deviation.

The inter individual variation in the implant group was even higher than that observed in the tooth sites.

These results suggest that, these is a greater variability in the bone turnover around implant when compared to natural tooth. In addition to the

masticatory load and the stomatognathic system that exists in natural teeth, the implant macro and micro design could have played a part in this variability in bone turnover [87].

Limitations of the study include the following;

(i) Sample size is relatively small, when we are trying to establish a baseline normal value.

(ii) Only two biomarkers, osteonectin and osteocalcin were assessed. Analysis of a group of biomarkers may provide more meaningful information.

Further longitudinal studies with a large sample size conducted as biomarker arrays may be necessary to elucidate the role of bone turnover proteins as biomarkers in assessing the peri implant status.

Discussion

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Further longitudinal studies with a large sample size conducted as biomarker arrays may be necessary to elucidate the role of bone turnover proteins as biomarkers in assessing the peri implant status.

Summary and Conclusion

SUMMARY AND CONCLUSION

The aim of the present was to evaluate the levels of osteocalcin and osteonectin obtained from the peri implant sulcular region around endosseous implants 6 months post loading.

24 patients who reported to out-patient Department of Periodontology, Ragas Dental College and Hospitals, Chennai were enrolled in the study. These patients were divided into two groups, 12 samples were obtained from the periodontally healthy subjects and 12 samples were obtained from the peri implant sulcular region of the implant which is loaded before six months. GCF and PISF samples were collected and analysed for osteocalcin and osteonectin using sandwich ELISA technique. Statistical analysis was done using Mann Whitney U test and independent t test.

Based on the results of the study, we may conclude that:

1. There was a statistically significant increase in the levels of osteocalcin and osteonectin in peri-implant sulcular fluid compared to GCF in periodontal health. This maybe a result of active bone remodeling taking place around the implant due to distant osteogenesis.

2. This increase in the osteocalcin and osteonectin levels implicates the need for establishing a baseline normal value in health and the importance of their sequential measurements to assess the underlying pathobiology.

Further longitudinal studies conducted as biomarker arrays on a larger population may be necessary to elucidate the role of bone turnover proteins as biomarkers in assessing the peri implant status and in affirming our results.

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Annexures

ANNEXURE- I

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA.
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TO WHOMSOEVER IT MAY CONCERN

DATE: 06.02.2019

Chennai.

From

The Institutional Review Board,

Ragas Dental College and Hospital,

Uthandi, Chennai- 600119.

The Dissertation topic titled **"EVALUATION OF OSTEOCALCIN AND OSTEONECTIN LEVELS FROM PERI IMPLANT SULCULAR FLUID AROUND ENDOSSEOUS IMPLANTS"** Submitted by **DR. ALI FIROOZI** has been approved by the Institutional Review Board of Ragas Dental College & Hospital.

Dr.N.S.Azhagarasan, M.D.S
Member Secretary,
Institutional Ethical Board,
Ragas Dental College and Hospital,
Uthandi, Chennai - 600119

ANNEXURE- II

URKUND

Urkund Analysis Result

Analysed Document:	Dr. ALI FIROOZI.docx (D47599423)
Submitted:	2/5/2019 7:36:00 AM
Submitted By:	alifirouzi17@gmail.com
Significance:	5 %

Sources included in the report:

https://www.sciencedirect.com/science/article/pii/S0968432805001137
https://slideplayer.com/slide/5997836/
https://www.science.gov/topicpages/b/bone+sialoprotein+osteocalcin.html
https://www.researchgate.net/publication/311844654_The_Mechanisms_of_Mineralized_Tissue_Resorption_by_Clast_Cells_in_Relation_to_Orthodontic_Tooth_Movement_and_Root_Resorption
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Instances where selected sources appear:

22

ANNEXURE – III

CONSENT FORM

I ……S/o, w/o, d/o……

aged about …….years, Hindu/Christian/Muslim …… …….residing at ……do solemnly And state as follows.

I am the deponent herein; as such I am aware of the facts stated here under

I state that I came to Ragas Dental College and Hospital, Chennai for my treatment for

……

……

I was examined by Dr…….. and I was requested to do the following

1. Full mouth Plaque Score

2. Full mouth bleeding score

3 Measurement of periodontal pocket depth and clinical attachment loss

I was also informed and explained about the collection of plaque during scaling in ……(language) known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequence of the dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I also authorise the Doctor to proceed with further treatment or any other suitable alternative method for the study,

I have given voluntary consent to the collection of plaque for approved research.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

Signature of the patient/Attendant

The patient was explained the procedure by me and has understood the same and with full consent signed in (English/Tamil/Hindi/Telugu?.....) before me

Signature of the Doctor