# Evaluation and Comparison of levels of Proinflammatory cytokine Interleukin-8 in Saliva and Gingival Crevicular Fluid during different phases of Orthodontic Treatment.

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# List of Abbreviations

| Sr. No. | Abbreviations | Full Form |
|---|---|---|
| 1. | IL | Interleukin |
| 2. | ELISA | Enzyme linked immunosorbent assay |
| 3. | GCF | Gingival Crevicular fluid |
| 4. | PDL | Periodontal ligament |
| 5. | Hrs | Hours |
| 6. | Pg/mm | Picogram/millimeter |
| 7. | BL | Baseline |
| 8. | TNF | Tumour Necrosis Factor |
| 9. | GM-CSF | Granulocyte Macrophage-colony stimulating factor |
| 10. | RANKL | Receptor activator of nucleotide kappa B ligand |
| 11. | SD | Standard Deviation |
| 12. | ANOVA | Analysis of Variance |
| 13. | P | Probability of occurring of an event |
| 14. | HS* | Highly Significant ($P < 0.0001$) |
| 15. | nm | Nanometer |
| 16. | MPO | Myeloperoxidase |
| 17. | OTM | Orthodontic Tooth Movement |
| 18. | OPG | Osteoprotegerin |

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# *Introduction*

Orthodontic tooth movement is the result of aseptic inflammatory response of periodontal tissue to the mechanical stimuli generated from orthodontic appliances. There is alteration in the homeostatic conditions causing the transient change, resulting in tooth movement into a more equilibrium environment through periodontal tissue adaptation including bone remodeling. This process involves, bone cell activities regulated via many signalling pathways and mechanisms.[1]

In 1962, Burstone stated that, if the rates of tooth movement were plotted against time, there would be 3 phases of tooth movement—an initial phase, a lag phase, and a postlag phase. The initial phase is characterized by rapid movement immediately after the application of force to the tooth. This rapid movement can be due to the displacement of the tooth in the PDL space. The first phase lasts 24 hours to 2 days and represents the initial movement of the tooth inside its bony socket. The initial

phase is immediately followed by the lag period which shows relatively low rates of tooth displacement or no displacement. It has been suggested that the lag is produced by hyalinization of the PDL in areas of compression. This second phase lasts for 20 to 30 days. The third phase of tooth movement which follows the lag period shows a gradual or sudden increase in rate of tooth movement. The third phase comprises most of the total tooth movement during orthodontic treatment.[2] Cellular and tissue reactions start in the initial phase of tooth movement, immediately after force application.[2] The level of cytokines are seen to be significantly increased at the early stages and decreased in 7–10 days, and it has been concluded that the early inflammatory response is the main trigger for bone remodeling processes.[3]

The velocity of orthodontic tooth movement is related to cytokine release which can be detected in gingival crevicular fluid. Cytokines play an important role in intercellular signaling and have been implicated in the pathology of periodontal diseases, bone destruction, and bone response to orthodontic treatment. Cytokines are biologically active substances expressed by the cells in periodontium in response to mechanical stress. One of the most important breakthroughs of bone biology has been the identification of the role of cytokines in bone remodeling. Bone remodeling is used by orthodontists when forces transmitted to the surrounding tissues of the periodontium initiate the remodeling process.[3] Cytokines that were found to affect bone metabolism, and thereby orthodontic tooth movement, include interleukin 1, interleukin 2, interleukin 3, interleukin 6, interleukin 8, tumor necrosis factor alpha, gamma interferon, and osteoclast differentiation factor.[2] Pro-inflammatory cytokines, such as interleukin (IL)- 1beta, IL-2, IL-5, IL-6, IL-8, TNF-alpha, interferon-gamma

and GM-CSF, induce the classical hallmarks of inflammation through vasodilation and tissue invasion by leukocytes.[5]

IL-8 is produced and secreted by many cells such as fibroblast, epithelial cell, endothelial cells and macrophages in response to inflammation. It is a potent proinflammatory cytokine that has a key role in the recruitment and activation of neutrophils during inflammation. It is secreted mainly by monocytes and is important in regulating alveolar bone resorption during tooth movement by acting early in the inflammatory response[3, 4]. As interleukin-8 affects osteoclastogenesis, various studies have been conducted to identify its role in orthodontic tooth movement. Studies have shown that interleukin -8 increased after 4 days (Maeda et al., 2007) but decreased to below-baseline levels by 7 days (Basaran et al., 2006).[5]

Gingival Crevicular Fluid is an exudate, the constituents of which are derived from a variety of sources, including microbial dental plaque, host inflammatory cells, host tissue and serum. GCF reflects the immune and inflammatory reactions deriving from host-parasite interactions and bio-mechanical stress. Among many inflammatory and immune mediators identified in GCF, cytokines have attracted particular attention[6]. During orthodontic treatment, the forces exerted on the tooth distort the PDL's extracellular matrix and alter the cellular shape and cytoskeletal configurations. These changes may modify the flow rate and composition of GCF. The analysis of specific constituents in the GCF provides a quantitative biochemical indicator for the evaluation of local cellular metabolism. In recent years, a number of GCF constituents have been shown to be diagnostic markers of active tissue destruction in periodontal

tissues, but only a few studies have focused on the GCF constituent's involvement in detection of inflammatory response during orthodontic tooth movement[4].

Saliva has been used as a potential diagnostic tool over the last decade due to its ease and non-invasive accessibility along with presence of abundance of biomarkers, such as genetic material and proteins[7]. During inflammatory conditions, there is increase in inflammatory exudate by more than 5 fold in gingival sulcus. Nearly half the cells in GCF are leukocytes which enter the oral cavity (saliva) through gingival sulcus by diffusion[4]. Inflammatory cytokines such as IL-1 β and TNF-α which are involved in bone and periodontal remodeling, have been quantified in the salivary crevicular fluid of patients undergoing orthodontic treatment. The mediators involved in alveolar bone remodeling are released in GCF which gets continuously washed into saliva , thus whole-saliva samples may constitute an easy alternative to individual gingival sulcular samples for determining analytes of bone turnover that are present within the periodontal environment, providing a sensitive and inexpensive detection technique[8].

While the increased levels of IL-8 during orthodontic tooth movement have been confirmed by GCF analysis (Tuncer et al., 1996), a definitive temporal pattern is yet to be established. Furthermore, a comparison between levels of IL-8 in GCF and saliva has not been examined during different phases of orthodontic tooth movement. The goal of this research, therefore, is to gain a better understanding of IL-8 during different phases of orthodontic tooth movement and whether there exists a relationship between its level in saliva and GCF. Also, future research may provide the clinician with novel techniques that make use of the complex role of IL-8 in orthodontic tooth movement.

Thus, the present study aims to determine the level and pattern of intrleukin-8 during different phases of orthodontic tooth movement and to establish and compare the levels of inteleukin-8 in GCF and saliva.

# *Aim and Objectives*

**AIM –**

The present study aimed to evaluate and compare the levels of proinflammatory cytokine Interleukin – 8 in saliva and gingival crevicular fluid during different phases of orthodontic treatment.

**OBJECTIVE –**

The objectives were assessing the levels of Interleukin – 8 in saliva and gingival crevicular fluid during various phases of orthodontic treatment as follows -

- To evaluate interleukin-8 in saliva at the base line time - before the start of orthodontic treatment, 24 hours after the initiation of leveling phase, 7 days after the initiation of leveling phase.
- To evaluate interleukin-8 in GCF at the base line time- before the start of

orthodontic treatment, 24 hours after the initiation of leveling phase, 7 days after the initiation of leveling phase.

- To evaluate interleukin-8 in saliva 24 hours after the beginning of space closure phase, 7 days after the beginning of space closure phase.

- To evaluate interleukin-8 in GCF 24 hours after the beginning of space closure phase, 7 days after the beginning of space closure phase.

- To compare the levels of interleukin-8 in saliva and GCF at baseline time, 24 hours and 7 days after start of leveling phase and 24 hours and 7 days after start of space closure phase.

# *Review of literature*

The literature has been reviewed under following headings

1. Biology of orthodontic treatment.
2. Expression of Cytokines in gingival crevicular fluid
3. Expression of cytokines and other inflammatory biomarkers in saliva

**Biology of orthodontic treatment**

The biologic changes in the animals after the placement of orthodontic appliances were studied by Carl Sandstedt, 1904 and he reported the following histologic changes[9]-

1. On the side of pull, with both weak and strong forces, a deposition of bone takes place on the old alveolar wall. The newly formed bone spicules follow the direction of the strained peridental fibres.

2. On the side of pressure the old alveolar bone is equally resorbed by weak forces. The surface of the tooth itself remains intact.

3. By strong forces the peridental soft tissue is compressed at first on the side of pressure and cannot resorb the old alveolar bone, because it is deprived of its vitality. Instead of this, an active resorption soon begins in the neighbouring marrow spaces of the alveolar bone; so the bone and the compressed soft tissue in the region of the greatest pressure are removed. When all the necrotic material is removed, the tooth assumes at one pull a new position. This process is called the undermining resorption.

**Schwartz AM (1932)**[9] stated that an optimal orthodontic force moves teeth efficiently into their desired position, without causing discomfort or tissue damage to the patient. Schwartz studied tissue changes incidental to orthodontic tooth movement, and proposed the concept of optimal forces. He defined optimal continuous force as ‘‘the force leading to a change in tissue pressure that approximated the capillary vessels blood pressure, thus preventing their occlusion in the compressed periodontal ligament’’. According to Schwarz, forces well below the optimal level cause no reaction in the periodontal ligament. Forces exceeding the optimal level would lead to areas of tissue necrosis, preventing frontal bone resorption. Tooth movement would thus be delayed until undermining resorption had eliminated the necrotic tissue obstacle.

**Reitan K (1967)**[10] studied the clinical and histologic changes on tooth movement during orthodontic treatment and concluded various stages of tooth movement during initial period (1) a gradual compression of the periodontal ligament which may last

from about 4 to 7 days (2) the hyalinization period, which may last from 4 or 5 days and up to 2 months or more in subjects that exhibit a high bone density (3) the secondary period during which there is mainly direct bone resorption so that the tooth will continue to move. After the hyalinization period, the orthodontic force will be resisted or counteracted by stretched fiber bundles on the tension side, further restricting the tooth movement. It has been proved that it is primarily the light continuous tension that may cause elongation of fiber bundles and, subsequently, bone formation.

**Buck DL and Church DH (1972)[11]** studied histologic change during orthodontic tooth movement and concluded that Light tipping forces created areas of cell loss in the pressure zone of the periodontal ligament. In areas of extreme compression, the rare phenomenon of an altered collagen metabolism or structure reaction may be present, which is termed "hyalinization". Morphologic evidence of signet ring cells characteristic of fatty tissue may appear in the periodontal ligament following light tipping force application. These changes are all readily reversible, and the rapidity of cell and fiber reorganization appears to render these phenomena relatively nonsignificant in a clinical tooth movement time sequence.

**Rygh P. (1976)[12]** studied the changes in tension zone of rat molar periodontium and found marked changes in the cells, fibrous systems, and ground substance and on the cementum and alveolar bone surfaces of the periodontal ligament during tension. A marked increase of interstitial spaces between the formed ligament structures of the periodontal ligament was characteristic of the initial phases of tension. The cells and the endoplasmic reticulum within the cells were spindle shaped and oriented in the

direction of strain. There was considerable increase in amount of elastic-like fibrils in the periodontal ligament during tension. The appearance of elastic-like fibers was different from that of the fine filaments which have been reported in hyalinized zones of orthodontically compressed periodontal ligaments. The new layers of cementum had a granular appearance.

**Roberts WE et al (1981)[13]** stated that Orthodontic force systems, by way of stress or strain, are transduced to a biologic signal that affects a multifaceted bone remodeling response. Tooth movement involves not only a periodontal ligament response (alveolus translocation), but also bursts of resorption associated with truncated remodeling events in the path of the advancing tooth. Osteoclasts are recruited primarily from the macrophage or monocyte series, whereas osteoblasts are produced by local periodontal ligament cell proliferation and differentiation.

**Tanne K et al (1995)[14]** studied the biomechanical behaviour of periodontium before and after orthodontic tooth movement. The amount of tooth mobility was measured immediately before and after canine retraction by use of a noncontact displacement sensor. Retraction was accomplished with varying distal forces of 0 to 500 grams applied to the mesial of canine. The following results were obtained:

**1.** Tooth mobility curves before and after canine retractions were nonlinear and exhibited two phase changes according to varying force levels. However the limit of force to distinguish the two phases was greater after tooth movement than before.

**2.** Before tooth movement, tooth mobility exhibited a substantial increase when forces ranging from 50 to 500 grams were applied; the rate of increase gradually decreased as the force rose to 500 grams.

**3.** At the end of tooth movement or 24 days after the initiation of movement, the amount of tooth mobility was significantly greater than it was before tooth movement in response to both the light and heavy forces.

**4.** The periodontal tissues – the PDL and the alveolar bone in particular – become more flexible at the end of tooth movement, indicating reduced support by periodontal tissues. These findings suggest that the elastic nature of the PDL and the alveolar bone may decrease substantially at the end of tooth movement.

**Melsen B (1999)[15]** explaining alveolar tissue reactions related to tooth movement has proposed that bone apposition is induced by –

a) The load exerted by stretched fibers of the PDL, which will consequently induce a slight bending of the alveolar bone.

b) Direct resorption by unloading of the alveolar wall in case of low forces.

c) Indirect resorption as repair due to ischemia after application of high forces.

**Rody WJ et al (2001)[16]** conducted study to quantify Osteoclast recruitment to sites of compression in orthodontic tooth movement. He stated during orthodontic force application, the periodontal ligament (PDL) undergoes hyalinization which prevents the tooth from moving until the adjacent bone and the necrotic tissue are removed by

osteoclasts. The solution would be either to prevent the formation of hyalinized tissue or to accelerate its removal by osteoclasts. The osteoclast formation process is accomplished through an orderly sequence of events which include stem cell division and osteoclast progenitor proliferation in hematopoietic tissues, migration of mononuclear osteoclast precursors to sites of bone resorption, differentiation into committed osteoclast precursors (preosteoclasts) guided by cell-to-cell interaction with osteoblasts, and fusion into multinuclear osteoclasts. Osteoclasts in the PDL originate by the fusion of recently recruited preosteoclasts from the marrow instead of from local PDL cells. Furthermore, the alveolar bone marrow plays a role in the formation of osteoclasts during orthodontic tooth movement.

**Melsen B. (2001)**[17] stated that the biological reaction was dependent on the force level and the stress/strain distribution. Histological examination demonstrated that the PDL fibres were stretched and formation activity was found along the major part of the alveolus. Only apical fibres were not observed to stretch. The stretched fibres will most likely lead to the delivery of strain values corresponding to modelling. The stretching of the fibres may induce a slight bending of the alveolar wall. This bending in terms of an increase in the curvature of the alveolar wall has previously been suggested by Epker and Frost (1965) as the cause of new bone formation, in an attempt to harmonize the orthodontic and the orthopaedic perception of the tissue reaction to mechanical load.

**Roberts WE et al (2004)**[18] stated catabolic bone remodeling at the periodontal ligament (PDL) surface is the rate-limiting step in tooth movement. In initiating tooth movement, connective tissue growth factor is expressed in osteoblasts and osteocytes,

and osteopontin is expressed by osteoclasts and osteocytes. Undermining resorption occurs by recruiting osteoclasts to the site of maximal PDL compression and a paravascular osteogenic response is initiated in widened areas of the PDL: (1) preosteoblast formation at 10 hours, (2) peak DNA synthesis at 20 hours, (3) maximum rate of mitosis at 30 hours, and (4) initiation of bone formation at 48 hours. Catabolic modeling is mediated by inflammatory cytokines. Osteoclast differentiation and activation is controlled by genes related to tumor necrosis factor (TNF) and its receptors.

**Meikle MC (2006)[19]** stated that in response to mechanical stimulation, fibroblasts, and osteoblasts in PDL as well as osteocytes in the bone are activated. The alveolar bone and the collagenous extracellular matrix of the PDL in both tension and compression sites are remodeled. Both soft and hard tissue metabolism can be modulated by cytokines and growth factors pointing to the possible involvement of such factors in tissue remodeling during orthodontic tooth movement.

**Krishnan V and Davidovitch ZE (2006)[2]** stated four stages of tooth movement. In the initial stage the fibers and cells within the PDL are compressed in one side and stretched in the other, thus creating areas of "compression" and "tension". Recruitment of osteoclast and osteoblast progenitors, as well as extravasation and chemoattraction of inflammatory cells begins. Hyalinized zones in the pressure area are present. This initial phase of orthodontic tooth movement is a typical acute inflammatory process, mainly exudative, with plasma and leukocytes leaving the capillaries and synthesis and release of various neurotransmitters, cytokines, growth factors and metabolites that act as second messengers on signal transduction pathways

(e.g. arachidonic acid and prostaglandins). In the second phase no tooth movement but formation and removal of necrotic tissue takes place. This phase is a chronic process replacing the acute inflammatory reaction and involves various cells, such as fibroblasts, endothelial cells, osteoblasts and alveolar bone marrow cells. In the areas of compression, the PDL fibers have a distorted appearance and recruitment of phagocytic cells to remove necrotic tissues from compressed PDL sites and adjacent alveolar bone, takes place. In the areas of tension, osteoblasts are enlarged and start producing new bone matrix. The third and fourth phases of orthodontic tooth movement, also known as the acceleration and linear phases, respectively, start about 40 days after the initial force application. The necrotic tissue is removed and tooth movement is accelerated. This phase corresponds to the moment when the orthodontist will activate the appliance, resulting in an acute inflammation which superimposes the on-going inflammation of the previous phase. A number of signalling molecules, such as prostaglandins, growth factors, cytokines, extracellular matrix proteins and neuropeptides are released during this phase. In the *fourth phase*, tooth movement is accelerated until total displacement.

**Cardaropoli D. et al (2007)**[20] stated when an orthodontic force is applied, tooth movement will occur in the direction of the force, by narrowing the periodontal ligament (PDL) at the site of compression, with subsequent bending and resorption of the alveolar bone. In this way teeth can be moved a small distance, until the resisting bone stops the movement. This resistance is eventually overcome by the ensuing resorption of the bone opposite the compressed PDL. At sites of force-induced tension in the PDL, a concomitant apposition of bone will occur, until the PDL has regained its normal width. Thus, tooth movement occurs as a direct outcome of force-induced

tissue remodeling around the dental root. Such a remodeling requires the presence of cells capable of resorbing and forming the extracellular matrix (ECM) of the PDL and alveolar bone.

**Henneman S. et al (2008)[21]** stated that the law in orthodontics is that a tooth can be moved through the alveolar bone when an appropriate orthodontic force is applied. This is based on the principle that a change in mechanical loading of a biological system results in strain, which subsequently leads to cellular responses aiming at adaptation of the system to the changed conditions. As a result of this principle, remodeling of the periodontal ligament (PDL) and the alveolar bone around a tooth takes place during orthodontic force application.

**Brooks PJ et al (2009)[22]** conducted a study on early markers of orthodontic tooth movement and concluded that after only 3 hours of orthodontic force, cells of the PDL are responding to the mechanical forces and are involved in osteoclast precursor signaling. Mechanical force distribution in the PDL due to orthodontic treatment can be determined through KI- 67 protein expression in periodontal cells.

**Zainal Ariffin SH. et al (2011)[23]** stated that when exposed to varying degrees of magnitude, frequency, and duration of mechanical loading, bone and adjacent periodontal tissues show extensive macroscopic and microscopic changes. Mechanical loading also alters periodontal tissue vascularity and blood flow, resulting in the local synthesis and release of various molecules such as neurotransmitters, cytokines, growth factors, colony-stimulating factors and arachidonic acid metabolites. The released molecules evoke cellular responses in the various cell types in and around teeth, providing a favourable microenvironment for tissue deposition or

resorption. Various cell-signalling pathways are activated, which ultimately stimulate PDL turnover, as well as localised bone resorption and bone deposition.

**Andrade I et al. (2012)**[24] when an orthodontic force is applied, the periodontal tissues express extensive macroscopic and microscopic changes, leading to alterations in five micro- environments: extracellular matrix, cell membrane, cytoskeleton, nuclear protein matrix, and genome. Capability of adaptive reaction to applied mechanical loading relies in the DNA of periodontal ligament (PDL) and alveolar bone cells. However, an inflammatory process is a precondition for these modifications to occur, which will lead to orthodontic tooth movement. PDL's vascularity and blood flow changes, as well as mechanical alterations in the cytoskeleton of PDL and bone cells, will result in local synthesis and release of various key mediators, such as chemokines, cytokines, and growth factors. These molecules will induce many cellular responses by various cell types in the periodontium, providing a favourable microenvironment for bone resorption or deposition and, consequently, for orthodontic tooth movement.

**Alikhani M. et al (2015)**[25] stated that Tooth movement occurs in response to orthodontic forces. However, this movement is not completely regulated by the laws of physics and therefore is not immediate or linear in response to the magnitude of the force. The biological response plays a central role in controlling orthodontic tooth movement. The rate of bone resorption in the direction of movement determines the rate of tooth movement. Bone resorption, in turn, is controlled by the rate of osteoclast formation. Events that lead to osteoclast formation at the early stages of tooth movement emphasize the importance of inflammatory cytokines and

chemokines in this process. In response to orthodontic forces, in non-hyalinized areas of the PDL, there is a temporary vasodilatation and release of chemokines, which recruit inflammatory cells and osteoclast precursors into the area. These release more inflammatory markers that directly or indirectly through mediators such as prostaglandins activate RANK–RANKL pathway, stimulating osteoclast precursor cells to differentiate into osteoclasts.

**Bumann EE. et al, (2017)**[26] stated that, the classic cascade of bone remodeling is (i) osteoclast precursors are attracted from the blood stream; (ii) the precursors form osteoclasts; (iii) localized bone resorption occurs; (iv) coupling—whereby, osteoclastic resorption occurs in parallel to the generation of osteoblasts and (v) bone formation occurs. In OTM, mechanical forces, which can be intermittent or continuous, can stimulate this bone remodeling cascade in alveolar bone. This occurs differently depending on whether the perspective is from the pressure or tension side of the tooth involved in OTM. The pressure side of OTM is characterized by bone resorption, while on the tension side bone formation occurs when forces are within a healthy biological range.

**Expression of Cytokines in gingival crevicular fluid–**

**Davidovitch Z et al (1988)**[27] demonstrated immunohistochemically that interleukin – 1 could be detected in the periodontal tissues of cat canine teeth following the application of a tipping force. Their study provided the first experimental evidence to support the hypothesis that neurotransmitters and cytokines play a regulatory role in orthodontic force-induced alveolar bone remodeling. The neurotransmitters vasoactive intestinal peptide, substance P and the cytokines IL-1 alpha and IL-1 beta

were localized immunohistochemically in paradental tissues of cat canines that had been treated by the application of an 80 g tipping force for 1 hour to 14 days. Consequently, determination of the cytokine synthetic activity by leukocytes of orthodontic patients may inform about their alveolar bone remodeling potential.

**Saito M. et al (1991)[28]** demonstrated that Prostaglandin E and 1L-1 are present in the unstressed cat PDL and that the application of mechanical forces to the teeth increases the levels of these molecules in stressed PDL cells in tension sites. These findings suggest that Prostaglandin E and IL-1 β play a regulatory role in the cellular response to mechanical force application in vivo. This hypothesis was supported by the findings in the in vitro part of this experiment, which has demonstrated that human PDL cells respond to the application of mechanical stress and IL-1β by increased synthesis of Prostaglandin E and by pro synthesis. The addition of IL-1 β to mechanical stress-stimulated cells elevated the synthesis of Prostaglandin E in a synergistic fashion. The production of Prostaglandin E at 24 hours after the removal of the mechanical stress plus IL-1 β was 20-fold that of control cells.

**Sandy JR. et al (1993)[29]** speculated a hypothesis regarding the formation or resorption of bone, which depends on (1) the cytokines produced locally by mechanically activated cells; and (2) the functional state of the available target cells. Cytokines that can influence connective tissue remodeling include the interleukins, tumor necrosis factors, interferons, polypeptide growth factors, and colony stimulating factors. The recent immunolocalization of interleukin-l α and interleukin-lβ in the periodontal tissues of cat canine teeth after the application of a tipping force has provided the first experimental evidence to support this hypothesis.

**Grieve WG et al (1994)**[30] examined gingival crevicular fluid (GCF) levels of two potent bone resorbing mediators, prostaglandin E (PGE) and interleukin-1 β (IL-1 β), during human orthodontic tooth movement. The GCF was sampled at control sites and treatment (compression) sites before activation and at 1, 24, 48, and 168 hours. Increases in IL-1 β levels at 1 and 24 hours preceded peak PGE levels which occurred at 24 and 48 hours after-activation. The results demonstrate that bone-resorbing PGE and IL-1 β produced within the periodontium are detectable in GCF during the early phases of tooth movement and return to baseline within 7 days.

**Lowney JJ. et al (1995)**[31] conducted study to measure tumor necrosis factor-α directly in the human gingival sulcus before and after the application of an orthodontic force. The samples were taken before force application (controls), and at a fixed time after force application. The amount of immunoabsorbed tumor necrosis factor-α was quantified with an immunochemical assay. There was a greater than twofold increase in tumor necrosis factor-α recoverable from the gingival sulcus after application of orthodontic forces. It was concluded that the quantity of tumor necrosis factor-α, found in human gingival sulcus is elevated during tooth movement. The source may be from the adjacent gingiva, but more likely the compressed periodontal ligament and the resorbing bone adjacent to the root surface.

**Uematsu S et al (1996)**[32] investigated the levels of IL- β, IL-6, tumor necrosis factor-α, epidermal growth factor and β2 micoglobulin in GCF at 0, 1, 24 and 168 hours after the initiation of orthodontic tooth movement. The concentrations of the cytokines were significantly higher in the experimental group at 24 hour after the experiment was initiated compared to the control or experimental sites at baseline.

Thus, cytokine production in the GCF by mechanically deformed tissues may account for many cellular effects associated with orthodontic tooth movement.

**Meager A. (1999)**[33] stated that Proinflammatory cytokines have been intimately involved in bone resorbing activities. The elevation of proinflammatory cytokines initiates signalling cascades that stimulate local vascularity, infiltration and activation of monocytes/ macrophages, and the production of potent secondary proinflammatory mediators, chemoattractants, proteases and reactive ions that collaborate to cause marked and irreversible breakdown. The role of cytokines in osteoclast formation and activation are central to focal bone destruction that occurs in osteolytic conditions.

**Alhashimi N. et al (1999)**[34] stated that in the early stage of orthodontic tooth movement, an acute inflammatory response characterized by the migration of leukocytes occurs. This response suggests the presence of specific chemotactic signals that may play a role in the mechanism of bone remodeling, in particular in resorption. It appears that a cascade of cellular events takes place after application of orthodontic movement. Cytokines, such as IL-1, IL-6, and TNF-a, will be released, and these cytokines will induce chemokines, such as monocyte chemoattractant protein-1, regulated on activation, normal T cell expressed and secreted, and macrophage inflammatory protein-2. These chemokines in turn will activate and recruit cells from the monocyte/macrophage line to the pressure side. These mononuclear cells mature and fuse into osteoclasts. Resident cells, such as fibroblasts and osteoblasts, may take part in the induction of monocyte chemoattractant protein-1, regulated on activation, normal T cell expressed and secreted, and macrophage inflammatory protein-2. It seems to be that there is a negative feedback system, as the chemokine mRNA reached their highest levels on day 3, followed by a decline to undetectable levels on day 10.

**Alhashimi N et al (2001)**[35] used in situ hybridization to measure the messenger RNA expression of IL- β, IL-6, and TNF- α at 3, 7, and 10 days after the application of orthodontic force on the maxillary first molars of 12 rats. The contralateral side and 3 untreated rats served as controls. Measurements of the messenger RNA expression were selected as the means to investigate the role of orthodontic force in de novo synthesis of proinflammatory cytokines. After the application of force, the induction of IL- β and IL-6 was observed to reach a maximum on day 3 and to decline thereafter. No messenger RNA induction of either cytokine was measured in the control teeth. The messenger RNA expression of TNF-α was not detected at any time point of this study in the experimental or contralateral sides or in the control animals.

**Ren Y. et al (2002)**[36] quantified three mediators (prostaglandin E, interleukin-6 and granulocyte macrophage - colony-stimulating factor (GM-CSF) in GCF during orthodontic tooth movement in juveniles and adults. The study recruited 43 juvenile patients and 41 adult patients. One of the lateral incisors of each patient was tipped labially, the other served as control. GCF samples were taken before force activation (t0) and 24 hours later (t24). Prostaglandin E concentrations were significantly elevated at t24 in juveniles and adults, while concentrations of IL-6 and GM-CSF were significantly elevated only in juveniles. The total amounts of all three mediators in GCF significantly increased at t24 in both groups. It was concluded that in early tooth movement, mediator levels in juveniles are responsive than levels in adults, which agrees with the finding that the initial tooth movement in juveniles is faster than in adults and starts without delay.

**Lee KJ. et al (2004)**[37] evaluated the effects of a light continuous force and an interrupted force with weekly reactivation on interleukin-1 (IL-1) and prostaglandin

E2 (PGE2); possible interactions between these 2 potent mediators of the bone resorption process were assessed in vivo. When a continuous force was provided, IL-1 levels showed a significant elevation at 24 hours and then decreased and maintained an insignificant but high mean concentration, compared with the control sites. Prostaglandin E2 levels showed a significant elevation at 24 hours and then decreased, showing temporary elevation. With an interrupted force, significant elevations of IL-1 levels were observed at 24 hours and a greater significant elevation at 24 hours after the first appliance reactivation, compared with the control sites. Prostaglandin E2 levels increased significantly at 24 hours and remained high for 1 week. The synergistic up-regulation of prostaglandin E2 by appliance reactivation and secreted IL-1 was not evident with either type of force after 1 week.

**Tuncer BB. et al (2005)[3]** evaluated the levels of IL-8 during mechanical forces on periodontal tissues at different stages of orthodontic therapy. A host-derived neutrophil-activating cytokine interleukin-8 (IL-8) is secreted mainly by monocytes and is considered to be important in regulating alveolar bone resorption during tooth movement. Gingival crevicular fluid was sampled from mesial and distal gingival crevices of each canine separately at baseline and one hour, 24 hours, six days, 10 days, and 30 days after the application of the force. An enzyme-linked immunosorbent assay for quantitative detection of IL-8 was used. Although there was an increase in the concentration of IL-8 at tension (mesial) sites after one hour, 24 hours, six days, and 10 days, a decrease was observed at 30 days. Pressure (distal) sites did not demonstrate such an increase at any period except at 10 days. However, the concentration of IL-8 at both sites showed a similar decrease and approached each other at day 30. It was concluded that local host response toward the orthodontic

forces might lead an increase in IL-8 and neutrophil accumulation, and this may be one of the triggers for bone remodeling processes.

**Basaran G et al (2006)**[38] studied levels of interleukins 2, 6, and 8 during tooth movement, and test whether they differed from each other with leveling and distalization forces in various treatment stages of standard orthodontic therapy. Increases were seen in the volume of gingival crevicular fluid and the concentrations of interleukins 2, 6, and 8. Although there were no statistical differences in IL-2 levels, it increased at the 7th and 21st days of leveling and distalization. IL-8 showed a statistically significant decrease on the 7th day of leveling. Again on the 7th and 21st days of the distalization, it increased to some extent. GCF volumes were greater on the 7th and 21st days of leveling and distalization, and returned to baseline levels after leveling. Leveling and distalization of the teeth evoke increases in interleukins 2, 6, and 8 levels in the periodontal tissues that can be detected in gingival crevicular fluid

**Krishnan V and Davidovitch ZE (2006)**[2] stated Cytokines as extracellular signaling proteins that act on nearby target cells in low concentrations in an autocrine or paracrine fashion in cell-to-cell communications. Cytokines that were found to affect bone metabolism, and thereby orthodontic tooth movement, include interleukin 1 (IL-1), interleukin 2 (IL-2) interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor alpha (TNF), gamma interferon, and osteoclast differentiation factor (ODF).

**Maeda A. et al. (2007)**[39] hypothesized that chemokines released from PDL cells under mechanical stress regulate osteoclastogenesis, and investigated the profiles and

mechanisms of chemokine expression by human PDL cells in response to mechanical stress. In vitro, shear stress and pressure force rapidly increased the gene and protein expressions of IL-8/CXCL8 by PDL cells. Consistently, amounts of IL-8 in the gingival crevicular fluid of healthy individuals increased within 2 to 4 days of orthodontic force application. Increased IL-8 levels in GCF during orthodontic tooth movement indicated that local IL-8 expression is evident in response to clinical orthodontic loads. Since IL-8 seems to activate osteoclastogenesis through various mechanisms, mechano-stress-induced IL-8 from PDL cells may play an essential role in efficient orthodontic tooth movement.

**Garlet TP. et al (2007)[40]** studied the pattern of expression of mRNA encoding several pro- and anti-inflammatory cytokines (Tumor Necrosis Factor-*α*, transforming growth factor- β, IL- 10) in relation to several extracellular matrix collagen I, matrix metalloproteinase and tissue inhibitor of metalloproteinase-1 and bone remodeling markers like osteocalcin, Receptor Activated Nucleotide Kappa Ligand, osteoprotegerin in tension (T) and compression (C) sides of the PDL of human teeth subjected to rapid maxillary expansion. The PDL of normal teeth was used as a control. The results showed that both T and C sides exhibited significantly higher expression of all targets when compared with controls, except for type I collagen and tissue inhibitor of metalloproteinase-1 on the C side. Comparing C and T sides, the C side exhibited higher expression of Tumor Necrosis Factor-*α*, Receptor Activated Nucleotide Kappa Ligand, and matrix metalloproteinase, whereas the T side presented higher expression of interleukin-10, tissue inhibitor of metalloproteinase-1, collagen-I, osteoprotegerin, and osteocalcin. The expression of transforming growth factor- β was similar in both C and T sides. The data demonstrated a differential expression of

pro- and anti-inflammatory cytokines in compressed and stretched PDL during orthodontic tooth movement.

**Ren Y, Vissink A (2008)**[41] reviewed among more than 100 regulatory proteins detected in GCF, cytokines are of interest. Cytokines refer to non-hormone signaling factors, including lymphocyte-derived factors, monocyte-derived factors, colony-stimulating factors, growth factors, and chemotactic chemokines. Cytokines are produced by periodontal tissue cells, such as fibroblasts and osteoblasts, and are involved in normal physiological turnover and remodeling of bone. The most consistent result was a peak of cytokine levels at 24 h. Associations existed between prostaglandin E2 (PGE2) and interleukin-1b (IL-1b) and pain, velocity of tooth movement, and treatment mechanics. Interleukin-1b and prostaglandin E2 showed different patterns of up-regulation, with IL-1b being more responsive to mechanical stress and prostaglandin E2 more responsive to synergistic regulation of IL-1b and mechanical force.

**Giannopoulou C et al (2008)**[6] conducted a study to evaluate the expression of IL-1 b, IL-4, and IL-8 in the gingival crevicular fluid (GCF) of children, adolescents, and young adults with and without fixed orthodontic appliances. Eighty systemically healthy children and adolescents participated in the study: 56 aged between 8 and 16 years without any orthodontic appliance (Group A) and 24 aged between 10 and 20 years having worn fixed orthodontic appliances for at least 12 months (Group B). The contents of interleukin-1 beta (IL-1 b), interleukin-4 (IL-4), and interleukin-8 (IL-8) were detected by ELISA. Statistically significant differences were noted for the mean log IL-1 b, IL-4, and IL-8 between the two groups: Group B showed significantly

higher mean levels in log IL-1 b and log IL-8 compared to Group A. Mean levels of log IL-4 were lower in Group B, although they did not reach statistical significance. Thus, it was concluded that fixed orthodontic appliances result in an increase in the expression of IL-1 b and IL-8. This may reflect biologic activity in the periodontium during orthodontic tooth movement.

**Kaya F.A et al (2010)[42]** determined tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β) and interleukin 8 (IL-8) levels in GCF in tooth early leveling movement orthodontic treatment. The patients were seen at baseline and between 1-7 days leveling of the teeth. The concentration of tumor necrosis factor alpha, IL-1β and IL-8 were evaluated. There were statistical differences between the observations at the 1-2 day of leveling for tumor necrosis factor alpha, IL-1β and IL-8. Orthodontic forced induces rapid release of the tumor necrosis factor alpha, 1L-1β and IL 8 levels during tooth movement in gingival crevicular fluid (GCF).

**Kaya AC et al (2011)[43]** conducted a study on late adult rats to evaluate the levels of interleukin1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in the samples of gingival crevicular fluid (GCF) during the orthodontic tooth movement. 19 adult (120 days) Spraque-Dawley rats were used. Approximately 15 g force applying open coil spring was applied actively between the upper incisors of the rats. Before and after the activation on the 3rd and 7th and 10th days GCF samples were taken from the vestibular surfaces of appliance fixed teeth using periopaper. The baseline levels for the concentration of interleukin-1β, interleukin 6 and tumor necrosis factor alpha increased on 3$^{rd}$ day and started to decrease on the 7th and 10th

days. The results of this study support the hypothesis that proinflammatory cytokines play a potent role in bone resorption after the application of orthodontic force.

**Salla JT et al (2011)[44]** investigated the effects of interleukin-1 receptor antagonist (IL-1Ra) on orthodontic tooth movement (OTM). OTM and TRAP-positive osteoclasts were evaluated after 12 days of mechanical loading and the levels of cytokines on periodontal tissues were analysed by ELISA after 12 and 72 h. Mice treated with IL-1Ra showed diminished OTM and decreased numbers of TRAP-positive osteoclasts. In line with this, lower levels of IL- β and TNFα, and higher levels of IL- 10, were observed on periodontal tissues of IL-1Ra-treated mice in relation to the vehicle- treated group. They suggested that IL-1Ra down regulates OTM, probably by its anti-inflammatory actions.

**Grant M et al (2012)[5]** conducted a study wherein cytokines GM-CSF, interferon-gamma, IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and Tumor Necrosis Factor alpha, tissue biomarkers (Matrix Metalloprotien Protien-9, tissue inhibitors of Matrix Metalloprotien Protien-1 & 2) and bone metabolism indicators (Receptor For NF Kappa B Ligand and osteopotegrin) were measured in GCF using multiplex assays. Tension sites adjacent to canines showed significant increases in IL-1beta, IL-8, TNF alpha, Matrix Metalloprotien Protien-9 and tissue inhibitors of Matrix Metalloprotien Protien-1 & 2 across all time points following force application, while compression sites exhibited increases in IL-1beta and IL-8 after 4 hours, Matrix Metalloprotien Protien-9 after 7 and 42 days and Receptor for nuclear factor Kappa B Ligand after 42 days. These data demonstrate that high levels of pro-inflmmatory cytokines and biomarkers of tissue and bone metabolism in GCF are associated with orthodontic

force application. Elevated levels were evident at 4 hours but continued for periods of up to 6 weeks. The data suggest that GCF biomarker analysis may help optimise orthodontic forces for individual patients.

**Madureira DF et al (2012)[45]** investigated the kinetics of interleukin-6 and chemokine ligands 2 and 3 levels in periodontal ligaments subjected to orthodontic forces. The experimental group consisted of premolars subjected to a force of 0.980 N in the apical direction for 3 hours, 15 hours, 3 days, 12 days, or 21 days. The contralateral teeth, without orthodontic appliances, were used as controls. Compared with the control group, an increase in chemokine ligand 2 was observed on days 3 and 12, and increases in interleukin-6 and chemokine ligand 3 were observed on day 12 in the experimental group. Thus, differential expressions of interleukin-6 and chemokine ligands 2 and 3 in periodontal ligaments after mechanical loading might reflect the distinct roles of these molecules in the bone remodeling process.

**Barbieri G et al (2013)[46]** conducted study To evaluate the expression of an Receptor activator of nuclear factor-kappa, osteoprotegerin, osteopontin, and transforming growth factor ß1 in gingival crevicular fluid (GCF) of teeth subjected to orthodontic forces. The GCF samples were collected from the tension and compression sites at baseline, 24 hours, and 7 days after the placement of separators. Levels of Receptor activator of nuclear factor-kappa, osteoprotegerin, osteopontin, and transforming growth factor ß1 were also analyzed using a multiplex enzyme-linked immunosorbent assay. The concentration of osteoprotegerin significantly decreased at the compression site by 24 hours, and the amount and concentration of Receptor activator of nuclear factor-kappa differed significantly between the control, the compression,

and the tension sites after 7 days. A significant increase in absolute transforming growth factor ß1levels was also detected at the compression site versus the control and tension sites after 7 days. Both increased expression of bone resorptive mediators (eg, Receptor activator of nuclear factor-kappa and transforming growth factor ß1) and decreased expression of a bone-forming mediator (eg, osteoprotegerin) on the compression side were detected. Thus, bone metabolism is affected by application of force to the teeth by elastic separators.

**Kapoor P. et al (2014)[47]** stated Cytokines are low-molecular weight proteins released in autocrine or paracrine environment in response to local signals like application of stress and are involved in normal physiological bone turnover and remodeling. Although cytokines have been extensively evaluated in GCF as quantitative biochemical indicators of inflammatory periodontal status, there has been an increasing interest on understanding their contributions as mediators of OTM owing to their role in bone and tissue remodeling. The secretion of cytokines in the local environment by cells activated on application of orthodontic force varies according to the force levels and functional state of available target cells. The first experimental evidence supporting role of cytokines in OTM was documented in periodontal tissues of cat canine teeth where IL-1α and IL-1β were identified after the application of a tipping force.

**Madureira DF et al (2015)[48]** analysed the expression of cytokines in GCF and PDL after mechanical stress. Interleukin (IL)-6 productions was significantly elevated in the PDL on day 1 after force application. Significantly strong positive correlations between GCF and PDL in experimental group were seen on days 3 (interferon-

gamma), days 7 (IL-10), days 14 (IL-17A), and days 28 (IL-17A, tumor necrosis factor-alpha), and significantly strong negative correlation were seen on days 14 (interferon-gamma) and days 21 (IL-2, IL-10). Different patterns of IL-6 expression were seen in the PDL and GCF after mechanical stress.

**Tsuge A. et al (2016)[49]** examined the early tissue reaction in the tension zone of periodontal ligament (PDL) during orthodontic tooth movement. The PDL in the tension zone was examined histologically, immunohistochemically and at a molecular level after 24 hours, 3 days and 7 days. Significantly high expressions of IL-1 β and Pentraxin-3 were characteristically observed not only in the endothelial cells and cells around the blood vessel, but also in fibroblasts throughout the PDL of the tension zone 24 hours after orthodontic force loading. Three and 7 days after loading, these showed tendencies to return to control levels.

**Nunes L. et al (2017)[50]** evaluated the gingival crevicular fluid (GCF) levels of ten cytokines, IL-6, IL-8, IL-10, IL-13, IL-17, IFN- γ, GMCSF, Monocyte Chemoattractant Protien-1, Macrophage Inflammatory Protien-1β and TNF- α, during initial orthodontic treatment. The GCF was collected from the test and control teeth before fixed appliance mounting (baseline) and after 1, 7 and 21 days. The total GCF cytokines levels were quantified using multianalysis Luminex technology. In the test teeth, the GCF levels of all the cytokines remained constant throughout the experimental term. On the contrary, significant reductions were seen in the control teeth for each cytokine. Moreover, significantly greater levels of IL-6, GM-CSF, Monocyte Chemoattractant Protien-1and TNF were seen in the test teeth as compared to the control teeth at 7 days.

**Expression of cytokines and other inflammatory biomarkers in saliva –**

**Burke JC et al (2002)[51]** conducted a study to determine if a reaction to local manipulation (minor tooth movement) is reflected in the composition of whole saliva (WS) and GCF. Orthodontic treatment alters the expression of secretory proteins like cytokines at the local level in bone and the oral cavity, but its systemic effects are not well understood. Changes in the expression of total and specific secretory proteins cyclic adenosine monophosphate (AMP)-dependent protein kinase subunit (RII) in Whole Saliva and GCF were measured in the initial phase of orthodontic tooth movement. Whole saliva and GCF were collected before and 1 day after treatment. Electrophoresis and Western blotting were carried out to establish the banding patterns of total proteins and to measure the isotype and amount of RII that serves as an apparent stress indicator. Digitized image files were used for densitometric analyses of the relative concentrations of RII and total protein. Individual protein values showed no statistically significant changes in saliva or GCF. Western blots, however, showed a dramatic difference in RII after the placement of separators: the 50-to-55 kilodaltons (kd) band virtually disappeared and was replaced by a fragment in the 20-kd range. Thus, the identification of secretory proteins in oral fluid as stress indicators could be useful in monitoring molecular responses in patients undergoing orthodontic treatment.

**Chiappin S et al (2007)[52]** stated Saliva, an attractive human biological fluid, contains a complex balance of secretions from the major and minor salivary glands as well as constituents from gingival crevicular fluid, oral microflora, food debris, and desquamated epithelial cells. Therefore, saliva contains abundant proteins, peptides,

small molecules, and other compounds. Thus, cytokines being protiens can be easily detected from the saliva.

**Teles RP et al (2009)**[53] conducted a study to evaluate and compare the levels of ten cytokines in whole saliva from periodontally healthy and chronic periodontitis subjects. Levels of GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN-γ and TNF-α were measured in whole saliva. The author has stated that several lines of evidence suggest that the primary source of cytokines in whole saliva is GCF and concluded that the inflammatory cytokines detected in whole saliva did not come from the secretions of major salivary glands, and proposed that GCF was the likely source of these cytokines. There was no association found between levels of salivary cytokines and clinical parameters of periodontal disease. Thus, the dilution of GCF components in saliva seems to mask existing differences in the levels of these biomarkers at the site level.

**Ariffin SH. et al (2010)**[54] conducted a study to determine enzymes profiles from patient's saliva during orthodontic tooth movement, i.e., lactate dehydrogenase for inflammation, tartrate resistant acid phosphatase for bone resorption and alkaline phosphatase for bone formation. Saliva samples were collected from 6 orthodontic patients. Activities of all enzymes were measured before placement of orthodontic fixed appliances (normal activity) followed with day 3, 7, 10, 14, 17, 21, 24, 28 and 31 after the braces were activated. All enzymes (lactate dehydrogenase, tartrate resistant acid phosphatase and alkaline phosphatase) showed significantly higher specific activities as compared to normal. lactate dehydrogenase showed the increment at the early phase of treatment (day 3, 7 and 10), tartrate resistant acid

phosphatase at day 14 and 17 followed by alkaline phosphatase at day 17, 21 and 24. As a conclusion, the profiles of enzymes activities showed that inflammation occured during early phase of treatment followed by bone resorption and bone formation. In addition, the inflammations, resorption and bone formation phases in orthodontic treatment were found to be completed within 24 days.

**Marcaccini AM. et al (2010)[55]** conducted a study to determine myeloperoxidase (MPO) activity in the gingival crevicular fluid (GCF) and saliva (whole stimulated saliva) of orthodontic patients at different time points after fixed appliance activation. GCF and saliva samples were collected at baseline, 2 hours, and 7 and 14 days after application of the orthodontic force. After appliance activation, increased MPO activity in the GCF was observed at 2 hours compared with baseline. The same trend was observed with MPO activity in whole saliva. This study showed that MPO activity is highly increased 2 hours after appliance activation, in both GCF and saliva, and that it decreases to baseline levels after 7 days. There was no statistically significant difference between MPO levels collected at 7 and 14 days, although a lower value was observed on day 14 in both saliva and GCF.

**Zhang J et al (2012)[56]** conducted a study to identify the discriminating protein profiles in unstimulated whole saliva of orthodontic patients with different treatment durations. Matrix-assisted laser desorption/ionization time-of- flight mass spectrometry (MALDI-TOF MS) combined with magnetic bead were used to analyse the salivary peptidome. Saliva samples from 40 patients (10 in each of four groups: the group without an appliance and groups under treatment for 2, 7, and 12 months) were analyzed. The potential discriminating biomarkers investigated in this study

reflected the complicated changes in periodontal tissues during orthodontic treatment and indicated dynamic interactions between orthodontic treatment and the saliva proteome. This study indicated that the peptide profiles changed as the orthodontic treatment proceeded by using magnetic bead-based Matrix-assisted laser desorption/ionization time-of- flight mass spectrometry.

**Ellias MF. et al (2012)**[57] stated that orthodontic treatment has been shown to induce inflammation, followed by bone remodeling in the periodontium. These processes trigger the secretion of various proteins and enzymes into the saliva. Thus, they conducted a study to identify salivary proteins that change in expression during orthodontic tooth movement. These differentially expressed proteins can potentially serve as protein biomarkers for the monitoring of orthodontic treatment and tooth movement. Whole saliva from three healthy female subjects were collected before force application using fixed appliance and at 14 days after 0.014 Niti wire was applied. Salivary proteins were resolved using two-dimensional gel electrophoresis (2DE) over a pH range of 3–10, and the resulting proteome profiles were compared. Differentially expressed protein spots were then identified by Matrix-assisted laser desorption/ionization time-of- flight mass spectrometry tandem mass spectrometry. Eight proteins were successfully identified using Matrix-assisted laser desorption/ionization time-of- flight mass spectrometry. Immunoglobulin J chain isoform, S100 calcium-binding protein A9, serum albumin precursor and Ig alpha-1 chain C region were down-regulated at day 14 of orthodontic treatment. On the other hand, cysteine-rich secretory protein 3 precursor was present only at day 14 while hemoglobin subunit beta and 14-3-3 protein σ (SFN) were only present at day 0.

**Flórez-Moreno GA et al. (2013)**[58] conducted study aimed to determine whether the variations in salivary concentrations of soluble receptor activator of nuclear factor kappa B ligand and osteoprotegerin, and their ratios, might be linked with the different phases of orthodontic tooth movement. Unstimulated whole saliva samples were collected from each patient before fitting the orthodontic appliances, and at 24 to 48 hours, 2 weeks, 5 weeks, and 8 weeks after the activation. Salivary soluble receptor activator of nuclear factor kappa B ligand and osteoprotegerin concentrations were determined by enzyme-linked immunosorbent assays. Overall, median values of soluble receptor activator of nuclear factor kappa B ligand showed significant increases, median osteoprotegerin salivary values showed a significant downward trend, and the soluble receptor activator of nuclear factor kappa B ligand / osteoprotegerin ratio tended to increase significantly over time after the activation visit. These findings thus indicate that variations in salivary concentrations of soluble receptor activator of nuclear factor kappa B ligand and osteoprotegerin and their ratios might be linked to the different phases of orthodontic tooth movement. Hence, these analytes might serve in a panel of salivary functional biomarkers that could assist in the screening of orthodontic treatment in clinical practice.

**Flórez-Moreno GA et al (2013)**[59] conducted study aimed to determine whether the variations in the concentration of bone remodeling biomarkers Deoxypyridinoline (DPD) and bone-specific alkaline phosphatase (BAP) as detected through a longitudinal follow – up with four consecutive visits may be linked with the different phases of OTM. Unstimulated whole saliva samples were collected from each patient prior to fitting the orthodontic appliances and 24–48 hours, 2 weeks, and 5 weeks after the activation. Salivary Deoxypyridinoline and bone-specific alkaline

phosphatase concentrations were determined by enzyme-linked immunosorbent assay. There were no statistically significant differences in salivary levels of biomarkers regarding demographic and clinical parameters. Deoxypyridinoline values revealed an increasing nature after force application and bone-specific alkaline phosphatase values showed a descending trend over time after activation visit. However, the results of the Friedman test showed that whereas salivary concentrations of bone-specific alkaline phosphatase did not significantly differ over time there were statistically significant quantitative changes in the mean ranks of the Deoxypyridinoline salivary levels through the different sampling times. Also, Deoxypyridinoline salivary levels revealed significant differences between every paired sampling times, except for the pair baseline test/ 24 –48 hours test. The findings indicate that although salivary levels of Deoxypyridinoline and bone-specific alkaline phosphatase may act as indicators of increased bone remodeling, it appears that Deoxypyridinoline dominates the earlier phases of OTM, whereas bone-specific alkaline phosphatase might serve as indicator of bone formation as soon as the tooth movement stops.

**Ghaib NH. (2013)[60]** carried out a study to estimate changes of the (interleukin-one beta, tumor necrosis factor – alpha and C-reactive protein) levels in unstimulated whole saliva during the leveling stage of orthodontic tooth movement. The unstimulated whole saliva was taken from each sample immediately before placement of the appliance (baseline), and at (after1hour, after one week and after two week) following placement of the fixed orthodontic appliance. The interleukin – one beta and tumor necrosis factoralpha were determined by enzyme linked immunosorbent assay, while the C-reactive protein was determined by latex agglutination. The result

of the present study showed that the mean value of salivary level IL-1 β and TNF-α were elevated to reach the peak at T1 following placement of the orthodontic appliance during the study periods followed by declined at T2 then T3. The mean value of salivary C-reactive protein was highly at the T1 with highly statistically significant difference ($P< 0.01$) among females and total sample and only significant difference ($P<0.05$) for males. The authors concluded that orthodontic force induces increasing the levels of interleukin – one beta, tumor necrosis factor -alpha, C-reactive protein in unstimulated whole saliva during orthodontic tooth movement.

**Kuroki H et al (2013)**[61] conducted a study to identify stress marker proteins during orthodontic treatment. Levels of receptor activator of nuclear factor kappa-B ligand and heat shock protein 70 in the gingival crevicular fluid (GCF) were analyzed as markers of mechanical stress, and levels of chromogranin-A and amylase in whole saliva were analyzed as markers of psychological stress. GCF was collected from control and experimental teeth at initiation of treatment and 24 h after treatment. Whole saliva was collected before treatment, at initiation of treatment and 24 h after treatment. Receptor activator of nuclear factor kappa-B ligand was expressed at 24 h after treatment in the experimental GCF, but not in the control GCF. Heat shock protein 70 appeared to be constitutively expressed in GCF, and its levels showed no major change between the control and experimental groups from initiation of treatment to 24 h after treatment. Amylase activity in whole saliva was enhanced at 24 h after treatment compared to control, but chromogranin-A levels showed little change between the groups. These results indicated that Receptor activator of nuclear factor kappa-B ligand and amylase may be the candidate markers for mechanical and psychological stress, respectively, during orthodontic treatment.

**Batool H. Al-Ghurabi et al (2014)[62]** conducted a study to investigate the effects of orthodontic tooth movement on the periodontium by analyzing proinflammatory cytokines (IL-1α, IL-8 and GM-CSF) levels within saliva. Saliva samples were collected from 18 subjects (12 females and 6males) at baseline time (BT), after 2weeks (T2) and after 4weeks (T4) from placement of orthodontic appliance. Salivary IL-1α, IL- 8 and GM-CSF were measured by means of enzyme-linked immune-sorbent assay. The results revealed no statistically significant differences in the levels of IL-1α at any times. Significant elevation in median salivary levels of IL-8 and GM-CSF was found after 2 weeks and after 4 weeks from placement of orthodontic appliance as compared to their levels at baseline time. In addition the levels of these cytokines were significantly decreased at 4weeks when compared to their levels at 2 weeks. These findings suggest that orthodontic force induces release of the proinflammatory cytokines (IL- 8 and GM-CSF), in addition proinflammatory cytokines play a crucial role in bone resorption after the application of orthodontic force.

**Luchian I et al (2016)[63]** conducted a study to evaluate the differences between the saliva levels of inflammation biomarkers of prostaglandin E2 in patients wearing orthodontic appliances who had a stabilised pre-existing periodontal pathology (chronic periodontitis localised in minimum 3 teeth) by comparison to patients with the same pathology who did not receive orthodontic treatment. The 60 patients included in the study were divided into three groups, as follows: group A, the control group, without periodontal disease; Group B, patients with periodontal disease (chronic periodontitis localised in minimum 3 teeth), who have received periodontal treatment; group C, patients with periodontal disease (chronic periodontitis localized

in minimum 3 teeth) who have received both orthodontic and periodontal treatment. The results of the post-treatment values of prostaglandin E2 have shown a significant decrease after both alternatives of treatment. To conclude, the values of prostaglandin E2 decreased more in the case of combined treatment. Thus the levels of prostaglandin E2 which increase during start of orthodontic treatment are seen to subside by the end of orthodontic treatment.

# *Materials and Methods*

The present study was carried out to evaluate the presence of interleukin – 8 and its comparison in the gingival crevicular fluid and saliva during different times of leveling and alignment phase and during the space closure phase. A total of 20 patients between the age group of 15 to 25 years requiring upper first premolar extraction, were selected from those visiting the Department of Orthodontics and Dentofacial Orthopaedics of our institute. The study was initiated after the clearance from the Institutional Ethics Committee of our institute. The levels of interleukin – 8 in gingival crevicular fluid and saliva were measured by using ELISA kit.

**Inclusion criteria –**

1. No prior orthodontic treatment
2. Patients requiring first premolar extraction in the maxillary arch.
3. Patients with no history of smoking.
4. Probing depths less than or equal to 3 mm

**Exclusion criteria –**

1. Patients with periodontal diseases.
2. Patients with history of systemic diseases.
3. Patients on anti-inflammatory drugs.

**The study was divided into two groups which were to be compared –**

**GROUP A – Gingival crevicular fluid group** – This group was subdivided into phases as per the time of sample collection.

Phase I – leveling phase –

Baseline phase (BL) - Collection of GCF at baseline time - before bonding of orthodontic appliance.

Phase I A (24 hrs) – collection of GCF 24 hours after the beginning of leveling and alignment phase.

Phase I B (7 days) – collection of GCF 7 days after the beginning of leveling and alignment phase.

Phase II – space closure phase –

Phase II A (24 hrs) – collection of GCF 24 hours after the start of canine retraction in space closure phase.

Phase II B (7 days) - collection of GCF 7 days after the start of canine retraction in space closure phase.

**GROUP B – Saliva group** – This group was also subdivided into phases as per the time of sample collection.

Phase I – leveling phase –

Baseline phase - Collection of saliva at baseline time (BT) - before bonding of orthodontic appliance.

Phase I a (24 hr) – collection of saliva 24 hours after the beginning of leveling and alignment phase.

Phase II b (7 days) – collection of saliva 7 days after the beginning of leveling and alignment phase.

Phase II – space closure –

Phase II a (24 hr) – collection of saliva 24 hours after the start of canine retraction in space closure phase.

Phase II b (7 days) - collection of saliva 7 days after the start of canine retraction in space closure phase.

Materials –

The materials and armamentarium used in this study for collection of GCF and for collection of Saliva, assessment of Interleukin-8 in GCF and saliva, application and assessment of force during canine retraction in space closure phase were as follows –

**For collection of GCF –**

1. Autoclaved cotton rolls and aspirator
2. Micropipettes (Figure 1)
3. Plastic vials

**For collection of saliva –**

Caliberated Eppendorf tubes (1.5 ml) (Figure 2)

Laboratory armamentarium –

1. Caliberated, volumetric transfer pipettes with 0-5 µl range, 5-50 µl range, 50-200 µl range and 200-1000 µl range.
2. Sterilized test tubes with test tube stand.
3. Distilled water.
4. Beakers, measuring cylinder.
5. Filter paper.
6. Sterile gloves.

**ELISA KITS: (Figure 3)**

Two ELISA kits for Human interleukin 8 ( krishgen biosystems, Whittier, CA,USA ) 96 wells each alongwith –

1. IL-8 Microtiter Coated Plate (12 strips X 8 wells)
2. Recombinant Human IL-8 Standard – 2 vials
3. Human IL-8 Biotin Conjugated Detection Antibody – 2 vials
4. Concentrated Streptavidin Horseradish Peroxidase - 1 vial
5. Wash Buffer (20X) – 25ml
6. Assay Diluent (5X) – 10ml
7. TMB Substrate – 12ml
8. Stop Solution – 12ml

**LABORATORY EQUIPMENTS –**

1. $-20^0$ C deep freezer (Quick freezer, REMI Equipments Pvt. Ltd.) (Figure 4)
2. Lab Centrifuge machine (R-8C, REMI Equipments Pvt. Ltd.) (figure 5)
3. Vortex Mixer (CM 101, REMI Eqipments Pvt. Ltd.)
4. Automated microplate washer (LISA wash, REMI Equipments Pvt. Ltd.) (figure 6)
5. ELISA reader (LISA plus multiplate reader, REMI Equipments Pvt. Ltd.) (figure 7)

**GCF and Saliva collection procedure –**

All the selected subjects were briefly informed about the procedure to be done and were seated comfortably in the dental chair. GCF and Saliva collection was done just before bonding (baseline phase) (figure 8), 24 hours and 7 days after start of initial leveling (figure 9, 10), followed by 24 hours and 7 days after the start of canine retraction during space closure phase (figure 12, 13). The canine retraction was done with active tiebacks and the force applied for the retraction of canine was 150 grams on each side. The force levels were checked using Dontrix gauge (Figure 11).

The GCF sample collection was done by using a caliberated micro-capillary pipette (1-5ul)[64]. Prior to collection of GCF, any supragingival soft deposits were removed without causing trauma to the gingival crevice. If any haemorrhage was evident after this procedure, no fluid was collected. The area was then thoroughly irrigated with distilled water, isolated by cotton rolls and dried by steam of air. About 10 min after isolation, an accumulation of fluid was seen at the marginal regions and interdental areas. The free ending of tubing was then brought in contact with the fluid and was

moved slightly backward and forward. The tube was partially and rapidly filled with liquid, rising into it by capillarity. From one of the side of the maxillary canines, a standardized volume of 5 µl was collected. Sites which did not express appropriate volume of fluid and micropipettes which were contaminated with blood and saliva were not included in the study. Collected GCF samples were immediately transferred to airtight plastic vials and were stored at $-20^0$C until assayed.

Saliva was collected using plastic calibrated 1.5 mL eppendorf tubes and a small straw segment. Participants were asked to rinse their mouth with water, to remove bacteria and food-related debris, and then wait for 10 minutes before giving a sample to reduce the possibility of saliva dilution. Participants were instructed to pool saliva in their mouth for three minutes before gently forcing it into the eppendorf tubes through the straw segment. Collected samples were immediately frozen at $-20^0$C until processing. In preparation for assay, samples were thawed and centrifuged at 1000 x g for 10 minutes in order to remove insoluble material and the supernatants were collected and used for assay.

**ESTIMATION OF INTERLEUKIN 8 LEVELS USING ELISA KITS –**

For quantitative estimation of IL-8, all reagents of the kit were allowed to warm to room temperature for atleast 30 minutes prior to opening. All reagents, standards and samples were prepared, according to manufacturer's instructions. Assay Diluent was diluted 5-fold with deionized or distilled water before use. Dilution of GCF and Saliva sample to 100 µl was carried out using Assay Diluent.

**METHODOLOGY –**

The Human IL-8 ELISA kit is an in vitro ELISA for the quantitative measurement of human interleukin-8. The assay employs an antibody specific for human IL-8 coated on a 96-well plate. Standards and samples are pipetted into the wells and IL-8 present in the sample is bound to the wells by the immobilized antibody. The wells are washed and then biotinylated anti-human IL-8 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to wells. The wells are again washed, a TMB substrate solution is added to the wells. The positive wells turn bluish in color. The stop solution is then added which changes the colour of solution from blue to yellow. The absorbance of each well is read on an ELISA reader using 450 nm as the primary wavelength.

**ASSAY PROCEDURE SUMMARY –**

1. Prepare all reagents, samples and standards as instructed

2. Add 100 µl standard or sample to each well. Incubate 2 hours at room temperature

Wash plate 4 times with wash buffer

3. Add 100 µl prepared biotin antibody to each well. Incubate 2 hour at room temperature.

Wash plate 4 times with wash buffer

4. Add 100 µl prepared streptavidin solution. Incubate 30 minutes at room temperature.

Wash plate 4 times with wash buffer

5. Add 100 µl TMB substrate reagent to each well. Incubate in dark for 15 minutes.

6. Add 50 µl stop solution to each well. Read absorbance at 450 nm immediately.

The colour of the solution in the wells changes from blue to yellow in proportion to the amount of human IL-8 bound to the substrate. The intensity of colour is measured on a microplate (ELISA) reader at a wavelength of 450 nm.

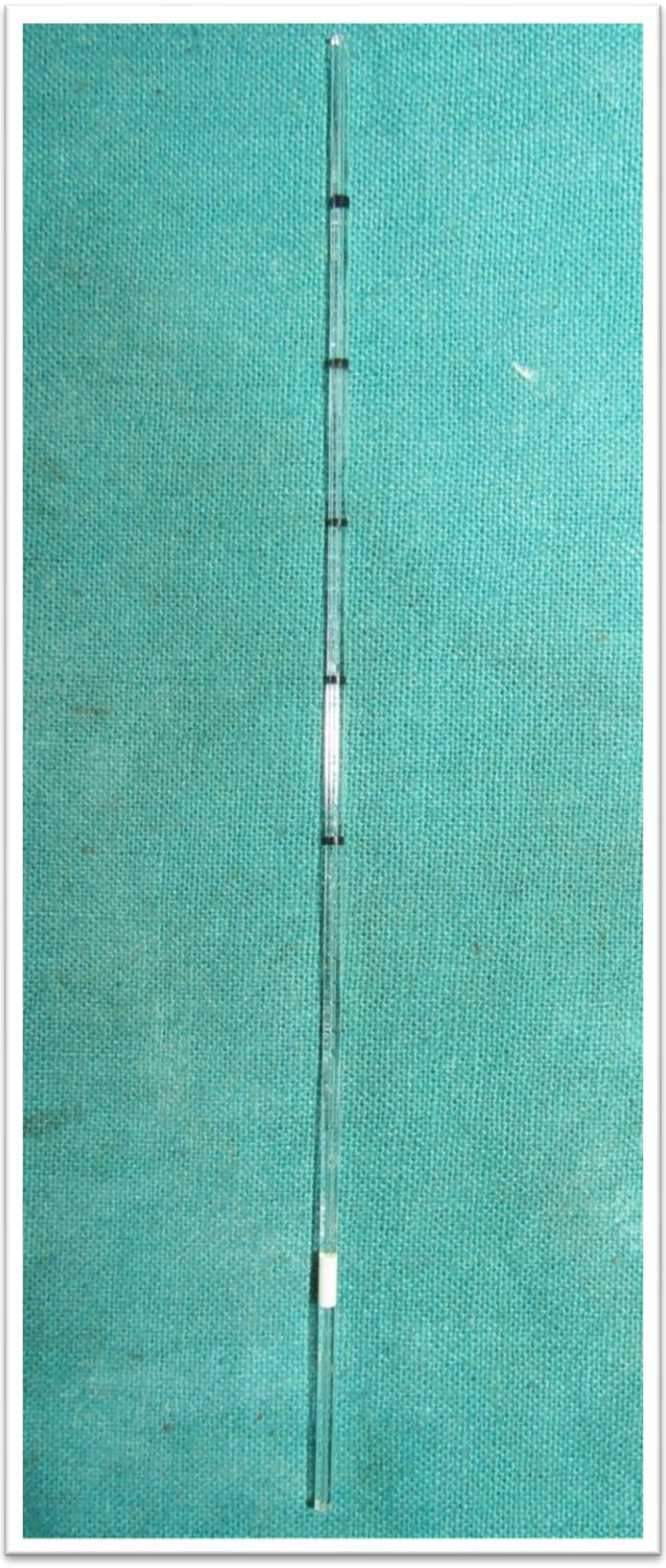


**Figure 1 – Micro capillary pipette**

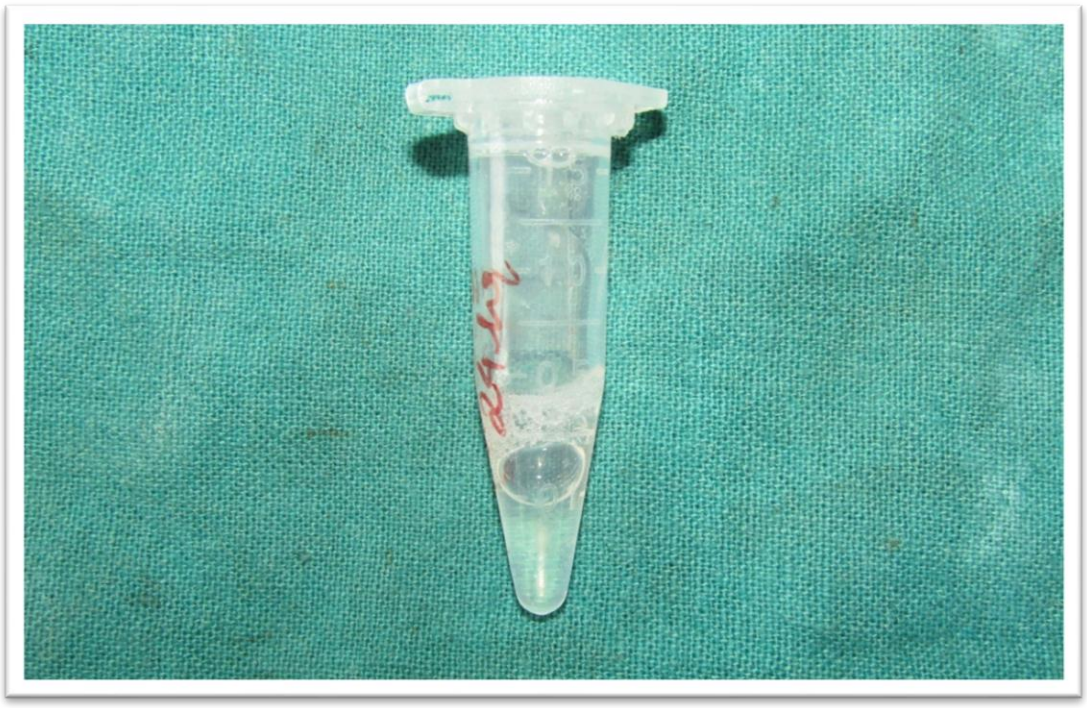


**Figure 2 – collection of saliva in eppendorf tubes**

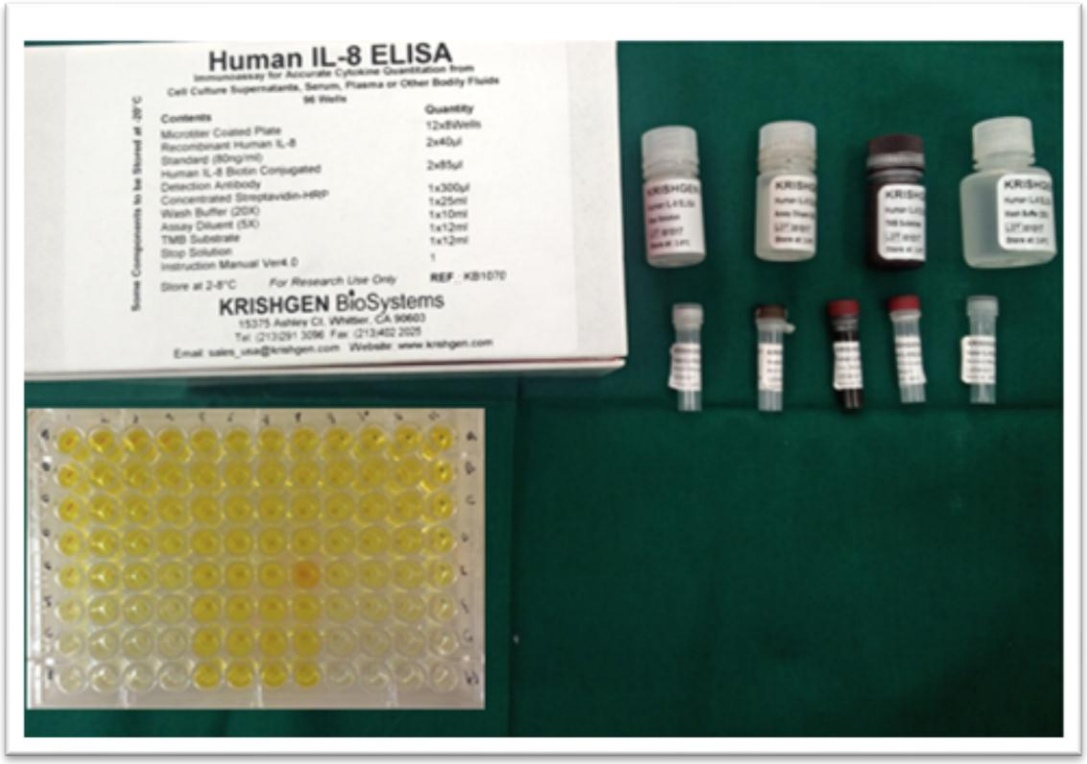



**Figure 3 – ELISA kit**


**Figure 4 – Laboratory Refrigerator**


**Figure 5 – Lab centrifuge machine**


**Figure 6 – Microplate washer**


**Figure 7 – ELISA reader**

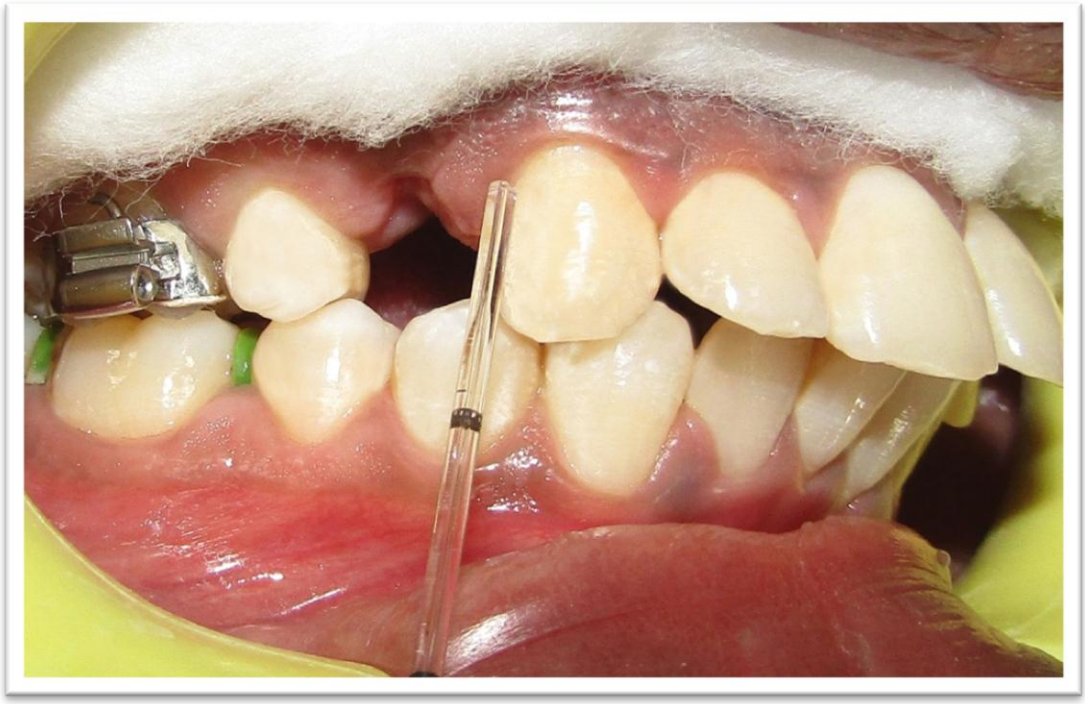


**Figure 8 – collection of GCF at the baseline i.e. before bonding.**

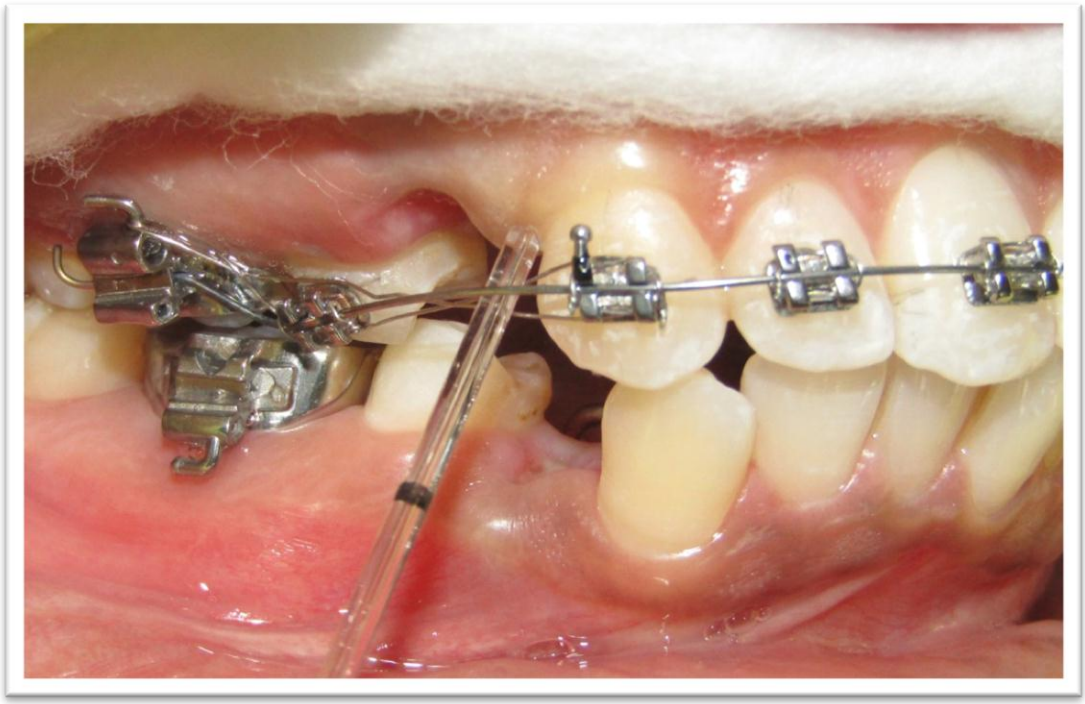


**Figure 9 – collection of GCF 24 hours after bonding.**

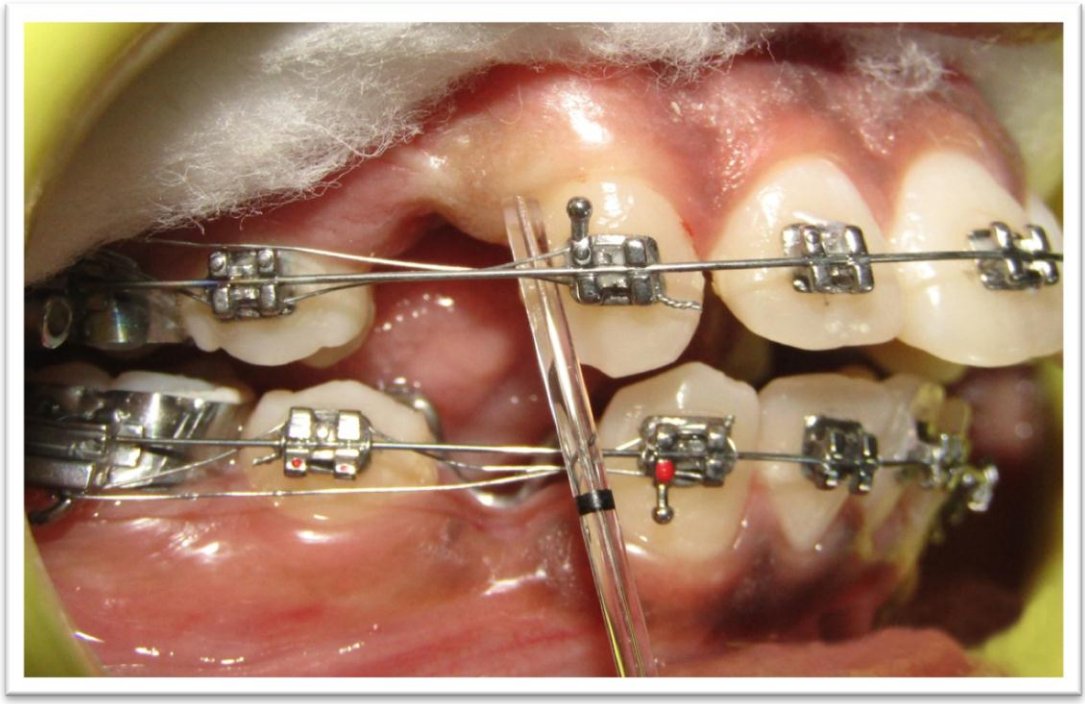


**Figure 10 – collection of GCF 7 days after bonding.**

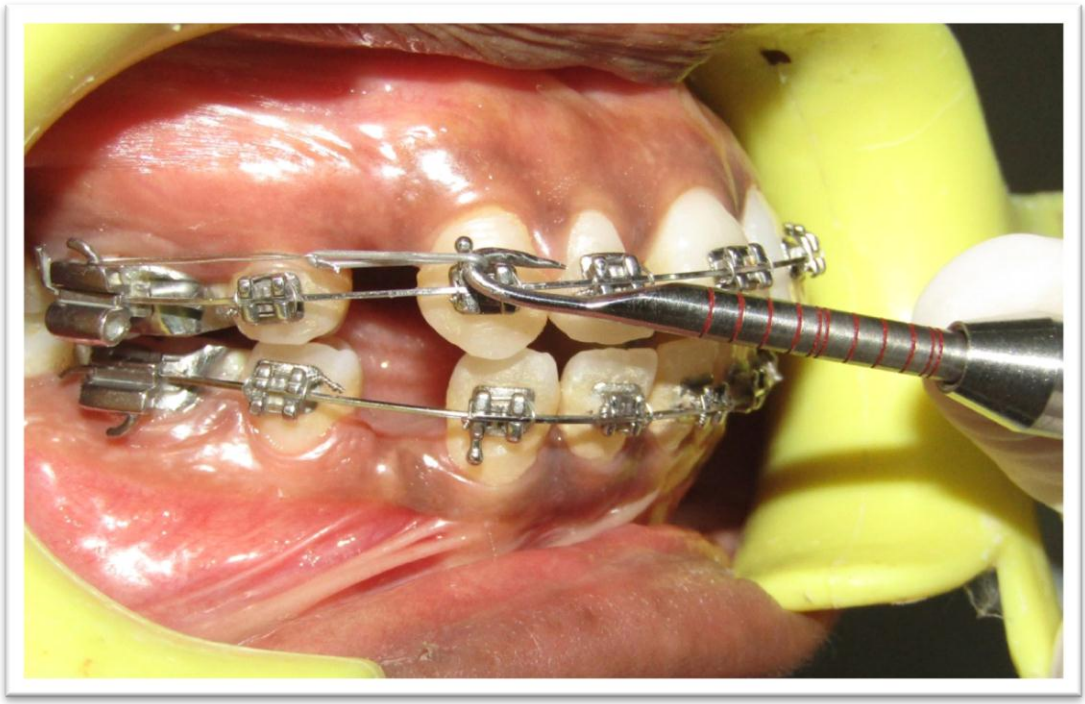


**Figure 11 – Individual canine retraction using Active tiebacks by application of 150 gms of force.**

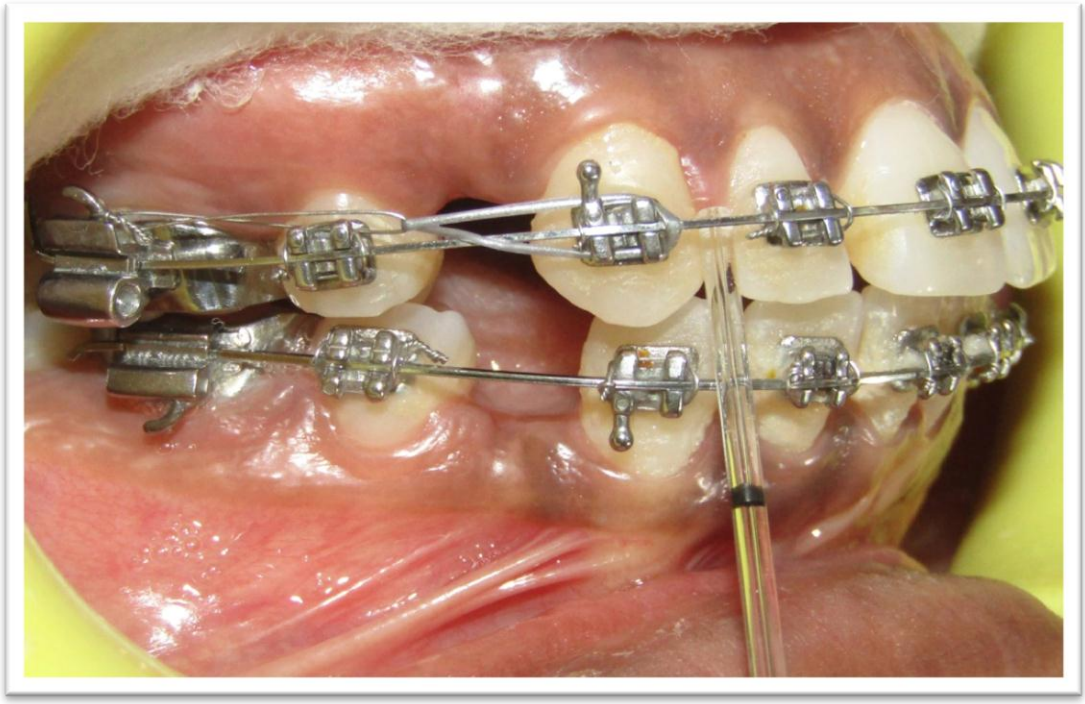


**Figure 12 – collection of GCF at 24 hrs after the start of retraction.**

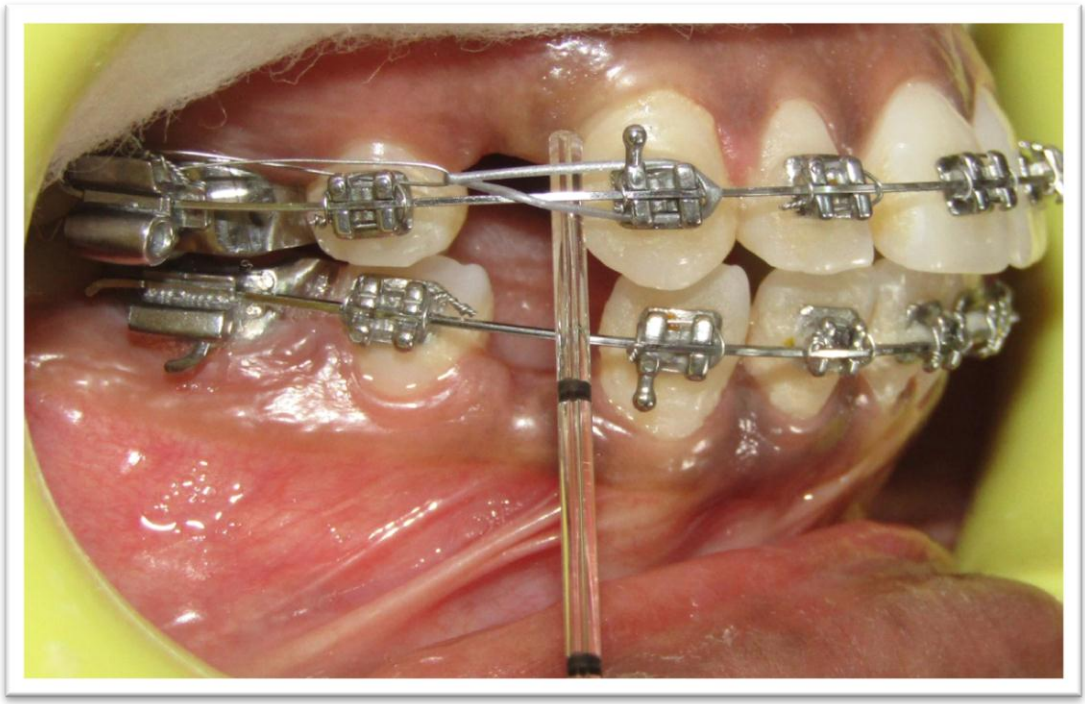


**Figure 13 – collection of GCF at 7 days after the start of retraction.**

## STATISTICAL METHODS

The data on IL-8 levels from GCF and Saliva were obtained before and after bonding at 24 hours and 7 days of leveling and space closure phase. Descriptive statistics like mean and standard deviation of IL-8 levels were obtained for each time point and each phase in two groups. The statistical comparison of mean IL-8 levels between leveling and space closure phases was performed using paired t-test, while across times in each phase was done using repeated measure analysis of variance. This analysis was performed independently for GCF and Saliva category. The paired comparison between time intervals was carried out using Tukey's post-hoc test. Also, the statistical comparison of mean IL-8 levels was performed between GCF and Saliva samples at different time points independently using t-test for independent samples. This analysis was performed separately for leveling and space closure phase. All the above analyses were performed using SPSS ver 20.0 (IBM Corp.) software and the statistical significance was tested at 5% level.

# *RESULTS*

In the present study the levels of interleukin – 8 were assessed in GCF and saliva at different times in leveling and space closure phase. The concentrations of IL-8 were assessed before bonding of appliance, followed by 24 hrs and 7 days after the bonding. Also IL-8 was assessed during space closure phase at 24 hrs and 7 days after the application of retraction force. The levels of IL-8 were assessed using ELISA test in both GCF and saliva. The concentrations of IL-8 were expressed in picograms/millilitre (pg/mL)

**Table 1: Mean and standard deviation for IL-8 in GCF and Saliva at different times**

| Time | Groups | | | |
|---|---|---|---|---|
| | Gingival Crevicular Fluid (GCF) | | Saliva | |
| | Leveling Phase | Space closure Phase | Leveling Phase | Space closure Phase |
| Baseline phase | 45.10 ± 3.24 | | 29.85 ± 2.98 | |
| 24 hours | 84.75 ± 5.02 | 119.90 ± 3.23 | 62.10 ± 3.04 | 102.20 ± 3.85 |
| 7 days | 67.10 ± 4.78 | 105.05 ± 4.95 | 40.05 ± 2.95 | 79.85 ± 3.30 |

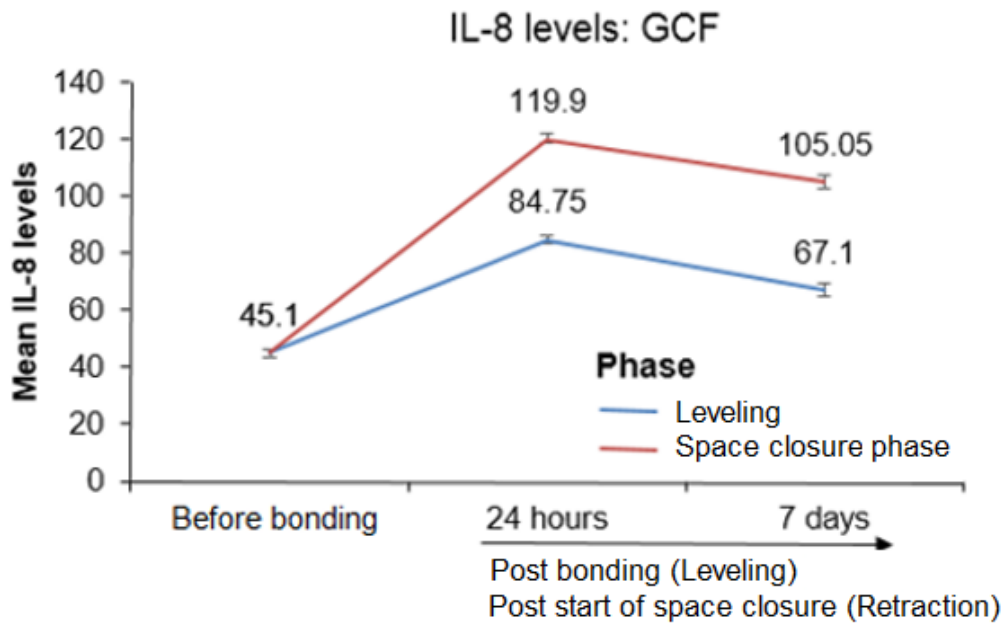
Table 1 provides the mean and standard deviation for IL-8 levels in GCF and saliva at different times at leveling and Space closure phases. In GCF at leveling phase, the mean IL-8 level before treatment was 45.10 ± 3.24 pg/mL, while after 24 hours it was 84.75 ± 5.02 pg/mL and after 7 days was 67.10 ± 4.78 pg/mL. In the Space closure phase, before treatment, after 24 hours, it was 119.90 ± 3.23 pg/mL and after 7 days, it was 105.05 ± 4.95 pg/mL. In Saliva, at leveling phase, the mean IL-8 level was 29.85 ± 2.98 pg/mL before treatment, 62.10 ± 3.04 pg/mL after 24 hours and 40.05 ± 2.95 pg/mL after 7 days. In the Space closure phase, after 24 hours, the mean was 102.20 ± 3.85 pg/mL and after 7 days was 79.85 ± 3.30 pg/mL.

**Table 2: Comparison of IL-8 levels of GCF between two phases as well as across times in each phase**

| Time | Phase | | P-value* |
|---|---|---|---|
| | Leveling | Space closure | |
| Baseline phase | 45.10 ± 3.24 | 45.10 ± 3.24 | - |
| 24 hours | 84.75 ± 5.02 | 119.90 ± 3.23 | < 0.0001 (HS) |
| 7 days | 67.10 ± 4.78 | 105.05 ± 4.95 | < 0.0001 (HS) |
| P-value** | < 0.0001 (HS) | < 0.0001 (HS) | |

**Calculated using paired t-test; **calculated using Repeated measures ANOVA; HS: Highly Significant*

Table 2 gives the comparison of mean IL-8 levels of GCF between two phases as well as across time interval in each phase. In leveling phase, the difference of means was statistically highly significant as indicated by p-value < 0.0001 using repeated measure analysis of variance. Similar was the observation in the space closure phase. Also, the comparison of levels was performed between leveling and space closure phases at 24 hours and 7 days using paired t-test. At both the times, the mean difference was statistically highly significant with p-values < 0.0001. The levels at space closure phase were significantly higher than that of leveling phase. Graph 1 provides a line chart representation of the mean levels at three time points and for each phase.




**Graph 1: Line plots showing mean IL-8 levels in GCF at different times for two phases**

**Table 2(a): Pair wise comparison of IL-8 of GCF between times in leveling phase**

| Leveling | Absolute mean difference | P-value |
|---|---|---|
| Baseline phase - 24 hours | 39.650 | < 0.0001 (HS) |
| Baseline phase - 7 days | 22.000 | < 0.0001 (HS) |
| 24 hours - 7 days | 17.650 | < 0.0001 (HS) |

*HS: Highly Significant*

The difference across time interval was statistically highly significant ($p < 0.0001$) in leveling phase and hence pair wise comparison was performed using Tukey's post-hoc test with the results shown in Table 2(a). The difference between the baseline and 24 hours as well as 7 days was statistically highly significant ($p < 0.0001$). Also the difference between 24 hours and 7 days was highly significant ($p < 0.0001$).

**Table 2(b): Pair wise comparison of IL-8 of GCF between times in space closure phase**

| Space closure | Absolute mean difference | P-value |
|---|---|---|
| Baseline phase - 24 hours | 74.800 | < 0.0001 (HS) |
| Baseline phase - 7 days | 59.950 | < 0.0001 (HS) |
| 24 hours - 7 days | 14.850 | < 0.0001 (HS) |

*HS: Highly Significant*

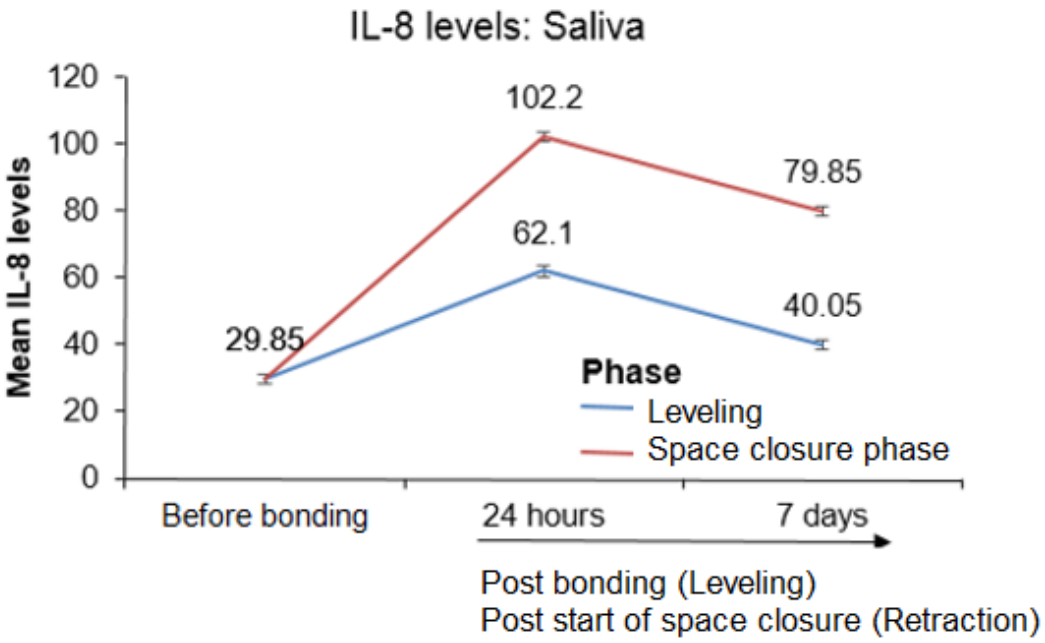
On similar lines, the difference across time interval was statistically highly significant ($p < 0.0001$) in space closure phase and hence pair wise comparison was performed using Tukey's post-hoc test with the results shown in Table 2(b). The difference between baseline and 24 hours as well as 7 days was statistically highly significant ($p < 0.0001$). Also the difference between 24 hours and 7 days was highly significant ($p < 0.0001$).

**Table 3: Comparison of IL-8 levels of Saliva between two phases as well as across times in each phase**

| Time | Phase | | P-value* |
|---|---|---|---|
| | Leveling | Space closure phase | |
| Baseline phase | 29.85 ± 2.98 | 29.85 ± 2.98 | - |
| 24 hours | 62.10 ± 3.04 | 102.20 ± 3.85 | < 0.0001 (HS) |
| 7 days | 40.05 ± 2.95 | 79.85 ± 3.30 | < 0.0001 (HS) |
| P-value** | < 0.0001 (HS) | < 0.0001 (HS) | |

**Calculated using paired t-test; **calculated using Repeated measures ANOVA; HS: Highly Significant*

Table 3 gives the comparison of mean IL-8 levels in saliva between two phases as well as across time interval in each phase. In leveling phase, the difference of means was statistically highly significant as indicated by p-value < 0.0001 using repeated measure analysis of variance. Similar was the observation in the space closure phase. Also, the comparison of levels was performed between leveling and space closure phases at 24 hours and 7 days using paired t-test. At both the times, the mean difference was statistically highly significant with p-values < 0.0001. The levels at space closure phase were significantly higher than that of leveling phase. Graph 2 provides a line chart representation of the mean levels at three time points and for each phase.




**Graph 2: Line plots showing mean IL-8 levels in saliva at different times for two phases**

**Table 3(a): Pair wise comparison of IL-8 of saliva between times in leveling phase**

| Leveling | Absolute mean difference | P-value |
|---|---|---|
| Baseline phase - 24 hours | 32.250 | < 0.0001 (HS) |
| Baseline phase - 7 days | 10.200 | < 0.0001 (HS) |
| 24 hours - 7 days | 22.050 | < 0.0001 (HS) |

*HS: Highly Significant*

The difference across time interval was statistically highly significant ($p < 0.0001$) in leveling phase and hence pair wise comparison was performed using Tukey's post-hoc test with the results shown in Table 3(a). The difference between the baseline and 24 hours as well as 7 days was statistically highly significant ($p < 0.0001$). Also the difference between 24 hours and 7 days was highly significant ($p < 0.0001$).

**Table 3(b): Pair wise comparison of IL-8 of saliva between times in space closure phase**

| **Space closure** | **Absolute mean difference** | **P-value** |
|---|---|---|
| Baseline phase - 24 hours | 72.350 | < 0.0001 (HS) |
| Baseline phase - 7 days | 50.000 | < 0.0001 (HS) |
| 24 hours - 7 days | 22.350 | < 0.0001 (HS) |

*HS: Highly Significant*

The difference across time interval was statistically highly significant ($p < 0.0001$) in space closure phase and hence pair wise comparison was performed using Tukey's post-hoc test with the results shown in Table 3(b). The difference between baseline

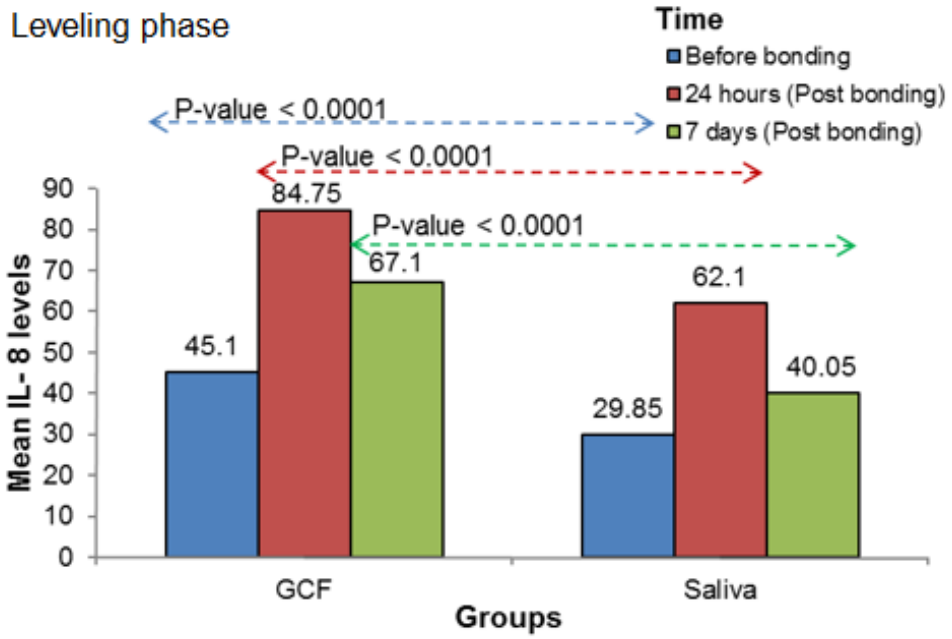
and 24 hours as well as 7 days was statistically highly significant ($p < 0.0001$). Also the difference between 24 hours and 7 days was highly significant ($p < 0.0001$).

**Table 4: Comparison of IL-8 levels at leveling phase between two groups at three time points**

| Time | Group | | P-value* |
|---|---|---|---|
| | GCF | Saliva | |
| Baseline phase | 45.10 ± 3.24 | 29.85 ± 2.98 | < 0.0001 (HS) |
| 24 hours | 84.75 ± 5.02 | 62.10 ± 3.04 | < 0.0001 (HS) |
| 7 days | 67.10 ± 4.78 | 40.05 ± 2.95 | < 0.0001 (HS) |

**Calculated using t-test for independent samples; HS: Highly Significant*

Table 4 gives the comparison of mean IL-8 levels between GCF and Saliva at leveling phase at three time points using t-test for independent samples. It is evident from the table that at all the three time points, the difference of mean levels between groups was statistically highly significant as indicated by p-values < 0.0001. The means for GCF were significantly higher than that of saliva. A graphical visualization of the comparison is given in graph 3.




**Graph 3: Comparison of mean IL-8 levels between GCF and Saliva at leveling phase for each time**

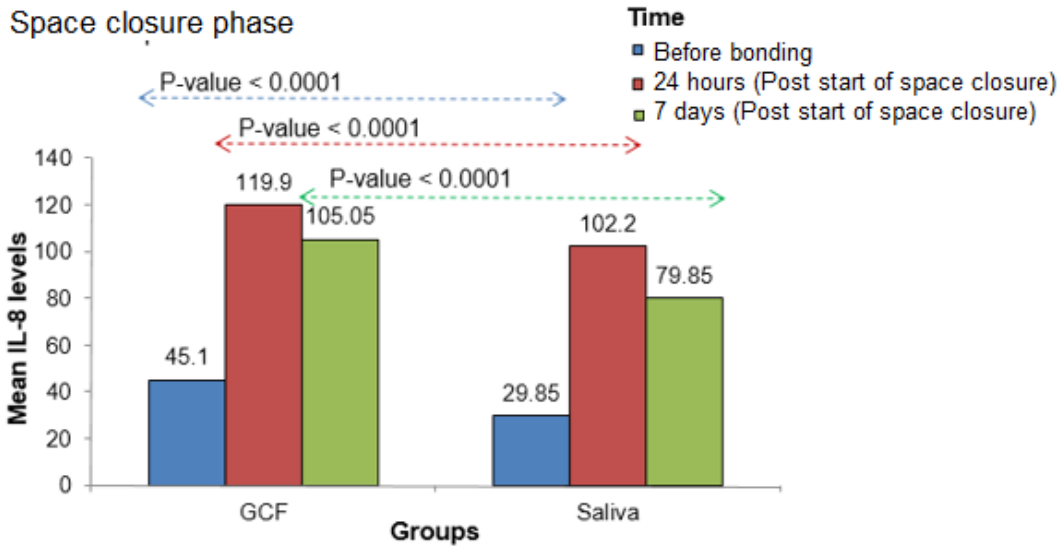
**Table 5: Comparison of IL-8 levels at space closure phase between two groups at three time points**

| Time | Group | | P-value* |
|---|---|---|---|
| | GCF | Saliva | |
| Baseline phase | 45.10 ± 3.24 | 29.85 ± 2.98 | < 0.0001 (HS) |
| 24 hours | 119.90 ± 3.23 | 102.20 ± 3.85 | < 0.0001 (HS) |
| 7 days | 105.05 ± 4.95 | 79.85 ± 3.30 | < 0.0001 (HS) |

**Calculated using t-test for independent samples; HS: Highly Significant*

Table 5 gives the comparison of mean IL-8 levels between GCF and Saliva at space closure phase at three time points using t-test for independent samples. It is evident from the table that at all the three time points, the difference of mean levels between groups was statistically highly significant as indicated by p-values < 0.0001. The

means for GCF were significantly higher than that of saliva. A graphical visualization of the comparison is given in graph 4.




**Graph 4: Comparison of mean IL-8 levels between GCF and Saliva at space closure phase for each time**

# *Discussion*

Orthodontic tooth movement is induced by mechanical stimuli and facilitated by remodeling of the periodontal ligament and alveolar bone. The remodeling activities and ultimately tooth displacement are the consequence of an inflammatory process[2]. The cellular, biochemical and molecular events that take place during orthodontic tooth movement have been extensively studied. Although inflammation is essential in the remodeling activities, it may result in several undesirable side effects. Thus, not only the aesthetic and functional outcome but also the adverse events for the teeth and the supporting tissue related to the orthodontic tooth movement should be considered. The local changes in surrounding periodontium are reflected in changes in the composition of GCF. Various biomarkers play a regulating role in the inflammatory process, amongst which group of cytokines have attracted particular attention as their levels may increase rapidly before the subsequent visible clinical inflammation,

suggesting that they may have a potential role as early markers of inflammatory changes[41].

The present study was carried to evaluate the levels of proinflammatory cytokine IL-8 in saliva and GCF during the start of leveling phase and space closure phase and to compare the concentrations between saliva and GCF. IL-8 is a proinflammatory cytokine found to affect the bone metabolism and thereby the orthodontic tooth movement. The orthodontic force activates the microenvironment of PDL; several proinflammatory cytokines are produced to trigger a cascade of cellular events[36]. IL-8 is identified as neutrophil activating cytokine, released by phagocytes and variety of tissue cells upon exposure to inflammatory stimuli[65]. It acts early in inflammatory response, thus playing a vital role in regulating alveolar bone resorption during tooth movement[62].

In literature various studies have demonstrated the presence of various biomarkers in GCF during orthodontic tooth movement. Proinflammatory cytokines are the important regulators in bone remodeling process on mechanical stimulation and are one of the early biomarkers in orthodontic treatment. These proinflammtory cytokines include interleukin (IL)-1beta, IL-2, IL-5, IL-6, IL-8, TNF-alpha, interferon-gamma and GM-CSF[47]. IL-8 was the biomarker of choice in this study due to its ability to stimulate human polymorphonucleocytes (PMN) to release enzyme like myeloperoxidase, which is one of the earliest markers to monitor the degree of periodontal inflammation[66,67].

It was found in this study that the concentrations of IL-8 significantly increased after the application of force by orthodontic appliance. This was in accordance to the

investigations done by **Catherine Giannopoulou[6], 2008** who compared the levels of IL-1beta, IL-8, IL-4 in subjects with and without fixed orthodontic appliances and found significant rise in levels of IL-1beta and IL-8 in subjects with fixed orthodontic appliances. Thus, indicating the role of IL-8 in the biologic activity in periodontium during orthodontic tooth movement.

There was significant increase in levels of IL-8 after 24 hrs, both during leveling and during space closure phase. This can be correlated to the initial phase of tooth movement which lasts for 24 hrs to 2 days and is characterized by rapid movement immediately after application of force to the teeth. The cellular and tissue reactions start in the initial phase of tooth movement, immediately after force application, resulting in synthesis and release of signalling molecules like cytokines[2]. In this study there was statistically significant increase in levels of IL-8 in GCF from the baseline to the 24 hrs. Also when the levels were evaluated at $7^{th}$ day of leveling, they were significantly higher with respect to baseline ($p < 0.0001$). But when compared across the time between 24 hrs to 7 days, there was statistically significant decrease in the levels of IL-8 by $7^{th}$ day.

These findings are consistent with those of **Kaya FA et al[42], 2010** who studied the levels of IL-8 in GCF at early leveling stage of tooth movement. Their study evaluated the levels of IL-8 from baseline till 7 days of leveling phase and found significant increase in concentration of IL-8 at 1-2 day of leveling. By $7^{th}$ day there was a gradual fall in levels of IL-8 but they found no significant differences in levels between $3^{rd}$ to $7^{th}$ day. Also **Maeda A et al[39], 2007** in their clinical investigations found significantly higher concentration of IL-8 levels at 24 hrs and 48hrs after the

orthodontic force application. The authors also stated that shear stress and pressure force rapidly increased the gene and protein expressions of IL-8/CXCL8 by PDL cells. Consistently, amounts of IL-8 in the gingival crevicular fluid of healthy individuals increased within 2 to 4 days of orthodontic force application. On the contrary **Basaran G. et al[38], 2006** studied the levels of IL-8 from baseline to 7th and 21st day of leveling and found statistically significant decrease on the 7th day of leveling from the baseline. This might be because the levels were not evaluated at short time intervals between 1st day to 7th day. The above results are supported by **Davidovitch Z et al[27], 1988** who in their experimental research have concluded that the levels of chemokines are significantly increased at the early stages and decreased in 7 – 10 days, thus confirming early inflammatory response of cytokines in bone remodeling process.

The levels of IL-8 during the retraction in space closure phase were found to be highly significant in GCF at both 24 hrs and 7th day ($p < 0.0001$). These results are in accordance to **Grant M. et al[5], 2012** who studied the levels of cytokines at 4hrs, 7 days, and 42 days after the application of distalizing forces to the maxillary canines. Tension sites adjacent to canines showed significant increases in levels of IL-8 from 4 hrs to 42 days after the application of force. Also **Basaran G. et al[38], 2006** in their study found significant increase in levels of IL-8 in GCF at 7th day followed by decrease in levels by 21st day after the application of distalization forces. Similarly in the study by **Tuncer BB. et al[3], 2005** the concentrations of IL-8 increased in 1 hr, 24 hrs, 6 days, and 10 days after application of distalizing force. The highest concentrations were observed on 6th day at the tension site whereas highest levels at the pressure site were observed in 1st hour and 24 hrs. In the present study the

concentrations of IL-8 were found to be highest at 24 hrs and reduced by 7 day, this might be because the collection of GCF was not specific to mesial or distal surface. The highly significant increase in levels of IL-8 during retraction is contributed by increased levels of forces which evoked an inflammatory response. About 150 gms of force was applied during retraction in space closure phase, which is reported as optimal range of pressure as studied for distal movement of canines in orthodontic patients by **Storey and Smith** in 1952. This optimal force provides an extrinsic mechanical stimulus that evokes a cellular response that aims to restore equilibrium by remodeling periodontal supporting tissues that leads to the maximum rate of tooth movement with minimal irreversible damage to root, PDL, and alveolar bone[2].

Also the mean levels of IL-8 were found to be significantly higher during space closure phase than during leveling phase. This may be because of the increased levels of forces applied during space closure phase. This is in accordance to **Kapoor P. et al[47], 2014** who stated that the secretion of inflammatory mediators in local environment by cells activated on application of orthodontic forces varies according to the force levels and functional state of available target cells.

The levels of interleukin-8 in saliva in this study were found to follow the similar trend of concentration as that of in GCF during the phases of orthodontic treatment. The salivary levels of IL-8 during leveling phase were seen to rise at 24 hrs and 7$^{th}$ day from the base line. This was in accordance to the study by **Batool H. Al-Ghurabi et al[62], 2014** who reported significant elevation of salivary levels of IL-8 after 2 weeks and 4 weeks from the placement of orthodontic appliance. Similarly the salivary levels of IL-8 during retraction in space closure phase were significantly high

after 24 hrs and 7 days of force application. **Teles R.P et al[53], 2009** investigated the levels of 10 cytokines in saliva between the periodontally healthy subjects and periodontitis patients and found no statistically significant in levels of 10 cytokines between the two groups. There was only weak statistically significant association among mean clinical parameters of periodontal disease and mean salivary levels of IL-8. Thus it can be stated that IL-8 levels rise significantly during inflammation and play an important role in bone remodeling process and can be assessed in saliva.

In present study, a comparison was done for the levels of IL-8 in GCF and saliva at all the three times i.e. baseline phase, 24 hrs and 7 days during both leveling and space closure phase. The mean levels of IL-8 between the groups were statistically highly significant as indicated by p-values < 0.0001 at all the three time points. The values for GCF were significantly higher than that of saliva. The lack of significance between the salivary levels and GCF levels of IL-8 here could be explained, in part, by the extensive dilution of the gingival crevicular fluid containing these cytokines in saliva. Importantly, inflammatory cytokines detected in whole saliva do not originate from major salivary glands secretions; instead, the GCF is the probable source of these cytokines. The amount of gingival crevicular fluid produced per site per hour has been estimated to be 3 µl/h for healthy sites, considering the turnover of the salivary compartment to be 20 ml/h, this would result in dilution factor of 1:6,666. Thus, there is limited contribution of GCF from sites to the composition of whole saliva for most patients. Hence, the mean levels for GCF were significantly higher than that of saliva[53]. Investigations by **Alejandro Navarro et al[67], 2014** show that Myeloperoxidase activity in GCF more accurately reflects inflammation due to orthodontic tooth movement than MPO activity in saliva. This indicated that GCF can

be a more confirmatory medium that accurately reflects inflammatory changes than saliva. GCF is produced directly in the gingival sulcus and by extravasation of circulating plasma. Saliva, in contrast is produced by the salivary glands. Although saliva contains substances similar to GCF, it reflects the buccal environment more than the tooth environment. Therefore, GCF likely reflects local tooth inflammation caused by orthodontic movement more accurately than saliva.

GCF is a unique biological exudate that has been found as a convenient medium to study these mediators with reasonable sensitivity. The biochemical mediators released sequentially at multiple stages during orchestration of tooth movement can be detected in gingival crevicular fluid (GCF). The major attraction of GCF as a diagnostic marker is the site-specific nature of the sample. GCF can be collected noninvasively with specifically designed filter paper or a micropipette or through magnetic beads placed in gingival crevice. GCF once collected may be cryopreserved or directly sent for chemical analysis. GCF can also be collected repeatedly at various stages of orthodontic treatment and therefore provides useful insight to biological events over the entire duration of observation[47]. During orthodontic treatment, the forces exerted produce a distortion of the PDL extracellular matrix, resulting in alterations in cellular shape and cytoskeletal configuration leading to cellular activation. These cells synthesize and secrete a wide variety of cytokines and growth factors. Such changes in the deeper periodontal tissues lead to synthesis and presence of extracellular matrix components, enzymes and inflammatory mediators may modify both the GCF flow rate and its components[69]. Thus, analysis of GCF samples may help in assessing tissue status around teeth undergoing orthodontic movement and provide a useful instrument for the modification of orthodontic treatment

procedures. The GCF collection was done using micropipette (T. Sueda) of known internal diameter and length. These are inserted into the entrance of the gingival crevice and the GCF will thus migrate into the tube by capillary action. The procedure may be repeated several times in the same crevice. The advantage of this method is that it allows the accurate volume determination of an undiluted "native" GCF sample. However, the technique is traumatic and time-consuming as in order to collect an adequate volume of GCF the procedure needs to be repeated several times[64].

Saliva is a perfect medium to be explored for health and disease surveillance. It is a simple and non-invasive diagnostic tool. It allows rapid screening and enables reliable evaluation of periodontal status. Saliva is readily available and easily collected without specialized equipment or personnel[68]. The identification of salivary biomarkers and its use as a diagnostic tool has many advantages. Most of the human studies concerning to the biology of orthodontic tooth movement have focused on the analysis of different mediators involved in alveolar bone remodeling in the GCF as it is more site specific in nature. But this study was designed to examine changes in the salivary concentrations of IL-8 as the mediators involved in bone remodeling during orthodontic treatment are released in GCF which is continuously washed in saliva and hence can serve as easy alternative to evaluate the levels of biomarkers. **Ellias MF et al[57], 2012** studied the protien profiles during orthodontic treatment in saliva and found a total of eight proteins to have changed in expression in relation to orthodontic treatment. **Flórez-Moreno GA. et al[58], 2010** reported variations in salivary concentrations of RANKL and OPG and their ratios during the different phases of

orthodontic tooth movement and concluded that these analytes might serve in a panel of salivary functional biomarkers that could assist in the screening of orthodontic treatment. **Marcaccini AM. et al[55], 2010** studied the levels of myeloperoxidase in GCF and saliva and reported no differences in the value of MPO levels in GCF and saliva, thus indicating saliva as an equally important diagnostic aid to assess the biomarkers of orthodontic tooth movement.

In this study, both leveling and retraction forces evoked changes in IL-8 levels indicating their role in alveolar bone remodeling and thereby the orthodontic tooth movement. Their levels were found to rise in both saliva and GCF with highly significant levels in GCF; this is because the salivary findings are the reflection of the cytokine profile in the GCF. The concentrations of IL-8 in saliva were seen to be correlated to those of GCF at each phase of orthodontic tooth movement with the peak concentration at 24 hrs in both saliva and GCF. Thus, suggesting that saliva can be an equally sensitive medium to assess the biomarkers, as the mediators involved in bone remodeling are continuously washed in saliva by GCF. The concentration of IL-8 in saliva was low, which might be due to high dilution of IL-8 in saliva, causing hindrance in detection of low levels of biomarkers. Altogether, the salivary findings might be reflection of the actual cytokine profile of GCF and consequently of the biologic activity within the periodontal environment during orthodontic tooth movement. This in vivo study provides information of saliva as reliable medium to study the mediators of bone remodeling during orthodontic tooth movement, as it is less technique sensitive, readily available and easily collected than GCF.

# *Limitation*

1. Research has observed the release of inflammatory mediators within 4 – 6 hrs of orthodontic force application. In this study the levels of interleukin -8 were assessed after 24 hrs of bonding. Thus further research is required to determine the levels of IL-8 for leveling and retraction forces at relatively short intervals from 1 hr to 24 hrs.

2. The decrease in the levels of IL-8 from peak to baseline was not assessed, which could have explained the need for subsequent force application.

3. Orthodontic forces affect the IL-8 levels on the compression and tension sites to different extent. Both the sites were not taken into account separately while assessing the IL-8 levels.

# *Summary*

The present study was carried out to evaluate and compare the presence of proinflammatory cytokine interleukin – 8 in the gingival crevicular fluid and saliva at 24 hrs and 7 days of leveling and space closure phase of orthodontic treatment. A total of 20 patients, between the age group of 15-25 years requiring upper first premolar extraction, were selected from those visiting the Department of Orthodontics and Dentofacial Orthopaedics of our institute. Interleukin – 8 levels in GCF and saliva were measured using ELISA kit.

Collection of GCF and saliva was done just before the bonding as the baseline data. This was followed by collection of GCF and saliva at the start of leveling phase ie, 24 hrs after bonding followed by 7 days after bonding. Further samples were collected after the start of retraction in space closure phase. Individual canine retraction was done using active tiebacks with 150 grms of force on each side. Samples were collected at 24 hrs and 7 days after the start of retraction. The amount of interleukin –

8 was measured using ELISA kit in both GCF and saliva and comparison was done at each phase between the two. Following observations were made –

1. It was found that levels of IL-8 rise at 24 hrs and 7 days from the baseline in both the leveling phase and during retraction in space closure phase.

2. The levels of IL-8 were significantly higher at space closure phase when compared to the leveling phase.

3. When comparison was done for the levels in saliva and GCF, there was a significant difference with higher levels in GCF than saliva.

4. However, the levels of IL-8 inspite being significantly lower in saliva, their concentration were comparable to those in GCF at each phase ie. In both GCF and saliva the levels were at peak at 24 hrs and gradually reduced by 7 day but were significantly higher than the baseline at leveling and the space closure phase.

# *Conclusion*

The findings for the study lead to following conclusion –

1. The levels of interleukin – 8 increased significantly in the gingival crevicular fluid during both leveling and space closure phase.

2. The levels of interleukin – 8 increased significantly in the saliva during both leveling and space closure phase.

3. The levels of interleukin – 8 rise with peak at 24 hrs of application of orthodontic force, suggesting their early involvement in the inflammatory process of orthodontic tooth movement.

4. The space closure phase shows significantly higher levels of interleukin – 8 than the leveling phase which reflects the rapid response of cellular and tissue reaction to the elevated orthodontic forces.

5. Significantly high levels of interleukin – 8 were seen in both the gingival crevicular fluid and saliva at all the phases of orthodontic tooth movement, suggesting saliva as equally sensitive medium for the study of biomarkers involved in orthodontic tooth movement.

6. Application of orthodontic force causes release in proinflammatory cytokine interleukin – 8, supporting its potent role in bone remodeling process of orthodontic tooth movement.

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# ANNEXURE

## Department of Orthodontics and Dentofacial Orthopaedics

## Informed Consent Form

## (Confidential)

## "Comparison and Evaluation of Proinflammatory cytokine Interleukin-8 in Saliva and Gingiva Crevicular Fluid during different phases of Orthodontic Treatment".

Mr./Mrs./Miss._____

Resident of: _____

_____ _____aged _____ years,

Exercising my free will/choice, without any pressure/lure of incentive in any form, hereby give my consent to be included as a subject in the said clinical study.

I acknowledge the receipt of "patient's information sheet", and also that the doctor has informed me about this research project suitably and sufficiently to my satisfaction. I agree to take part in this project and will not mix any other projects during the period of this trial. I permit to publishing the results of my participation in this study. I shall not be given any reimbursement or compensation. I hereby record my consent for participation in the said trial.

I hereby record my consent for participation in the said trial.

| | | | |
|---|---|---|---|
| 1. _____ | _____ | _____ | _____ |
| Patient's Name | Signature/Thumb Print | Date | Time |
| 2. _____ | _____ | _____ | _____ |
| Witness' Name | Signature/Thumb Print | Date | Time |
| 3. _____ | _____ | _____ | _____ |
| Principal/ Investigator Name | Signature/Thumb Print | Date | Time |

# MASTER SHEET

| Sr. No. | Group A – Gingival Crevicular Fluid | | | | |
|---|---|---|---|---|---|
| | Leveling Phase | | | Space closure Phase | |
| | Before Treatment | 24 hours | 7 Days | 24 hours | 7 Days |
| 1 | 42 | 89 | 67 | 124 | 100 |
| 2 | 44 | 85 | 63 | 119 | 97 |
| 3 | 47 | 84 | 73 | 122 | 110 |
| 4 | 45 | 81 | 61 | 124 | 102 |
| 5 | 41 | 93 | 71 | 120 | 104 |
| 6 | 40 | 79 | 69 | 117 | 106 |
| 7 | 47 | 86 | 65 | 118 | 108 |
| 8 | 44 | 88 | 72 | 114 | 112 |
| 9 | 49 | 89 | 66 | 117 | 107 |
| 10 | 45 | 82 | 75 | 125 | 115 |
| 11 | 43 | 88 | 60 | 121 | 99 |
| 12 | 44 | 95 | 71 | 116 | 105 |
| 13 | 51 | 82 | 65 | 119 | 100 |
| 14 | 46 | 80 | 69 | 117 | 110 |
| 15 | 47 | 82 | 62 | 125 | 102 |
| 16 | 49 | 85 | 66 | 118 | 107 |
| 17 | 45 | 91 | 63 | 124 | 110 |
| 18 | 39 | 77 | 69 | 119 | 98 |
| 19 | 50 | 80 | 60 | 121 | 103 |
| 20 | 44 | 79 | 75 | 118 | 106 |

| Sr. No. | Group B – Saliva | | | | |
|---|---|---|---|---|---|
| | Leveling Phase | | | Space closure Phase | |
| | Before Treatment | 24 hours | 7 Days | 24 hours | 7 Days |
| 1 | 32 | 65 | 45 | 105 | 77 |
| 2 | 29 | 59 | 41 | 99 | 80 |
| 3 | 30 | 64 | 39 | 107 | 79 |
| 4 | 27 | 66 | 40 | 102 | 82 |
| 5 | 33 | 59 | 37 | 97 | 75 |
| 6 | 25 | 62 | 43 | 109 | 81 |
| 7 | 29 | 66 | 35 | 104 | 77 |
| 8 | 31 | 58 | 39 | 98 | 83 |
| 9 | 33 | 62 | 38 | 100 | 85 |
| 10 | 26 | 58 | 41 | 104 | 79 |
| 11 | 31 | 61 | 42 | 107 | 74 |
| 12 | 34 | 64 | 44 | 102 | 81 |
| 13 | 28 | 59 | 38 | 96 | 75 |
| 14 | 30 | 65 | 35 | 98 | 84 |
| 15 | 31 | 62 | 40 | 100 | 85 |
| 16 | 35 | 59 | 45 | 103 | 79 |
| 17 | 33 | 64 | 41 | 101 | 80 |
| 18 | 25 | 59 | 42 | 99 | 83 |
| 19 | 28 | 62 | 37 | 105 | 77 |
| 20 | 27 | 68 | 39 | 108 | 81 |