

**“ASSESSMENT OF BIOMARKER LEVELS IN GCF
DURING FIXED ORTHODONTIC TREATMENT IN
GROWING AND NON-GROWING SUBJECTS –
AN IN VIVO STUDY.”**

DISSERTATION

**Submitted to
BABU BANARASI DAS UNIVERSITY, LUCKNOW, UTTAR
PRADESH**

*In the partial fulfillment of the requirements for the degree
Of*

MASTER OF DENTAL SURGERY

**In
ORTHODONTICS AND DENTOFACIAL ORTHOPAEDICS
By**

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**Under the guidance of
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**BABU BANARASI DAS COLLEGE OF DENTAL
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(Faculty of Babu Banarasi Das University)

YEAR OF SUBMISSION: 2025

BATCH: 2022-2025

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I bow my head to the **Almighty God (Lord Shiva)**, who blessed me with his blessings, bestowed me with his kind grace, provided me with strength, courage and good health to reach this stage and made it possible for me to bring out this manuscript. The satisfaction and euphoria that accompany the successful completion of a task could be incomplete without the mention of the people who made it possible.

It is my privilege and honour to express my most sincere and heartfelt thanks to my head **Dr. Shantanu Khattri** MDS, Professor & Head and my guide **Dr. Kamna Srivastava** MDS, Reader, Department of Orthodontics and Dentofacial Orthopaedics, Babu Banarasi Das College of Dental Sciences, Lucknow whose brilliant foresight and practical approach have been the guiding force behind the present shape of my project. His inestimable calm and ineffable wisdom, continuous encouragement and earnest attention have always been a driving force throughout the project.

*“I extend my sincere thanks to my advisor, mentor **Dr. Kamna Srivastava**, MDS, Reader, Department of Orthodontics and Dentofacial Orthopaedics, Babu Banarasi Das College of Dental Sciences, Lucknow, for her constant support and guidance. Her insightful advice helped me navigate the challenges of this research project. I am deeply grateful for their patience, motivation, and expertise that she shared with me”.*

I am also extremely thankful to my Co-Guide **Dr. Tripti Tikku**, MDS, Reader, Department of Orthodontics and Dentofacial Orthopaedics, Babu Banarasi Das College of Dental Sciences, Lucknow and **Dr. Kalpana Singh** prof. and HOD, Department of Biochemistry, KGMU. I am deeply grateful for their patience, motivation, and expertise that she shared with me. Most importantly, I am thankful for her unwavering support, collaboration, and mentorship throughout the past three years. Without her support nothing would have been possible for me. It was her understanding, valuable suggestions, unstinted help and personal attention that have provided good and smooth basis for this work.

I am equally indebted to **Dr. Sneh Lata Verma**, MDS, Professor, Department of Orthodontics and Dentofacial Orthopaedics, Babu Banarasi Das College of Dental Sciences, Lucknow for her valuable advice throughout, unbiased support and constant help rendered not just for dissertation, but always. At every stage, her wise and logical

suggestions and meticulous attention to the fine details have helped me give this dissertation its final shape.

I would also like to express my gratitude to **Dr. Rana Pratap Maurya**, MDS for his encouragement, constant moral support and inspiration.

I would also like to thank all my Senior Lecturers **Dr. Srishti Aditi, Dr. Prateek, Dr. Anjali Dr. Sparsh and Dr Rashi** MDS, Department of Orthodontics and Dentofacial Orthopaedics, Babu Banarasi Das College of Dental Sciences, Lucknow, for their valuable suggestions, time to time guidance, encouragement and constant moral support during the period of my study. Without their support nothing would have been possible for me. It was their understanding, valuable suggestions, unstinted help and personal attention that have provided good and smooth basis for this work. I feel motivated and encouraged by their presence. It's honour to have to have such teachers and shall forever be indebted to them.

Thanks to **Dr. Pratibha Taneja** maam for doing statistical analysis for the results, valuable suggestions, time to time guidance and constant support during the period of my study.

Last, but not the least, I express my gratitude to the staffs at **KGMU, Lucknow** who directly or indirectly provided their esteemed cooperation that made me complete my thesis. My journey would have been difficult without their encouragement and patience through the constant highs and lows.

Thanks to **Dr. Kalpana maam and Dr. Rizvi** for doing lab analysis and for his valuable suggestions, time to time guidance and constant support during the period of my study.

I gratefully acknowledge my present standing to my dear parents **Mr. Manik Debnath**, my mother **Mrs. Aparna Debnath**, elder brother **Tushar Kanti Debnath** for their love and constant support throughout my life. Thank you for giving me sincere encouragement and inspiration throughout my project and for giving me strength to reach for the star and chase my dreams. I owe you everything. Without your blessings it would never have been possible.

*I would like to pay high regards to all my loving seniors. Thank you, my dear seniors **Dr. Jaya, Dr. Shireen, Dr. Monika, Dr. Aliya and Dr. Sarbajit**. I would also like to thank my colleagues **Dr. Abhishek E P, Dr. Anupama Chatterjee, Dr. Sineen Khan, Dr. Nidhi Tiwari and Dr. Ashima Verma** for their help and encouragement throughout my work sailing together in the same boat.*

*I would also like to thank my juniors **Mudasir, Ankita, Aditya, Upasna, Pooja, and Safa** for their constant support and help in all my work.*

*It is a pleasure at this point to thank my principal, **Dr. Puneet Ahuja** for providing me with the opportunity to conduct the research and use the facilities of this college.*

*I would like to express my heartfelt gratitude to the non-teaching personnel **Mrs. Nirmala, Mr. Narendra, Mr. Abhishek and Amma** in the Department of Orthodontics and Dentofacial Orthopaedics for their compassion and assistance.*

Last, but not the least, I express my gratitude for all who directly or indirectly provided their esteemed cooperation that made me complete my dissertation.

~Dr. Ashim Debnath.

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LIST OF ABBREVIATIONS

S.NO	ABBREVIATEDFORM	FULLFORM
1.	GCF	Gingival crevicular fluid.
2.	PBS	Phosphate buffer solution.
3.	ELISA	Enzyme linked immunosorbent assay.
4.	Lat. Ceph	Lateral cephalogram.
5.	CVMI	Cervical vertebrae maturity indicator.
6.	Ni-Ti	Nickel Titanium.
7.	KGMU	King George medical university.
8.	µl	Microlitre.
9.	Mpa	MegaPascal
10.	Mm	Millimetre
11.	RANKL	Receptor activator of nuclear factor kappa- B ligand
12.	IL-6	Interlukin 6
13.	Pg/ml	Picogram / mililitre.
14.	OTM	Orthodontic tooth movement.
15.	N	Newton.
16.	G	Grams.

17.	ALP	Alkaline phosphatase
18.	IGF	Insulin-like Growth Factor
19.	PGE2	Prostaglandin E2
20.	GM-CSF	Granulocyte macrophage colony stimulating factor.
21.	BALP	Bone Alkaline Phosphatase
22.	MMPs	Matrix metalloproteinase
23.	OPG	Osteoprotegerin
24.	DHEA-S	Dehydroepiandrosterone sulfate
25.	PTHrP	Parathyroid hormone-related protein
26.	CRP	C-reactive protein
27.	DCR3	Decoy receptor 3
28.	TNF- α	Tumor necrosis factor alpha
29.	PGDF	Platelet derived growth factor
30.	CA-125	Cancer antigen- 125

ABSTRACT

Aim: To assess biomarker levels in GCF during fixed Orthodontic treatment in growing and non-growing subjects.

Material and method: Sample for this study consisted of 30 subjects undergoing fixed orthodontic treatment and equally divided into two groups – Group-I (N=15 growing subjects with mean age 13.72+_1.27 years) and Group-II (N=15 non-growing subjects with mean age 20.36+_4.64 years) based on CVMI staging described by Hassel and Farman. Gingival crevicular fluid (GCF) samples were collected from the distal site of the maxillary canine at different time intervals: T0 (Baseline), T1 (after 24 hours), T2 (after 1 week), and T3 (after 2 months) using absorbent paper points. The levels of IL-6 and RANKL biomarkers in the GCF samples were assessed using the ELISA kit following standard protocol of storage, transportation and ELISA analysis. Data was tabulated and adequate statistical comparison was made.

Results:

Trends for various biomarkers were – IL-6 in group I (**T0>T2>T3>T1**) and for Group II (**T0>T3>T2>T1**), RANKL in Group I (**T0>T1>T3>T2**) and for Group II (**T0>T1>T3>T2**) with statistically non-significant difference on intragroup comparison of both biomarker in each group.

Inter group comparison showed higher values of IL-6 and RANKL in Group I > Group II at all time intervals, though the difference were statistically non-significant.

Conclusion: The main clinical implication of present study would be that, as biomarker levels during tooth movement were found to be increased in growing subjects as compared to non-growing subjects, hence in non-growing subjects, other non-surgical or surgical methods of accelerating tooth movement could be employed to increase biomarker levels at molecular level for faster rate of tooth movement.

Keywords: Biomarker, GCF, alignment and levelling, growing and non-growing subjects.

Orthodontic treatment corrects skeletal and dental malrelationships, enhances facial aesthetics and establishes occlusal harmony and structural balance¹. The fixed orthodontic treatment is limited not only to children but adults are equally seeking orthodontic treatment for desirable aesthetic outcome². However, the response to orthodontic treatment varies with age³. Orthodontic treatment is faster in growing individuals in comparison to adult as vascularity is more and inflammatory system is more activated in growing children that can react faster to these local changes^{3,4,6}.

The mechanical forces exerted during fixed orthodontic treatment initiates a cascade of movements like initiation of acute inflammatory response (Vasodilation of capillaries of PDL, release of inflammatory mediators like IL-6, RANKL, PGE2, Cytokines, IL-1B) at molecular level⁵. This brings about bone remodeling. Also inflammatory markers are activated because of growth as well, hence additional stimulation due to orthodontic forces might result in variable response in growing and non-growing subjects during fixed orthodontic treatment^{4,6,24}.

The release of inflammatory mediators like arachidonic acid metabolites, cytokines, neurotransmitters released during fixed orthodontic tooth movement can be evaluated quantitatively as biomarkers and have enhanced our knowledge of underlying histological changes at molecular level⁷.

Biomarkers are substances that can be measured and evaluated objectively as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention^{7,8}.

Biomarkers had been used as diagnostic tool for the identification of disease, as a tool for staging of disease, as an indicator for disease prognosis and used for prediction and monitoring of clinical response to an intervention in medical field⁹.

The assessment of biomarkers in dentistry has found numerous application in diagnosing and preventing periodontal diseases, dental caries and oral cancer and in understanding response to therapeutic intervention for these conditions¹⁰.

In orthodontics biomarkers have helped us in assessing puberty, bone turnover or understanding orthodontic tooth movement at molecular level¹¹.

Serum biomarkers like IGF, BALP, PTHrP, DHEA-S (dehydroepiandrosterone sulfate) are used to assess puberty and growth status, fibroblast growth factor (FGF) for skeletal development, parathyroid hormone-related protein, Indian hedgehog, Sox9, and bone-specific alkaline phosphatase to evaluate bone turnover^{12,15}.

Besides this, various inflammatory mediators like Interlukins, tumor necrosis factor (TNF)- α , epidermal growth factor, matrix metalloproteinases (MMP), osteoprotegerin (OPG), and sclerostin (SOST) are released during orthodontic tooth movement and are evaluated quantitatively to understand variable responses to different types of orthodontic tooth movement or to different mechanics employed in adults and children. These biomarker levels can be evaluated from serum, saliva and GCF¹².

Serum biomarkers are widely recognized as valuable tools for diagnosing diseases, identifying therapeutic targets, and predicting disease prognosis. They are used in a range of conditions, such as serum LDL for cholesterol levels in subject with blood pressure, the P53 gene, and matrix metalloproteinases (MMPs) as tumor markers for cancer, and HbA1c for diabetes etc¹³.

In dentistry various serum biomarkers like BALP, ALP, IGF-1, CRP, DCR3 etc had been used to evaluate periodontal disease, dental caries, pulpal inflammation, bone metabolism, systemic oral health condition and to monitor post treatment outcomes^{12,14}.

Various serum biomarker had been assessed in orthodontics for detection of pubertal growth (IGF, FGF) or to assess bone cell turn over (PTHrP, BALP)^{12,15}.

Anusuya et al¹⁶, found the mean serum DHEA-S (Dehydroepiandrosterone sulfate) levels followed a typical pattern from the CS-1 till CS-6 which was comparable and similar to the mean serum IGF-1 levels in respect to CS stages.

Hussain et al¹⁵, found that Mean serum PTHrP levels were significantly higher in the late pubertal stages than in the early pubertal stages and had a positive correlation with cervical vertebral maturation stages from the prepubertal to the late pubertal stages, and a negative correlation from the late pubertal to the post pubertal stages.

Tripathi et al¹⁷, found that Serum IGF-1 and BALP attained highest levels at CVMI stage 3 with peak BALP levels observed earlier than IGF1. Although a positive

correlation was determined between IGF-1 and BALP but BALP followed skeletal growth pattern more precisely.

In a study done by **Kapoor et al**¹², found that Pubertal growth spurts were correlated with peak serum IGF-1 and BALP in both sexes individually.

Based on these studies, it can be stated that the levels of biomarkers to assess puberty differ during growing phase of an individual, hence their effect on underlying changes at molecular level during orthodontic tooth movement will also differ. Considering this it was decided to evaluate commonly released biomarkers in growing and non-growing children during initial stage of orthodontic treatment.

The main limitation of any serum biomarkers is that their collection requires invasive procedures, also has risk of contamination or cross infection and may cause anxiety in certain individuals. Their levels could fluctuate because of associated inflammatory conditions in other parts of body or due to durational variations. As they are not considered truly representative of changes occurring in periodontal ligament during orthodontic tooth movement, hence serum was not selected to quantify biomarkers in present study¹⁸.

Biomarkers can also be derived from saliva like levels of sorbitol and fructosamine in saliva correlate with levels of glucose in capillary blood, increased levels of isoprostane and oxidized low density lipoproteins in saliva of individuals with coronary heart disease etc.

In dentistry, various biomarkers have been studied in saliva, including TNF- α , IL-1, IL-6, IL-8, IgA, IgM, α -glucosidase, acid phosphatase, alkaline phosphatase, MMP-13, MMP-8, MMP-9, PDGF, and cancer antigen-125 (CA-125) in individuals with oral cancer. Several studies have assessed release of salivary biomarkers in orthodontics^{19,20}.

In orthodontics salivary biomarkers like (IGF-1, ALP Alkaline phosphatase) bone specific alkaline phosphatase (B-ALP), had been used to assess pubertal growth spurt²⁰.

Amparo et al²¹, also salivary biomarkers like sRANKL (soluble receptor activator of nuclear factor kappa B ligand) and OPG (osteoprotegerin), as well as their ratios had been associated with the different phases of orthodontic tooth movement.

However, similar to serum, salivary biomarkers are present in very low concentrations, requiring highly sensitive tests to detect them. The composition of saliva is influenced by its origin and various environmental and psychological factors. Thus, qualitative analysis of saliva markers can be reliably performed but the detection of level of these markers in saliva remains a significant challenge¹⁸.

To overcome these limitations of serum and salivary biomarkers, GCF biomarkers are often preferred due to their proximity to periodontal tissues, reducing the influence of other systemic inflammatory processes. Gingival crevicular fluid (GCF) is an osmotically mediated inflammatory exudate found in the gingival sulcus, where its volume increases with inflammation and greater capillary permeability. Hence assessment of biomarkers in GCF collected non-invasively would be used in present study²².

Navarro-Palacios et al²³, had investigated myeloperoxidase activity in gingival crevicular fluid (GCF) and saliva and found that GCF levels more accurately reflected inflammatory changes than saliva.

Gingival Crevicular Fluid (GCF) collection is an easy yet sensitive method, with various techniques employed to obtain samples. These methods include the use of micropapillary tubes, intra-crevicular washing, pre-weighted twisted threads, absorbent filter paper strips, and paper points. However, each method has its limitations. For instance, the micropapillary tube technique may take over 30 minutes to collect an adequate sample, posing a risk of tissue trauma, which can increase the possibility of saliva and blood contamination. Additionally, removing the complete sample from the tubing can be challenging^{18,22}.

Intra-crevicular washing is less commonly used due to its technique sensitivity and the high risk of blood contamination. The irritation caused to the sulcular epithelium can lead to increased GCF production, further complicating the process²².

On the other hand, the non-invasive absorbent paper point technique for GCF collection offers several benefits. It is easy to use, allows for sampling from individual sites, and is quick. This approach minimizes tissue trauma, making it suitable for collecting samples across different age groups. Furthermore, it allows for the collection of samples

from multiple sites within the oral cavity. These advantages make it a preferred method in most of the previous studies¹⁸.

Various biomarkers associated with bone resorption, such as IL-1, IL-6, and TNF- α , play a role in upregulating the expression of RANKL by stromal cells and osteoblasts, thereby stimulating bone resorption. IL-6 is a multifunctional cytokine that promotes osteoclast differentiation and is the potent inflammatory mediators induced in initial stages²⁴. RANKL, a proinflammatory cytokine produced by osteoblasts, is activated by T lymphocytes and serves as a key regulatory factor. RANKL binds to its specific receptor (RANK) on the surface of osteoclasts, triggering their maturation and leading to the resorption of alveolar bone. However, when RANKL binds to OPG, bone deposition occurs instead. This process of bone remodeling could be better understood by evaluating levels of biomarker in GCF²⁵.

Various studies have been conducted in animals and humans demonstrating various cytokines and their variations at different time periods in the periodontal tissues and also in the gingival crevicular fluid during different phase of orthodontic tooth movement²⁶.

However not many studies have been conducted to evaluate changes at molecular level during orthodontic tooth movement between growing and non-growing children.

In a study by **Jayaprakash et al**²⁷, after force application for distal movement of maxillary canine both Interlukin-1 & Interlukin-6 were assessed in growing subjects (15.6 $\pm$.9 years) and it was found that Il-1 and IL-6 increased as early as 1 minute or 1 hour and attained peak in 24 hours and decreased subsequently.

Kumar et al²², found that the volume of fluid and IL-1 levels increased 24 hours after treatment began. IL-1 levels also varied between adolescents and young adults, showing different tissue reactions, with tooth movement in teenage than in young adults.

Ren et al⁴, found that concentrations of biomarker (PGE2, IL-6, GM-CSF) were more in juveniles than the adults and all three mediators increased significantly from baseline to 24 hours.

Amongst various biomarker IL-6 released initially during orthodontic tooth movement that is responsible for induction of process of osteoclastogenesis was selected in present study also RANKL which is a potent biomarker that attaches to RANK to promote bone resorption would be evaluated in present study for both growing and non-growing children.

Considering this it was decided to evaluate and compare the levels of IL-6 and RANKL at different time intervals in growing and non-growing subjects at initial alignment and levelling phase of fixed orthodontic treatment.

AIM

To assess biomarker levels in GCF during fixed Orthodontic treatment in growing and non-growing subjects.

OBJECTIVES

1. To evaluate the level of RANKL in growing and non-growing subjects before start of treatment and during course of fixed orthodontic treatment at different time intervals (24 hour, 20 days, 2 months).
2. To evaluate the level of IL-6 in growing and non-growing subjects before start of treatment and during course of fixed orthodontic treatment at different time intervals (24 hour, 20 days, 2 months).
3. To make intra group comparison for mean level of biomarkers between different time intervals, both for growing and non-growing subjects.
4. To make inter group comparison for mean levels of both biomarker (RANKL and IL-6) between growing and non-growing subjects at each time intervals.

Hassel B and Farman. G Allan (1995)²⁸: focused on skeletal maturation assessment, particularly using cervical vertebrae. Various methods for determining growth stages, such as hand-wrist radiographs and biological age indicators, are discussed. The study acknowledges previous research showing a strong correlation between facial and statural growth. It highlights the historical development of skeletal maturation indicators, including methods developed by Todd, Greulich, Pyle, Fishman, and Hagg. The review also emphasizes the importance of cervical vertebrae in growth assessment, citing studies on vertebral maturation, ossification patterns, and their clinical relevance in orthodontics. Ultimately, the study aims to provide orthodontists with an alternative diagnostic tool by evaluating skeletal maturity through lateral cephalometric radiographs.

Uematsu S, Mogil M, and Deguchi T (1996)²⁹: conducted a study to evaluate IL-1 β , IL-6, TNF- α , epidermal growth factor and β 2 microglobulin levels and found significantly elevated levels of IL-6 in GCF during separate canine retraction in subjects with mean age of 14.4 $\pm$ 0.9 years by elastic chain exerting an initial force of 250 g. The mean levels of IL-6 was significantly higher at 24 hr (0.063 $\pm$ 0.004 pg / μ g) than at baseline (0.020 $\pm$ 0.007 pg / μ g) $p = 0.0441$. At 168 hr (7days) mean level of IL-6 was (0.009 $\pm$ 0.006 pg / μ g.) $p = 0.0147$ was significantly lesser than baseline values.

Y Ren, JC Maltha, MA Vant, JW Von den, AM Kuijpers (2002)⁴: conducted a study to determine the relation of bone remodelling during orthodontic tooth movement to the expression of mediators (prostaglandinE2, Interlukin-6 and granulocyte-Macrophage colony-stimulating factor) in gingival crevicular fluid (GCF). PGE2 concentrations were significantly elevated at t24 in juveniles and adults, while concentrations of IL-6 and GM-CSF were significantly elevated only in juveniles. In early tooth movement, mediator level in juveniles were more responsive than levels in adults, which agrees with the finding that the initial tooth movement in juveniles is faster than in adults.

Yamamoto T, Kita M, Oseko F, Terauchi R, Takahashi K (2006)³⁰: investigated the effect of mechanical stress as hydrostatic pressure (HP) on cytokine expression in hPDL cells and concluded that the exposure to 6 MPa of HP caused no morphological changes of hPDL cells, and did not affect the cellular viability. No expression of IL-1b, IL-6, IL-8, TNF-a, receptor activator of NF-kB (RANK), receptor activator of NF-kB ligand

(RANKL), or osteoprotegerin mRNA was observed in the control cells under atmospheric pressure, whereas, in hPDL cells treated with HP, a pressure-dependent enhancement of IL-6, IL-8, RANKL, and OPG mRNA expression was observed between 10 and 60 min after the exposure to HP. These results suggest that hPDL cells may play a role in the production of cytokines in response to mechanical stress *in vivo*.

Krishnana V and Davidovitch Ze'ev (2006)³¹: assessed the biology of tooth movement and the role of laboratory experiments in orthodontics. It highlights how orthodontics has evolved from mechanical force applications to incorporating biological insights, including cellular and molecular mechanisms. Early studies relied on histology, but modern research focuses on cellular responses to mechanical stress. The review emphasizes the importance of optimal orthodontic force and explores ways to accelerate tooth movement using biochemical agents and physical stimuli. Additionally, it discusses diagnostic advancements like biomarkers in gingival crevicular fluid. Overall, the paper underscores the need for integrating biological research with clinical orthodontic practices.

Yamaguchi M, N Kojima, Aihara (2006)³²: conducted a study to Evaluate RANKL and OPG level in 10 patients with age range of (14.5-15.4 years) in GCF at 0, 1, 24, and 168 h after the retracting force was applied (canine retraction) along a 0.018 inch archwire with an elastomeric chain delivered an initial force of 250gm. The mean values of RANKL were significantly higher on experimental side after 24 h (219.3 ± 26.9 pg/ μ l) than control sites (7.7 ± 5 pg/ μ l).

Kawasaki K, Takahashi T, Yamaguchi M, Kasai K (2006)³³: conducted a study to evaluate RANKL and OPG levels in GCF in 30 patients (15 juveniles, mean age 15.1 ± 2.8 years, and 15 adults, mean age 31 ± 3.6 years) during canine retraction using elastomeric chains on Edgewise brackets (0.018 x 0.025-inch slot) with a 250-gram initial force. The study found that tooth movement was greater in juveniles (1.23 ± 0.15 mm) than in adults (0.82 ± 0.22 mm). RANKL levels were significantly elevated at experimental sites in both groups at all time points 1 hr (T1), 24hr (T2), 168 hr (T3) except for 24 hours (baseline). In juveniles, RANKL levels at experimental sites were (210.3 ± 27.6 pg/ μ l), compared to (9.7 ± 2.3 pg/ μ l) at control sites. In adults, the RANKL level at experimental sites was 140.5 ± 25.5 pg/ μ l, while at control sites it was 8.2 ± 2.6 pg/ μ l.

Basaran G, Ozer T, Kaya Filiz (2006)³⁴: Conducted a study to compare fifteen patients (9 female, 6 male, ages, 15-19 years; mean age, 16.7 $\pm$ 2.3 years) who underwent a session of professional oral hygiene and received oral hygiene instructions. The patients were seen at baseline, at days 7 and 21, and as the teeth were levelled. Records of the baseline scores for the distalization forces were taken at the sixth month. Scores of days 7 and 21 after 6 months of the distalization treatment were also recorded. Increases were seen in the volume of gingival crevicular fluid and the concentrations of interleukins 2, 6, and 8. Leveling and distalization of the teeth evoke increases in interleukins 2, 6, and 8 levels in the periodontal tissues that could be detected in gingival crevicular fluid.

Nakao k, Goto T, Gunjigake K.K, Konoo. T, Kobayashi S (2007)³⁵: discussed the role of periodontal ligament (PDL) cells in bone remodeling during orthodontic tooth movement. The study builds on previous findings that PDL cells express RANKL and OPG, key regulators of osteoclast differentiation. Mechanical stress, particularly intermittent force, has been shown to modulate these expressions, with IL-1 β playing a significant role in enhancing RANKL expression. Prior research has demonstrated that continuous force can cause more cell damage, whereas intermittent force allows for tissue recovery, making it more effective for orthodontic movement. The study aims to clarify the molecular mechanisms by comparing the effects of continuous and intermittent forces on PDL cell responses.

David D, Wong T (2008)¹⁰: discussed advancements in salivary diagnostics, emphasizing its potential for non-invasive disease detection and health monitoring. Saliva is described as a "mirror of the body," capable of reflecting systemic health through biomarkers detectable via modern genomic and proteomic technologies. Challenges, including sensitivity and accessibility, are being addressed through innovative platforms like microfluidics and MEMS. The document highlights significant applications, such as detecting oral cancer and monitoring systemic diseases, showcasing saliva's advantages over traditional biofluids like blood due to its ease of collection and patient compliance. Salivary diagnostics promise transformative impacts on global healthcare by enabling portable, cost-effective tools for early detection and monitoring.

AR Prabhakar, Gulati A, Mehta Deepak, S Sugandhan (2009)³⁶: conducted a study to assess the diagnostic applications of Saliva in Dentistry and found that the ability to monitor health status, disease onset and progression, and treatment outcome through non-invasive means is the most desirable goal in health-care promotion and delivery. There are three prerequisites for this goal to be realised: specific biomarkers associated with a health or disease state, a non- invasive approach to detect and monitor these biomarkers, and the technologies to discriminate between and among biomarkers. The ability to utilize saliva to monitor the health and disease state of an individual is a highly desirable goal for health promotion and health-care research. However, saliva diagnostics is a 'late bloomer', since only recently has there been a growing appreciation of saliva as a mirror of the body which can reflect virtually the entire spectrum of normal and disease states.

Grant M, Wilson Jay, Rock Peter and Chapple Iain (2012)³⁷: highlighted how orthodontic forces induce remodeling in the periodontal ligament (PDL) and alveolar bone, driven by changes in cytokine and biomarker levels within GCF. Cytokines such as IL-1 β , IL-6, and TNF- α play crucial roles in inflammation and bone metabolism, with elevated levels correlating with phases of tooth movement. The study draws from prior findings on cytokine involvement in osteoclastogenesis through RANKL and OPG balance, showing similar temporal patterns of expression. Furthermore, enzymes like MMP-9 and its inhibitors (TIMPs) are linked to extracellular matrix remodeling, essential for tissue adaptation during orthodontic treatment. These findings align with past research that emphasizes the interplay of inflammatory mediators and mechanical forces in facilitating controlled orthodontic tooth movement.

Barbieria G, Solano P, Alarcon Jose. A (2013)³⁸: Conducted a randomized, pilot clinical trial including 10 healthy volunteers was conducted using a split-mouth design. Orthodontic elastic separators were placed between the second premolar and first molar, with the contralateral quadrant serving as a control. The GCF samples were collected from the tension and compression sites at baseline, 24 hours, and 7 days after the placement of separators. The GCF sample volumes were measured using a Periotron 8000, and total protein concentrations were determined. Levels of RANK, OPG, OPN, and TGF- β 1 were also analyzed using a multiplex enzyme-linked immunosorbent assay.

Hussain M, Kumar A, Prasad M (2013)¹⁵: focused on understanding the role of skeletal maturation as a key determinant of growth in orthodontic treatment. It highlights the importance of systemic and local factors, such as growth hormones and parathyroid hormone-related protein (PTHrP), in regulating bone growth and craniofacial development. Previous research has established that skeletal maturation indicators, such as cervical vertebral stages, are reliable tools for assessing growth potential during adolescence. The study also emphasizes the interplay between local and systemic growth factors, including the influence of PTHrP-Ihh signaling on chondrocyte differentiation and endochondral ossification. Despite advancements, the review notes ongoing debates about the accuracy of hormonal markers, such as PTHrP, in predicting peak growth velocity, and it calls for further research to confirm their reliability and clinical application.

Tuncer B Baloş, Ozdemir B Çakılcı, Boynuegri Duygu (2013)³⁹: evaluated RANKL level in GCF in 9 adults (age range 17.5-18.9 year) and found non-significant difference in levels of RANKL with time on retracting the maxillary canines by sentalloy closed coil springs with a continuous force of 200gm on the segmental 016x.022 S.S wire, four weeks after 1st premolar extraction. The mean value was highest at 7 days (1.29 ± 1.0 pmol/l) > at baseline (pre-treatment- 1.19 ± 0.71 pmol/l) > at 1st month ($1.04 \pm .50$ pmol/l) > then at 24hrs ($.96 \pm .55$ pmol/l) least at 1 hr ($0.83 \pm .30$ pmol/l).

Florez-Moreno G Amparo, Isaza-Guzman D Maria (2013)²¹: explored the variations in salivary levels of sRANKL and OPG during orthodontic tooth movement, aiming to determine their role as biomarkers for different treatment phases. A longitudinal study was conducted on 20 participants with fixed orthodontic appliances. Key findings include a significant increase in sRANKL levels and a downward trend in OPG levels, with a resultant increase in the sRANKL/OPG ratio. These changes were linked to the biomechanical phases of orthodontic movement, indicating that sRANKL and OPG may serve as reliable markers for bone remodeling during treatment. The study underscores the potential of saliva as a diagnostic medium in orthodontics.

Rody Wellington J, Wijegunasinghe Manjula, A William (2014)⁴⁰: investigated differences in the gingival crevicular fluid (GCF) composition between adolescent and

adult patients undergoing orthodontic treatment with fixed appliances. Ten adolescents (14.4 6 1.43) and 10 adults (28.5 6 7.83) with Class I malocclusions and minor upper incisor crowding were allocated to two different age groups. Samples of GCF were collected from the labial sides of the upper incisors (experimental sites) and lower incisors (control sites) of each subject at five time points. Aliquots from diluted GCF were screened for the presence of receptor activator of nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG), interleukin-1 (IL-1), interleukin-1 receptor antagonist (IL-1RA), and metalloproteinase-9 (MMP-9) using a microarray technique. In adults, the ratio of IL-1 to IL-1RA decreased significantly ($P = 0.033$) in experimental sites 3 weeks after appliance placement and first archwire activation. In adolescents, the ratio of RANKL to OPG peaked 6 weeks after the insertion of the first rectangular archwire. This ratio peak found in adolescents was a consequence of a decrease in the mean concentration of OPG. This study demonstrates age trends in the GCF levels of IL-1, IL-1RA, RANKL, and OPG that may be used to track differences in tissue response between adults and adolescents undergoing orthodontic treatment.

Navarro-Palacios A, Garcia-Lopez E, Meza-Rios A (2014)²³: Investigated the role of myeloperoxidase (MPO) enzymatic activity as an indicator of inflammation in orthodontic patients with varying levels of dental crowding. MPO activity in gingival crevicular fluid (GCF) and saliva was measured after initial orthodontic activation. The results demonstrated that while inflammation was evident post-activation, the level of dental crowding did not significantly influence MPO activity. However, GCF proved to be a more accurate reflection of local inflammatory changes compared to saliva. The study suggests that MPO activity could serve as a useful, non-invasive biomarker for monitoring orthodontic-induced inflammation.

Alhadlaq M Adel (2015)⁵: analysed that gingival crevicular fluid (GCF) may be an acceptable way to examine the ongoing biochemical processes associated with bone turnover during orthodontic tooth movement. Each biomarker was discussed separately based on the available evidence. Several sensitive GCF markers are available to detect the biomechanical changes occurring during orthodontic tooth movement.

Tarvade S, Ramkrishna S, Sarode S (2015)⁴¹: evaluated the salivary alkaline phosphatase (ALP) activity in growing subjects in relation to the stages of individual skeletal Maturation using middle phalanx of third finger MP3. The study was conducted

on 60 girls and 60 boys who were age group of 10yrs to 15yrs were selected using simple random sampling technique. Salivary ALP were estimated by the enzymatic method using p- nitro phenyl phosphate as substrate and MP3 stages were assessed using Hagg and Taranger method. Mean salivary ALP and MP3 were compared by analysis of variance ANOVA test. Results showed highly significant differences between ALP and MP3 stages with p value 0.033. Salivary ALP levels showed good association with skeletal age in 13yrs in boys subjects with highest mean ALP and significant value p value of 0.015 and in 12yrs girls and boys at 13yrs at G stage with maximum growth. Salivary ALP levels can be used as an additional diagnostic tool to optimize orthodontic treatment timing.

Kumar A, Saravanan K, Kohila K, Kumar S Satesh (2015)⁸: GCF analysis is a non-invasive method for studying the cellular response of the underlying periodontium. They Concluded that on the basis of sequential reactions and released substances, numerous substances have been proposed as biomarkers. This will give a better understanding of the ongoing cellular process during orthodontic treatment. Thus, the data suggest that knowledge of all the biomarkers present in the GCF that can be used to mark the changes in tooth that is undergoing orthodontic treatment may be of clinical usefulness leading to proper choice of mechanical loading, to improve and to shorten the period of treatment, avoiding adverse consequences.

Madureira D, Mayra J, Sliva D, Teixeira A (2015)⁴²:evaluated levels of IL-6 in GCF and PDL in 23 patients (mean age 13.43 ± 2.64) with a 0.017x0.025 - in beta-titanium alloy cantilever attached on premolars that had to be extracted and were subjected to a force of 0.980 N in the apical direction for 3 hours, 15 hours, 3 days, 12 days, or 21 days. They found that there was an increase in IL-6 levels from 15hrs to day 3 and after that there was a subsequent decrease in IL-6 level at day 7 with mild increase till day 14 and concentration of IL-6 was levelled from day 14 to day 21.

Majeed Z N, Philip K, Alabsi A.M (2016)²²: evaluated the most common and precise method for gingival crevicular fluid (GCF) sample collection, biomarker analytical methods, and the variability of biomarker quantification, even when using the same analytical technique. An electronic search was conducted on in vivo studies that presented clinical data on techniques used for GCF collection and biomarker analysis.

The results showed that 71.1%, 24.7%, and 4.1% of the studies used absorption, microcapillary, and washing techniques, respectively, in their gingival crevicular fluid collection. 73.1% of the researchers analyzed their samples by using enzyme-linked immunosorbent assay (ELISA). 22.6%, 19.5%, and 18.5% of the researchers included interleukin-1 beta (IL-1 β), matrix metalloproteinase-8 (MMP-8), and tumor necrosis factor-alpha (TNF- α), respectively, in their studies as biomarkers for periodontal disease. IL-1 β can be considered among the most common biomarkers that give precise results and can be used as an indicator of periodontal disease progression.

Wen Xi, Gu Yan and Chen Feng (2016)⁴³: focused on the importance of identifying pubertal growth peaks in orthodontics, highlighting the limitations of traditional methods like hand-wrist analysis and cervical vertebral maturation (CVM). Gingival crevicular fluid (GCF) has emerged as a promising non-invasive biomarker source due to its rich protein content and site-specific nature. Prior research has explored mass spectrometry techniques like MALDI-TOF/MS to analyze biomarkers for various conditions, including periodontal diseases and orthodontic tooth movement. However, no studies had previously used GCF proteomic analysis to identify pubertal growth markers. This study aims to fill that gap by leveraging MALDI-TOF/MS to detect novel GCF biomarkers that differentiate pubertal from post-pubertal subjects.

Fernandez-Olavarria A, Mosquera-Perez R, Diaz Sanchez Rosa (2016)¹³: explored the significance of various serum biomarkers in detecting and predicting the progression of oral cancer. The study analyzed 29 selected articles, revealing that, biomarkers like Cyclin D1, CRP, DcR3, VEGF, and SCC-Ag show potential in diagnosis and prognosis. However, some biomarkers exhibited inconsistent clinical utility. Despite the promising findings, the review highlights the need for standardized monitoring protocols and further research to validate the clinical applicability of these biomarkers for improved oral cancer management.

Chouinard M Claude, K. Taranpreet, Uribe Flavio (2016)²⁵: discussed the average treatment duration and the potential drawbacks of prolonged orthodontic therapy, such as white spot lesions, root resorption, and gingival inflammation. The review outlines three main approaches to accelerating tooth movement—pharmacological, surgical, and mechanical. Among these, mechanical vibration has gained interest for its potential to enhance bone remodeling and reduce treatment time. Previous studies on vibration

devices have shown mixed results, with some suggesting increased tooth movement and others finding no significant difference. The review also examines the impact of vibration on bone remodeling biomarkers, including RANKL/OPG signaling and inflammatory cytokines, and highlights the need for further clinical trials to clarify the biological mechanisms involved.

Vujacic A, Konic A, Pavlovic Jasna (2016)⁶: evaluated the levels of IL-6 and IL-1b in GCF, 10 children (mean age 13) and 10 adults (mean age 20) during the early phase of orthodontic tooth movement (OTM) by using elastomeric separators and found higher level of IL-6 in children than adults at each time interval. Level of IL-6 in children were 2.2 ± 0.5 pg/ml, at 24 hours, 0.4 ± 0.1 pg/ml at 72 hrs and 1.7 ± 1.1 pg/ml at 168 hours whereas in adults increase was much smaller, averaging 0.5 ± 0.2 pg/ml at 24 hours, 0.10 ± 0.04 pg/ml at 72 hrs and 0.2 ± 0.1 pg/ml at 168 hours when compared to control teeth. The amount of tooth movement was statistically greater in children than in adults 168 hours after the initiation of OTM.

G Perinetti, Baccetti L, Contardo R Di (2016)⁴⁴: highlighted the importance of identifying skeletal maturity for optimal orthodontic treatment planning, especially during the pubertal growth spurt. Biomarkers, such as alkaline phosphatase (ALP) in gingival crevicular fluid (GCF), are emerging as non-invasive tools to assess this critical phase. Traditional methods, like radiographic imaging, have been reliable but involve radiation exposure. Recent studies suggest that GCF ALP activity significantly increases during the pubertal growth phase, making it a promising indicator irrespective of dental eruption stages. This approach not only reduces radiation risks but also aligns with patient physiology for precise treatment timing.

Caires M, De Aguiar S, G Perinetti, and Capelli Jonas (2017)²: evaluated whether the GCF biomarkers in growing subjects reflect both the stages of individual skeletal maturation and the local tissue remodelling triggered by orthodontic force. Present evidence is still little regarding whether and which GCF biomarkers are correlated with the growth phase (mainly pubertal growth spurt), while huge investigations have been reported on several GCF biomarkers (for inflammation, tissue damage, bone deposition and resorption, and other biological processes) in relation to the orthodontic tooth movement. The GCF biomarkers are helpful to assess the growth phase in children and juvenile patients.

Liu Yi, Song F, Meng Mingmei (2017)²⁶: highlighted the role of interleukin-6 (IL-6) in orthodontic tooth movement, emphasizing its regulatory function in bone remodeling. Previous research has established that IL-6 influences osteoblast and osteoclast activity through key signaling pathways, including glycoprotein 130 (gp130), signal transducer and activator of transcription 3 (STAT3), and Src homology phosphotyrosine phosphatase (SHP2). Studies suggest that IL-6 plays a dual role in both bone formation and resorption, impacting periodontal ligament (PDL) responses to mechanical forces. While prior work has confirmed IL-6 expression in periodontal tissues during orthodontic treatment, the downstream effects of its signaling pathways remain underexplored. This study aims to bridge this gap by investigating the expression patterns of IL-6 and its key signaling molecules under mechanical stress, providing insights into the molecular mechanisms of orthodontic tooth movement.

Srivastava N, Nayak P A, Rana S (2017)¹⁸: discussed advancements in periodontal diagnosis, focusing on Point-of-Care (POC) diagnostics. Traditional clinical methods such as probing depth and radiographic assessment do not accurately reflect disease activity or predict future progression. Therefore, newer diagnostic approaches emphasize the detection of biomarkers in oral fluids like saliva and gingival crevicular fluid (GCF). Various POC diagnostic tools, including microfluidic platforms, nanosensor tests, and enzyme-based assays, have been developed to detect periodontal disease more efficiently. These tools enable early detection, risk assessment, and monitoring of disease progression, ultimately improving clinical outcomes. However, challenges such as cost, validation against existing methods, and integration into routine practice must be addressed for widespread adoption.

Irham F, Bahirrah S ((2017)²⁰: focused on the use of alkaline phosphatase (ALP) levels in saliva as a biomarker for determining the pubertal growth phase in orthodontics. Various methods, such as skeletal age assessment via cervical vertebral maturation (CVM), have traditionally been used to evaluate growth stages. However, ALP in saliva provides a less invasive alternative for tracking growth phases, as it reflects bone mineralization activities. Studies indicate that ALP levels peak during the pubertal phase and decrease in prepubertal and postpubertal stages. This biomarker could enhance orthodontic diagnosis and treatment timing, reducing reliance on radiographic methods.

Castroflorioa T, Eugenio F (2017)⁴⁵: discussed the biological effects of orthodontic tooth movement induced by aligners, focusing on biochemical markers in gingival crevicular fluid (GCF). It highlights the role of cytokines such as interleukin-1 beta (IL-1b), transforming growth factor-beta (TGF- β 1), receptor activator of nuclear factor kappa B ligand (RANKL), osteoprotegerin (OPG), and osteopontin (OPN) in bone remodeling. Previous studies suggest that orthodontic forces trigger inflammatory responses, leading to bone resorption and formation. However, limited research exists on how aligners influence these processes. The study aims to fill this gap by examining the expression of these markers in response to early orthodontic forces exerted by aligners, providing insights into their impact on bone metabolism.

Gurbaxani H, Shenoy U, Banerjee S, Hazarey A (2017)⁴⁶: evaluated myeloperoxidase (MPO) activity in gingival crevicular fluid (GCF) during orthodontic tooth movement, with varying force levels. MPO, an inflammatory biomarker, is crucial for tracking periodontal inflammation. Using controlled forces of 50, 75, 100, and 150 grams, GCF samples were collected at 2 hours, 7 days, and 14 days. The results indicated a significant increase in MPO levels at 14 days, correlating with inflammatory responses critical for bone remodeling. This highlights MPO's potential as a sensitive marker for monitoring orthodontic force effects and inflammation. The findings align with prior research linking GCF biomarkers to periodontal changes during orthodontics.

Tripathi T, Gupta P, Sharma J, Rai P (2017)¹⁷: assessed "Bone-specific alkaline phosphatase – a potential biomarker for skeletal growth assessment", reviews the use of serum Bone-specific alkaline phosphatase (BALP) and Insulin-like growth factor-1 (IGF-1) as biomarkers for skeletal maturity. It compares these biomarkers with the cervical vertebral maturation index (CVMI). Findings show significant correlations between serum IGF-1 and BALP across CVMI stages, suggesting their potential as reliable indicators of growth status. BALP demonstrated a sharp peak at CVMI stage 3 for both genders, emphasizing its relevance in skeletal growth assessment. The study advocates for the integration of biochemical markers like BALP into clinical orthodontic practices to enhance growth prediction accuracy.

Kaur A, Kharbanda O.P, Kapoor P (2017)⁴⁷: explored the role of biomarkers in perimniscrew implant crevicular fluid (PMICF) as indicators of inflammation, bone turnover, and implant stability in orthodontic treatments. Miniscrew implants (MSIs),

used for temporary anchorage, generate inflammatory responses upon insertion and loading, reflected in the levels of cytokines such as interleukins (IL-1 β , IL-6, IL-8), tumor necrosis factor (TNF- α), and the RANKL/OPG ratio. These biomarkers are analyzed to monitor tissue remodeling, with studies indicating variations correlating with implant success or failure.

Amanda J, Widayati R, Soedarsono N and Purwanegara M K (2018)⁴⁸: conducted a study to determine the differences in RANKL concentrations in early orthodontic treatment between preadjusted edgewise appliances (PEA) and self-ligating (SL) bracket systems. GCF was retrieved before treatment and at 1, 24, and 168 hours after treatment from five maxillary anterior proximal sites on the mesiolabial side of the upper right-to-left canines. The results showed differences in RANKL concentrations between the SL and PEA groups at 1, 24, and 168 hours after bracket insertion. The RANKL concentration at 168 hours in the SL group was higher compared to that in the PEA group, whereas that in the PEA group decreased to the baseline value after 24 hours. RANKL concentration in the passive SL bracket system was higher compared to that in the PEA system due to differences in the force and mechanotherapy of the SL brackets.

Alikhani M, Y Michelle, Khoo E, Alansari S, Kwal R (2018)³: Conducted a study to assess RANKL levels in GCF in 18 patients, 9 adolescents, (age range of 11-14 years), and 9 adults (age range of 21-45 years) during canine retraction using Ni-Ti closed coil spring on 0.017x0.025 s.s wire (MBT (0.022 inch slot) with a 50 CN of initial force before orthodontic treatment, 1,7,14 and 28 days after canine retraction. The study found that equivalent orthodontic forces stimulated age dependent increases in GCF levels in adults compared to adolescents. However, the higher levels of GCF cytokines in adults did not result in faster rate of tooth movement compared to adolescent.

P Jayaprakash, Mugur Basavanna (2019)²⁷: focused on to identify and quantify the various cytokines in human gingival crevicular fluid (GCF), and to investigate the changes in their levels during orthodontic tooth movement (OTM). A statistically significant no. of subjects (n = 10 and mean age = 15.6 years) were included in the study. A maxillary cuspid of each subject having one treatment for distal orthodontic

tooth movement served as the experimental tooth, whereas the contralateral cuspids were used as controls. Gingival crevicular fluid (GCF) around the experimental and the two control teeth was collected from each subject immediately before activation, and at 1, 24, and 168 hours after the initiation of tooth movement. The concentrations of interleukin (IL)-1 β , IL-6, tumor necrosis factor- α , epidermal growth factor, and β 2-microglobulin were significantly higher in the experimental group than in the controls at 24 hr after the experiment was initiated.

Chang H. Joy, Chen P Jung, Michael R. Arul Eliane H. Dutra (2019)⁴⁹: conducted a study to evaluate an injectable formulation containing receptor activator of nuclear factor kappa-B ligand (RANKL) on the OTM and found that these formulations were able to sustain the release of RANKL for more than 30 days, and the released RANKL showed a positive effect on mice osteoclast precursor cells (RAW 264.7). These were effective in accelerating OTM compared with other control groups, with 129.2 per cent more tooth movement than no formulation group and 71.8 per cent more than placebo formulation group. There was corresponding a significant increase in the amount of TRAP activity and there was no significant differences in root resorption between the groups.

Mohammed A, Saidath K, Mohindroo A, Shetty A (2019)⁵⁰: evaluated IL-6 in PDL of extracted 1 premolar each tooth was assigned a time point for its extraction at 2 hours, 15th day or the 30th day after application of force. Brackets were bonded to the experimental teeth at 5 degrees to the long axis of the tooth, 200-250 g of force was exerted on placing 0.014 Ni-Ti wire. They found a significant increase on experimental teeth in the level of IL-6 on 15th day (14.91) than on 30th day (11.1) and least was at 2hrs (3.4) which was non- significant in comparison to control.

Agarwal P, Goel D (2020)¹: biomarker is a substance that is measured and evaluated objectively as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Tooth movement by orthodontic treatment is characterized by remodeling changes in the periodontal ligament, alveolar bone and gingiva. A reflection of these phenomena can be found in the gingival crevicular fluid (GCF) of moving teeth, with significant elevations in the concentrations of its components like cytokines, neurotransmitters, growth factors and arachidonic acid metabolites. The orthodontic displacement of a tooth is the result of a mechanical

stimulus, generated by a force applied to the crown of a tooth, which results in an acute inflammatory response in periodontal tissues, which in turn may trigger the cascade of biological events associated with bone remodeling.

Muhammad N, Ahmad S, Muhammad S, Hussain A (2020)⁵¹: evaluated the correlation between serum IGF-1 levels and cervical vertebral maturation stages (CVMS) to assess skeletal maturity. Traditional methods like CVMS and hand-wrist radiographs have limitations, including radiation exposure and inconsistent growth indicators. The study highlights that IGF-1, a hormone regulated by growth hormone, peaks at specific puberty stages, offering a potential non-radiographic marker for skeletal maturity. The findings demonstrate that IGF-1 levels are highest in females at CVMS stage III and in males at stage IV, correlating strongly with pubertal growth spurts. This positions IGF-1 as a reliable biochemical indicator for orthodontic treatment planning.

Ahmed N, K Ranjan, Bhat R, Joseph R, Younus A (2020)⁷: studied that examination of Gingival crevicular fluid (GCF) may be considered an acceptable way to depict the biochemical changes occurring during orthodontic tooth movement. On Correlating the changes taking place in GCF with different types of orthodontic forces, the patient can be managed based on individual patients' tissue response. There was little evidence regarding which GCF biomarkers are associated with the growing phase. Most of the earlier reports provided information about correlation of GCF biomarkers with inflammation, bone remodeling and tissue damage and other processes associated with orthodontic tooth movement. These biomarkers provide vital information about the micro-environment and reflect the changes occurring during orthodontic treatment. Knowledge of biomarkers gives information about the proper choice of mechanical loading, which thereby helps in improving patient comfort and reducing treatment time.

Desai P, Donovan L, Janowitz E, Kim J (2020)⁵²: reviewed the clinical utility of salivary biomarkers for identifying type 2 diabetes risk and metabolic syndrome. Traditionally, type 2 diabetes is diagnosed through invasive blood tests. The study explores saliva as a non-invasive alternative, focusing on six biomarkers: adiponectin, leptin, C-reactive protein, interleukin-6, tumor necrosis factor-alpha, and 1,5-anhydroglucitol. While saliva sampling offers advantages like accessibility and reduced invasiveness, the findings on its diagnostic reliability remain inconsistent due to

challenges in standardizing collection and analysis methods. Further research and standardization are recommended to establish saliva's role in clinical diagnostics.

V. Anusuya, Nagar A, Tandon P, Singh G.K, Singh G.P (2020)¹⁶: focused on evaluating the relationship between serum DHEA-S (dehydroepiandrosterone-sulfate) and IGF-1 (insulin-like growth factor) levels and their potential as biomarkers for assessing skeletal maturity during the pubertal growth spurt in orthodontic patients. Traditionally, skeletal age is determined using hand-wrist radiographs, but such methods involve additional radiographic exposure. The study highlights the increasing interest in non-invasive biomarkers for skeletal growth, such as serum hormones, which reflect growth and hormonal changes during puberty. Previous research established IGF-1 as a reliable indicator, but its high testing costs prompted the exploration of DHEA-S as a more accessible alternative. Both hormones are critical in bone growth, with DHEA-S also stimulating IGF-1 and influencing skeletal development. The study underscores the need for precise timing in orthodontic interventions to correct skeletal discrepancies effectively.

Chelarescu S, Petra S, Decusara M, Oprica M (2021)⁵³: conducted a study to investigate comparatively IL1 β and IL6 in the gingival crevicular fluid of clinically healthy adolescents and young adults during the acute phase of orthodontic treatment. Gingival crevicular fluid was collected from 20 patients (aged between 11 and 28) undergoing orthodontic treatment. Measurements were taken before (T0) and after 24 h after distalization forces were activated (T1). IL1 β and IL 6 were analyzed using Elisa tests. Between the two time periods there was a significant raise both in the crevicular fluid rate (0.57 μ L at T0 vs. 0.95 μ L at T1, $p = 0.001$) and in IL1 β levels (15.67 pg/ μ L at T0 vs. 27.94 pg/ μ L at T1, $p = 0.009$). There was a significantly increased level of IL β at T1 in adolescents, more than in young adults (42.96 pg/ μ L vs. 17.93 pg/ μ L, $p = 0.006$). In the early stage of orthodontic treatment, the periodontal tissues of adolescents were more responsive to orthodontic forces than those of young adult.

Kahlon SS, Aggarwal V, Ahluwalia KS, Narang RS (2021)¹⁴: conducted a study to assess serum insulin like growth factor -1 levels (IGF-1) in female and male subjects in relation to various stages of cervical vertebrae maturation in 240 subjects (120 male and 120 female) and found that gender was a significant factor associated with the age

of attainment of each CVMI stages. Male subjects showed on average attainment of the CVMI stages by about 14 months later as comparison to females. Serum IGF-1 levels are at peak in CVMI stage 3 in both males and females. IGF-1 serve as an alternate method for growth assessment which is non-invasive to x-ray exposure, simple and gives a quantitative value to find pubertal growth peak.

Luchian I, Moscalu M, Goriuc A, Nucci L(2021)⁵⁴: conducted a study Using Salivary MMP-9 to successfully quantify periodontal inflammation during Orthodontic Treatment and results indicating salivary MMP-9 as a strong candidate for quantifying inflammation in affected periodontal tissues during orthodontic treatment. It further indicates that MMP-9 can be used to accurately predict the level of inflammation associated with the type of malocclusion, which makes it a real and viable diagnosis instrument in monitoring the evolution of the periodontium during orthodontic treatment.

Bhat S, V Ameet, M. Basavaraddi (2021)¹⁹: Assessed the presence of the salivary physiological biomarkers that would benefit the clinician in appropriate pain diagnosis and management objectively of various problems encountered during the orthodontic procedures and for better outcome of biomechanical therapy. Several sensitive salivary and GCF biomarkers are available to detect the biomechanical changes occurring during orthodontic tooth movement and pain occurring during fixed orthodontic therapy. There was a significant increase in OTM with injectable formulations containing RANKL. Present dissertation deals with brief insight into the biomarkers, connected to orthodontic treatment. The knowledge of the ongoing process occurring in periodontal tissues during orthodontic and dentofacial orthopaedic therapies help us to make proper choice of mechanical loading, adverse consequences, the amount of force application during the treatment and period of treatment may be shortened. The essential goal is to develop a screen test based on these factors or biological markers that could be used non-invasively and easily by the orthodontist at chairside to monitor the ongoing process and detect early root resorption. There is an enormous scope for research in this field which may be a boon for future orthodontic treatment and modalities.

Marahleh A, Kitauro H, Ohori F (2021)⁵⁵: focused on the effect of TNF- α on osteocyte RANKL expression during orthodontic tooth movement (OTM) highlights

the crucial role of osteocytes in bone remodeling. Osteocytes, as the primary source of RANKL, regulate osteoclastogenesis, which is essential for bone resorption. TNF- α , a pro-inflammatory cytokine, enhances RANKL expression in osteocytes, contributing to bone remodeling during OTM. Previous studies have shown that TNF- α is associated with pathological bone loss in diseases like rheumatoid arthritis and periodontal disease, emphasizing its role in osteoclast differentiation. Research involving TNF receptor-deficient mice demonstrated that the absence of TNF- α signaling leads to reduced RANKL expression and osteoclast formation, confirming its importance in OTM. This review underscores the interplay between TNF- α and RANKL in osteocytes, suggesting a potential therapeutic target for controlling bone remodeling in orthodontic and pathological conditions.

GV Veena and Tripathi T (2021)¹¹: assessed the biomarkers level during orthodontic tooth movement and correlation with skeletal maturity indicator. Traditional methods include hand-wrist radiography and cervical vertebral maturation index (CVMI). Though skeletal maturation has been assessed quantitatively through biomarkers obtained from saliva, gingival crevicular fluid (GCF), and urine. 10 studies were selected and showed that the concentration of biomarkers in saliva increased during the pubertal growth peak. On the contrary, 2 articles showed no significant difference between the levels of biomarkers and pubertal growth peak. It can be concluded that the level of biomarkers increases during the pubertal growth spurt and can provide a quantitative way of assessing skeletal maturity.

Kumar B, Singh N, Verma S.K, Singh S (2022)²⁴: conducted a study to assess IL-1 and IL-6 levels in GCF in 20 patients with the age range of 11-16 years (8 patient) and 17-24 year (12 patient) during canine retraction using a closed coil spring on a 19x25 SS wire. IL-6 was measured at T0 (Baseline) and T1(24 hours after activation). They were not able to found IL-6 in all the samples as other values were below the detection threshold and the mean overall IL-6 levels were 0.14 pg/ μ l at T0 and 1.27 pg/ μ l at T1, that were not subjected to statistical test.

Shetty A, Jain Mahima, K Sneha, Shetty V (2022)⁵⁶: conducted a study to assess level of IL-6 in 20 patients with the age range of 18-30 years in PDL in a split mouth study from the premolar region just before the extraction on control side and on the experimental site. Brackets (0.022 slot MBT mechanotherapy) were bonded on

premolar to be extracted after 3 days and an initial intrusive force of 100-150 g was applied using a cantilever spring on this premolar for 3 days. They found insignificant difference in mean level of IL-6 in GCF after 3rd day of force application 4.595 ± 1.6668 in the control group and 4.3975 ± 1.5948 in the experimental group.

Kapoor P, Balachandran R, Chowdhry A (2023)¹²: aimed to appraise the evidence for biomarkers in blood serum, gingival crevicular fluid (GCF), saliva, and urine in comparison with standard radiographic indices for skeletal maturation assessment. A total of 344 articles were screened, of which 33 met the inclusion criteria and quality assessment. The skeletal maturity indicators included insulin-like growth factors (IGF-1), alkaline phosphatase (ALP), bone-specific alkaline phosphatase (BALP), dehydroepiandrosterone Sulfate (DHEAS), vitamin D binding protein (DBP), parathormone-related protein (PTHrP), osteocalcin, metalloproteins, and serotransferrin (TF) along with different metabolites. At puberty, a significant rise was seen in IGF-1, DBP, ALP, osteocalcin, TF, and BALP. However, the serum DHEAS and PTHrP increased from pre-pubertal to post-pubertal stages.

Alqerban.A, Asiri S.N, Alharbi .F, Almalki .A, Alqhtani .N, Alenazi .A(2023)⁵⁷: conducted a study to assess the effect of ten different biomarkers in the gingival crevicular fluid of obese and non-obese undergoing fixed orthodontic treatment. The total number of participants included was 44. In the obese and non-obese groups, the male/female ratio was the same i.e., (n=11 each). Among the levels of biomarkers adiponectin ($p<0.006$) and leptin ($p<0.028$) demonstrated a significant difference between obese and non-obese participants. Also, a significant difference was noted between obese and non-obese in tissue remodeling biomarker MMP9 ($p<0.03$). A surge in the level of the biomarkers, i.e., MMP9, leptin, and adiponectin in the gingival crevicular fluid is found in obese undergoing fixed orthodontic treatment.

Song M, Bai H, Zhang. P, Ying B (2023)⁵⁸: evaluated Promising applications of human-derived saliva biomarker testing in clinical diagnostics. Saliva as a diagnostic tool due to its non-invasive collection and rich biological components. It highlights the utility of saliva testing in identifying biomarkers for various diseases, including cancer, oral diseases, neurodegenerative conditions, and psychological disorders. The review explores categories of biomarkers like DNA, RNA, proteins, exosomes, and hormones,

emphasizing their diagnostic, prognostic, and therapeutic implications. It also covers advances in saliva collection and preservation techniques to enhance the reliability of biomarker analysis.

Abdullah M, Abdullah A. (2023)⁵⁹: assessed IL-6 levels in GCF during relief of crowding of 2-4 mm in 50 patients (mean age 25±4 years) using aligners and conventional labial appliances and found higher level of IL-6 with aligner at 3.79±1.82 pg/ml at T0(baseline), 17.88±1.52 pg/ml at T1(after 1 month), whereas in conventional labial appliances were 3.65±1.21 pg/ml at T0(baseline) and 12.25±1.26 pg/ml at T1(after 1month).IL-6 showed significant difference from baseline to follow-up. Highest level of RANKL with conventional labial appliances 0.7±0.1 pg/ml at T0(baseline), 1.9±0.2 pg/ml at T1(after 1 month), whereas in aligners 0.6±0.1 pg/ml at T0(baseline) and 1.3±0.1 pg/ml at T1(after 1month). A significant difference between conventional labial appliances and aligners was noted in both the biomarkers (IL-6 and RANKL).

Bodaghi Ali, Fattahi N, Ramazani A (2023)⁹: focused on the significance of biomarkers in disease diagnosis, prognosis, and treatment, with a particular emphasis on COVID-19 and other illnesses. Biomarkers serve as crucial indicators of biological and pathological processes, aiding in early disease detection and personalized medicine. The article categorizes biomarkers based on molecular, cellular, and imaging properties, as well as their clinical applications. It highlights various technological advancements in biomarker discovery, including genomics, proteomics, and metabolomics, which contribute to improved medical diagnostics. Additionally, the review discusses the challenges in biomarker validation and implementation, emphasizing the need for standardized protocols and clinical trials for effective integration into healthcare. Overall, biomarkers hold promising potential in revolutionizing medical diagnostics and therapeutic strategies.

Kumar S, Kumar S, Singh G.P (2023)⁶⁰: explored the potential of salivary biomarkers—Insulin-Like Growth Factor-1 (sIGF-1) and Alkaline Phosphatase (sALP)—as indicators of skeletal maturity. Conducted on 80 North-Indian subjects aged 7-21 years, the research correlates these biomarkers with cervical vertebral maturation stages (CVMS), mandibular, and maxillary base lengths. Results revealed that both sALP and sIGF-1 peak during pubertal growth phases (CVMI Stage 3 for

males and Stage 4 for females), indicating sexual dimorphism. Salivary ALP also showed dependence on chronological age, highlighting its diagnostic potential. This study suggests non-invasive biomarkers can complement traditional radiographic assessments for evaluating skeletal development.

Yezdani A, Kumar K, Padmavathy K (2023)⁶¹: conducted a study on Serum osteocalcin – A biochemical marker for pubertal growth assessment. Growth evaluation methods have made great strides in the shift from radiologic to non-radiologic biomarkers. Osteocalcin (OC), a bone protein, has been reportedly used as a biomarker for osteoblastic bone formation. The present study aimed at evaluation of serum OC in Class II skeletal patterns for accurate assessment of pubertal growth spurt to facilitate functional jaw orthopedics during the growth period. Eighty subjects, comprising 38 males and 42 females with skeletal Class II malocclusion in the age range of 11–18 years, were recruited for the study. Human serum OC was quantitatively assessed with enzyme-linked immunosorbent assay. The cervical vertebral stages were assessed from lateral cephalograms. Gender-wise comparison of mean serum OC levels revealed that it was highest in CS2 in both males (72.24 ng/mL) and females (74.71 ng/mL) with another discernible peak in CS5 in males (66.82 ng/mL) and in CS6 in females (63.78 ng/mL), exhibiting thereby a circadian rhythm in bone modeling during the entire adolescent growth spurt. Despite a pre-pubertal and a late pubertal spike in both the genders, the mean OC serum levels actually exhibited a circadian rhythmicity across all the CSs, exhorting thereby the importance of bone remodeling during the complete circumpubertal growth period.

Vaghela N, Sardana R, Banerjee M, Kumar P, Singh S (2024)⁶²: evaluated salivary biomarkers during en-masse retraction of protruded teeth into recent and healed extraction sites and highlights the role of these biomarkers in understanding biological responses to orthodontic forces and their effect on tooth movement rates. The study found that salivary RANKL levels increased while OPG decreased in both groups, with significant changes observed in the recent extraction group. This suggests faster tooth movement in recent extraction sites, supported by a higher RANKL/OPG ratio. The findings emphasize the potential of salivary analysis as a non-invasive diagnostic tool in orthodontics.

Chen Y, Qian Y, Zhou X, Zhao Z, Zheng W (2024)⁶³: explored the dynamic molecular mechanisms underlying orthodontic tooth movement (OTM), focusing on the role of biomarkers in gingival crevicular fluid (GCF). Over the past decade, clinical studies identified 13 differentially expressed proteins (DEPs) in GCF, such as IL-1 β , TNF- α , MMPs, and TGF- β , which are critical in regulating bone remodeling during OTM. Using bioinformatics tools like the STRING database, researchers mapped protein-protein interactions to reveal key regulatory nodes. These findings emphasize the potential of GCF as a non-invasive medium for real-time monitoring and personalized orthodontic treatment, enhancing efficiency while minimizing adverse effects.

Cazzolla A, Brescia V, Lovero R, Fontana A (2024)⁶⁴: evaluated biomarkers of bone Metabolism on salivary matrix in the remodeling of periodontal tissue during orthodontic treatment, explores how bone turnover markers (BTMs) in saliva can reflect bone remodeling during orthodontic treatment. The study highlights the dynamic changes in biomarkers like PINP, TRAcP, and PTHrP in response to mechanical forces applied during treatment. These biomarkers, collected at various intervals, demonstrated significant shifts, reflecting the remodeling processes of bone deposition and resorption. The non-invasive method of salivary sampling offers potential for monitoring treatment progress and optimizing orthodontic strategies, providing insights beyond clinical observations or imaging.

This in vivo study was conducted in the Department of Orthodontic & Dentofacial Orthopedics, Babu Banarasi Das College of Dental Sciences, BBD university in collaboration with Department of Biochemistry KGMU, Lucknow with the aim to assess biomarker levels in GCF during fixed orthodontic treatment in growing and non-growing subjects. The sample for this study consisted of 30 subjects who were undergoing fixed orthodontic treatment and equally divided into two groups – Group-I included 15 growing subjects with mean age (13.72+_1.27 years) and Group-II included 15 non-growing subjects with mean age (20.36+_4.64 years). The sample was divided as Growing and Non-growing based on CVMI staging described by Hassel and Farman²⁸.

Criteria for sample selection: Following inclusion and exclusion criteria was taken for sample selection.

Inclusion criteria

1. Patients with Angle's Class I Bi-Maxillary protrusion and Class II division 1 malocclusion.
2. All the subjects with good general health and healthy periodontium.
3. Age group of 10-15 years for growing subjects with CVMI stage 3,4,5.
4. Age group of 16 years and beyond considered as non-growing subjects with CVMI stage 6.

Exclusion criteria

1. History of systemic disease affecting growth and development.
2. Patients having history of previous orthodontic treatment (fixed or removable)
3. Patients having history of smoking or tobacco use.
4. Patients having history of taking drugs that can affect orthodontic tooth movement like contraceptive pills, antihistamines, non-steroidal anti-inflammatory drugs, Bisphosphonates few months prior to study onset or continuing at the time of start of study.

Study sample

Based on above mentioned inclusion and exclusion criteria subjects having class I Bimaxillary protrusion and class II Div 1 malocclusion from patients undergoing fixed orthodontic treatment in the department were selected and were divided as Growing (n=15) and non-growing (n=15) on the basis of CVMI staging.

Table 1: Sample distribution for study

Group	Number subject (N)	Mean Age	CVMI Stage
Group I (Growing)	15	13.72+_1.27 years	CVMI stage3- CVMI stage4- CVMI stage5-
Group II (Non-growing)	15	20.36+_4.64 years	CVMI stage6-

Ethical Committee Approval

Approval was taken from the Ethical Committee of BBDCODS, BBDU, Lucknow prior to the start of the present study. Written informed consent was obtained from the selected subjects participating in this study before beginning of the study and was explained about the study.

Materials and Equipments (Fig 1)

1. Material used for clinical examination

A. Diagnostic instrument

a. Gloves

b. Mouth mirror

c. Explorer

d. Tweezer

e. Kidney tray



Fig 1. Material used for diagnosis

a. Gloves b. Mouth mirror c. Explorer d. Tweezer e. Kidney tray

2. Material used for Cephalometric tracing (Fig 2)

- a. Lateral Cephalogram.
- b. Tracing Paper.
- c. Cephalometric tracing board (LED).
- d. Sharp Pencil (0.5mm).
- e. Eraser.

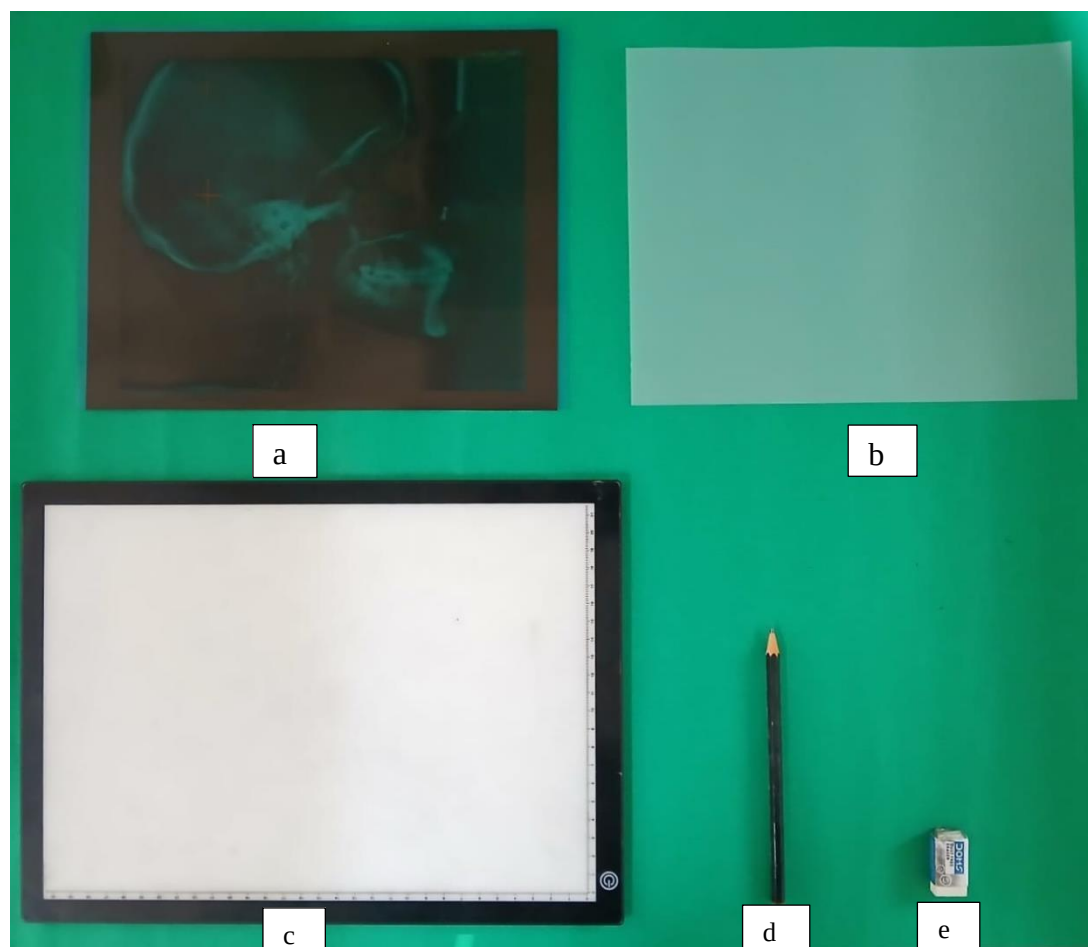


Fig 2. Material used for cephalometric tracing.

a. Lateral Cephalogram **b.** Tracing Paper **c.** Cephalometric tracing board (LED) **d.** Sharp Pencil (0.5mm) **e.** Eraser.

3. Material used for fixed orthodontic treatment (Fig 3)

- a. Separators (Garmy).
- b. Preformed bands with buccal tube on molar as per size.
- c. Glass Ionomer Cement- Luting (GC corporation Tokyo Japan).
- d. Cheek retractor.
- e. MBT gauge (GDC).
- f. Bonding materials (d-tech) (etchant, primer, adhesive, applicator tip).
- g. Light cure gun (Woodpecker).
- h. MBT Brackets (.022x.028) (3M Unitek)
- i. Bracket positioning tweezer
- j. Initial alignment arch wire Ni-Ti (0.012, 0.014, 0.016)
- k. Weingart plier
- l. Mathieu haeden plier
- m. Distal end cutter
- n. Ligature wire cutter Steel ligature wire
- o. Elastomeric module
- p. Steel ligature wire

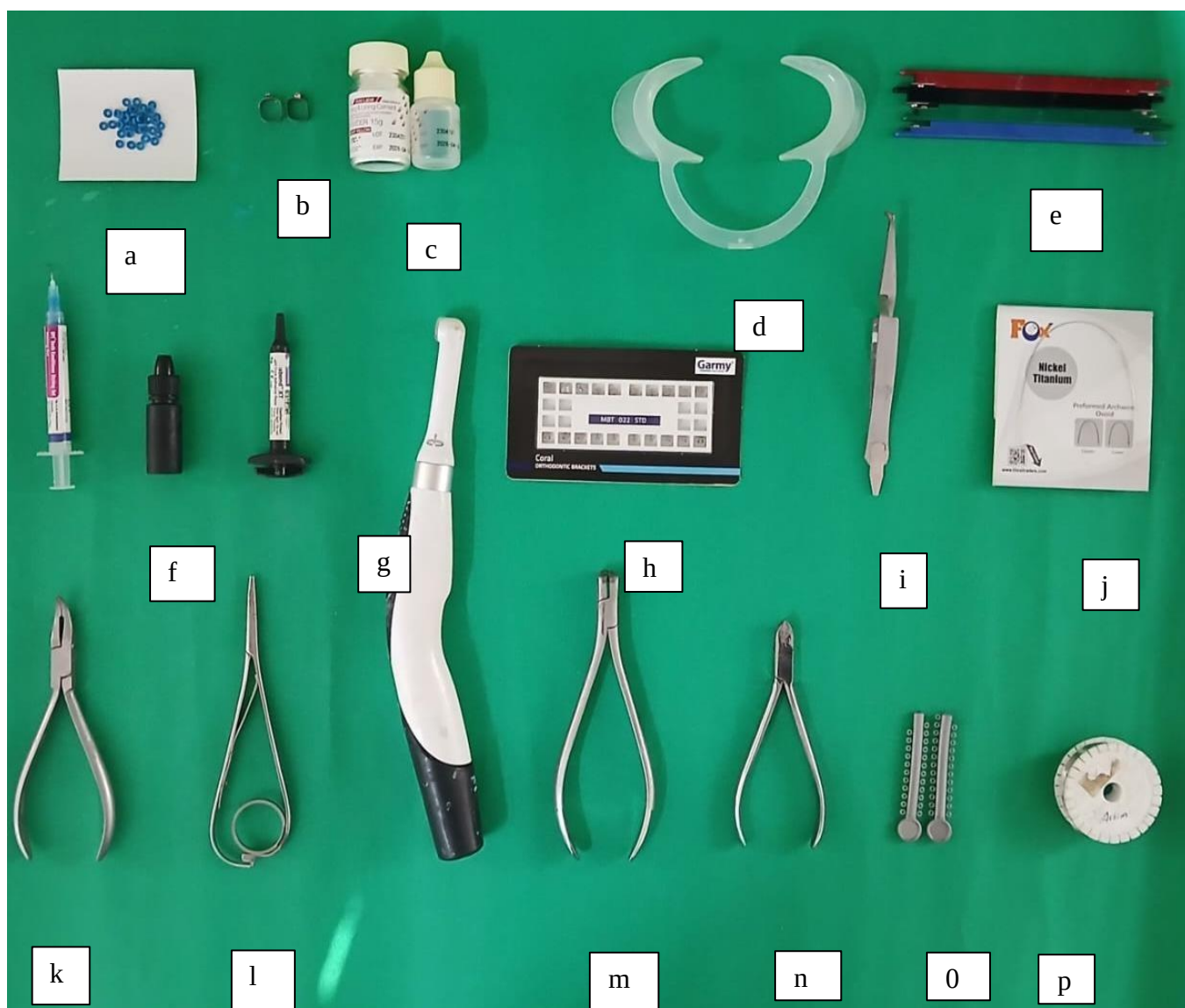


Fig 3. Material used for fixed orthodontic treatment

a. Separators **b.** Preformed bands with buccal tube on molar as per size **c.** Glass Ionomer Cement **d.** Cheek retractor **e.** MBT gauge **f.** Bonding materials (etchant, primer, adhesive, applicator tip) **g.** Light cure gun (Woodpecker) **h.** MBT racks (.022x.028) (3M Unitek) **i.** Bracket positioning tweezers **j.** Initial alignment arch wire Ni-Ti (0.012, 0.014, 0.016) **k.** Weingart plier **l.** Mathieu haeden plier **m.** Distal end cutter **n.** ligature wire cutter **o.** Elastomeric Modules **p.** Steel ligature wire.

4. Material used for GCF collection (Fig 4)

- a. Mouth wash.
- b. Gloves.
- c. cotton roll.
- d. Cheek retractor.
- e. Phosphate buffer solution (SRL).
- f. Absorbent paper points (Meta Biomed).
- g. Eppendrof tube.

**Fig 4. Material used for GCF collection.**

a. Mouth wash **b.** Gloves **c.** cotton roll **d.** Cheek retractor **e.** Phosphate buffer solution (SRL)
f. Absorbent paper points (Meta Biomed) **g.** Eppendrof tube.

5. Material used for storage and transfer (Fig 5)

- a. Ice box for interim storage of sample at 4° C.
- b. Deep freezer at -80°.



Fig 5. Material used for sample storage and transportation.

a. Ice box for interim storage of sample at 4° C **b.** Deep freezer at -80°.

6. Material used for ELISA test (Fig 6)

- a. Incubator
- b. ELISA analyser system (Thermo scientific).
- c. Centrifugation machine (GMI)
- d. ELISA kit for RANKL (Elabsciences)
- e. ELISA kit for IL-6 (Elabsciences).

**Fig 6. Material used for ELISA test**

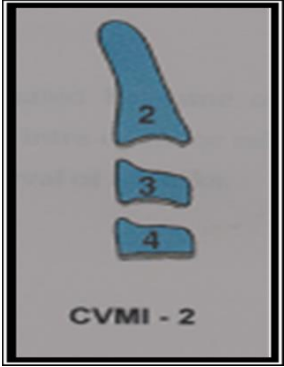
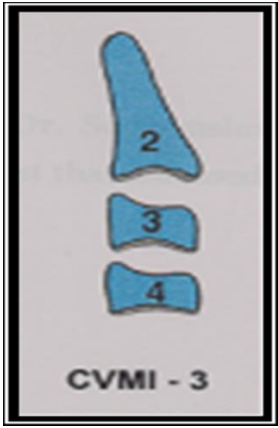
a. Incubator **b.** ELISA analyser system (Thermo scientific) **c.** Centrifugation machine (GMI) **d.** ELISA kit for RANKL (Elabsciences) **e.** ELISA kit for IL-6 (Elabsciences).

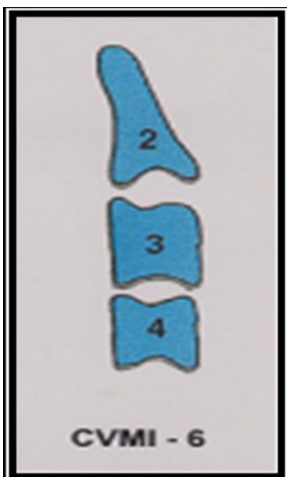
Methodology

A. For division of sample

The subjects who came to the Department of Orthodontics and Dentofacial Orthopaedics, BBDCODS, Lucknow seeking orthodontic treatment and had Angles class 1 Bi-maxillary protrusion or Class II division 1 malocclusion on clinical examination were selected. Pre-treatment records including (lateral cephalogram, orthopantomogram, study models, extra oral and intra oral photographs) of all patients were taken. As seen on lateral cephalogram taken for each subjects. Cervical vertebrae (C2, C3, C4, C5, C6) were traced on tracing paper with 0.5 mm diameter pencil after marking proper orientation marks. The morphological shape of each Vertebrae was assessed based on CVMI staging given by Hassel and Farman (Table 2). The subjects with stage 3,4,5 were considered as growing (group I) and those with CVMI stage 6 were considered as non-growing (group II).

Table 2: CVMI staging by Hassel and Farman²⁸

CVMI stage	Shape of vertebrae	Concavity of lower border	Expected adolescent growth
CVMI stage I 	Wedge shaped	Inferior border of C2, C3, C4 were flat.	80%-100% of adolescent growth expected.
CVMI stage II 	Bodies of C3 and C4 are nearly rectangular in shape.	Concavities developing in the inferior border of C2 and C3.	65%-85% of adolescent growth expected.
CVMI stage III 	Border of C3 and C4 are rectangular in shape.	Distinct concavities seen in the inferior border of C2 and C3.	25%-65% of adolescent growth expected.

CVMI stage IV		Vertebral bodies of C3 and C4 are nearly square in shape.	Distinct concavities are seen in the lower border of C2, C3 and C4	10%-25% of adolescent growth expected.
CVMI stage V		The bodies of C3 and C4 were nearly square in shape.	More accentuated concavities were seen in the inferior border of C2, C3 and C4.	5%-10% of additional growth expected.
CVMI stage VI		The bodies of C3, C4 were square or greater in vertical dimension than in horizontal dimension.	Deep concavities were seen in the inferior borders of C2, C3 and C4.	Growth was considered to be complete at this stage.

B. For fixed orthodontic treatment

- After case analysis, separators were placed and patient was recalled after 2 days.
- The preformed band with buccal tube as selected for each subject were cemented to molars using GIC.
- Brackets of conventional MBT prescription (0.022 slot S.S) (3M Unitek) was bonded following standard protocol for both the groups.
- Depending on individual case requirement alignment and levelling was initiated using preformed Ni-Ti arch wire (0.012/0.014/0.016).
- Lace backs were given from canine to first molar and arch wire was placed using elastomeric modules. (Fig 7).



Fig 7. Intra oral photograph of the selected participants

a. Occlusal view **b.** Frontal view **c.** Lateral view

C. Pre collection procedure

The participants were seated comfortably in a dental chair and the procedure for GCF collection was explained to them (Fig 8a). The subjects were asked to rinse their mouths with 0.012% chlorhexidine mouthwash (Fig 8b). Before collection of GCF, any supragingival soft deposits were removed without causing trauma to the gingival crevice. If any haemorrhage was evident during this procedure, no fluid was collected that day. The target site was then thoroughly irrigated with distilled water, isolated by cotton rolls, cheek retractors and dried by an air syringe (Fig 8c).



Fig 8. Pre collection procedure

a. Subjects seated comfortably in dental chair. **b** Subjects rinsing the mouth with 0.012 % chlorhexidine mouth wash **c.** Isolation of target site before collection of GCF.

D. GCF collection

Initial GCF were collected from the distal site of the maxillary canine on both right and left side of growing and non-growing subjects before placement of initial alignment arch wire at T0 (baseline). For collection of GCF absorbent paper points (size 30) were gently inserted into the gingival crevice until slight resistance was felt, then left in place for 30 seconds. (Fig 9). Blood stained absorbent paper point was discarded.

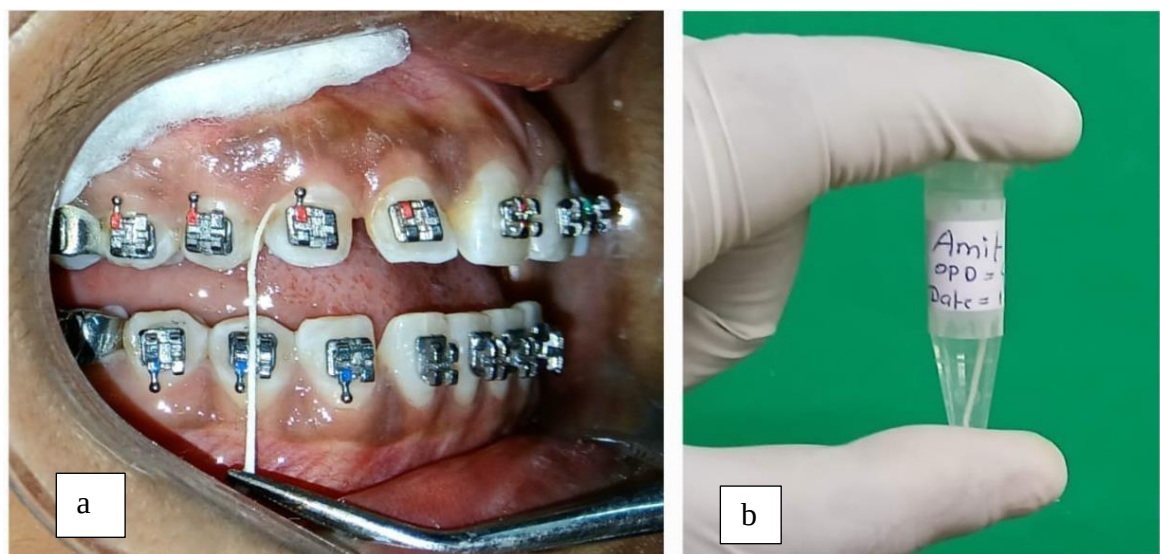
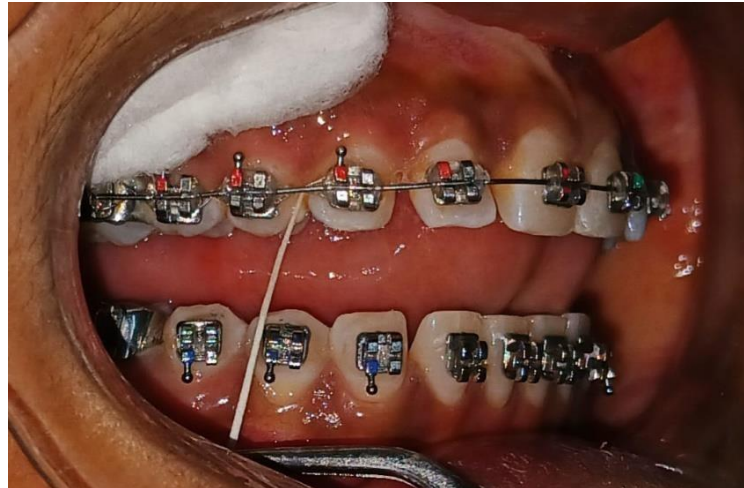


Fig 9. Initial GCF collection

- a.** GCF collection before placement of initial alignment arch wire at (T0).
- b.** Eppendorf tube with collected absorbent paper points.

After collection of GCF, paper points were individually inserted into an eppendorf tube containing 200 μ l, phosphate buffer saline (Fig 9b). Similar procedure was used for collection of GCF after placement of initial arch wire at T1(after 24 hour), T2 (after 3 week) and T3 (after 8 week). (Table 3) (Fig 10).

**Fig 10.**

GCF collection after placement of initial alignment Arch wire at T1(after 24 hour).

Table 3: Different time at which GCF was collected

Group I (N=15)	T0 (at baseline)	T1 (after 24 hour)	T2 (after 1 week)	T3 (after 8 week)
Group II (N=15)	T0 (at baseline)	T1 (after 24 hour)	T2 (after 1 week)	T3 (after 8 week)

E. Storage and Transportation of GCF

Eppendorf tube with collected GCF were then kept in a ice box (Fig 11. a) and placed in a standard freezer for interim storage until they could be transported to Biochemistry lab on same day for final storage in a sample box (Fig 11. b) at -80° until further analysis.



Fig 11. Material used for sample storage and transportation

a. Ice box for interim storage of sample at 4° C. **b.** Sample box with eppendorf tube for

F. For Processing of sample:

After collection of all samples of study, samples were processed at lab. Frozen GCF samples were thawed at room temperature (18° - 25° C) before use. GCF samples were mixed fully to avoid foaming. Each sample was vortexed for 30 seconds using centrifuge machine to ensure the complete elution of GCF from the paper points (**Fig 12.a**). The paper points were then removed, and the collected supernatants were transferred into new Eppendorf tubes and kept in incubator (**Fig 12. b**).

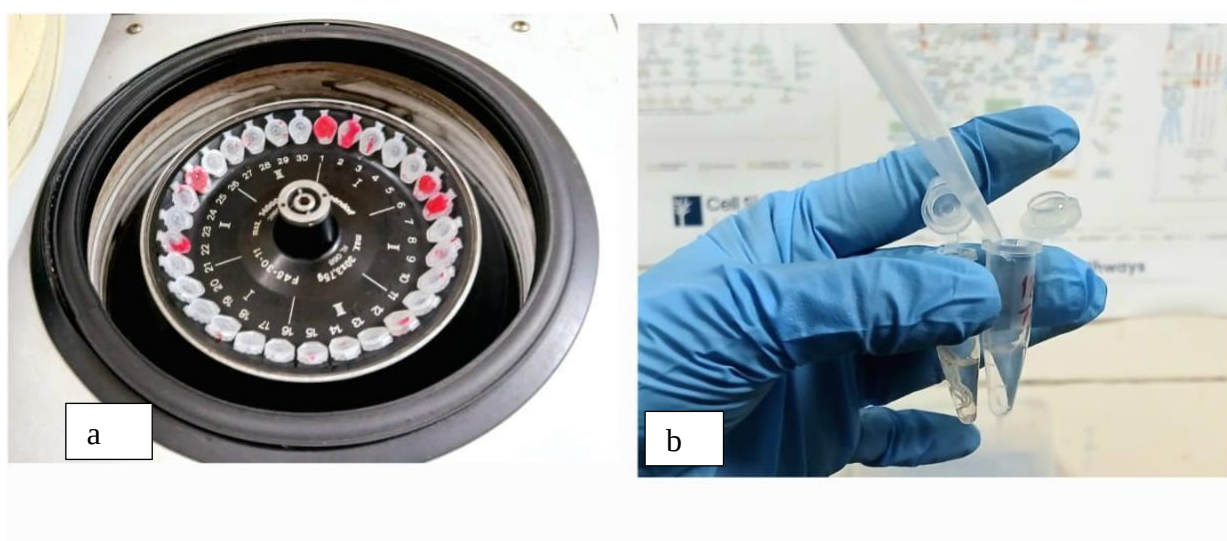


Fig 12. material used for sample processing

a. Centrifuge machine with eppendrof tubes for elution of GCF **b.** Transfer of supernatants to new Eppendorf tubes.

G. For Biomarker Analysis (ELISA):

After completion of elution of GCF for all the samples, analysis for the levels of IL-6 and RANKL in the GCF was done. The processing of reagent preparation is described as follows.

1. The kit out from the refrigerator 20 minutes in advance to bring all reagents to room temperature (18-25 C) before use for either of the biomarker to be tested was taken. The samples to be tested for particular biomarker were brought and were mix fully. **(Fig 13).**
2. Dilute the 25x Concentrated Wash Buffer to 1x working solution.
3. Dilute the Reference Standard of particular biomarker.
4. Dilute the 100x Biotinylated Detection Ab/Ag to 1x working solution 15 minutes earlier.

5. Dilute the 100x Concentrated HRP Conjugate to 1x working solution 15 minutes earlier.

After these preliminary steps of reagent preparation, microplate reader of ELISA analyser system was turned on and assay procedure summary shown in flow chart as followed.



Fig 13. Reagents as obtained from ELISA KIT of RANKL and IL-6

a.ELISA kit for IL-6 b. ELISA kit for RANKL

Assay procedure summary (Fig 14)

Add 100 µl of standard or sample to each well. Incubate for 90 minutes at 37°C and biomarker molecule present in sample were bound to the well.



Remove the liquid. Add 100 µl of Biotinylated Detection Ab/Ag Incubate for 1 hour at 37°C



Aspirate and wash for 3 times to remove unbound Biotinylated Ab/Ag.



Add 100 µL of HRP Conjugate, Incubate for 30 minutes at 37°C and the positive wells turn bluish in color.



Aspirate and wash for 5 times.



Add 90 µl of Substrate Reagent. Incubate for 15 minutes at 37°C which changes the colour of solution from blue to yellow.



Add 50 µl of Stop Solution. The absorbance of each well was read immediately in ELISA reader using 450 nm of wavelength.

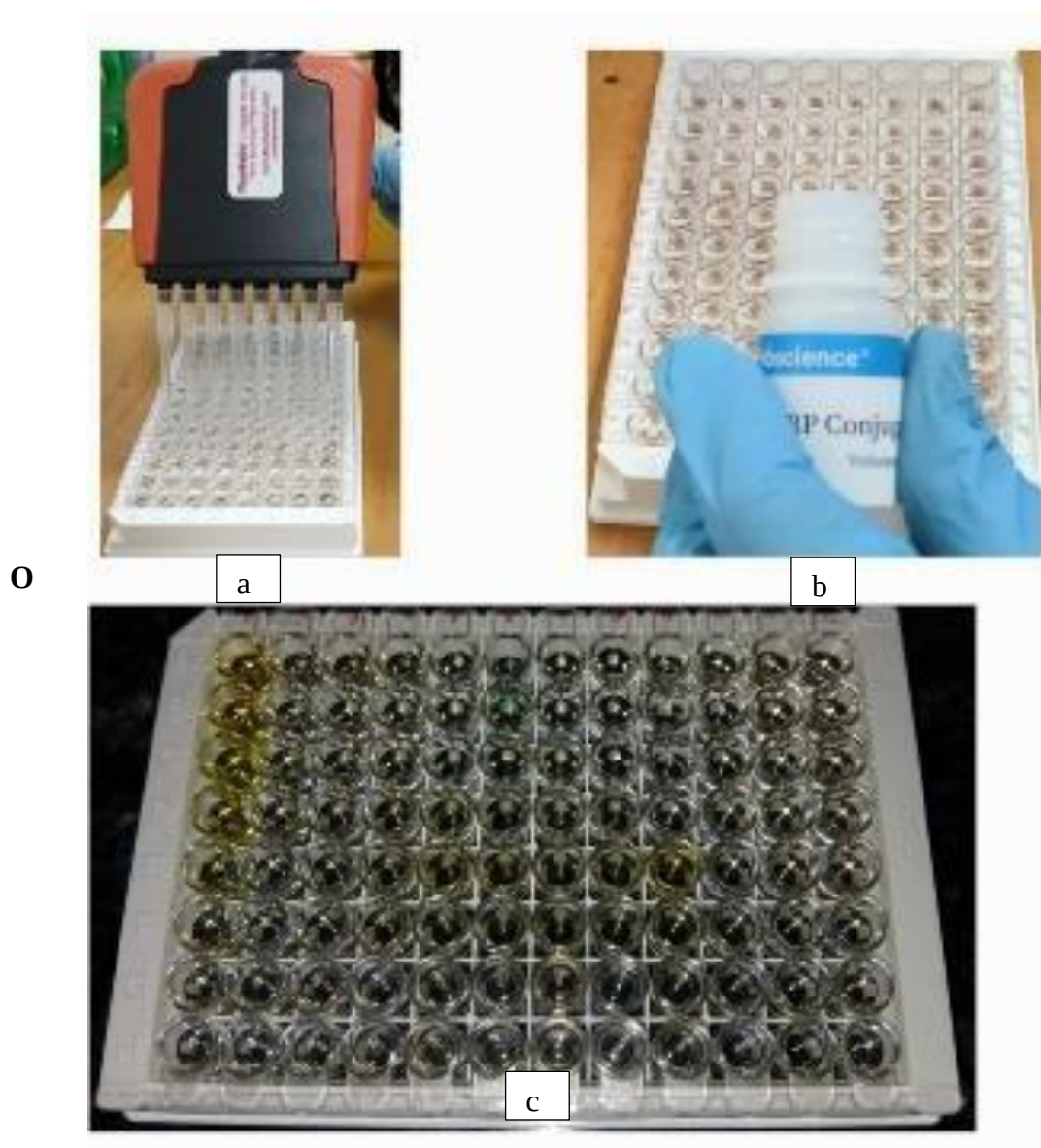


Fig 14. Preparation of assay for sandwich ELISA procedure.

a. washing of wells **b.** Horseradish peroxidase (HRP)-conjugated streptavidin is pipetted to wells **c.** Colour changes of positive solution from blue to yellow.

The present study was conducted in the Department of Orthodontics and Dentofacial Orthopaedics, Babu Banarasi Das College of Dental Sciences, Lucknow in collaboration with Department of Biochemistry KGMU, Lucknow with the aim to assess biomarker levels in GCF during fixed orthodontic treatment in growing and non-growing subjects. The Sample for this study consisted of 30 subjects who were undergoing fixed orthodontic treatment and equally divided into two groups – Group-I included 15 growing subjects with mean age (13.72 ± 1.27 years) and Group-II included 15 non-growing subjects with mean age (20.36 ± 4.64 years).

The results of present study are summarized as follows :-

Table 4: Descriptive statistics for mean value of IL-6 and RANKL as obtained for Group I and Group II at different time.

Table 5: Intra group comparison of IL-6 for Group I at different time.

Table 6: Intragroup comparison of IL-6 for Group II at different time.

Table 7: Intergroup comparison of IL-6 between Group I and Group II at T0, T1, T2, T3.

Table 8: Intragroup comparison of RANKL for Group I at different time.

Table 9: Intragroup comparison of RANKL for Group II at different time.

Table 10: Intergroup comparison of RANKL between Group I and Group II at T0, T1, T2, T3.

Table 11: overall comparison between Group I and Group II for IL-6 and RANKL.

Table 4: Descriptive statistics for mean value of IL-6 and RANKL in Group I and Group II.

N=15	IL-6			RANKL		
Group I	MIN pg/ml	MAX pg/ml	MEAN +_ SD pg/ml	MIN pg/ml	MAX pg/ml	MEAN+_SD pg/ml
T0	2.056	2.771	2.762+_1.540	26.768	72.650	45.914+_14.628
T1	2.113	2.746	2.343+_228	29.197	82.999	44.259+_18.422
T2	2.031	3.211	2.444+_.414	30.887	52.217	38.101+_6.094
T3	2.034	2.857	2.392+_263	33.31	59.662	39.454+_8.439
Group II						
T0	2.074	3.612	2.588+_505	26.768	86.642	40.294+_16.755
T1	2.011	2.485	2.202+_162	25.976	63.147	39.954+_12.869
T2	2.027	3.100	2.250+_297	27.508	47.201	34.875+_6.515
T3	1.913	4.173	2.400+_649	28.141	75.079	38.326+_13.980

- For Group I, Mean level of IL-6 was highest at **T0**(2.762+_1.540 pg/ml), followed by **T2**(2.444+_.414 pg/ml) than **T3**(2.392+_263 pg/ml) and least in **T1**(2.343+_228 pg/ml). (T0>T2>T3>T1).
- For Group II, Mean level of IL-6 was highest in **T0**(2.588+_505 pg/ml) followed by **T3**(2.400+_649pg/ml) than **T2**(2.250+_297pg/ml) and least in **T1**(2.202+_162pg/ml). (T0>T3>T2>T1).

- For Group I, Mean level of RANKL was highest in **T0**(45.914+_14.628pg/ml), followed by **T1**(44.259+_18.422 pg/ml) than **T3**(39.454+_8.439pg/ml) and least in **T2**(38.101+_6.094 pg/ml). (T0>T1>T3>T2).
- For Group II, Mean level of RANKL was highest in **T0** (40.294+_16.755pg/ml) followed by **T1**(39.954+_12.869pg/ml) than **T3**(38.326+_13.980pg/ml) and least in **T2**(34.875+_6.515pg/ml). (T0>T1>T3>T2).

TABLE 5: Intra group comparison of IL-6 for Group I at different time intervals.

		Paired differences				t	df	P value
		Mean +_std. D (Pg/ml)	Std. error mean	95% confidence interval of the difference				
				lower	Upper			
Group I	T0-T1	.419+_1.601 pg/ml	.482	-.656	1.495	.868	10	.406
	T0-T2	.318+_1.738 pg/ml	.524	-.849	1.486	.608	10	.557
	T0-T3	.370+_1.392 pg/ml	.419	-.564	1.305	.883	10	.398
	T1-T2	-.100+__.392 pg/ml	.118	-.364	.163	-.851	10	.415
	T1-T3	-.048+__.382 pg/ml	.115	-.305	.208	-.423	10	.682
	T2-T3	.052+-.513 pg/ml	.154	-.292	.396	.336	10	.744

On comparing mean levels of IL-6 between **T0** and **T1**, it was seen that values were higher for **T0**(2.762+_1.540pg/ml) than **T1** (2.343+_228 pg/ml) With mean difference of (.419+_1.601 pg/ml) and that difference was not statistically significant. (p=.406).

On comparing mean level of IL-6 between **T0** and **T2**, it was seen that values were higher for **T0** (2.762+_1.540pg/ml) than **T2** (2.444+_414pg/ml) with mean difference of (.318+_1.738 pg/ml) but that difference was not statistically significant. (p=.557).

On comparing mean level of IL-6 between **T0** and **T3**, it was seen that values were higher for **T0** (2.762+_1.540pg/ml) than **T3**(2.392+_263 pg/ml) with mean difference of (.370+_1.392 pg/ml) but that difference was not statistically significant. (p=.398).

On comparing mean level of IL-6 between **T1** and **T2**, it was seen that values were higher for **T2**(2.444+_414pg/ml) than **T1**(2.343+_228 pg/ml) with mean difference of (-.100+_392 pg/ml) but that difference was not statistically significant. (p=.415).

On comparing mean level of IL-6 between **T1** and **T3**, it was seen that values were higher for **T3**(2.392+_263 pg/ml) than **T1**(2.343+_228 pg/ml) with mean difference of (-.048+_382 pg/ml) but that difference was not statistically significant. (p=.682)

On comparing mean level of IL-6 between **T2** and **T3**, it was seen that values were higher for **T2**(2.444+_414pg/ml) than **T3**(2.392+_263 pg/ml) with mean difference of (.052+-.513 pg/ml) but that difference was not statistically significant. (p=.744)

Table 6: Intragroup comparison of IL-6 For Group II at different time.

		Paired differences				t	df	P value
		Mean +_std. D (pg/ml)	Std. error mean	95% confidence interval of the difference				
				Lower	Upper			
Group II	T0-T1	.386+_577(pg/ml)	.174	-.001	.775	2.220	10	.051
	T0-T2	.338+_554(pg/ml)	.167	-.034	.710	2.023	10	.071
	T0-T3	.188+_864(pg/ml)	.260	-.391	.769	.725	10	.485
	T1-T2	-.048+-.296(pg/ml)	.089	-.247	.150	-.546	10	.597
	T1-T3	-.197+_678(pg/ml)	.204	-.654	.258	-.967	10	.356
	T2-T3	-.149+_777(pg/ml)	.234	-.671	.373	-.636	10	.539

On comparing mean levels of IL-6 between **T0** and **T1**, it was seen that values were higher for **T0**(2.588+_.505pg/ml) than **T1**(2.202+_.162pg/ml) With mean difference of .386+_.577(pg/ml) and that difference was not statistically significant. (p=.051).

On comparing mean level of IL-6 between **T0** and **T2**, it was seen that values were higher for **T0** (2.588+_.505pg/ml) than **T2** (2.250+_.297pg/ml) with mean difference of (.338+_.554 pg/ml) but that difference was not statistically significant. (p=.071).

On comparing mean level of IL-6 between **T0** and **T3**, it was seen that values were higher for **T0** (2.588+_.505pg/ml) than **T3** (2.400+_.649pg/ml) with mean difference of (.188+_.864 pg/ml) but that difference was not statistically significant. (p=.485).

On comparing mean level of IL-6 between **T1** and **T2**, it was seen that values were higher for **T2** ($2.250 \pm .297$ pg/ml) than **T1** ($2.202 \pm .162$ pg/ml) with mean difference of $(-.048 \pm .296$ pg/ml) but that difference was not statistically significant. ($p=.597$).

On comparing mean level of IL-6 between **T1** and **T3**, it was seen that values were higher for **T3** ($2.400 \pm .649$ pg/ml) than **T1** ($2.202 \pm .162$ pg/ml) with mean difference of $(-.197 \pm .678$ pg/ml) but that difference was not statistically significant. ($p=.356$).

On comparing mean level of IL-6 between **T2** and **T3**, it was seen that values were higher for **T3** ($2.400 \pm .649$ pg/ml) than **T2** ($2.250 \pm .297$ pg/ml) with mean difference of $(-.149 \pm .777$ pg/ml) but that difference was not statistically significant. ($p=.539$)

Table :7 Intergroup comparison of IL-6 between Group I and Group II at T0, T1, T2, T3.

N=15	TIME	Group	Mean+_ Std. D (pg/ml)	Std. error mean	P value
	T0	I	2.762 ± 1.540 (pg/ml)	.464	0.726
		II	$2.588 \pm .505$ (pg/ml)	.152	
	T1	I	$2.343 \pm .228$ (pg/ml)	.068	0.110
		II	$2.202 \pm .162$ (pg/ml)	.048	
	T2	I	$2.444 \pm .414$ (pg/ml)	.124	0.223
		II	$2.250 \pm .297$ (pg/ml)	.089	
	T3	I	$2.392 \pm .263$ (pg/ml)	.0793	0.971
		II	$2.400 \pm .649$ (pg/ml)	.195	

On comparing Group I with Group II at **T0**, mean value of IL-6 was higher in Group I (2.762+_1.540 pg/ml) than Group II (2.588+_505 pg/ml) and difference was not statistically significant. (p=0.726).

On comparing Group I with Group II at **T1**, mean value of IL-6 was higher in Group I (2.343+_228pg/ml) than Group II (2.202+_162pg/ml) and difference was not statistically significant. (p=0.110).

On comparing Group I with Group II at **T2**, mean value of IL-6 was higher in Group I (2.444+_414pg/ml) than Group II (2.250+_297pg/ml) and difference was not statistically significant. (p=0.223).

On comparing Group I with Group II at **T3**, mean value of IL-6 was higher in Group II (2.400+_649 pg/ml) than Group I (2.392+_263 (pg/ml) and difference was not statistically significant. (p=0.971).

TABLE 8: Intra group comparison of RANKL for Group I at different time.

	Time	Paired differences				t	df	P value
		Mean +_ Std. D (pg/ml)	Std. error mean	95% confidence interval of the difference				
				Lower	Upper			
Group I	T0 - T1	1.654+_15.078 pg/ml	4.546	-8.475	11.784	.364	10	.723
	T0 - T2	7.813+_13.822 pg/ml	4.167	-1.473	17.099	1.875	10	.090
	T0 - T3	6.459+_17.913 pg/ml	5.401	-5.575	18.493	1.196	10	.259
	T1 - T2	6.158+_15.991 pg/ml	4.821	-4.585	16.901	1.277	10	.230
	T1 - T3	4.804+_19.740 pg/ml	5.952	-8.457	18.066	.807	10	.438
	T2 - T3	-1.353+_8.788 pg/ml	2.649	-7.257	4.550	-.511	10	.621

On comparing mean levels of **RANKL** between **T0** and **T1**, it was seen that values were higher for **T0**(45.914+_14.628 pg/ml) than **T1** (44.259+_18.422 pg/ml) With mean difference of (1.654+_15.078 pg/ml and that difference was not statistically significant. (p=.723).

On comparing mean level of **RANKL** between **T0** and **T2**, it was seen that values were higher for **T0**(45.914+_14.628 pg/ml) than **T2** (38.101+_6.094 pg/ml) with mean difference of (7.813+_13.822 pg/ml but that difference was not statistically significant. (p=.090).

On comparing mean level of **RANKL** between **T0** and **T3**, it was seen that values were higher for **T0**(45.914+_14.628 pg/ml) than **T3**(39.454+_8.439pg/ml) with mean difference of (6.459+_17.913 pg/ml) but that difference was not statistically significant. (p=.259).

On comparing mean level of **RANKL** between **T1** and **T2**, it was seen that values were higher for **T1**(44.259+_18.422 pg/ml) than **T2**(38.101+_6.094 pg/ml). with mean difference of (6.158+_15.991 pg/ml) but that difference was not statistically significant. (p=.230).

On comparing mean level of **RANKL** between **T1** and **T3**, it was seen that values were higher for **T1** (44.259+_18.422 pg/ml) than **T3** (39.454+_8.439pg/ml) with mean difference of (4.804+_19.740 pg/ml) but that difference was not statistically significant. (p=.438).

On comparing mean level of **RANKL** between **T2** and **T3**, it was seen that values were higher for **T3**(39.454+_8.439pg/ml) than **T2**(38.101+_6.094 pg/ml) with mean difference of (-1.353+_8.788 pg/ml) but that difference was not statistically significant. (p=.621)

TABLE 9: Intra group comparison of RANKL for Group II at different time.

	Time	Paired differences				T	df	P value
		Mean +_Std. D (pg/ml)	Std. error mean	95% confidence interval of the difference				
				Lower	upper			
Group II	T0 - T1	.339+_11.529(pg/ml)	3.476	-7.405	8.085	.098	10	.924
	T0 - T2	5.418+_20.217(pg/ml)	6.095	-8.164	19.0005	.889	10	.395
	T0 - T3	1.967+_23.938(pg/ml)	7.217	-14.115	18.049	.273	10	.791
	T1 - T2	5.078+_13.793(pg/ml)	4.159	-4.188	14.345	1.221	10	.250
	T1 - T3	1.627+_21.576(pg/ml)	6.505	-12.867	16.122	.250	10	.808
	T2 - T3	-3.451+_ 14.730(pg/ml)	4.441	-13.347	6.445	-.777	10	.455

On comparing mean levels of **RANKL** between **T0** and **T1**, it was seen that values were higher for **T0** (40.294+_16.755pg/ml) than **T1**(39.954+_12.869pg/ml) With mean difference of (.339+_11.529pg/ml) and that difference was not statistically significant. (p=.924).

On comparing mean level of **RANKL** between **T0** and **T2**, it was seen that values were higher for **T0**(40.294+_16.755pg/ml) than **T2** (34.875+_6.515pg/ml) with mean difference of (5.418+_20.217pg/ml) but that difference was not statistically significant. (p=.395).

On comparing mean level of **RANKL** between **T0** and **T3**, it was seen that values were higher for **T0**(40.294+_16.755pg/ml) than **T3**(38.326+_13.980pg/ml) with mean difference of (1.967+_23.938pg/ml) but that difference was not statistically significant. (p=.791).

On comparing mean level of **RANKL** between **T1** and **T2**, it was seen that values were higher for **T1**(39.954+_12.869pg/ml) than **T2** **T2**(34.875+_6.515pg/ml) with mean difference of (5.078+_13.793pg/ml) but that difference was not statistically significant. (p=.250).

On comparing mean level of **RANKL** between **T1** and **T3**, it was seen that values were higher for **T1**(39.954+_12.869pg/ml) than **T3**(38.326+_13.980pg/ml) with mean difference of (1.627+_21.576 pg/ml) but that difference was not statistically significant. (p=.808).

On comparing mean level of **RANKL** between **T2** and **T3**, it was seen that values were higher for **T3** (38.326+_13.980pg/ml) than **T2** (34.875+_6.515pg/ml) with mean difference of (-3.451+-14.730 pg/ml) but that difference was not statistically significant. (p=.455).

Table 10: Intergroup comparison of RANKL between Group I and Group II at T0, T1, T2, T3.

N=11	Time	Group	Mean +_Std. D (pg/ml)	Std. error mean	P value
	T0	I	45.914+_14.628 pg/ml	4.410	0.412
		II	40.294+_16.755pg/ml	5.052	
	T1	I	44.259+_18.422pg/ml	5.554	0.532
		II	39.954+_12.869pg/ml	3.880	
	T2	I	38.101+_6.094pg/ml	1.837	0.244
		II	34.875+_6.515pg/ml	1.964	
	T3	I	39.454+_8.439pg/ml	2.544	0.821
		II	38.326+_13.980pg/ml	4.215	

On comparing Group I with Group II at **T0**, mean value of **RANKL** was higher in Group I (45.9145+_14.628 pg/ml) than Group II (40.294+16.755 pg/ml) and difference was not statistically significant. (p=0.412).

On comparing Group I with Group II at **T1**, mean value of **RANKL** was higher in Group I (44.259+_18.422pg/ml) than Group II (39.954+_12.869 pg/ml) and difference was not statistically significant. (p=0.532).

On comparing Group I with Group II at **T2**, mean value of **RANKL** was higher in Group I (38.101+_6.094 pg/ml) than Group II (34.875+_6.515 pg/ml) and difference was not statistically significant. (p=0.244).

On comparing Group I with Group II at **T3**, mean value of **RANKL** was higher in Group I (39.454+_8.439 pg/ml) than Group II (38.326+_13.980 pg/ml) and difference was not statistically significant. (p=0.821).

Table 11: Comparison between both groups of IL-6 and RANKL (Total).

	Group	Mean+_Std. D (pg/ml)	Std. error mean	P value
IL-6	I	9.942+_1.605 pg/ml	.483	0.364
	II	9.441+__.792 pg/ml	.239	
RANKL	I	167.730+_34.425 pg/ml	10.379	0.294
	II	153.450+_27.380 pg/ml	8.255	

On overall comparison of **IL-6** level in Group I with Group II, mean value of IL-6 was higher in **Group I** (9.942+_1.605 pg/ml) than **Group II** (9.441+__.792 pg/ml), however difference was not statistically significant. (p=0.364).

On overall comparison of **RANKL** level in group I with Group II, mean value of RANKL was higher in **Group I** (167.730+_34.425 pg/ml) than **Group II** (153.450+_27.380 pg/ml), however difference was not statistically significant. (p=0.294).

The results of the present study showed variable levels of Biomarkers in both Group I and Group II at different time intervals (T0, T1, T2, T3), suggestive of the fact that fluctuations of these Biomarkers are a part of intricate process of OTM at molecular level.

The trend of level of **IL-6** in Group I was **T0**(2.762+_1.540 pg/ml)>**T2**(2.444+__.414 pg/ml) >**T3**(2.392+__.263 pg/ml) >**T1**(2.343+__.228 pg/ml) whereas for Group II trend was **T0**(2.588+__.505 pg/ml) >**T3**(2.400+__.649pg/ml) >**T2**(2.250+__.297pg/ml) >**T1**(2.202+__.162pg/ml).

On intragroup comparison of level of **IL-6** statistically non-significant difference was noted between T0-T1, T0-T2, T0-T3, T1-T2, T1-T3 and T2-T3 for both Group I and Group II.

The trend for levels of **RANKL** in Group I was **T0**(45.914+_14.628pg/ml), >**T1**(44.259+_18.422 pg/ml) >**T3**(39.454+_8.439pg/ml >**T2**(38.101+_6.094 pg/ml) whereas for Group II trend was **T0**(40.294+_16.755pg/ml) >**T1**(39.954+_12.869pg/ml) >**T3**(38.326+_13.980pg/ml) >**T2**(34.875+_6.515pg/ml).

On intragroup comparison of level of **RANKL** statistically non-significant difference was noted between T0-T1, T0-T2, T0-T3, T1-T2, T1-T3 and T2-T3 for both Group I and Group II.

Orthodontic treatment corrects skeletal and dental malrelationships, enhances facial aesthetics and establishes occlusal harmony and structural balance¹. The fixed orthodontic treatment is limited not only to children but adults are equally seeking orthodontic treatment for desirable aesthetic outcome². The mechanical forces exerted during fixed orthodontic treatment initiates a cascade of movements like initiation of acute inflammatory response that involves vasodilation of capillaries of PDL, release of inflammatory mediators/biomarker like IL-6, RANKL, PGE2, Cytokines, IL-1 β , Tumor necrosis factor (TNF)- α etc⁵. In growing subjects, additional stimulation to orthodontic forces because of growth might result in variable response to fixed orthodontic treatment in comparison to non-growing subjects. Clinically, it is seen that orthodontic treatment is faster in growing individuals in comparison to adults, at molecular level, the reason could be that as vascularity is more and inflammatory system is more activated in growing children that can react faster to local changes on application of mechanical force⁴.

Biomarkers are substances that can be measured and evaluated objectively as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention^{7,8}.

In Orthodontics biomarkers have helped us in assessing puberty, bone turnover or understanding Orthodontic tooth movement at molecular level. Biomarkers can be obtained from Serum, Saliva and GCF^{12,15}.

The Serum Biomarkers have limitations, including the need for invasive collection, risk of contamination, and potential anxiety. Their levels can also fluctuate due to inflammation in other body parts over time.

Similarly Salivary Biomarkers are present in low concentrations, requiring sensitive tests for detection and are influenced by various environmental and psychological factors¹⁸.

To overcome these limitations of Serum and Salivary Biomarkers, GCF Biomarkers are preferred due to their proximity to periodontal tissues, minimizing systemic influences²².

Various studies evaluated Biomarkers in Serum and Saliva to assess pubertal growth spurt and found difference in levels of Biomarker during growing phase of an individual^{15,16}.

The levels of various Biomarkers that bring about changes at molecular level had been assessed during various phases of orthodontic tooth movement in non-growing subjects⁵⁶.

Other studies compared level of different biomarker like IL-1 β , TNF- α , GM-CSF in growing and non-growing subjects⁴.

Amongst various Biomarkers IL-6 released initially during Orthodontic tooth movement that is responsible for induction of process of Osteoclastogenesis was selected in present study²⁴. Also RANKL which is a potent biomarker that attaches to RANK to promote bone resorption would be evaluated in present study for both growing and non-growing children²⁵.

However not many studies have compared different types of biomarker level in growing and non-growing subjects. Considering this the aim of present study was to assess Biomarkers (IL-6 and RANKL) levels in GCF during fixed Orthodontic treatment in growing and non-growing subjects.

The present study was conducted in the Department of Orthodontics and Dentofacial Orthopaedics, Babu Banarasi Das College of Dental Sciences, BBD University in collaboration with Department of Biochemistry KGMU, Lucknow.

For present study, 30 subjects with Angle's Class 1 bi-maxillary protrusion or Class II division 1 malocclusion were selected, who had to undergo fixed orthodontic treatment in the department. The sample was divided into two groups: Group-I (15 growing subjects, age range 13.72+_1.27 years) and Group-II (15 non-growing subjects, age range 20.36+_4.64 years). The groups were categorized based on CVMI staging by Hassel and Farman²⁸.

Pre-treatment records, including lateral cephalogram, orthopantomogram, study models and photographs were taken and skeletal maturation was assessed based on CVMI

staging by Hassel and Farman²⁸. Subjects with stages 3,4,5 were categorized as growing (Group I), while those at stage 6 were classified as non-growing (Group II).

Fixed orthodontic treatment was started using MBT brackets following standard protocol. Alignment and leveling were initiated using preformed Ni-Ti arch wires (0.012/0.014/0.016) as per case requirement with lace backs from canine to hook on tubes of maxillary first molar and arch wires were secured using elastomeric modules. The GCF samples were collected at T0 (at baseline) i.e before placing initial alignment wire, T1 (at 24 hours), T2 (at 3 week), T3 (at 8 week) following standard protocol and using absorbent paper points.

GCF samples were collected from the distal site of the maxillary canine at selected time intervals. After GCF collection, paper points were placed in Eppendorf tubes with 200 µl phosphate buffer saline and transported to Biochemistry lab for final storage at -80° until further analysis.

After collection, frozen GCF samples were thawed at room temperature and centrifuged. The paper points were removed and the supernatants were transferred to new Eppendorf tubes and kept in an incubator.

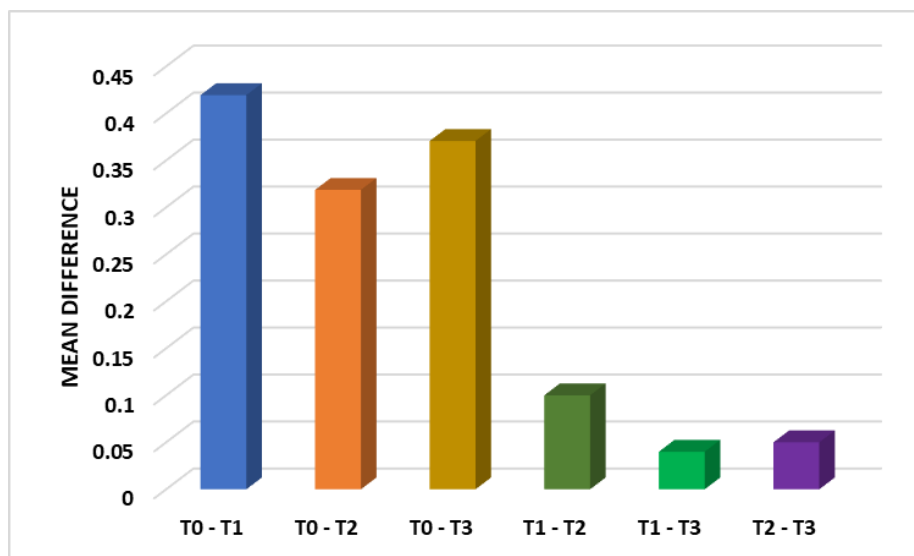
After elution, GCF samples were analyzed for IL-6 and RANKL levels. Reagents were prepared by bringing the kit out 20 minutes before use to room temperature (18-25°) the steps were followed as per manufacturer's instruction. The ELISA analyzer was then turned on and the assay procedure was followed as per protocol.

The absorbance of each sample, after sequential adding of reagents as per manufacturers instruction measured in pg/ml, Mean level of IL-6 and RANKL was read at 450 nm using an ELISA analyser after comparing with standard curve as obtained with kit. Data was tabulated and adequate statistical comparison were made.

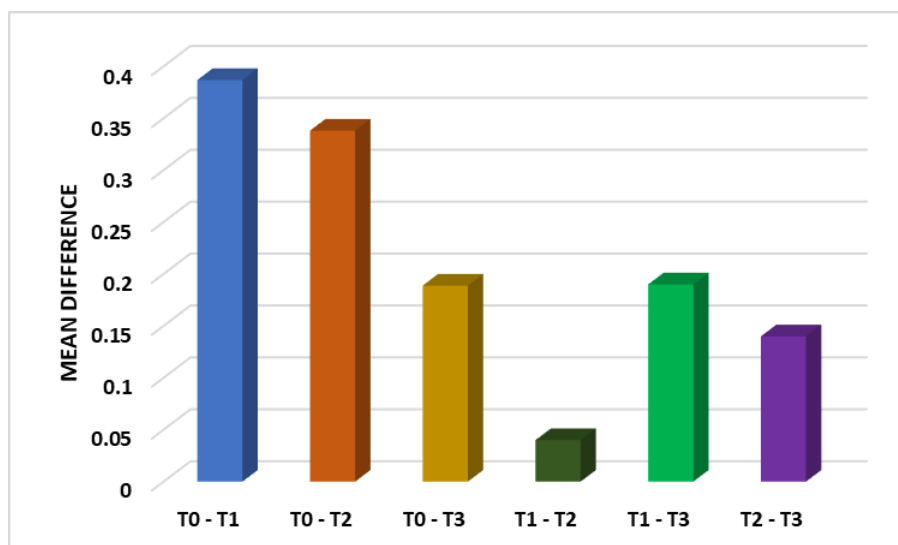
The results of the present study showed variable levels of Biomarkers, both in Group I and Group II at different time intervals (T0, T1, T2, T3), suggestive of the fact that fluctuations of these biomarkers are a part of intricate process of Orthodontic Tooth Movement at molecular level, both for growing and non-growing subjects.

The trend of level of **IL-6** in Group I was **T0**(2.762+ $\pm$ 1.540 pg/ml)>**T2**(2.444+ $\pm$.414 pg/ml) >**T3**(2.392+ $\pm$.263 pg/ml) >**T1**(2.343+ $\pm$.228 pg/ml) whereas for Group II trend was **T0**(2.588+ $\pm$.505 pg/ml) >**T3**(2.400+ $\pm$.649pg/ml) >**T2**(2.250+ $\pm$.297pg/ml) >**T1**(2.202+ $\pm$.162pg/ml).

On intragroup comparison of level of **IL-6**, statistically non-significant difference was noted between T0-T1, T0-T2, T0-T3, T1-T2, T1-T3 and T2-T3, for both Group I and Group II (Bar diagram 1 and 2).



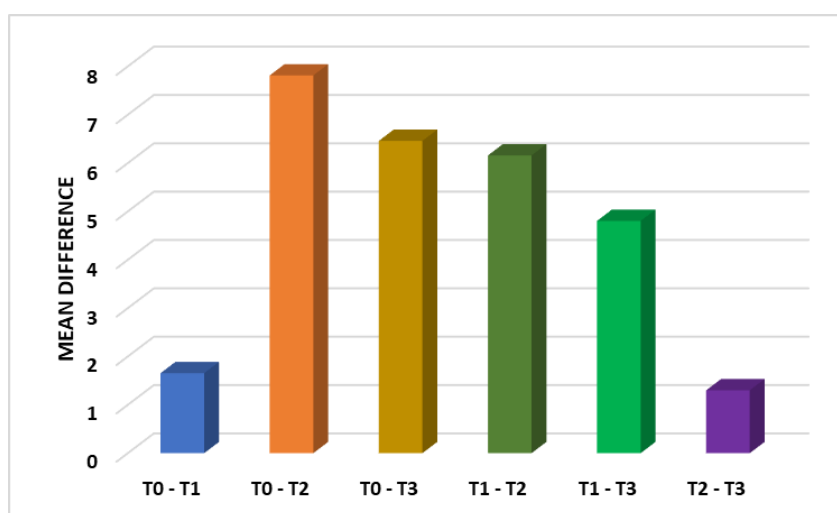
Bar diagram 1. Intra group comparison of IL-6 in Group I.



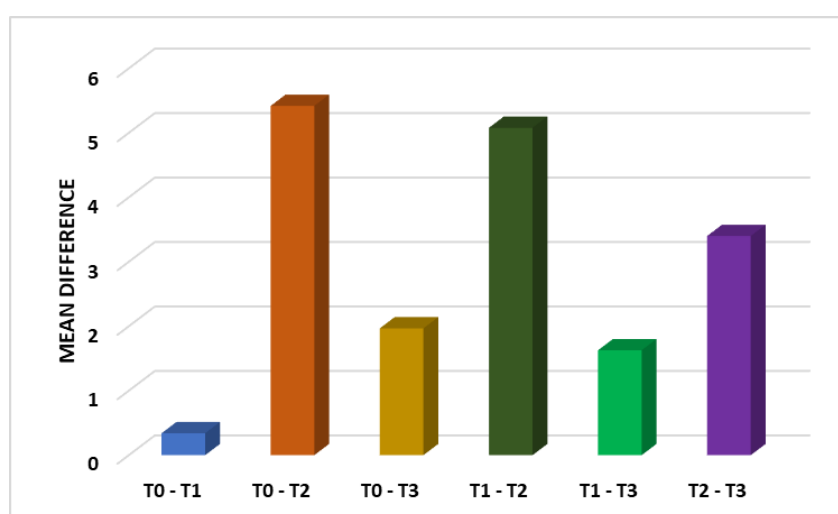
Bar diagram 2. Intra group comparison of IL-6 in Group II.

The trend for levels of **RANKL** in Group I was **T0**(45.914+_14.628pg/ml), >**T1**(44.259+_18.422 pg/ml) >**T3**(39.454+_8.439pg/ml) >**T2**(38.101+_6.094 pg/ml) whereas for Group II trend was **T0**(40.294+_16.755pg/ml) >**T1**(39.954+_12.869pg/ml)>**T3**(38.326+_13.980pg/ml)>**T2**(34.875+_6.515pg/ml).

On Intragroup comparison of level of **RANKL** statistically non-significant difference was noted between T0-T1, T0-T2, T0-T3, T1-T2, T1-T3 and T2-T3 for both Group I and Group II (Bar diagram 3 and 4).

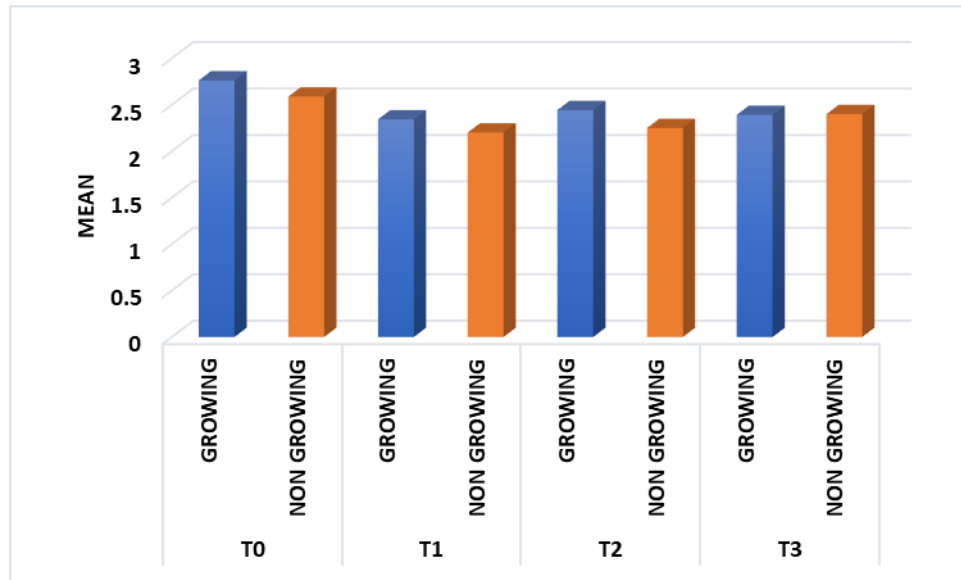


Bar diagram 3. Intra group comparison of RANKL in Group I.

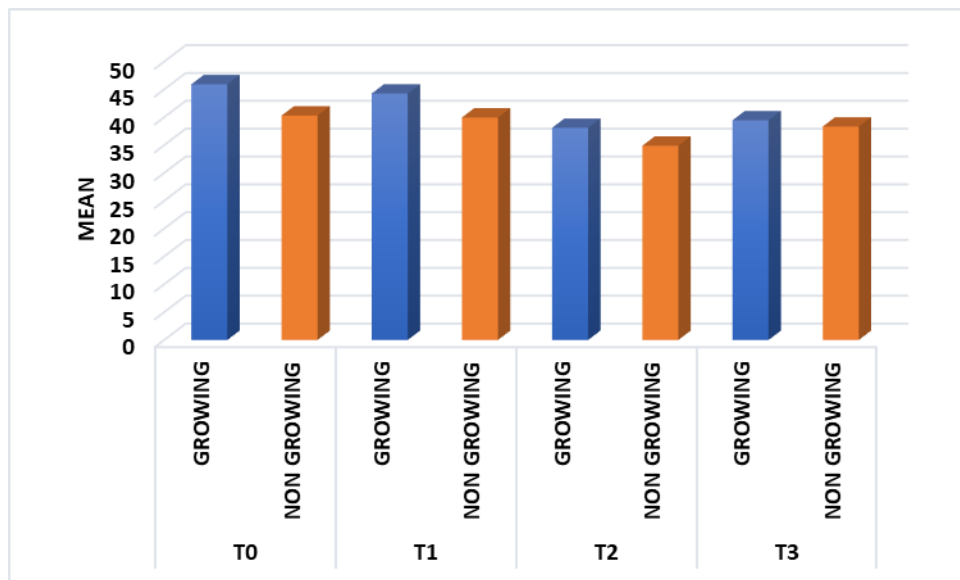


Bar diagram 4. Intra group comparison of RANKL in Group II.

Inter group comparison showed higher values of both the biomarkers at all time intervals for Group I (growing subjects) in comparison to non-growing subjects (Group II) but difference were statistically non-significant at each time intervals. (Bar diagram 5 and 6).



Bar diagram 5. Inter group comparison of IL-6 between Group I and Group II.



Bar diagram 6. Inter group comparison of RANKL between Group I and Group II.

In present study, distinct trends were observed in the IL-6 levels at various time intervals for both the groups, highest values were at T0 (Group I > Group II) and least were at T1(Group I > Group II). The reason could be that, on application of Orthodontic force, lag phase would have started resulting in decreased levels at T1 than T0 in both the groups.

Growing subjects had highest concentration of IL-6 at T2 than T3, whereas non growing subject had highest concentration of IL-6 at T3 than T2. This is suggestive of fact that after initial decrease of biomarker level at T1 in both the groups, levels increased earlier at T2 in Growing subjects (T2>T3) than non-growing subjects who attained increased levels at T3 (T3>T2), though difference was not statistically significant.

Levels of RANKL had a trend of hyper values at 2 months, i.e T3 than T2 (20 days) suggestive of its role in sustaining osteoclastogenesis during bone remodeling due to Orthodontic tooth movement.

On Inter-group comparison, both for IL-6 and RANKL showed higher values of both the biomarkers at all time intervals for Group I (growing subjects) in comparison to non-growing subjects (Group II) but difference were statistically non-significant at each time intervals. The increased growth potential in Group 1 accelerated the inflammatory response and cytokine production, whereas in Group 2, the absence of growth led to trend of lesser values at each time interval.

However, as levels were evaluated in initial stage of Alignment and Levelling only, concentration of levels may not be to that extent to cause considerable statistical difference between growing and non-growing subjects.

As levels of biomarker during Alignment and Leveling were not assessed in many studies, results would be compared to studies that measured biomarker level during various stages of orthodontic tooth movement between growing and non-growing subjects (Simiana et al⁵³, Vujacic A et al⁶, Ren et al⁴, Barun et al²⁴, Grant m et al³⁷, Kawasaki et al³³, Alikhani et al³) or in growing subjects (Jayaprakash et al²⁷, Madureira et al⁴², S. Uematsu et al²⁹, Nishijima et al³²) or in non-growing subjects(Shetty A et al⁵⁶, Mohammad et al⁵⁰, Basaran et al³⁴, Muhammad et al⁵¹, j Amanda et al⁴⁸, Tuncer et al³⁹).

Simiana et al (2021)⁵³, found significantly elevated level of IL-1 β in GCF, both at T0 and T1 in adolescents (11-16 years) than young adults (17-28 year) during retraction on 0.019x 0.025 S.S wire with closed coil spring .They were not able to detect IL-6 in all the sample as the values were being below the test threshold and the mean value identified for IL-6 at T0 was 0.14pg/ μ l and at T1 it was 1.27pg/ μ l but did not mention groupwise values separately hence levels of IL-6 were not subjected to statistical test.

Vujacic A et al (2016)⁶, evaluated the levels of IL-6 in GCF, 10 children (mean age 13) and 10 adults (mean age 20) during the early phase of orthodontic tooth movement (OTM) by using elastomeric separators and found higher level of IL-6 in children than adults at each time interval. Level of IL-6 in children were 2.2+_0.5pg/ml, at 24 hours, 0.4+_0.1pg/ml at 72 hrs and 1.7+_1.1pg/ml at 168 hours whereas in adults increase was much smaller, averaging 0.5+_0.2pg/ml at 24 hours, 0.10+-0.04pg/ml at 72 hrs and 0.2+_0.1pg/ml at 168 hours when compared to control teeth. The amount of tooth movement was statistically greater in children than in adults 168 hours after the initiation of OTM. This time period must have coincided with completion of lag phase resulting in acceleration of OTM. However, the excretory response of cytokines, particularly IL-6, was significantly higher in children's GCF, which is similar to the observation of present study.

Ren et al (2002)⁴, found significantly elevated levels of IL-6 in GCF from 64 pg/ μ l at T0 to 76 pg/ μ l at T1 in juveniles (mean age 11 $\pm$ 0.7 years) during labial movement of the maxillary lateral incisor using a 70 CN of force. Other mediators like PGE2, GM-CSF levels also increased significantly in juveniles from T0-T1 whereas only PGE2 increased significantly in adults (mean age 24 $\pm$ 1.6 years). IL-6 showed non-significant increase from T0-T1 in adults. Contrary to trend of present study, they focused significantly hyper levels of IL-6 in adults then juveniles at T0 whereas at T1of adults had hyper levels than juveniles with non-significant difference.

Barun et al (2022)²⁴, assessed IL-6 levels in GCF in 20 patients with the age range of 11-16 years (8 patient) and 17-24 year (12 patient) during canine retraction using a closed coil spring on a 19x25 SS wire. IL-6 was measured at T0 (Baseline) and T1(24 hours after activation). They were not able to found IL-6 in all the samples as other values were below the detection threshold and the mean overall IL-6 levels were 0.14 pg/ μ l at T0 and 1.27 pg/ μ l at T1, that were not subjected to statistical test.

Grant M et al (2012)³⁷, evaluated IL-6 level in 20 patient with the age range of (12-18 years) in GCF at pretreatment, four hours, 7 days; and 42 days after application of distalising forces to maxillary canine teeth using 9 mm nickel-titanium closed coil spring on 0.018 S.S wire and force produced was 100 g.

IL-6 level was highest at T1(at 4 hrs) 25.79 ± 13.91 > T2(at 7 days) 9.71 ± 3.15 > T0(baseline) 4.48 ± 1.10 , T3(42 days) 4.44 ± 0.86 in pg/30s, however there was statistically nonsignificant difference in levels of IL-6 with time. There was slight increase in RANKL level within 4 hrs(T1) of force application, after that it reduced till 7th day (T2) and then there was significant increase in RANKL level from 7th to 42 days days(highest) after force application.

Kawasaki et al (2006)³³, assessed RANKL levels in GCF in 30 patients (15 juveniles, mean age 15.1 ± 2.8 years, and 15 adults, mean age 31 ± 3.6 years) during canine retraction using elastomeric chains on Edgewise brackets (0.018 x 0.025-inch slot) with a 250-gram initial force. The study found that tooth movement was greater in juveniles (1.23 ± 0.15 mm) than in adults (0.82 ± 0.22 mm). RANKL levels were significantly elevated at experimental sites in both groups at all time points 1 hr(T1), 24hr (T2), 168 hr (T3) except for 24 hours (baseline). In juveniles, RANKL levels at experimental sites were (210.3 ± 27.6 pg/ μ l), compared to (9.7 ± 2.3 pg/ μ l) at control sites. In adults, the RANKL level at experimental sites was 140.5 ± 25.5 pg/ μ l, while at control sites it was 8.2 ± 2.6 pg/ μ l.

Alikhani et al (2018)³, assessed RANKL levels in GCF in 18 patients, 9 adolescents, (age range of 11-14 years), and 9 adults (age range of 21-45 years) during canine retraction using Ni-Ti closed coil spring on 0.017x0.025 s.s wire (MBT (0.022 inch slot) with a 50 CN of initial force before orthodontic treatment, 1,7,14 and 28 days after canine retraction. The study found that equivalent orthodontic forces stimulated age dependent increases in GCF levels in adults compared to adolescents. However, the higher levels of GCF cytokines in adults did not result in faster rate of tooth movement compared to adolescent.

Jayprakash P et al (2019)²⁷, found significantly elevated levels of IL-6 in GCF on moving canine distally by elastic chain exerting an initial force of 250 g on experimental side only in subject with age range of 15.9 years at 24 hours concentration of IL-6 was

higher ($0.065 \pm 0.005 \text{ pg} / \mu\text{g}$) than at baseline ($0.030 \pm 0.008 \text{ pg} / \mu\text{g}$). At 168 hr (7days) mean level of IL-6 ($0.044 \pm 0.007 \text{ pg} / \mu\text{g}$) were higher than baseline but difference was not significant. On other side where no forces was applied, there was decrease in level of IL-6 at 24 hr ($0.016 \pm 0.006 \text{ pg} / \mu\text{g}$) than at baseline ($0.034 \pm 0.0091 \text{ pg} / \mu\text{g}$), at 168 hr ($0.036 \pm 0.0091 \text{ pg} / \mu\text{g}$) and the difference was statistically non-significant between 168hr and baseline.

Madureira et al (2015)⁴², evaluated levels of IL-6 in GCF and PDL in 23 patients (mean age 13.43 ± 2.64) with a 0.017×0.025 - in beta-titanium alloy cantilever attached on premolars that had to be extracted and were subjected to a force of 0.980 N in the apical direction for 3 hours, 15 hours, 3 days, 12 days, or 21 days. They found that there was an increase in IL-6 levels from 15hrs to day 3 and after that there was a subsequent decrease in IL-6 level at day 7 with mild increase till day 14 and concentration of IL-6 was levelled from day 14 to day 21.

S. Uematsu et al (1996)²⁹, found significantly elevated levels of IL-6 in GCF during separate canine retraction in subjects with mean age of 14.4 ± 0.9 years by elastic chain exerting an initial force of 250 g. The mean levels of IL-6 was significantly higher at 24 hr ($0.063 \pm 0.004 \text{ pg} / \mu\text{g}$) than at baseline ($0.020 \pm 0.007 \text{ pg} / \mu\text{g}$) $p = 0.0441$. At 168 hr (7days) mean level of IL-6 was ($0.009 \pm 0.006 \text{ pg} / \mu\text{g}$) $p = 0.0147$ was significantly lesser than baseline values.

Nishijima et al (2006)³², evaluated RANKL level in 10 patients with age range of (14.5-15.4 years) in GCF at 0, 1, 24, and 168 h after the retracting force was applied (canine retraction) along a 0.018 inch archwire with an elastomeric chain delivered an initial force of 250gm. The mean values of RANKL were significantly higher on experimental side after 24 h ($219.3 \pm 26.9 \text{ pg} / \mu\text{l}$) than control sites ($7.7 \pm 5. \text{ pg} / \mu\text{l}$).

Shetty A et al (2022)⁵⁶, assessed level of IL-6 in 20 patients with the age range of 18-30 years in PDL in a split mouth study from the premolar region just before the extraction on control side and on the experimental site. Brackets (0.022 slot MBT mechanotherapy) were bonded on premolar to be extracted after 3 days and an initial intrusive force of 100-150 g was applied using a cantilever spring on this premolar for 3 days. They found insignificant difference in mean level of IL-6 in GCF after 3rd day

of force application 4.595 ± 1.6668 in the control group and 4.3975 ± 1.5948 in the experimental group.

Mohammed et al (2019)⁵⁰, evaluated IL-6 in PDL of extracted 1 premolar each tooth was assigned a time point for its extraction at 2 hours, 15th day or the 30th day after application of force. Brackets were bonded to the experimental teeth at 5 degrees to the long axis of the tooth, 200-250 g of force was exerted on placing 0.014 Ni-Ti wire. They found a significant increase on experimental teeth in the level of IL-6 on 15th day (14.91) than on 30th day (11.1) and least was at 2hrs (3.4) which was non-significant in comparison to control.

Basaran G et al (2006)³⁴, assessed the levels of IL-6 in 15 patients with the mean age of 16.7 ± 2.3 years during canine distalization on 0.016 x 0.022-inch S.S archwire using 7mm sentalloy closed coil spring with 150 g of force and found highest values at 21 days T2(2.433 ± 0.399 pg/ml) followed by 7th day T1($2.387 \pm .594$ pg/ml) and least was at baseline T0(2.32 ± 0.528 Pg/ml) just before starting canine retraction. However, difference was not statistically significant at any time interval.

Muhammad et al (2023)⁵¹, assessed IL-6 levels in GCF during relief of crowding of 2-4 mm in 50 patients (mean age 25 ± 4 years) using aligners and conventional labial appliances and found higher level of IL-6 with aligner at 3.79 ± 1.82 pg/ml at T0(baseline), 17.88 ± 1.52 pg/ml at T1(after 1 month), whereas in conventional labial appliances were 3.65 ± 1.21 pg/ml at T0(baseline) and 12.25 ± 1.26 pg/ml at T1(after 1 month). IL-6 showed significant difference from baseline to follow-up. Highest level of RANKL with conventional labial appliances 0.7 ± 0.1 pg/ml at T0(baseline), 1.9 ± 2 pg/ml at T1(after 1 month), whereas in aligners 0.6 ± 0.1 pg/ml at T0(baseline) and 1.3 ± 0.1 pg/ml at T1(after 1 month). A significant difference between conventional labial appliances and aligners was noted in both the biomarkers (IL-6 and RANKL).

J Amanda et al (2018)⁴⁸, found significant difference in RANKL level in patient with the age range of 15-35 years in GCF during early leveling and aligning phase using self-ligating and conventional preadjusted brackets at T0(before bracket insertion), T1(at 1hr), T2 (at 24 hr) and T3 (at 168 hours) after bracket insertion. In intergroup comparison for PEA bracket could be compared to results of present study. They found

trend of RANKL in this group as t0 t1 t2 t3 and found statistical significant difference between T1-T2, T2-T3.

Tuncer et al (2013)³⁹, evaluated RANKL level in GCF in 9 adults (age range 17.5-18.9 year) and found non-significant difference in levels of RANKL with time on retracting the maxillary canines by sentalloy closed coil springs with a continuous force of 200gm on the segmental 016x.022 S.S wire, four weeks after 1st premolar extraction. The mean value was highest at 7 days (1.29 ± 1.0 pmol/l) > at baseline (pre-treatment- 1.19 ± 0.71 pmol/l) > at 1st month ($1.04 \pm .50$ pmol/l) > then at 24hrs ($.96 \pm .55$ pmol/l) least at 1 hr ($0.83 \pm .30$ pmol/l).

Similar to present study, all of the above mentioned studies showed fluctuation of levels of biomarker like IL-6 and RANKL on application of orthodontic forces. However, the differences in results, regarding the difference at each time interval between groups or between each time interval in same group could be attributed to variations in methodologies, such as the timing, appliances (mechanotherapy), force directions, and magnitudes applied. Additionally, factors like age, sex, bone mineral density, anatomic structures, and cellular activity in the periodontal ligament (PDL) and alveolar bone of the individual may also play a role.

The major limitation of the present study may be linked to differences in the composition of the gingival crevicular fluid (GCF) or the biological response of an individual. Since GCF is sensitive to contamination from plaque and saliva, potential biases should be considered when interpreting the results. Other sources of cytokines, such as those from bone, gingivae, blood vessels, and external factors like plaque accumulation, may also influence the GCF and PDL microenvironments.

Despite of these limitations results of present study would help us in understanding Orthodontic tooth movement at molecular level. According to the blood flow theory of orthodontic tooth movement, the stimulus for cellular differentiation is generated by alterations in blood flow within the periodontal ligament (PDL). When a continuous force is applied, blood flow decreases in the compression site and increases in the tension site, creating changes in the chemical environment and leading to differences in cellular responses on compression site in comparison to tension site. We analyzed

biomarker level only on compression site, where decrease of blood flow must have influenced release of proinflammatory cytokine IL-6 and RANKL.

Disruption in blood flow on compression site leads to an arrest of tooth movement, which can last for 3 to 4 days depending on amount of force applied. Movement only resumes after the necrotic tissue is removed and cells for bone resorption are removed from adjacent marrow spaces occurs. On application of heavy forces, lag phase is longer and causes undermining resorption⁴¹.

On application of lighter forces, as used for Alignment and Leveling in present study, IL-6 levels must have started bone remodeling that was sustained by RANKL at later stages. Tightening of Laceback for each appointment must have gradually increase IL-6 levels after T1, with hyper levels at T2 in growing subjects and T3 in non-growing subjects.

The main clinical implication of the present study is to know magnitude of forces that are light and optimum for each individual based on assessment of biomarker levels at compression site and to understand influence of growth on fixed orthodontic treatment. As biomarker levels are relatively higher in growing subjects, hence, lighter forces of lower magnitude would be more appropriate in growing subjects than non-growing subjects.

Future scope of the present study included validating results on a larger sample size, by assessing biomarker levels in subjects undergoing growth modification treatment using myofunctional or orthopaedic appliances. Also use of different bracket systems, modification of time intervals for sample collection, use of different sizes of arch wires, assessment of other inflammatory biomarkers, use of different orthodontic mechanics, technological advancement in biomarker measurement could be done, both for growing and non-growing subjects.

By addressing these areas, future studies will provide more comprehensive insights into the biological responses during orthodontic treatment, ultimately helping to refine treatment protocols and improve patient outcomes.

Following conclusions are drawn from the present study with the aim to assess the level of biomarker in GCF during fixed orthodontic treatment in growing and non-growing subjects.

1. Application of mechanical stress during orthodontic treatment altered the level of biomarkers (IL-6 and RANKL) in both growing and non-growing subjects at different time intervals.

2. On intragroup comparison, mean level of **IL-6** in Group I was (T0>T2>T3>T1) whereas in Group II (T0>T3>T2>T1), however difference were statistically non-significant between different time intervals. Levels of IL-6 were attained earlier in growing subjects (T2>T3) than non-growing subjects, where levels at T3 was higher than T2 (T3>T2).

3. On intragroup comparison, mean level of **RANKL** in Group I was (T0>T1>T3>T2) whereas in Group II (T0>T1>T3>T2), however difference were statistically non-significant between different time intervals. Higher level of RANKL at T3 than T2 in both group suggested its role in sustaining osteoclastogenesis during orthodontic tooth movement.

4. Inter group comparison showed higher values of both the biomarkers at all time intervals for Group I than Group II at all intervals. Growth may additionally stimulate levels of biomarkers during orthodontic tooth movement, though the difference was observed statistically non-significant.

The main clinical implication of present study would be that, as biomarker levels during tooth movement were found to be increased in growing subjects as compared to non-growing subjects, hence in non-growing subjects, other non-surgical or surgical methods of accelerating tooth movement could be employed to increase biomarker levels at molecular level for faster rate of tooth movement.

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Metabolism on Salivary Matrix in the Remodeling of Periodontal Tissue during Orthodontic Treatment. Dent J (Basel). 2024;12(7):209.

Orthodontic treatment corrects skeletal and dental malrelationships, enhances facial aesthetics and establishes occlusal harmony and structural balance. The fixed orthodontic treatment is limited not only to children but adults are equally seeking orthodontic treatment for desirable aesthetic outcome. The mechanical forces exerted during fixed orthodontic treatment initiates a cascade of movements like initiation of acute inflammatory response that involves vasodilation of capillaries of PDL, release of inflammatory mediators/biomarker like IL-6, RANKL, PGE2, Cytokines, IL-1 β , Tumor necrosis factor (TNF)- α etc. In growing subjects, additional stimulation to orthodontic forces because of growth might result in variable response to fixed orthodontic treatment in comparison to non-growing subjects. Clinically, it is seen that orthodontic treatment is faster in growing individuals in comparison to adults, at molecular level, the reason could be that as vascularity is more and inflammatory system is more activated in growing children it reacts faster to local changes on application of mechanical force.

Biomarkers are substances that can be measured and evaluated objectively as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention.

In Orthodontics biomarkers have helped us in assessing puberty, bone turnover or understanding Orthodontic tooth movement at molecular level.

The Serum Biomarkers have limitations, including the need for invasive collection, risk of contamination, and potential anxiety and can be fluctuate due to inflammation in other body parts over time.

Similarly Salivary Biomarkers are present in low concentrations, requiring sensitive tests for detection and are influenced by various environmental and psychological factors.

To overcome these limitations of Serum and Salivary Biomarkers, GCF Biomarkers are preferred due to their proximity to periodontal tissues, minimizing systemic influences.

Various studies evaluated Biomarkers in Serum and Saliva to assess pubertal growth spurt and found difference in levels of Biomarker during growing phase of an individual.

The levels of various Biomarkers that bring about changes at molecular level have been assessed during various phases of orthodontic tooth movement in growing and non-growing subjects.

However not many studies have compared different types of biomarker level in growing and non-growing subjects.

Other studies compared level of different biomarker like IL-1 β , TNF- α , GM-CSF in growing and non-growing subjects.

Amongst various Biomarkers IL-6 released initially during Orthodontic tooth movement that is responsible for induction of process of Osteoclastogenesis was selected in present study. Also RANKL which is a potent biomarker that attaches to RANK to promote bone resorption would be evaluated in present study for both growing and non-growing children.

Considering this, the aim of present study was to assess Biomarkers (IL-6 and RANKL) levels in GCF during fixed Orthodontic treatment in growing and non-growing subjects.

The present clinical study was conducted in the Department of Orthodontics and Dentofacial Orthopaedics, Babu Banarasi Das College of Dental Sciences, BBDU Lucknow, in collaboration with department of Biochemistry, King George's Medical University Lucknow.

For present study, 30 subjects with Angle's Class 1 bi-maxillary protrusion or Class II division 1 malocclusion were selected, who had to undergo fixed orthodontic treatment in the department. The sample was divided into two groups: Group-I (15 growing subjects, age range 13.72+_1.27 years) and Group-II (15 non-growing subjects, age range 20.36+_4.64 years). The groups were categorized based on CVMI staging by Hassel and Farman.

Pre-treatment records, including lateral cephalogram, orthopantomogram, study models and photographs were taken and skeletal maturation was assessed based on CVMI staging by Hassel and Farman. Subjects with stages 3,4,5 were categorized as growing (Group I), while those at stage 6 were classified as non-growing (Group II).

Fixed orthodontic treatment was started using MBT brackets following standard protocol. Alignment and leveling were initiated using preformed Ni-Ti arch wires (0.012/0.014/0.016) as per case requirement with lace backs from canine to hook on tubes of maxillary first molar and arch wires were secured using elastomeric modules. The GCF samples were collected at T0 (at baseline) i.e before placing initial alignment wire, T1 (at 24 hours), T2 (at 3 week), T3 (at 8 week) following standard protocol and using absorbent paper points.

GCF samples were collected from the distal site of the maxillary canine at selected time intervals. After GCF collection, paper points were placed in Eppendorf tubes with 200 µl phosphate buffer saline and transported to Biochemistry lab for final storage at -80° until further analysis.

After collection, frozen GCF samples were thawed at room temperature and centrifuged. The paper points were removed and the supernatants were transferred to new Eppendorf tubes and kept in an incubator.

After elution, GCF samples were analyzed for IL-6 and RANKL levels. The absorbance of each sample was read at 450 nm using an ELISA analyser after comparing with standard curve as obtained with kit in pg/ml. Data was tabulated and adequate statistical comparison were made.

Following conclusions are drawn from the present study with the aim to assess the level of biomarker in GCF during fixed orthodontic treatment in growing and non-growing subjects.

1.Application of mechanical stress during orthodontic treatment altered the level of biomarkers (IL-6 and RANKL) in both growing and non-growing subjects at different time intervals.

2.On intragroup comparison, mean level of **IL-6** in Group I was (T0>T2>T3>T1) whereas in Group II (T0>T3>T2>T1), however difference were statistically non-significant between different time intervals. Levels of IL-6 were attained earlier in growing subjects (T2>T3) than non-growing subjects, where levels at T3 was higher than T2 (T3>T2).

3. On intragroup comparison, mean level of **RANKL** in Group I was ($T_0 > T_1 > T_3 > T_2$) whereas in Group II ($T_0 > T_1 > T_3 > T_2$), however difference were statistically non-significant between different time intervals. Higher level of RANKL at T_3 than T_2 in both group suggested its role in sustaining osteoclastogenesis during orthodontic tooth movement.

4. Inter group comparison showed higher values of both the biomarkers at all time intervals for Group I than Group II at all intervals. Growth may additionally stimulate levels of biomarkers during orthodontic tooth movement, though the difference was observed statistically non-significant.

The main clinical implication of present study would be that, as biomarker levels during tooth movement were found to be increased in growing subjects as compared to non-growing subjects, hence in non-growing subjects, other non-surgical or surgical methods of accelerating tooth movement could be employed to increase biomarker levels at molecular level for faster rate of tooth movement.

ANNEXURE-I

BABU BANARASI DAS UNIVERSITY
BBD City, Faizabad Road, Lucknow – 226028 (INDIA)

Dr. Lakshmi Bala

Professor and Head, Deptt of Biochemistry and
Member-Secretary, Institutional Ethics Committee (IEC) of BBD University, Lucknow

Communication of the Decision of the VIIIth Institutional Ethics Committee Meeting

IEC Code: 32

BBDU/MDS/32/2024

Date: 07/03/2024

Title of the Project: Assessment of Biomarker Levels In GCF During Fixed Orthodontic Treatment In Growing And Non-Growing Subjects.

Principal Investigator: Dr. ASHIM DEBNATH

Department: Department of Orthodontics
and Dentofacial Orthopaedics

Name and Address of the Institution: BBD University, Lucknow

Type of Submission: New, MDS Dissertation proposal

Dear Dr. Ashim Debnath

The meeting of the Institutional Ethics Committee (IEC) was held on 06-02-2024 in Conference room, First Floor, BBDCODS, BBD University, Lucknow. Following members were present:

1	Dr. Chandishwar Nath Rtd. Chief Scientist, CDRI, Lucknow.	Chairman
2	Dr. JS Srivastava, Rtd. Chief Scientist, CDRI, Lucknow.	Member
3	Dr. Manodeep Sen, Professor, Department of Microbiology, RMLIMS, Lucknow	Member
4	Dr. Shaleen Chandra, Professor & Dean, Dental Sciences, Atal Bihari Vajpai Medical University (ABVM University), Lucknow	Member
5	Dr. Manuka Khanna, Professor, Deptt. of Political Science, Lucknow University, Lucknow	Member
6	Mr Abhishek Chaudhary, Advocate, Lucknow	Member
7	Dr. Puneet Ahuja, Professor of Oral Pathology and Principal, BBD College of Dental Sciences, BBD University, Lucknow	Member
8	Dr. Lakshmi Bala, Professor & Head, Department of Biochemistry, BBD College of Dental Sciences, BBD University, Lucknow	Member-Secretary

The committee reviewed and discussed your submitted documents of the research proposal in the meeting.

Following comments were suggested and communicated.

Comments:

1. To enclose simple translation of child information document in Hindi.
2. The specific method of obtaining GCF at different time intervals along with the time interval of visits of patients should be mentioned in PID.

Thereafter, the proposal was revised by Principal Investigator and duly approved by the Supervisor and Head of the Department.

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

Lakshmi Bala
07/03/24
(Dr. Lakshmi Bala)
Member-Secretary IEC
BBD University
Ethics Cell (Vth Floor, BBDCODS)
ethics@bbdu.ac.in

Dr. LAKSHMI BALA
Member-Secretary
Institutional Ethics Committee
BBD University, Lucknow-28

ANNEXURE-II

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

1. Title of the project: **ASSESSMENT OF BIOMARKER LEVELS IN GCF DURING FIXED ORTHODONTIC TREATMENT IN GROWING AND NON-GROWING SUBJECTS.**

2. Name of the department/ address of the investigator: **Department of orthodontics and dentofacial orthopaedics.**

3. Name of Faculty (Guide/Co-Guide) with designation and department: **GUIDE -Dr. Kamna Srivastava (Reader) Department of Orthodontics ,Co-Guide- prof.Dr.Tripti Tikku,- Department of orthodontics and dentofacial orthopaedics, Co-guide-Dr.Kalpna Singh, Prof.&Head -Department of biochemistry ,KGMU ,Lucknow.**

4. Date of approval by Institutional Research Committee (IRC) (PI enclose approval letter along with finally approved research proposal):

5. Sources of funding: Nil

6. Study related information:

(i) Place of Study:
(a) BBDU-BBDCODS
(b) Other Institution:-Biochemistry department of KGMU, Lucknow

Kindly attached Consent letter from the concerned faculty/clinician of other Institution.

(ii) *In-vitro studies on human subjects:* Please specify if it is body fluid blood/tissues/ teeth.
(a) Bile, Saliva etc.
(b) Teeth, please specify type
(c) Tissue, please specify type
(d) Use of stored or left over specimens
(e) Any other

(iii) *In-vivo study on human subjects:*
(a) Intervention
(b) Drugs
(c) Implants
(d) Any other e.g. X-rays/Ultrasound/etc-GCF

(iv) Vulnerable subjects:
(a) Pregnant Woman
(b) Elderly
(c) Terminally ill
(d) Physically/ mentally challenged
(e) Children under 18
(f) Students
(g) Orphans

(v) Survey of human subject:
(a) Verbal questionnaire
(b) Non- invasive examination
(c) Invasive procedures

(vi) SEA (Severe Adverse Events) reporting:

7. Ethical issues involved in the study:
Less than minimal risk/ minimal risk/ more than minimal risk to the study subjects (for guidance please consult ICMR guidelines 2006)-Less than Minimal risk

8. Do you need exemption from obtaining Informed Consent from study subject – if no give justifications - **NO**
In following cases exemption can be requested:
a. Audits of educational practices.
b. Research on microbes cultured in the laboratory.
c. Research on immortalized cell lines.
d. Computer Simulation and Dental Materials.
e. Analysis of data freely available in public domain.
f. Any other.

9. Whether Consent forms and Participant Information Document in English and in Hindi are enclosed? **YES**

10. Conflict of interest for any other investigator(s) (if yes, please explain in brief)-**NO**

11. We the undersigned, have read and understood this protocol and hereby agree to conduct the study in accordance with this protocol and to comply with all requirements of the ICMR guidelines (2006)

Signature of the Investigator: _____ Date: _____

Signature of the Guide: Dr. Kamna srivastava _____ Date 28-12-23
Reader, Department of Orthodontics
BBDCODS,BBDU

Co-Guide : prof.Dr. Tripti Tikku _____ Date 28-12-23
Department of Orthodontics
BBDCODS,BBDU

Co-Guide : Prof.Dr. Kalpna Singh _____ Date _____
Prof. & Head, Department of Biochemistry
KGMU ,Lucknow

Signature of the Head of the Department: Prof.Dr Rohit Khanna _____ Date 27-12-23

(Note: The investigator must provide information to the subjects in a simple language, and it should address the subjects, in a dialogue format)

इसमें भाग लेना है या नहीं के यह तनणषय लेना आपके ऊपर है। यतद आप भाग लेने का तनणषय लेते हैं कि आपको यह सूचना पत्रक देने के लिए तदया जाएगा और एक सहमति प्रपत्र पर हस्ताक्षर करने के लिए कहा जाएगा। यतद आप भाग लेने का तनणषय लेते हैं कि आप तकसी भी समय तबना क ई कारण बिए आपसे लेने के लिए स्वीकृत हैं।

6. मुझे क्हा जाएगा यतद मैं इस अध्ययन में भाग लेता हूँ।

आपका नुकसान नहीं होगा। तनति ओर्थोडोंटिक उपचार के लिए आपको हर महीने आना होगा उस अतिथि के दौरान अध्ययन पूरा हो जाएगा।

7. मुझे क्या करना है?

आपको दी गई तनयुक्ति पर आना होगा और ऑर्थोडोंटिक उपचार के अपने पाठ्यक्रम का पालन करना होगा।

8. तकस प्रतक्रया का अध्ययन तकया जा रहा है?

ऑर्थोडोंटिक दंतों में लेने के दौरान बाय मक्सर के स्तर के लिए आपसे एकत्र तकए गए जीसीएफ नमूने का प्रयोग शांति में विश्लेषण तकया जाएगा।

9. इस शोध में कौन से हस्तक्षेप तकए जाएंगे?

हस्तक्षेप अध्ययन नहीं होगा।

10. इस अध्ययन में भाग लेने के क्या दुष्प्रभावि हैं ?

शोध प्रतक्रया का क ई दुष्प्रभाव नहीं है, क्योंकि हम सामान्य ऑर्थोडोंटिक उपचार प्रतक्रया का पालन कर रहे हैं

11. इस अध्ययन में भाग लेने के संभावित निमित्त और नुकसान

क्या है? क ई निमित्त नहीं होगा।

12. अध्ययन में भाग लेने के सौभाग्य का क्या है?

अध्ययन में भाग लेने से रोगी/स्वयंसेवक को कई तत्कालीन लाभ नहीं मिलेंगे, यह स्पष्ट रूप से बताया जाएगा।

13. क्या होगा यदि कोई जानकारी उपलब्ध हो जायेगी ?

कभी-कभी एक शोध परियोजना के दौरान, अध्ययन तकिए जा रहे शोध के बारे में नई जानकारी उपलब्ध हो जायेगी है। यदि ऐसा होगा है, तो आपका शोधकर्ता आपको इसके बारे में बताएगा और आपसे चर्चा करेगा कि क्या आप अध्ययन जारी रखना चाहते हैं। यदि आप

विपक्ष लेने का तय कर लेंगे हैं, तो आपका शोधकर्ता/अनुवर्क आपकी तनकासी को स्थिर करेगा। यदि आप अध्ययन जारी रखने का तय कर लेंगे हैं, तो आपको एक अग्रिम सहमति फॉर्म पर हस्ताक्षर करने के लिए कहा जा सकता है।

14. क्या होगा जब अध्ययन / शोध परीक्षण बंद हो जायेगा ?

यदि शोध अध्ययन समाप्त/बंद हो जायेगा है तो आपके ऑर्थोडॉन्टिक उपचार के कार्यक्रम पर कुछ भी असर नहीं पड़ेगा।

15. क्या होगा अगर कुछ गलत हो जायेगा ?

चूंकि यह गैर-आक्रामक प्रक्रिया है जहाँ जीसीएफ के बिना आपसे एकत्र किया जायेगा है, इसलिए कुछ भी गलत नहीं हो सकता है, हालांकि तब तक भी समस्या के मामले में सौस्थान या आईईसी से संपर्क करें।

16. क्या इस अध्ययन में मेरा सहसागनीयता जाएगा?

हो।

17. अध्ययन / शोध परीक्षण के परिणाम का क्या होगा?

परिणामों का उपयोग बढ़ी और गैर-बढ़ी दंत चिकित्सा के दोनों की गति की दर को ठीक करना करने के लिए किया जाएगा।

18. इस अध्ययन क कौन आय तजि कर रहा है और इस परीक्षणकेलिए धन कहाँ से आएगा।

यह शोध अध्ययन शैक्षणिक संस्थान (बीबीडीसीओडीएस) द्वारा आय तजि तकया जाँ है।

19. क्या संशोधन हमें जानेकेबाद उपस्थित रहेगी या नहीं?।

हों।

20. अध्ययन की समीक्षा तकसने की है?

अध्ययन की समीक्षा की गई है और तिभागकेप्रमुख, और आईईसी/आईआरसीकेद्वारा अनुमति दी तकया गया है। तनम्र िंग केसे संशोधन करें

21. अतधक जानकारीकेलिए संशोधन करें।

डॉ आतशम देवनार्थ

ऑर्थोडोंटिक्स और डेंटिफेतशिय ऑर्थोपेडिक्स तिभाग

बाबू बनारसी कॉलेज ऑफ डेंटिफेतशिय।

फ़ोन-226028-

म ब- 7895833847

डॉ कामना श्रीवास्ति (पाठक)

ऑर्थोडोंटिक्स और डेंटिफेतशिय ऑर्थोपेडिक्स तिभाग

बाबू बनारसी कॉलेज ऑफ डेंटिफेतशिय।

फ़ोन-226028

म ब-9956099502

डॉ. तृनिनिक्कू (प्रोफेसर)

ऑर्थोडोन्टिक्स और डेंटि फेतशियि ऑर्थोपेटडक्स तिभाग

बाबू बनारसी कॉलेज ऑफ

डेंटि साइंसेज। िनिऊ-

227105

म ब-9554832799

डॉ. कल्पना (एच

डी) जैरिसायनति

भगकेजीएमयूम ब-

9532155750

डॉ. शोनिनु त्री (एचओडी)

ऑर्थोडोन्टिक्स और डेंटि फेतशियि ऑर्थोपेटडक्स

तिभाग बाबू बनारसी कॉलेज ऑफ डेंटि

साइंसेज।

िनिऊ-226028

म ब-9936424084

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पीआईकाहस्ताक्षर

नाम

तदनॉक.....

ANNEXURE- IV

To,
Prof. Dr. Kalpana Singh
KGMU
Lucknow.

Subject: Request to be Co-guide for thesis.

Respected Ma'am,

I, Dr Ashim Debnath, MDS student from the Department of Orthodontics and Dentofacial Orthopaedics BBD, is planning to conduct a study titled **"Assessment of Biomarker levels in GCF during fixed orthodontic treatment in both growing and non-growing subjects"**, for which collaboration from your department is needed.

I wish and request you to kindly be my Co-guide for the above study.

I will be highly obliged for the same.

Thanking you,

Yours faithfully,
Dr. Ashim Debnath
MDS 1st Year,
Department of Orthodontics and Dentofacial Orthopaedics,
BBDCODS, Lucknow.

for Dr. Ashim
HOD
Dept. of Orthodontics & Dentofacial Orthopaedics
B.B.D. College of Dental Sciences
Lucknow

Kalpana Singh
Dr. Kalpana Singh
Professor & Head
Department of Biochemistry
KGMU Lucknow

I, Prof. Dr. Kalpana Singh (H.O.D - Biochemistry) KGMU Lucknow giving consent to be the Co- guide for the study titled **"Assessment of Biomarker levels in GCF during fixed orthodontic treatment in both growing and non-growing subjects"** which will be conducted by Dr. Ashim Debnath, MDS student, from the Department of Orthodontics and dentofacial Orthopaedics BBDCODS, Lucknow.

Signature

Date

Kalpana Singh
Prof. Dr. Kalpana Singh
(HOD-Biochemistry)
KGMU, LUCKNOW
Dr. Kalpana Singh
Professor & Head
Department of Biochemistry
KGMU Medical University U.P.
Lucknow

ANNEXURE-V

CHILD SATISFACTION FORM

NAME-

AGE/SEX-

TOOTH NUMBER-

How satisfied are you with our work?



Very dissatisfied



Dissatisfied



Neutral



Satisfied



Very Satisfied

ANNEXURE- VI

Ashim Debneth Thesis

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Ren Y, Amila V, Alikhani M, Kapoor P, Hussain.M, Krishnan V, Grant M, Chelarescu S, "Biomarkers in Body Fluids as indicators of Skeletal Maturity", Rambam Maimonides Med J, 2021

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