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Age-associated changes in salivary biomarkers and periodontal ligament fibroblast senescence in vitro

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BOSTON UNIVERSITY
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DISSERTATION

**AGE-ASSOCIATED CHANGES IN SALIVARY BIOMARKERS AND
PERIODONTAL LIGAMENT FIBROBLAST SENESENCE IN *VITRO***

by

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BDS, KING SAUD UNIVERSITY, 2010

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DEDICATION

I dedicate this work to my first role models and lifelong heroes; my dear parents,

Fatimah and Ahmed,

To my biggest blessings from God; my dear brothers and sisters

To my sweet nephews and nieces

To my amazing friends

I would never have succeeded without your love and prayers.

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PERIODONTAL LIGAMENT FIBROBLAST SENESENCE IN *VITRO*

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ABSTRACT

OBJECTIVES:

Aging is characterized by a progressive loss of function and presents a primary risk factor for major human pathologies. There is a positive correlation between aging and periodontal destruction. Saliva is a complex fluid that contains numerous biological markers and has an important diagnostic value. D-Galactose has been used to induce senescence in multiple cell types. The effect of D-Galactose on human periodontal ligament fibroblasts (hPDLF) has not been demonstrated. The first part of this study investigated the impact of age on a wide range of salivary biomarkers of inflammation and tissue turnover. The second part of this study investigated the induced senescence effect of D-Galactose on hPDLF.

METHODS:

Part I included 74 healthy subjects undergoing orthodontic treatment. Whole saliva samples were collected prior to starting orthodontic treatment and were studied by multiplex immunoassay for several inflammatory and tissue turnover markers. For part II, hPDLF were cultured and passaged. At p3, cells were assigned to control and test groups. The test group received D-Galactose-supplemented media in different

concentrations. Cells were incubated for 24 and 48 hrs. (SA- β -Gal) staining, Western blotting and supernatant analysis were done to investigate senescence-associated changes in hPDLF.

RESULTS:

For part I, the final number of participants included 30 males and 44 females, with the age range 8-63 years old. Subjects were categorized into quartiles (<13 y.o., 13-16 y.o., 17-27 y.o., >27 y.o.). Significant difference among the four age groups was found in BMP-4, fibronectin, VEGF, IGFBP-2, IGFBP-4, IGFBP-6, IGFBP-7, IL-1 β , lactoferrin, MMP-2 ($p < 0.05$). In most of these analyses, the young adults in the third quartile (17-27 y.o.) were significantly different from others ($p < 0.05$). For part II, D-Galactose treated cells showed a dose-dependent increased expression of (SA- β -Gal), higher expression of pMAPK and decreased expression of fibronectin ($p < 0.05$).

CONCLUSIONS:

From part I, the data suggested that salivary biomarkers of inflammation and tissue turnover showed significant variation in a systemically and periodontally healthy group of individuals when different age groups were compared. From part II, the data suggested that D-Galactose induced hPDLF to express senescence-related changes including higher expression of SA- β -Gal and increased pMAPK activity.

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LIST OF ABBREVIATIONS

AGEs.....	Advanced Glycation End-products
β -Actin	Beta Actin
BMP-2.....	Bone Morphogenic Protein-2
D-Gal.....	D-Galactose
FN	Fibronectin
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IL-1 β	Interleukin-1 Beta
IQ	Inter-quartile range
LF.....	Lactoferrin
MAPK.....	Mitogen-activated Protein Kinase
MMP	Matrix Metalloprotease
NADPH.....	Nicotinamide Adenine Dinucleotide Phosphate
NFKB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
PI3k.....	Phosphoinositide 3-Kinases
Q.....	Quartile
RAGE.....	Receptor for advanced glycation End-products
SA- β -Gal.....	Senescence Associated Beta Galactosidase
SD	Standard Deviation
TNF- α	Tumor Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth Factor

PART I: AGE-ASSOCIATED CHANGES IN SALIVARY BIOMARKERS

INTRODUCTION

Aging and its Biological Consequences

Aging is a biological process characterized by a gradual decline in the physiological function of the living organism (Brosel et al., 2018; François et al., 2018). Aging is known to be a predisposing factor for multiple diseases such as Diabetes, Alzheimer's diseases and certain types of cancers (López-Otín et al., 2013; Field et al., 2018). There is an overall deterioration of physical and cognitive ability with aging (Ambrose, 2015).

The population of elderly is increasing globally (Sander et al., 2015). Due to the universal trend of increased longevity and reduced fertility levels, there is an expected increase in the number of people 65 years and older, along with a decrease in the number of people 25 years old and younger. In 2019, the elderly (>65 years old) form 11% of the total world population and are expected to increase to 16% by 2050. Higher numbers are expected for Europe and Northern America, with elderly population size increasing from 18% by 2019 to be 25% of the total population by 2050 (United Nations, 2009).

Multiple diseases are associated with advancing in age (Jaul et al., 2017). Due to numerous functional and structural changes in the body, older individuals become more susceptible to degenerative diseases. For example, physiological aging is associated with loss of elasticity of blood vessels (J. C. Wang et al., 2012); which in turn, increases the risk of vascular diseases such as atherosclerosis, cardiovascular diseases and hypertension (J. C. Wang et al., 2012; Jaul et al., 2017). Muscle formation and fat metabolism also show a marked decline with aging, leading to reduced muscular strength, muscle mass and increased fat deposition (A. B. Newman et al., 2006). With

chronic progression, this could lead to sarcopenia, physical disability and overall increased morbidity among elderly (Xue et al., 2010; Dodds et al., 2017; Jaul et al., 2017). With aging, there is a change in insulin regulation by a decrease in function and increase in tissue resistance (Klötting et al., 2005). Along with the decline in muscle mass and increase in fat storage, metabolic capacity decreases (Jaul et al., 2017). These factors contribute to the increased prevalence of diabetes mellitus and metabolic diseases in the elderly (Fournet et al., 2018). Prevalence of diabetes among American older adults is expected to increase more than 400% by 2050 (Kirkman et al., 2012).

Osteopenia is the normal loss of bone density with aging (Jaul et al., 2017). Many elderly people develop osteoporosis, a more severe form of bone weakening and decline in bone density (Jaul et al., 2017). Unlike osteopenia, osteoporosis is associated with an increased rate of bone fractures among the elderly, especially above 85 years old (Jaul et al., 2017). In terms of cognitive function, short-term memory loss, sentence-forming abilities and ability to understand complicated concepts have been associated with normal aging while some cognitive functions were found to be increased with normal aging such as wisdom, knowledge, altruism and empathy (Blazer et al., 2015; Rosen et al., 2016; Jaul et al., 2017).

Another effect of aging on tissue is the decline in immune system function. This decline leads to increased susceptibility to infections and autoimmune diseases among elderly and increased severity of disease complications along with a decreased capacity of healing (Zapata et al., 2014; Bandaranayake et al., 2016; Jaul et al., 2017).

Aging studies are getting an increased attention in the scientific community (Hosokawa et al., 1997; Jylhävä et al., 2017; Brosel et al., 2018; Jiang et al., 2019). Multiple questions arise about the changes occurring in tissues with age; what could be the origin of these changes, when do they start to happen, and what causes them to

happen earlier and more evidently in certain subjects than others? On the tissue and cellular levels, several theories tried to explain how aging occurs and what are the main hallmarks of cell aging, including:

- **Genomic Damage of DNA:**

With advancing age, multiple forms of DNA damage occur to the cells (Moskalev et al., 2013). This damage could occur from an exposure or multiple exposures to a variable range of physical, chemical and oncogenic insults that could occur throughout the cell's lifetime. This damage is usually resolved with self-corrective mechanisms; however, as cells age, their capacity to resolve DNA damage reduces (Lavdaniti, 2009). Some of this damage remains unresolved and accumulates leading to an overall deterioration and reduced function of the cells (Norwood et al., 1996; López-Otín et al., 2013).

- **Telomere shortening:**

Telomeres are the terminal ends of DNA strands. They function as protective caps to chromosomes, where DNA genetic material resides (Blackburn et al., 2006). With every cell division, a part of the DNA telomere is lost. When telomeres shorten to a certain level that creates a risk to chromosomal integrity, cell division decreases as a part of the protective mechanism in the cell (de Magalhães et al., 2018). Cell division and replication continue to decline until the cells ultimately stop dividing and go into a stage of "cellular senescence" (Tigges et al., 2014).

Cellular senescence:

Cellular senescence is a phenomenon that describes several changes that occur to cells as they "age", and mainly characterized by their lack of ability to divide (Crews, 1993; Cristofalo et al., 1996). Cell senescence is a normal fate of somatic mortal cells. Cells can stop dividing due to multiple reasons, most important ones include: reaching

the "Hayflick limit" and telomere shortening as previously mentioned. Hayflick limit is the number of replications somatic cells can reach before they stop dividing and enter into senescence (Hayflick et al., 1961). This number varies depending on cell type. Only certain types of cells have long telomeres making them capable of continuously replicating. This includes stem cells, as a part of their regenerative capacity (Fathi et al., 2019) and cancer cells, contributing to their "immortal" features (Jafri et al., 2016). Along with short telomeres and lack of proliferation capacity, senescent cells also show a change in morphology; cells appear bigger and flatter with hyperchromatic large nuclei (Marmary et al., 2016). Cell function decreases and cells start releasing inflammatory and tissue-damaging cytokines as part of their senescence-associated secretory phenotype. As senescent cells accumulate in the body, their pro-inflammatory effect expands to adjacent cells leading to overall tissue frailty and vulnerability to disease (J. P. Coppé et al., 2008; Young et al., 2009; Özcan et al., 2016; Noren Hooten et al., 2017; Eustachio, 2019).

- **Decreased mitochondrial function:**

Mitochondria are the main organelles responsible for cellular respiration and energy generation. With advancing in age, mitochondrial function diminishes. It is thought that with aging, mitochondria dysfunction causes increased production of reactive oxygen species (ROS); which in turn, affects the mitochondrial function and cause a deteriorative effect on the whole cell via oxidative damage (Harman, 1965; Freitas-Rodríguez et al., 2017). Irrespective of damage from oxidative free radicals, mitochondria has its own DNA which is susceptible to age-related DNA damage as well (Malik et al., 2013). This contributes to further dysfunction of the mitochondria, leading to cell aging and reduced life span (Malik et al., 2013).

- **Stem cell exhaustion:**

Aging is marked by a decrease in tissue repair and regeneration capacity, which is the main function of stem cells (López-Otín et al., 2013). There is a global decrease in stem cell proliferation and function with age. Similar to other cell types, stem cells face changes in their DNA and intercellular signaling mechanisms, which affect their proliferation and ability to function. This leads to a marked decline in the body's potential for maintenance and repair (Bonab et al., 2006; López-Otín et al., 2013; Fathi et al., 2019).

- **Epigenetic changes:**

This term refers to alterations happening to a DNA strand without changing its the nuclear base sequence (Bocklandt et al., 2011). Epigenetic changes occur to cells throughout their lifetime, including DNA methylation. In DNA hyper-methylation, a methyl group is attached to one carbon atom on the DNA causing that area (gene) to be silent. It has been reported that aging is accompanied by complex changes in DNA methylation. These changes are thought to affect gene transcription patterns, contributing to alterations in cell function and ultimately affecting life span (Crews, 1993; Bocklandt et al., 2011; Horvath, 2013).

- **Inflammation and immune system dysfunction:**

There is a strong link between inflammation and aging (López-Otín et al., 2013). The accumulation of senescent cells with age acts as a source of inflammation markers, leaving the tissues in a state of low-grade chronic inflammation. This process is thought to provide the basis to multiple age-related pathologies such as atherosclerosis, diabetes, osteoporosis, arthritis, cancer and neurodegenerative diseases (de Gonzalo-Calvo et al., 2010).

In parallel, aging is associated with multiple changes in the immune system, often referred to as Immunosenescence (Olivieri et al., 2018). There is a marked decline in B cell function, dysregulation of T cell generation and function, and decline in the body's innate immunity (López-Otín et al., 2013). These changes make the body more susceptible to different bacterial, viral and fungal infections (Gould et al., 2015). Another source of age-related inflammation -Inflammaging- is the imbalance of reactive oxygen free radicals in the body. As previously mentioned, oxidative damage is expected to increase with age, along with a reduction in the scavenging system by the cells. This causes a trigger to further inflammation in the tissue and affects the cells major functions and capacity for maintenance, such as wound healing, inflammatory resolution and tissue repair (López-Otín et al., 2013; Jaul et al., 2017; Olivieri et al., 2018).

Recent studies have discussed the concept of "biological age", and how aging could occur differently among people from the same chronological age depending on their individual differences (Crews, 1993; Bocklandt et al., 2011; Kanasi et al., 2016). There is a general correlation between chronological age and the underlying aging mechanism happening in the body. However, the functional deterioration could occur earlier in some subjects compared to others in their age range, dependent on their genetics, biological characteristics, environmental and even psychological conditions (Horvath, 2013; McHugh et al., 2018; Ridout, Parade, et al., 2019).

The importance of studying aging is that aging is a process that precedes and forms a risk factor to multiple degenerative conditions as we advance in age. The underlying mechanisms of aging are shared among the most prevalent chronic diseases such as inflammation and oxidative damage. Studying aging and its underlying processes can help us understand how to intervene and prevent such deterioration from

occurring. This can provide space for drug delivery research and treatment applications. Research has targeted multiple forms of biological environments to further understand the process of aging, including animal and *in vitro* cell models (Kiyoshima et al., 2012; Laberge et al., 2015; Shwe et al., 2018; Waaijer et al., 2018). It also focused on studying human biological samples for age-related markers, such as samples isolated from tissue biopsies and swabs, in addition to body fluids like: serum, blood, urine and saliva (H. L. Lee et al., 2008; Vanhooren et al., 2008; M. Nassar et al., 2014; Moreno-Villanueva et al., 2015; Ji et al., 2017).

According to current predictions of world population demographics, there will be an increase in number of elderly (United Nations, 2009). The patient pool for dental and orthodontic treatment will expand to include older adults. Thorough understanding of the biological changes with aging is essential. It is important to help in diagnosis, treatment planning, prognosis and prevention of age-related complications

Saliva and its Value as a Diagnostic Biological Fluid

Saliva is composed of water, mucus, electrolytes, enzymes, immunoglobulins, leukocytes, and buccal epithelial cells (Bahar et al., 2007; Tiwari, 2011). It originates from salivary glands and circulates in the oral cavity participating in hydration, lubrication and masticatory functions (Tiwari, 2011). Saliva has gained a substantial interest as a diagnostic measure (Fleissig et al., 2010; Goodson et al., 2014). Some of its advantages over other body fluids as a diagnostic platform include the easy availability, relatively high stability, reduced risk of cross-contamination and non-invasive, inexpensive methods of acquisition and storage (Bahar et al., 2007; Tóthová et al., 2015; Nam et al., 2019). Saliva is shown to provide an alternative to blood, as the collection process is painless, suitable for subjects from different ages and health conditions, and does not require high caliber skills or equipment (Bahar et al., 2007; Tóthová et al., 2015). Saliva testing has increased in the field of forensic as it helps in the identification of subjects in crime scenes (Martí-Álamo et al., 2012; S. R. Hong et al., 2017).

Saliva is a complex fluid that contains both local and systemic sources of biological markers. Whole saliva consists of secretions from different salivary glands, mucosal and periodontal tissue, epithelial cells, microorganism, gingival crevicular fluid, plaque and food debris (Martí-Álamo et al., 2012). Saliva contains molecules that transfer from the bloodstream through ultrafiltration, diffusion, and active transport (Y. H. Lee et al., 2009; Pfaffe et al., 2011; Nam et al., 2019).

Salivary components can be affected by local inflammation of the oral tissues. Reports have shown that poor oral hygiene, gingivitis and periodontal disease can cause higher inflammatory marker levels in saliva. Some reports mentioned significantly higher levels of cytokines in saliva compared to blood due to the pool of these markers

from both the bloodstream and local oral tissues (Miller et al., 2010; Rahnama et al., 2013; Slavish et al., 2015). Other reports mentioned variable correlation between saliva and blood markers of inflammation (Dillon et al., 2010; Riis et al., 2014). This variability might exist as some molecules do not pass from bloodstream to saliva due to their molecular weight (Bosch, 2014). Another reason might be individual factors; such as oral tissue integrity and permeability and the presence of local wounds and tissue damage. These variables might ultimately affect saliva composition (Bosch, 2014; Slavish et al., 2015).

Similar to other body fluids, salivary analysis serves two main vital functions: it can help diagnose the presence of specific diseases, and monitor the current status and progress of treatment. It has been reported to largely contribute in the investigation of viral and bacterial infections, autoimmune diseases, endocrine pathologies and cancers such as oral squamous cell carcinoma (J. Y. Wu et al., 2010; Medina et al., 2010; Hu et al., 2011; M. Nassar et al., 2014)

Saliva shows changes in quantity and quality with advancing age (Xu et al., 2019). Age-related changes that can be detected in saliva can be summarized as follows:

- **Decreased salivary flow rate:**

Like other body organs, salivary glands go through physiological changes with age. Decreased flow rate is most evident in the submandibular salivary gland as the secretory tissue ability decreases with age, while parotid salivary glands show less change in function and anatomy with age (M. Nassar et al., 2014). This finding supports the reported increased risk of xerostomia, periodontal disease and oral infections in the elderly (W. I. Chang et al., 2011; François et al., 2018).

- **Decreased mucin content with aging:**

Mucin is an important protein released mainly by minor salivary glands. It gives saliva a mucous form and participates in the lubrication and anti-microbial protection of the oral cavity (Tabak et al., 1982). It has been reported that epithelial cells isolated from saliva showed a reduced mucin expression in older compared to younger subjects (W. I. Chang et al., 2011).

- **Senescence-associated telomere shortening:**

Aging is associated with an increase in DNA telomere shortening, which, as mentioned, relates to the decreased replicative ability in cells with age (Tigges et al., 2014). Multiple reports discussed the correlation of salivary telomere length with aging, stress and stress-related diseases (Lahnert, 2005; Lapham et al., 2015; Ridout, Ridout, et al., 2019). Some studies reported that salivary cells showed telomere shortening increase with aging (Lapham et al., 2015), while other papers indicated no significant difference was found in salivary telomere length between young and old individuals (Brown et al., 2018; François et al., 2018).

- **Increased tissue degradation markers:**

Matrix metalloproteases are a group of enzymes that degrade cellular and extracellular proteins. They are a part of the senescence-associated secretory phenotype. They work by degrading fibrotic plaques in the extracellular matrix and help in tissue remodeling (Bäck et al., 2007; Serban et al., 2016; Freitas-Rodríguez et al., 2017). These markers are increased with aging and age-related diseases, particularly MMP's 1, 2, 13 (Kim et al., 2016; Özcan et al., 2016; McHugh et al., 2018). Similar to MMPs, cathepsins are a group of proteases that are involved in tissue destructions and turnover (Mathews et al., 2016). Cathepsin serum levels have also been found to be increased with advancing in age (Goseki et al., 1996).

- **Changes in inflammatory cytokine levels:**

Inflammatory cytokines are signaling protein secreted by immune system cells and other cell types in response to stimuli to regulate the process of inflammation in the body (Zheng et al., 2009; de Gonzalo-Calvo et al., 2010). As aging is characterized by a generalized increase in inflammatory activity, multiple studies reported changes in levels of these circulating cytokines in elderly compared to young individuals, particularly: IL-1 β , IL-6 and TNF- α (Wei et al., 1992; Ershler, 1993; Maggio et al., 2006).

Interleukin-1 beta (IL-1 β) and Tumor necrosis alpha (TNF- α) are among the earliest signaling molecules activated in the inflammatory process (de Gonzalo-Calvo et al., 2010). They stimulate the release of Interleukin-6 (IL-6), which when increased, works on decreasing the production of IL-1 β and TNF- α , therefore having a dual function as a pro-inflammatory and anti-inflammatory cytokine (Morley et al., 2004; de Gonzalo-Calvo et al., 2010).

These markers play an important role in regulating bone turnover, muscle degeneration, and overall tissue inflammation (Morley et al., 2004). Multiple studies reported an increase in IL-1 β , TNF- α and IL-6 in older individuals (Ershler, 1993; Maggio et al., 2006; J. P. Coppé et al., 2008; W. I. Chang et al., 2011). These markers were also found to have higher levels in salivary tissue and saliva in cases of inflammation and senescence (Gümüş et al., 2014; Marmary et al., 2016).

- **Increased Oxidative Stress Markers:**

As mentioned earlier, the increased oxidative damage and reactive free radicals have been associated with aging (Z. Wang et al., 2013). One form of these oxidative reactions is lipid peroxidation that happens when free radicals cause degradation of lipids in cell membranes causing overall cell damage (Z. Wang et al., 2013). Some

markers for oxidative stress include 8-Hydroxydeoxyguanosine (8-OHdG, Malondialdehyde (MDA) and 4-Hydroxy-2-nonenal (HNE) (Hendek et al., 2015). These markers were reported to be increased in saliva in cases of increased metabolic stresses, smoking and inflammation (Gursoy et al., 2013; Kurgan et al., 2015).

Changes in growth factors and tissue forming markers:

Tissue growth factors (TGFs) are a family of polypeptides that regulate multiple mechanisms, including tissue development, immune regulation, wound healing, and inflammation (Kastin, 2013). This family includes a group of proteins called bone morphogenic proteins (BMP) that are responsible for signaling for skeletal tissue development and homeostasis (Wan et al., 2005).

TGF- β is a marker from the TGF superfamily. It is responsible for bone formation and regulating osteoblast function (M. Wu et al., 2016). The expression levels of TGF- β , BMP-2 and BMP-4, showed to decrease with aging (Abdallah et al., 2006; Grzibovskis et al., 2010; van Caam et al., 2016).

Vascular Endothelial Growth Factor (VEGF) is a marker for vasculogenesis and angiogenesis activity in the tissue. It is an important indicator of the vascular system development and regeneration potential. Research shows it there is a decline in VEGF activity with aging (Ahluwalia et al., 2014; Andisheh-Tadbir et al., 2014).

Fibronectin is a glycoprotein that is found both intracellular and extracellular in the blood plasma. It plays an important role in cell adhesion and wound healing (Pankov et al., 2002). Fibronectin plasma levels are reported to increase with age (Labat-Robert et al., 1988; Feist et al., 2013; Lemańska-Perek et al., 2013) while tissue expression of fibronectin varied with age between different tissue types (Li-Korotky et al., 2007; Lemańska-Perek et al., 2013).

- **Changes in insulin-like growth factors and associated proteins:**

Aging is associated with changes in the secretion of insulin-like growth factor (IGF) and insulin-like growth factor binding proteins (IGFBP's) (Acosta et al., 2008). IGFBP's bind to circulating IGF and therefore regulate its availability and uptake by tissue. Some studies reported an age-related decrease in certain IGFBP's (Gelato et al., 1997), while others reported increased IGFBP levels with age and cellular senescence (Hayden et al., 1995; S. Wang et al., 1996; Gelato et al., 1997; Conover et al., 2003; Carrington, 2005; J.-P. Coppé et al., 2010). Others reported no changes in IGFBPs with age (Chan et al., 1997). Along from their effect on IGF, some reports indicated IGFBP's could have a direct effect on tissue and cellular senescence (Acosta et al., 2008; Wajapeyee et al., 2008; S. Hong et al., 2018).

Changes in Lactoferrin levels:

Lactoferrin is an iron-binding glycoprotein present in plasma and other major exocrine fluids including tears, saliva and breast milk (Shugars et al., 2001; Farnaud et al., 2003). It has an important anti-microbial effect and plays an important role in the immune system fighting fungal, bacterial and viral infections (Farnaud et al., 2003; Ammons et al., 2013). It was found to also play an anti-inflammatory role by facilitating neutrophils adherence to endothelial cells (Oseas et al., 1981; Ammons et al., 2013). Due to its high affinity to iron, it plays a role in transferring iron to cells. The excess of free iron molecules is known to contribute to the formation of oxidative free radicals. Therefore, lactoferrin helps in reducing the level of oxidative damage in the body (Farnaud et al., 2003; Ammons et al., 2013). An increase in lactoferrin levels in the plasma of the elderly with infections has been reported (Adeyemi et al., 1992). Similarly, high lactoferrin levels were in the plaque of older subjects, along with an increase in immune-related markers (Cole et al., 1981). Others reported that lactoferrin

was decreased in the whole saliva of healthy elderly along with histological changes in salivary gland tissue (Lyng Pedersen et al., 2019). Studies on age-related neurological diseases indicated saliva lactoferrin might be a strong predictor of the early development of Alzheimer's disease, as patients showed lower levels compared to healthy controls. Moreover, these patients showed a correlation between levels of lactoferrin in saliva and in the cerebral spinal fluid CSF (Y. W. H. Yang et al., 1989; François et al., 2018; Cardoso et al., 2018). Some reports indicated that an increase in glycation, which is known to increase with age and age-related diabetes, inhibits the anti-microbial effect of lactoferrin (Fournet et al., 2018).

HYPOTHESIS

Null hypothesis: There is no difference between the levels of salivary markers of inflammation and various biological processes between young and old individuals.

Alternative hypothesis: Salivary content of markers of inflammation and biological processes will change between young and older individuals.

OVERALL AIM:

The overall goal of this study was to assess the relationship between inflammatory and senescence-related markers in saliva and age.

SPECIFIC AIMS:

1. To measure the expression of inflammatory and senescence-related markers in saliva.
2. To assess whether there is a specific age showing a significant change in senescence-related and inflammatory markers.
3. To identify differences in the expression of salivary markers between males and females

METHODS

Saliva sample collection:

The study sample included saliva specimens collected from 127 patients from the Department of Orthodontics and Orofacial Orthopedics at the Henry M. Goldman School of Dental Medicine at Boston University in the period between April 2017 and March 2018. After the IRB was approved, patients were introduced to the study idea and provided with oral and written consents for their agreement to participate. Subjects were asked about their medical history and their intake of any medications, vitamins or supplements. If subjects reported positively, they were excluded from the study. According to the orthodontic treatment guidelines, a patient must have a controlled periodontal status prior to starting orthodontic treatment. Therefore, study sample excluded any subjects with active periodontal disease or those with extensive bone loss due to previous periodontal disease. The sample group included patients with variable degrees of malocclusion and different orthodontic treatment plans.

While not studied in this project, as a part of the longitudinal assessment of treatment, saliva samples were collected on every orthodontic appointment for the period from treatment plan visit until the end of the leveling and alignment phase. Every participant was provided with a 15 ml polypropylene tube and asked to provide at least 3 ml of saliva. Whole saliva samples were then immediately preserved at -80°C. After collection, saliva samples were transferred to the Forsyth Institute on dry ice and stored in -80°C freezer until ready for analysis.

Out of 127 patients recruited, 74 subjects provided their saliva samples. Only initial saliva samples from the treatment plan visits were analyzed for the purpose of this study.

Markers investigated:

A group of biological markers were chosen to be analyzed with Luminex 200® including BMP-2, BMP-4, Fibronectin, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-6, IGFBP-7, IL-1 β , IL-2, IL-6, IL-8, Lactoferrin, TNF- α , VEGF, MMP-1, MMP-2, MMP-13.

A multiplex analysis kit was customized to include all these markers and purchased from the R&D systems® (Catalog#: LXSAHM-19plex incl BMP-2, BMP-4, Fibronectin, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-6, IGFBP-7, IL-1 β , IL-2, IL-6, IL-8, Lactoferrin, TNF- α , VEGF, MMP-1, MMP-2, MMP-13).

Sample Processing:

Samples were thawed at 4°C prior to assay and kept on ice throughout the assay procedure. Manufacturers' instructions were followed with a general protocol as follows: all kit components were brought to room temperature. Reagents were prepared as per kit instructions (including wash buffers, beads, and standards). Assay plates (96-well) were loaded with assay buffer, standards, samples, and beads and then covered and incubated on a plate shaker (500 rpm) overnight at 4°C. After primary incubation, plates were washed twice, and then detection antibody cocktail was added to all wells; the plates were covered and left to incubate at room temperature for one hour on a plate shaker. After the one-hour incubation, streptavidin-phycoerythrin fluorescent reporter was added to all wells; the plate was covered and incubated for 30 minutes at room temperature on a plate shaker. Plates were then washed twice and beads were resuspended in sheath fluid, placed on a shaker for 5 minutes, and then read on Bio-Plex®200 following manufacturers' specifications and analyzed using Bio-Plex Manager software v6.0.

Statistical Analysis:

Statistical analysis was performed by SAS® software [9.4] (SAS Institute Inc., Cary, NC, USA). Depending on samples normality (tested by Shapiro-Wilk test), parametric or non-parametric statistical tests were used, including T-test, Kruskal-Wallis and multiple comparison analysis as needed. The level of significance was noted at $p < 0.05$ for all analyzed data.

RESULTS

Sample description and distribution

We had a total of 74 subjects: 44 females and 30 males. The subjects' age ranged between 8 years old and 63 years old. After running a statistical normality analysis of the study population, Shapiro-Wilks test was significant ($p < 0.05$) indicating a non-normal distribution. Subjects were ranked based on age and then divided into four quartiles. Each quartile indicated an age group: age group I (Q1): subjects younger than 13 years old ($n=16$), age group II (Q2): 13 -16 years old ($n=20$), age group III (Q3): 17 -27 years old ($n=18$) and age group IV (Q4): subjects older than 27 years old ($n=20$). This classification of the age variable divided the sample into subgroups that are approximately balanced in size. The age 13 years old was chosen as end of childhood stage (1st age group; Q1) based on reported time of puberty in U.S boys and girls in 2019 (Brix et al., 2019) [**Table 1**].

Table 1: Sample distribution based on age and gender

	1st Quartile (Q1)	2nd Quartile (Q2)	3rd Quartile (Q3)	4th Quartile (Q4)	
	<13 years old	13 -16 years old	17- 27 years old	>27 years old	Total
Mean age ±SD (years)	11.19 ±1.33	14.50 ±1.24	22.50 ±3.323	35.90 ±9.74	
No. of subjects	16	20	18	20	
Male	5	11	10	4	30
Female	11	9	8	16	44

Descriptive statistics of the study populations

Data was described with both mean and median and their corresponding variance measures (SD and IQ range, respectively). Due to the non-normal distribution of the data, median and inter-quantile range were found as better measures to describe the data [**Table 2**], and non-parametric statistical analysis was used to analyze the data.

Table 2: Descriptive results of the study populations

Variable	Mean \pm SD	Median	IQ Range
BMP-2	65.25 \pm 44.24	52.05	61.24
BMP-4	91.56 \pm 64.98	80.92	101.18
Fibronectin	151273.69 \pm 150742.08	97180.68	201751.38
VEGF	1595.03 \pm 1248.32	1126.63	1118.75
IGFBP-1	1656.12 \pm 1460.16	1294.47	1157.98
IGFBP-2	19506.63 \pm 39486.03	11665.81	9856.08
IGFBP-3	25583.43 \pm 26714.24	20934.97	21920.49
IGFBP-4	1126.24 \pm 1142.90	845.72	1252.02
IGFBP-6	1351.30 \pm 556.02	1284.22	615.74
IGFBP-7	5590.16 \pm 4696.43	3600.21	4393.29
IL-1β	441.94 \pm 429.95	301.24	395.13
IL-2	40.15 \pm 33.34	30.69	24.99
IL-6	24.26 \pm 55.25	10.33	10.97
IL-8	965.54 \pm 975.70	700.68	564.30
TNF-α	105.20 \pm 114.48	73.12	151.82
Lactoferrin	23029.41 \pm 2278.10	23787.80	677.50
MMP-1	147.53 \pm 461.09	34.81	65.36
MMP-2	2496.48 \pm 954.84	2340.01	1495.34
MMP-13	294.33 \pm 215.55	255.74	229.96

The difference in sample population measures between males and females:

Comparing the whole sample group, no statistically significant difference was found between males and females in the levels of BMP-2, BMP-4, VEGF, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-6, IGFBP-7, IL-1 β , IL-2, IL-6, IL-8, TNF- α , lactoferrin, MMP-1, MMP-2 and MMP-13 ($p < 0.05$). An evident difference was found in fibronectin levels among gender, with females having higher values (Median: 138785.61, IQ: 213494.55) than males (median 57651.72, 106044.99). This difference; however, was not significant at $p < 0.05$ ($p = 0.063$) [**Table 3**]. Gender comparison was not done in each age quartile.

Table 3: Difference in sample population measures based on gender

Variable	Female (n=44)				Male (n=30)				P-Value
	Mean	SD	Median	IQ Range	Mean	SD	Median	IQ Range	
BMP-2	68.48	47.39	61.28	64.59	60.34	39.26	50.49	56.15	0.57
BMP-4	93.63	67.67	95.86	98.69	88.53	61.84	69.91	101.49	0.85
Fibronectin	166475.73	138288.52	138785.61	213494.55	128977.37	167269.13	57651.72	106044.99	0.06
VEGF	1658.78	1171.22	1190.36	1444.58	1504.91	1366.06	1065.52	862.14	0.52
IGFBP-1	1746.04	1562.65	1430.77	1123.81	1524.23	1309.94	1143.91	1189.83	0.48
IGFBP-2	15627.77	19162.07	10149.69	9280.38	25195.61	57654.08	12685.00	9121.71	0.36
IGFBP-3	25223.42	30190.97	21224.70	22933.51	26198.45	19999.39	20789.18	21210.71	0.59
IGFBP-4	1011.89	904.70	845.72	1233.65	1290.13	1417.91	885.35	1352.17	0.69
IGFBP-6	1385.52	520.22	1259.03	583.81	1301.11	610.37	1343.79	666.98	0.53
IGFBP-7	5343.01	4859.81	3221.19	3799.18	5952.64	4502.60	5089.87	5443.73	0.60
IL-1β	423.23	293.96	337.79	351.14	469.39	579.57	249.53	461.19	0.38

IL-2	42.74	36.57	30.56	29.98	36.35	28.10	31.44	20.00	0.70
IL-6	15.72	13.77	11.25	8.38	36.79	84.45	8.79	14.01	0.25
IL-8	1007.60	1154.86	635.54	813.60	904.97	654.61	713.16	440.38	0.58
TNF-α	109.22	113.90	78.97	153.62	99.31	117.02	65.89	125.50	0.44
Lactoferrin	23211.66	1827.43	23724.30	767.50	22762.10	2826.27	23818.80	671.00	0.51
MMP-1	159.39	564.60	38.80	64.34	130.14	248.98	30.16	99.53	0.73
MMP-2	2658.27	988.52	2611.58	1333.73	2259.18	864.95	2069.10	1264.68	0.08
MMP-13	289.87	214.78	235.60	219.80	301.94	221.28	263.57	253.53	0.97

Difference in sample population measures between different age groups

Kruskal-Wallis statistical test was used to compare medians of tested markers among the four different age groups. Data showed significant differences with subjects' age in the level of following markers: BMP-4, fibronectin, VEGF, IGFBP-2, IGFBP-4, IGFBP-6, IGFBP-7, IL- β , lactoferrin and MMP-2 ($p < 0.05$). Levels of BMP-2, IGFBP-1, IGFBP-3, IL-6, IL-8, TNF- α , MMP-1 and MMP-13 showed no statistically significant difference between the four age groups ($p < 0.05$) [**Table 4**].

To investigate which groups showed a significant difference, non-parametric multiple comparison analysis of the medians (Dwass, Steel, Critchlow-Fligner test) was done for markers that showed significant differences across age groups.

Table 4: Difference in markers' level between age groups

Variable	1 st Quartile (Q1) (Age: <13 years old) n=16			2 nd Quartile (Q2) (Age: 13 -16 years old) n=20			3 rd Quartile (Q3) (Age: 17 -27 years old) n=18			4 th Quartile (Q4) (Age: >27 years old) n=20			P-value
	Mean ±SD	Median	IQ Range	Mean ± SD	Median	IQ Range	Mean ± SD	Median	IQ Range	Mean ±SD	Median	IQ Range	
BMP-2	53.03 ± 37.84	42.84	39.86	61.90 ± 48.22	48.91	59.57	77.56 ± 39.02	80.92	39.91	67.12 ± 49.36	38.94	78.50	0.30
BMP-4	66.80 ± 39.94	49.51	62.43	80.20 ± 69.55	54.84	87.68	124.46 ± 58.79	124.25	44.07	93.12 ± 73.16	53.91	108.16	0.05*
Fibrone	186276. 01 ±	154197. 69	148598. 42	188869. 02 ±	121351. 82	251040. 48	61986. 96 ±	33958. 40	65016. 04	166034.5 7	129952. 62	193314. 77	0.008 *

	163091. 26			178325. 53			66928. 89			±142114. 28			
VEGF	1769.78 ± 1194.36	1382.63	1526.68	1847.78 ± 1487.75	1595.39	1106.20	816.56 ± 323.52	784.61	401.95	1955.13 ±1357.39	1279.83	2207.13	0.001 *
IGFBP-1	1892.61 ± 1482.61	1540.36	961.65	1642.62 ± 2062.82	1045.60	1088.02	1560.4 8 ± 1056.1 2	1313.8 5	939.16	1566.49 ±1067.05	1434.97	1429.07	0.46
IGFBP-2	22512.1 7 ± 26722.8 5	13341.8 7	10486.4 4	34836.5 4 ± 69376.3 4	16021.2 8	15024.7 2	7660.3 1 ± 4034.0 0	7028.8 9	5669.9 1	12433.96 ±12586.0 9	9133.62	7509.86	0.000 8*

IGFBP-3	20946.4 5 ± 18147.5 3	17295.0 5	24458.8 5	33835.5 1 ± 46047.8 5	16081.1 3	30184.6 2	25770. 84 ± 15564. 09	23333. 64	15163. 36	22004.60 ±15087.4 3	21224.7 0	21343.7 5	0.69
IGFBP4	653.46 ± 398.50	599.06	517.91	876.20 ± 717.44	905.25	1211.23	1956.4 9 ± 1634.8 6	1312.6 5	1954.3 3	983.63 ±1005.00	655.89	1410.83	0.02*
IGFBP-6	1211.21 ± 389.80	1237.30	479.54	1508.23 ± 675.99	1420.96	658.63	1057.4 7 ± 313.59	1028.5 7	352.78	1570.90 ±589.82	1417.86	695.61	0.01*
IGFBP-7	6558.95 ± 4757.75	4465.04	8939.00	7710.00 ± 5920.37	6673.70	6912.15	3747.0 2 ±	2539.7 1	1486.6 8	4354.12 ±3239.52	3214.57	3803.64	0.02*

							3503.1 1						
IL-1β	622.51 \pm 608.47	487.11	592.96	500.75 \pm 511.51	263.17	424.49	233.65 \pm 155.24	180.90	189.39	426.15 \pm 249.56	351.58	389.87	0.02*
IL-2	31.42 \pm 13.68	30.81	8.24	39.77 \pm 36.75	25.06	29.26	43.17 \pm 35.61	33.56	25.48	44.79 \pm 39.25	27.56	41.71	0.88
IL-6	20.1 5 \pm 35.71	9.56	5.91	19.69 \pm 31.78	10.63	14.27	12.49 \pm 13.00	9.01	7.73	42.72 \pm 95.06	13.11	26.14	0.11
IL-8	1272.32 \pm 1720.42	594.29	725.84	922.62 \pm 623.34	723.07	1019.79	690.94 \pm 590.17	583.04	421.28	1072.09 § 730.04	871.12	1069.47	0.38

TNF-α	92.38 \pm 103.05	76.55	92.67	95.34 \pm 135.85	12.90	130.21	124.96 § 103.39	84.11	104.54	107.54 \pm 115.48	45.58	167.14	0.35
LF¹	23744.9 6 \pm 311.53	23821.5 5	401.50	23847.4 7 \pm 310.39	23962.3 0	233.25	21110. 02 \pm 3993.3 0	23137. 80	2779.0 0	23366.35 \pm 867.95	23801.8 0	848.00	0.000 2*
MMP-1	144.18 \pm 211.85	45.37	105.95	322.93 \pm 851.11	27.96	117.92	60.49 \pm 79.95	32.35	26.86	53.14 \pm 48.79	36.99	59.74	0.81
MMP-2	2753.07 \pm 1003.32	2803.08	1615.15	2628.72 \pm 973.44	2604.51	1917.38	1933.9 2 \pm 589.20	1950.3 2	916.40	2665.27 \pm 1022.91	2497.48	1310.21	0.037 *

MMP-13	255.41± 222.61	190.35	291.95	296.91 ±195.97	259.51	168.74	319.41 ± 229.91	272.16	226.97	304.91 ± 227.09	271.40	232.55	0.83
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* Statistically significant at $p < 0.05$, ¹LF: lactoferrin

BMP-2: Descriptive measures for BMP-2 in the study population are in presented in **Table 5**. Data showed the overall difference between females and males was not significant ($p < 0.05$) [**Table 6**]. Females had higher values than males in Q3 and lower values in Q1 and Q4. BMP-2 levels showed a mild increase with age across age groups, being highest in Q3, followed by a decrease in Q4. These differences; however, were not statistically significant at $p < 0.05$ [**Figure 1**] [**Table 7**].

Table 5: BMP-2 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
BMP-2	65.25 \pm 44.24	52.05	61.24

Table 6: BMP-2 level among different gender groups

BMP-2	N	Mean	SD	Median	IQ Range
Female	n=44	68.48	47.39	61.28	64.59
Male	n=30	60.34	39.26	50.49	56.15
P-value	0.57				

Table 7: BMP-2 among different age groups

BMP-2	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	53.03 \pm 37.84	42.84	39.86
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	61.90 \pm 48.22	48.91	59.57
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	77.56 \pm 39.02	80.92	39.91
4th Quartile (Q4) (Age: >27 years old)	n=20	67.12 \pm 49.36	38.94	78.5

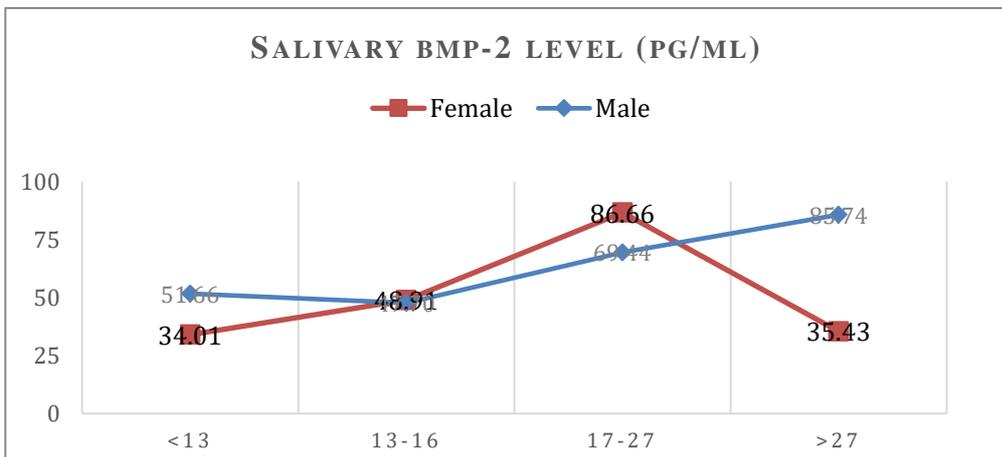
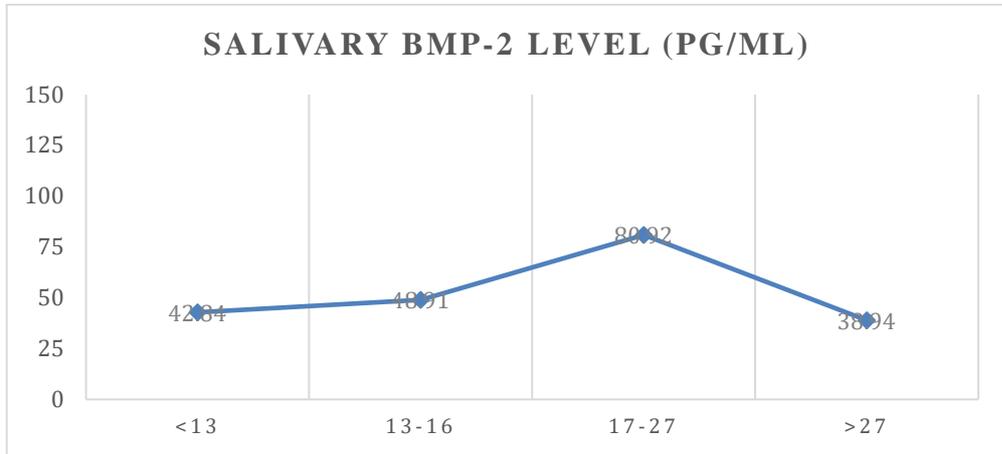


Figure 1: Median BMP-2 levels among different age groups (top), and among females and males separately (bottom).

BMP-4: Descriptive measures for BMP-4 in the study population are in **Table 8**. Data showed an increase in BMP-4 with age in the sample group. The difference was statistically significant between Q1 and Q3 (<13 years old and 17 – 27 years old respectively) ($p < 0.05$). Difference between other age groups was found to be statistically non-significant [**Tables 10 and 11**] [**Figure 2**]. Females showed higher overall levels than males, that was statistically non-significant [**Table 9**]. Per age quartiles, females showed higher levels of BMP-4 in Q2 and Q3 while males showed higher levels in Q4 [**Figure 2**].

Table 8: BMP-4 levels in the study population

Variable	Mean \pm SD.	Median	IQ Range
BMP-4	91.56 \pm 64.98	80.92	101.18

Table 9: BMP-4 level among different gender groups

BMP-4	N	Mean	SD	Median	IQ Range
Female	n=44	93.63	67.67	95.86	98.69
Male	n=30	88.53	61.84	69.91	101.49
P-value	0.85				

Table 10: BMP-4 among different age groups

BMP-4	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	66.80 \pm 39.94	49.51	62.43
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	80.20 \pm 69.55	54.84	87.68
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	124.46 \pm 58.79	124.25	44.07
4th Quartile (Q4) (Age: >27 years old)	n=20	93.12 \pm 73.16	53.91	108.16
P-value	0.0468*			

*Statistically significant at $p < 0.05$

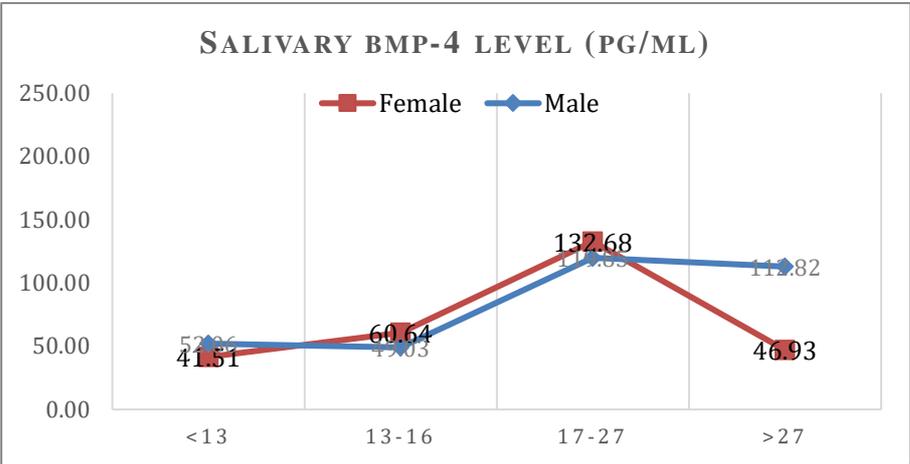
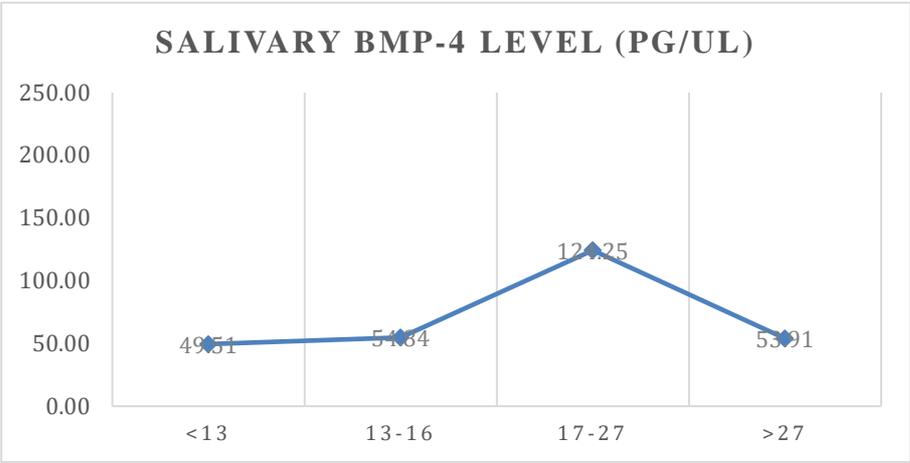


Figure. 2: Median BMP-4 levels among different age groups (top) and among females and males ,separately (bottom).

Table 11: Multiple comparison analysis of BMP-4 levels between the sample age groups

Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method			
BMP-4			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	1.0000
Q1 vs. Q3	-2.8297	4.0018	0.0241*
Q1 vs. Q4	-0.8596	1.2157	0.8256
Q2 vs. Q3	-2.2369	3.1634	0.1134
Q2 vs. Q4	-0.6900	0.9758	0.9010
Q3 vs. Q4	1.4620	2.0676	0.4607

Age groups based on quartiles: Q1: (<13 years old), Q2: (13-16 years old), Q3: (17-27 years old), Q4: (<27 years old)

*: Significant at P<0.05

^ marginally significant

Fibronectin: Descriptive measures for fibronectin in the study population are in **Table 12**. Data showed a decrease in fibronectin levels with age, with Q3 showing lowest values. Fibronectin levels increased from Q3 to Q4, but were still lower than Q1 and Q2. The difference was found statistically significant comparing the third quartile with first and fourth quartiles of age ($p < 0.05$), and marginally significant between Q3 and Q2 ($P = 0.055$) [**Tables 14 and 15**] [**Figure 3**]. Females had higher fibronectin levels than males [**Table 13**] and this was consistent across all age quartiles. Gender difference was found to be marginally significant ($p < 0.06$).

Table 12: Fibronectin levels in the study population

Variable	Mean \pm SD.	Median	IQ Range
Fibronectin	151273.69 \pm 150742.08	97180.68	201751.38

Table I3: Fibronectin level among different gender groups

Fibronectin	N	Mean	SD	Median	IQ Range
Female	n=44	166475.73	138288.52	138785.61	213494.55
Male	n=30	128977.37	167269.13	57651.72	106044.99
P-value	0.063				

Table 14: Fibronectin among different age groups

Fibronectin	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	186276.01 \pm 163091.26	154197.69	148598.42
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	188869.02 \pm 178325.53	121351.82	251040.48
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	61986.96 \pm 66928.89	33958.40	65016.04
4th Quartile (Q4) (Age: >27 years old)	n=20	166034.57 \pm 142114.28	129952.62	193314.77
P-value	0.0081*			

*Statistically significant at $p < 0.05$

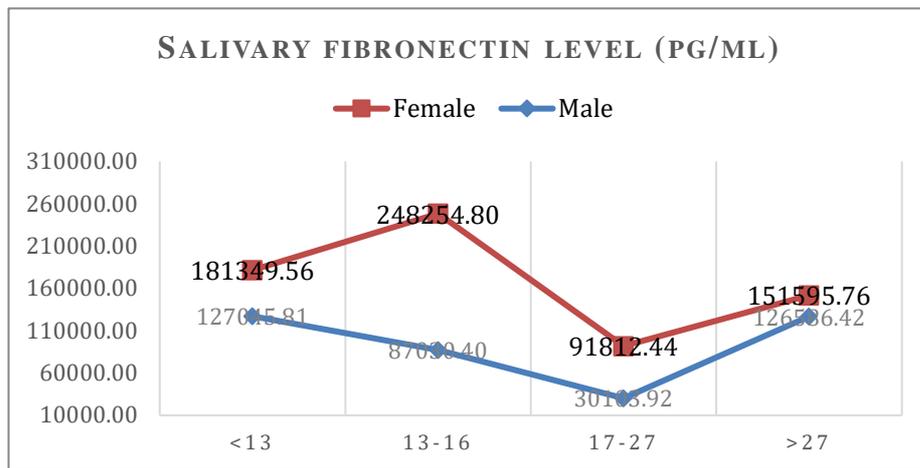
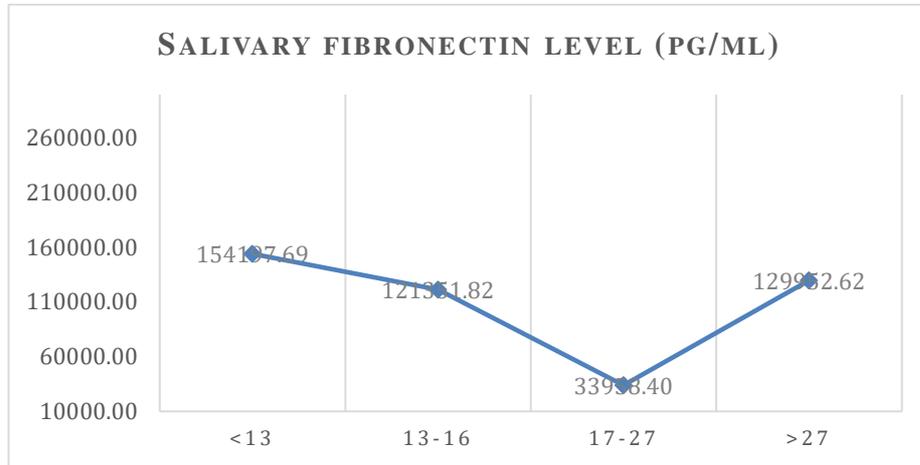


Figure 3: Median Fibronectin levels among different age groups (top) and among females and males, separately (bottom).

Table 15: Multiple comparison analysis of Fibronectin levels between the sample age groups.

Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method			
Fibronectin			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	0.9975
Q1 vs. Q3	-2.8297	4.0018	0.0159*
Q1 vs. Q4	-0.8596	1.2157	0.9961
Q2 vs. Q3	-2.2369	3.1634	0.0556
Q2 vs. Q4	-0.6900	0.9758	0.9985
Q3 vs. Q4	1.4620	2.0676	0.0199*

Age groups based on quartiles: Q1: (<13 years old), Q2: (13-16 years old), Q3: (17-27 years old), Q4: (<27 years old)

*: Significant at P<0.05

VEGF: Descriptive measures for VEGF in the study population are presented in **Table 16**. The data showed a slight increase in VEGF levels from Q1 to Q2, followed by a decrease in Q3, then a marked increase in Q4. Levels in Q4 were still lower than Q1 and Q2. The difference in VEGF level was found to be statistically significant comparing Q3 with all other age quartiles Q1, Q2 and Q3 ($p < 0.05$) [**Tables 18 and 19**] [**Figure 4**]. Females showed overall higher values than males, but this was statistically not significant ($p < 0.05$) [**Table 17**]. Gender differences were variable across age groups; females and males showed similar VEGF levels in Q2 and Q3, males had higher levels in Q1 while females levels were higher in Q4.

Table 16: VEGF levels in the study population

Variable	Mean \pm SD	Median	IQ Range
VEGF	1595.03 \pm 1248.32	1126.63	1118.75

Table 17: VEGF levels among gender

VEGF	N	Mean	SD	Median	IQ Range
Female	n=44	1658.78	1171.22	1190.36	1444.58
Male	n=30	1504.91	1366.06	1065.52	862.14
P-value	0.52				

Table 18: VEGF among different age groups

VEGF	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	1769.78 \pm 1194.36	1382.63	1526.68
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	1847.78 \pm 1487.75	1595.39	1106.20
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	816.56 \pm 323.52	784.61	401.95
4th Quartile (Q4) (Age: >27 years old)	n=20	1955.13 \pm 1357.39	1279.83	2207.13
P-value	0.0011*			

*Statistically significant at p<0.05

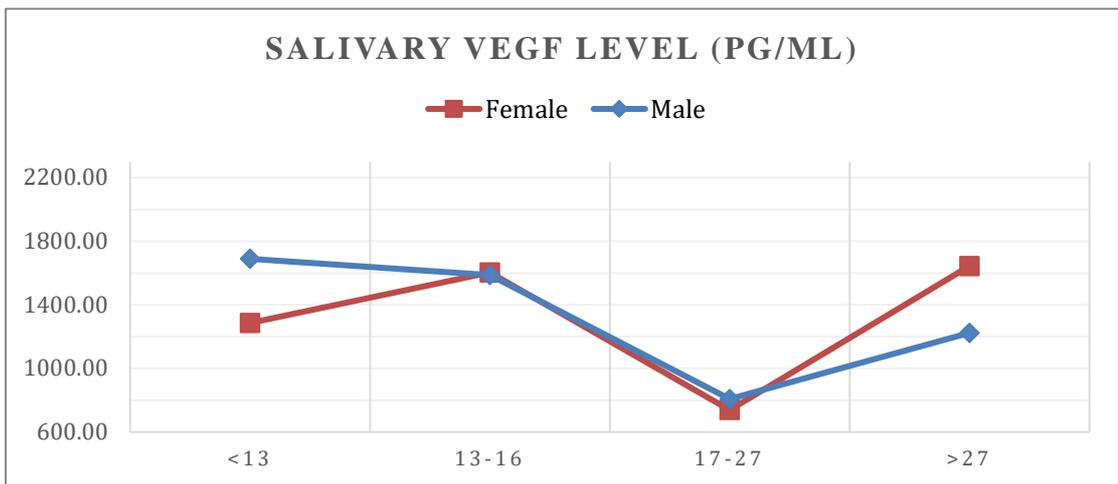
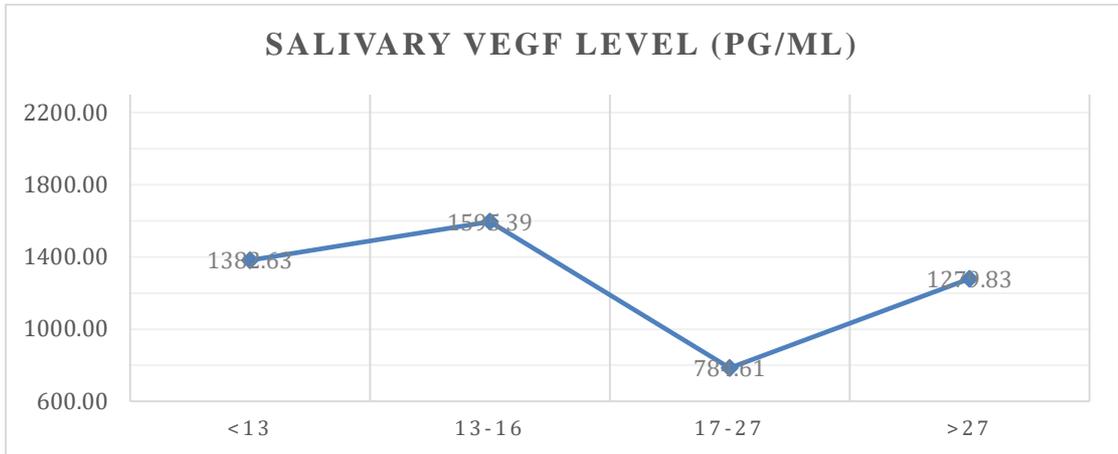


Figure 4: Median VEGF levels among different age groups (top), and among females and males, separately (bottom).

Table 19: Multiple comparison analysis of VEGF levels between the sample age groups.

Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method			
VEGF			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	0.9943
Q1 vs. Q3	-2.8297	4.0018	0.0179*
Q1 vs. Q4	-0.8596	1.2157	0.9960
Q2 vs. Q3	-2.2369	3.1634	0.0035*
Q2 vs. Q4	-0.6900	0.9758	0.9999
Q3 vs. Q4	1.4620	2.0676	0.0057

Age groups based on quartiles: Q1: (<13 years old), Q2: (13-16 years old), Q3: (17-27 years old), Q4: (<27 years old)

*: Significant at P<0.05

IGFBP-1: Descriptive measures for IGFBP-1 in study population are presented in **Table 20**. Although not statistically significant ($p < 0.05$), an overall decrease was seen in IGFBP-1 with age, with lowest values in Q2 [**Table 22**] [**Figure 5**]. Data showed overall higher levels of IGFBP-1 in females than males (not Significant at $p < 0.05$) [**Table 21**]. Per age quartiles, female values were higher in Q3 while males showed higher values in Q4 [**Figure 5**].

Table 20: IGFBP-1 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
IGFBP-1	1656.12 \pm 1460.16	1294.47	1157.98

Table 21: IGFBP-1 among different gender groups

IGFBP-1	N	Mean	SD	Median	IQ Range
Female	n=44	1746.04	1562.65	1430.77	1123.81
Male	n=30	1524.23	1309.94	1143.91	1189.83
P-value	0.48				

Table 22: IGFBP-1 among different age groups

IGFBP-1	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	1892.61 \pm 1482.61	1540.36	961.65
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	1642.62 \pm 2062.82	1045.60	1088.02
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	1560.48 \pm 1056.12	1313.85	939.16
4th Quartile (Q4) (Age: >27 years old)	n=20	1566.49 \pm 1067.05	1434.97	1429.07
P-value	0.46			

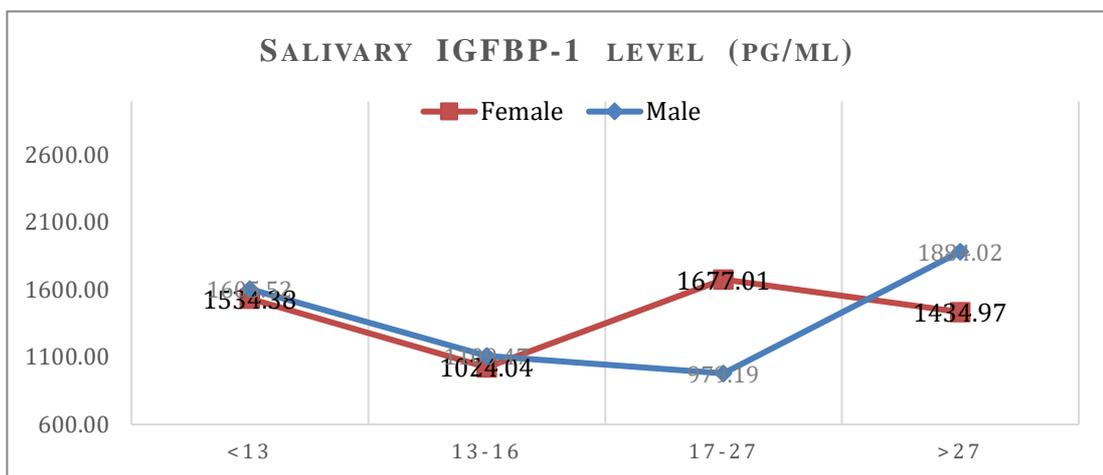
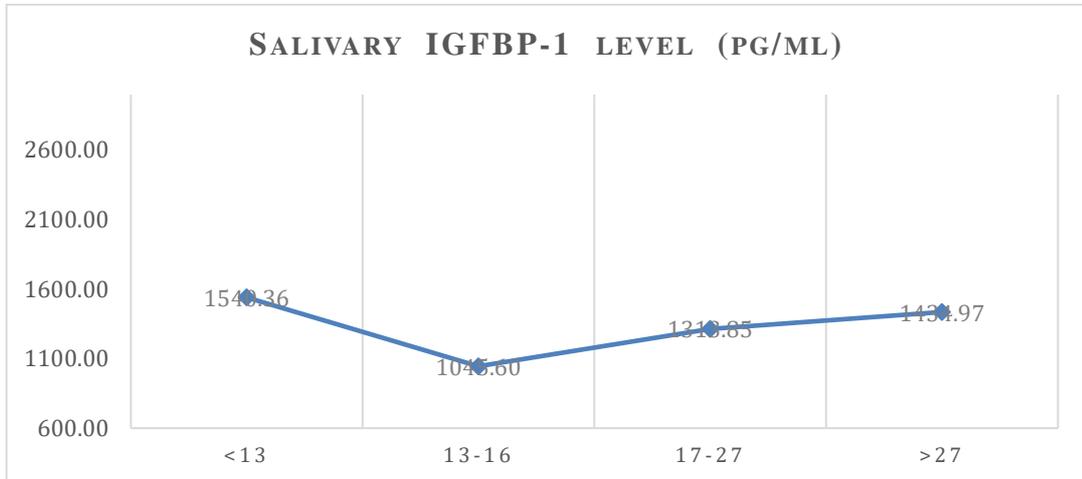


Figure 5: Median IGFBP-1 levels among different age groups (top), and among females and males , separately (bottom).

IGFBP-2: Descriptive measures for IGFBP-2 in the study population are presented in **Table 23**. There was a statistically significant change in IGFBP-2 among age groups ($p < 0.05$). There was an overall decrease with age except with some variability across age groups. Levels increased from Q1 to Q2, then decreased to their lowest levels in Q3, then a mild increase was found between Q3 and Q4, that was still lower than Q1 and Q2 levels. The decline in IGFBP-2 was found significant when comparing Q1 to Q3, and Q2 to Q3 and Q4 ($p < 0.05$) [**Tables 25 and 26**] [**Figure 6**]. IGFBP-2 levels were overall higher in males than females (not statistically significant at $p < 0.05$) [**Table 24**]. The difference varied among age groups, being higher in males in Q2, Q3 and Q4, while Q1 showed slightly higher values in females [**Figure 6**].

Table 23: IGFBP-2 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
IGFBP-2	19506.63 \pm 39486.03	11665.81	9856.08

Table 24: IGFBP-2 among gender groups

IGFBP-2	N	Mean	SD	Median	IQ Range
Female	n=44	15627.77	19162.07	10149.69	9280.38
Male	n=30	25195.61	57654.08	12685.00	9121.71
P-value	0.36				

Table 25: IGFBP-2 among different age groups

IGFBP-2	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	22512.17 \pm 26722.85	13341.87	10486.44
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	34836.54 \pm 69376.34	16021.28	15024.72
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	7660.31 \pm 4034.00	7028.89	5669.91
4th Quartile (Q4) (Age: >27 years old)	n=20	12433.96 \pm 12586.09	9133.62	7509.86
P-value	0.0008*			

(*) Statistically significant at $p < 0.05$

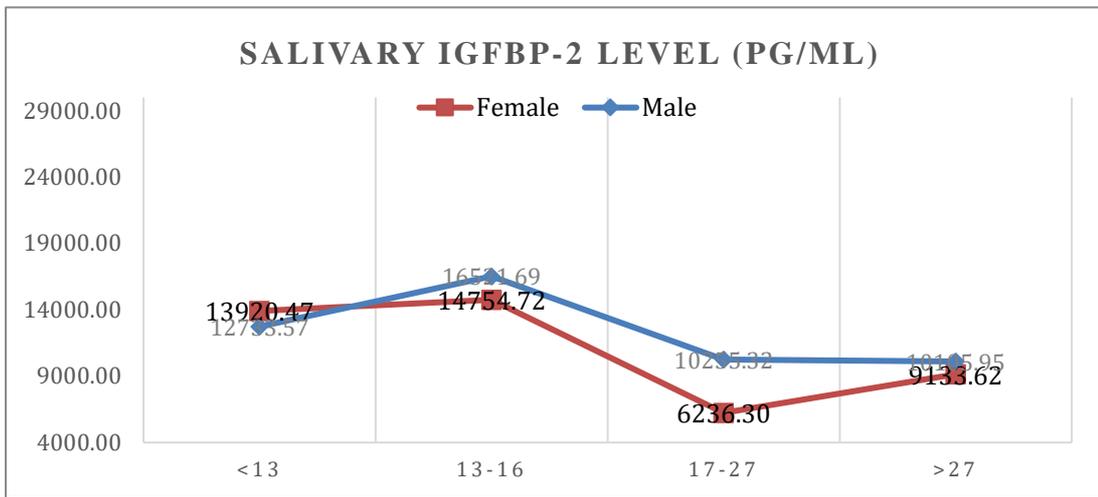
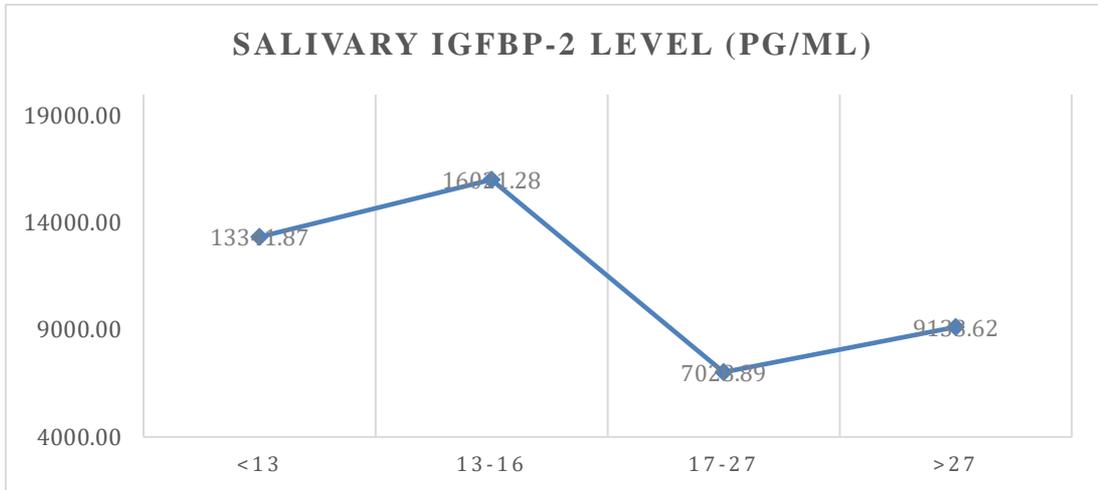


Figure 6: Median IGFBP-2 levels among different age groups (top), and among females and males separately (bottom).

Table 26: Multiple comparison analysis of IGFBP-2 levels between the sample age groups.

(Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method)			
IGFBP-2			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	0.8706
Q1 vs. Q3	-2.8297	4.0018	0.0358*
Q1 vs. Q4	-0.8596	1.2157	0.4206
Q2 vs. Q3	-2.2369	3.1634	0.0015*
Q2 vs. Q4	-0.6900	0.9758	0.0464*
Q3 vs. Q4	1.4620	2.0676	0.3908

Age groups based on quartiles: Q1: (<13 years old), Q2: (13-16 years old), Q3: (17-27 years old), Q4: (<27 years old)

*: Significant at P<0.05

IGFBP-3: Descriptive measures for IGFBP-3 in the study population are presented in **Table 27**. Data showed an overall increase in values with age with some variability across age groups. Levels slightly decreased from Q1 to Q2, then increased in Q3, then decreased mildly in Q4. Levels in Q4 were still higher than Q1 and Q2. These differences; however, were not statistically significant at $p < 0.05$. [**Table 29**] [**Figure 7**]. Females showed overall higher values (not statistically significant at $p < 0.05$) [**Table 28**]. Within the age groups, females had higher values in Q3, while males showed higher values in Q1, Q2 and Q4 [**Figure 7**].

Table 27: IGFBP-3 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
IGFBP-3	25583.43 \pm 26714.24	20934.97	21920.49

Table 28: IGFBP-3 among gender groups

IGFBP-3	N	Mean	SD	Median	IQ Range
Female	n=44	25223.42	30190.97	21224.70	22933.51
Male	n=30	26198.45	19999.39	20789.18	21210.71
P-value	0.59				

Table 29: IGFBP-3 among different age groups

IGFBP-3	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	20946.45 \pm 18147.53	17295.05	24458.85
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	33835.51 \pm 46047.85	16081.13	30184.62
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	25770.84 \pm 15564.09	23333.64	15163.36
4th Quartile (Q4) (Age: >27 years old)	n=20	22004.60 \pm 15087.43	21224.70	21343.75
P-value	0.69			

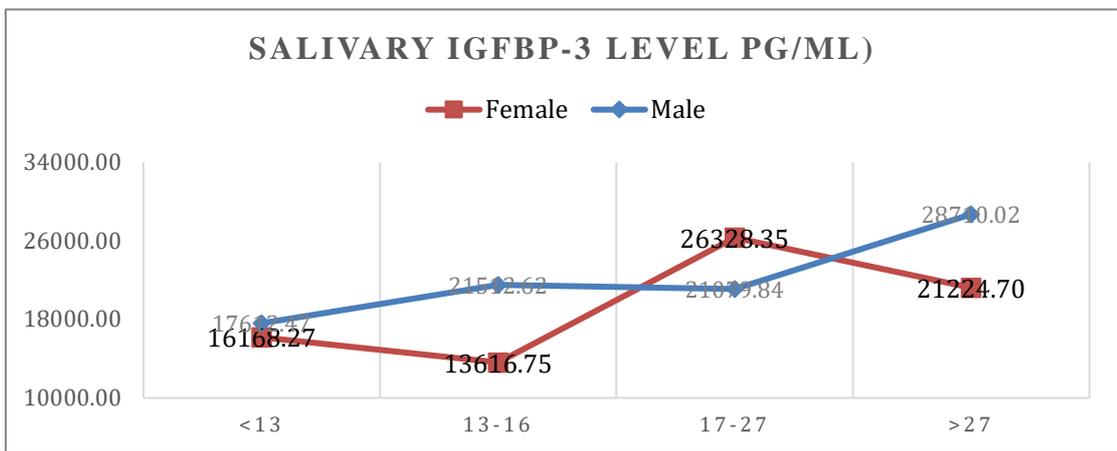
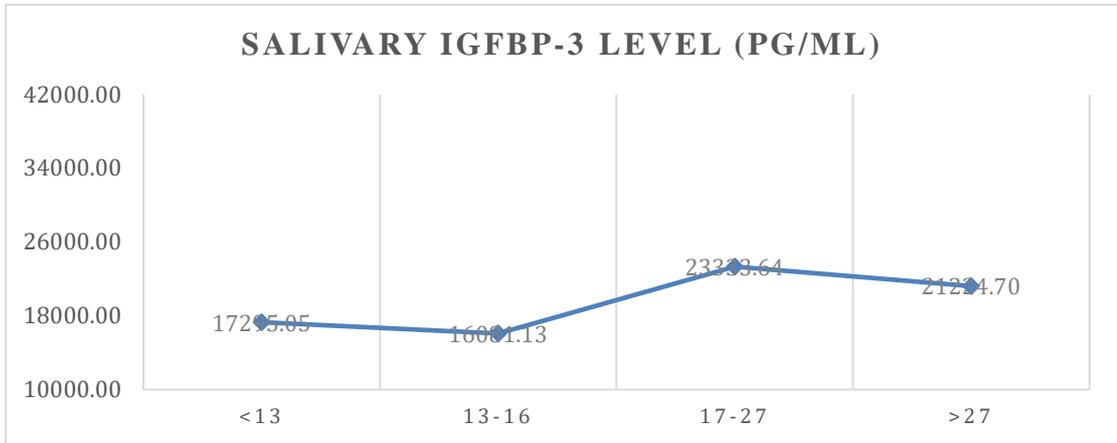


Figure 7: Median IGFBP-3 levels among different age groups (top), and among females and males separately (bottom).

IGFBP-4: Descriptive measures for IGFBP-4 in the study population are presented in **Table 30**. Statistically significant difference in IGFBP-4 levels was found among age groups. There was an overall increase from Q1 to Q3 (significant at $p < 0.05$), then a decrease in Q4. Levels in Q4 were lower than Q2, but slightly higher than Q1 [**Tables 32 and 33**] [**Figure 8**]. Gender difference showed that males had higher values than females, but the difference was not found to be statistically significant [**Table 31**] [**Figure 8**]. Per age quartiles, males had higher values in Q3 and Q4.

Table 30: IGFBP-4 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
IGFBP-4	1126.24 \pm 1142.90	845.72	1252.02

Table 31: IGFBP-4 among gender groups

IGFBP-4	N	Mean	SD	Median	IQ Range
Female	n=44	1011.89	904.70	845.72	1233.65
Male	n=30	1290.13	1417.91	885.35	1352.17
P-value	0.69				

Table 32: IGFBP-4 among different age groups

IGFBP-4	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	653.46 \pm 398.50	599.06	517.91
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	876.20 \pm 717.44	905.25	1211.23
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	1956.49 \pm 1634.86	1312.65	1954.33
4th Quartile (Q4) (Age: >27 years old)	n=20	983.63 \pm 1005.00	655.89	1410.83
P-value	0.0229*			

(*) Statistically significant at $p < 0.05$

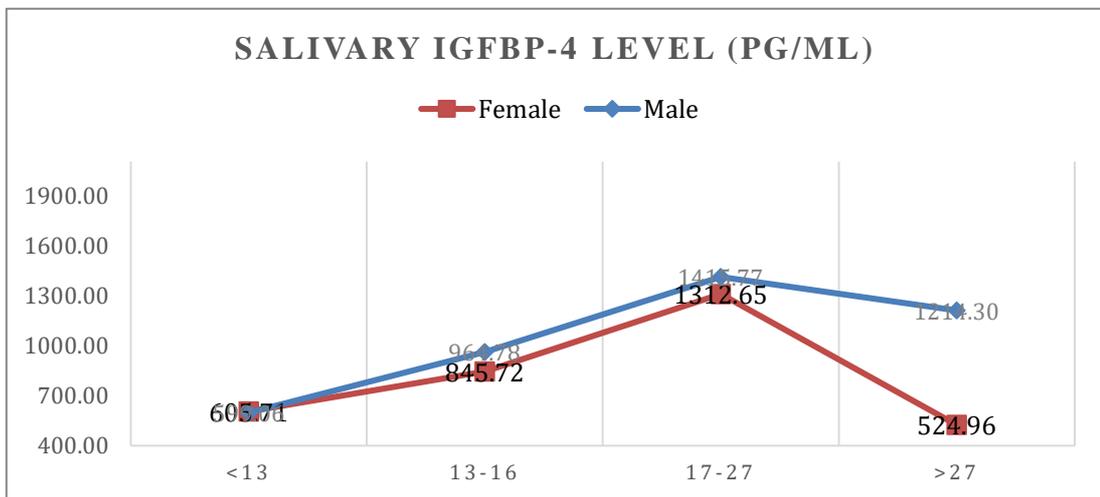
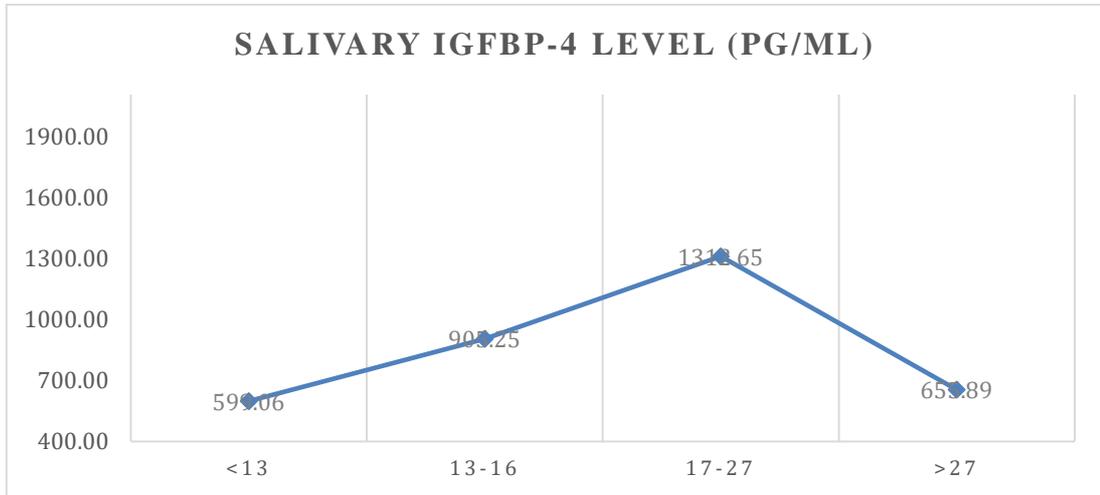


Figure 8: Median IGFBP-4 levels among different age groups (top), and among females and males, separately (bottom).

Table 33: Multiple comparison analysis of IGFBP-4 levels between the sample age groups

(Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method)			
IGFBP-4			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	0.9371
Q1 vs. Q3	-2.8297	4.0018	0.0143*
Q1 vs. Q4	-0.8596	1.2157	0.9955
Q2 vs. Q3	-2.2369	3.1634	0.0958
Q2 vs. Q4	-0.6900	0.9758	0.9998
Q3 vs. Q4	1.4620	2.0676	0.1293

Age groups based on quartiles: Q1: (<13 years old) , Q2: (13-16 years old) , Q3: (17-27 years old) , Q4: (<27 years old)

*: Significant at P<0.05

IGFBP-6: Descriptive measures for IGFBP-6 in the study population are presented in **Table 34**. Data showed a significant difference between age groups. There was an increase with age from Q1 to Q2, then a decrease from Q2 to Q3 (marginally significant at $p < 0.05$), then another increase in Q4 (significant at $p < 0.05$) [**Tables 36 and 37**] [**Figure 9**]. There was no statistically significant difference between males and females [**Table 35**]. Females showed lower values than males in Q3 and Q4 [**Figure 9**].

Table 34: IGFBP-6 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
IGFBP-6	1351.30 \pm 556.02	1284.22	615.74

Table 35: IGFBP-6 among gender groups

IGFBP-6	N	Mean	SD	Median	IQ Range
Female	n=44	1385.52	520.22	1259.03	583.81
Male	n=30	1301.11	610.37	1343.79	666.98
P-value	0.53				

Table 36: IGFBP-6 among different age groups

IGFBP-6	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	1211.21 \pm 389.80	1237.30	479.54
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	1508.23 \pm 675.99	1420.96	658.63
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	1057.47 \pm 313.59	1028.57	352.78
4th Quartile (Q4) (Age: >27 years old)	n=20	1570.90 \pm 589.82	1417.86	695.61
P-value	0.0105*			

(*) Statistically significant at $p < 0.05$

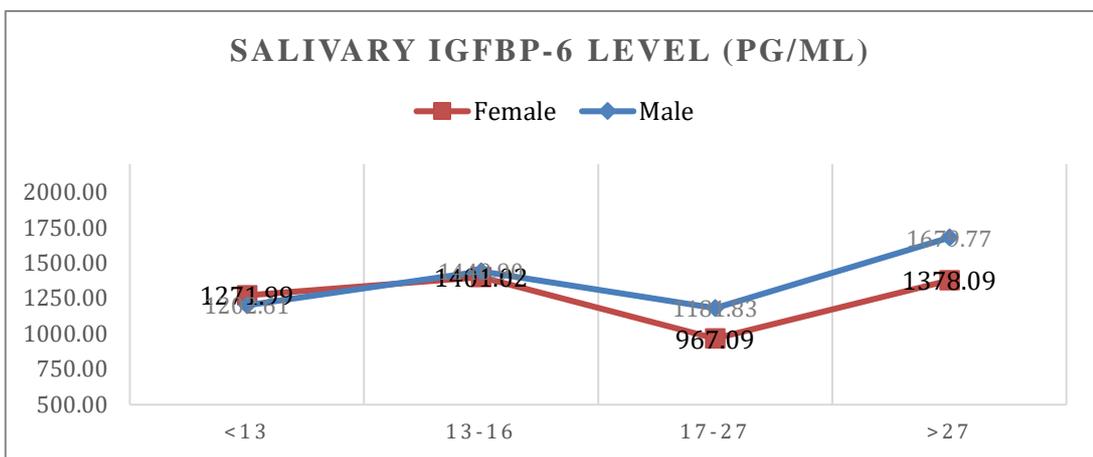
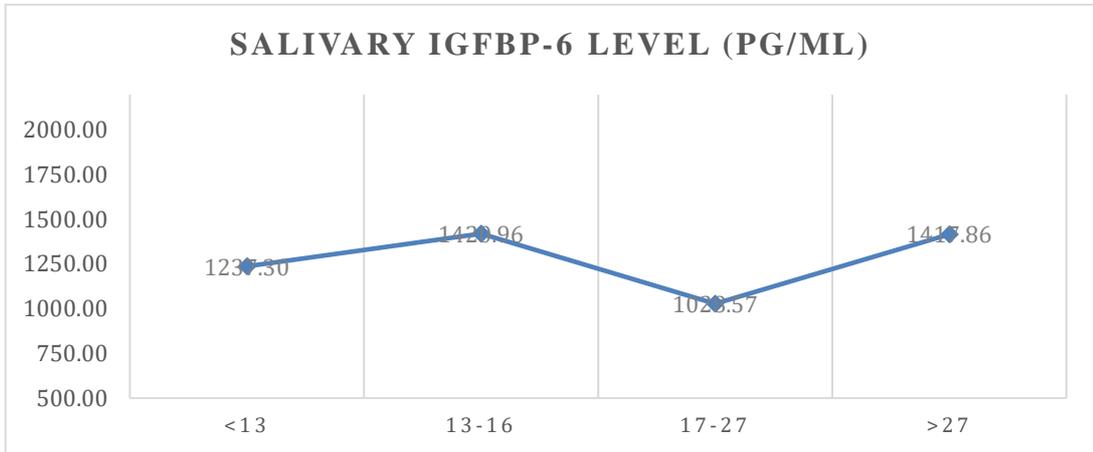


Figure 9: Median IGFBP-6 levels among different age groups (top), and among females and males, separately (bottom).

Table 37: Multiple comparison analysis of IGFBP-6 levels between the sample age groups.

(Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method)			
IGFBP-6			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	0.4988
Q1 vs. Q3	-2.8297	4.0018	0.6439
Q1 vs. Q4	-0.8596	1.2157	0.2663
Q2 vs. Q3	-2.2369	3.1634	0.0622
Q2 vs. Q4	-0.6900	0.9758	0.9815
Q3 vs. Q4	1.4620	2.0676	0.0139*

Age groups based on quartiles: Q1: (<13 years old) , Q2: (13-16 years old) , Q3: (17-27 years old) , Q4: (<27 years old)

*: Significant at P<0.05

IGFBP-7: Descriptive measures for IGFBP-7 in the study population are presented in **Table 38**. Data showed an overall decrease in IGFBP-7 with age. In Q2, there was an increase compared to Q1(not significant at $p < 0.05$), then a marked decrease in Q3 (significant at $p < 0.05$). IGFBP-7 levels increased mildly between Q3 and Q4 (not significant at $p < 0.05$) with values still lower than Q1 [**Tables 40 and 41**] [**Figure 10**]. Males showed higher levels than females but this was not statistically significant [**Table 39**]. Gender difference across age groups showed that males had higher IGFBP-7 values than females in Q2 and Q4, lower values in Q1, and almost equal values in Q3 [**Figure 10**].

Table 38: IGFBP-7 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
IGFBP-7	5590.16 \pm 4696.43	3600.21	4393.29

Table 39: IGFBP-7 among gender groups

IGFBP-7	N	Mean	SD	Median	IQ Range
Female	n=44	5343.01	4859.81	3221.19	3799.18
Male	n=30	5952.64	4502.60	5089.87	5443.73
P-value	0.60				

Table 40: IGFBP-7 among different age groups

IGFBP-7	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	6558.95 \pm 4757.75	4465.04	8939.00
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	7710.00 \pm 5920.37	6673.70	6912.15
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	3747.02 \pm 3503.11	2539.71	1486.68
4th Quartile (Q4) (Age: >27 years old)	n=20	4354.12 \pm 3239.52	3214.57	3803.64
P-value	0.0157*			

(*) Statistically significant at $p < 0.05$

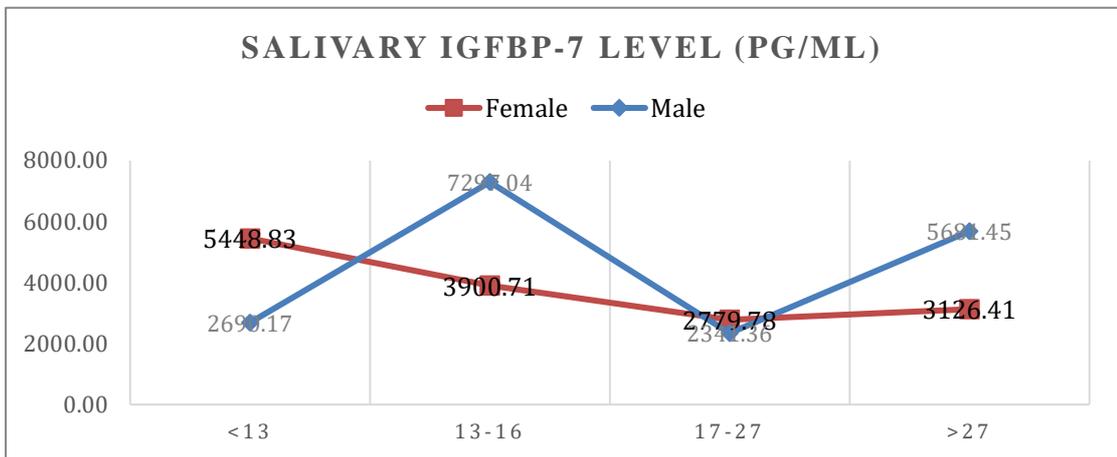
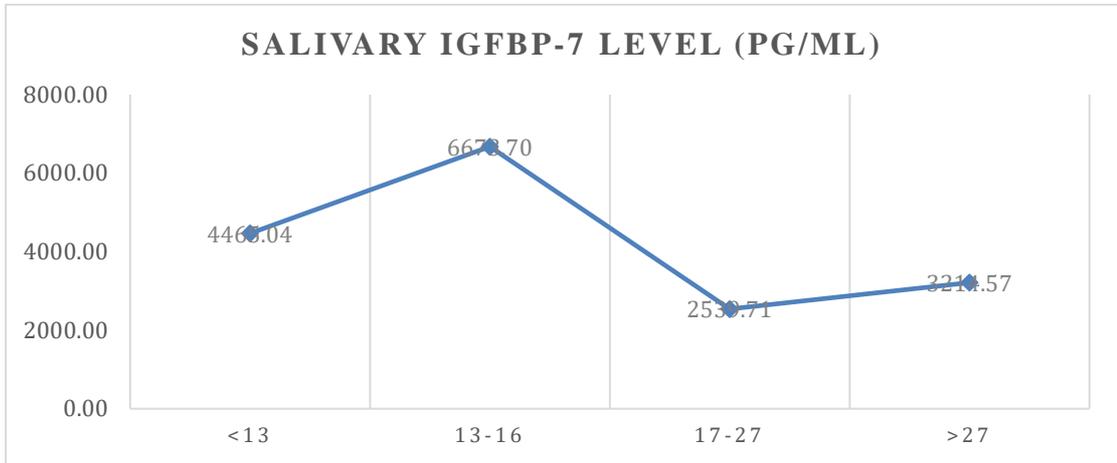


Figure 10: Median IGFBP-7 levels among different age groups (top), and among females and males separately (bottom).

Table 41: Multiple comparison analysis of IGFBP-7 levels between the sample age groups.

(Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method)			
IGFBP-7			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	0.9705
Q1 vs. Q3	-2.8297	4.0018	0.1407
Q1 vs. Q4	-0.8596	1.2157	0.4988
Q2 vs. Q3	-2.2369	3.1634	0.0237*
Q2 vs. Q4	-0.6900	0.9758	0.1294
Q3 vs. Q4	1.4620	2.0676	0.8168

Age groups based on quartiles: Q1: (<13 years old) , Q2: (13-16 years old) , Q3: (17-27 years old) , Q4: (<27 years old)

*: Significant at $p < 0.05$

IL-1 β : Descriptive measures for IL-1 β in the study population are presented in **Table 42**. Data showed an overall decrease in IL-1 β with age. There was significant decrease in IL-1 β from Q1 to Q3 ($p < 0.05$), and a marked increase between Q3 and Q4 (significant at $p < 0.05$) [**Tables 44 and 45**] [**Figure I.11**]. Females showed higher values than males which was found not to be statistically significant [**Table 43**]. Female values were higher in Q2 and Q3 [**Figure 11**].

Table 42: IL-1 β levels among the study population

Variable	Mean \pm SD	Median	IQ Range
IL-1β	441.94 \pm 429.95	301.24	395.13

Table 43: IL-1 β among gender groups

IL-1β	N	Mean	SD	Median	IQ Range
Female	n=44	423.23	293.96	337.79	351.14
Male	n=30	469.39	579.57	249.53	461.19
P-value	0.38				

Table 44: IL-1 β among different age groups

IL-1β	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	622.51 \pm 608.47	487.11	592.96
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	500.75 \pm 511.51	263.17	424.49
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	233.65 \pm 155.24	180.90	189.39
4th Quartile (Q4) (Age: >27 years old)	n=20	426.15 \pm 249.56	351.58	389.87
P-value	0.0209*			

(*) Statistically significant at p<0.05

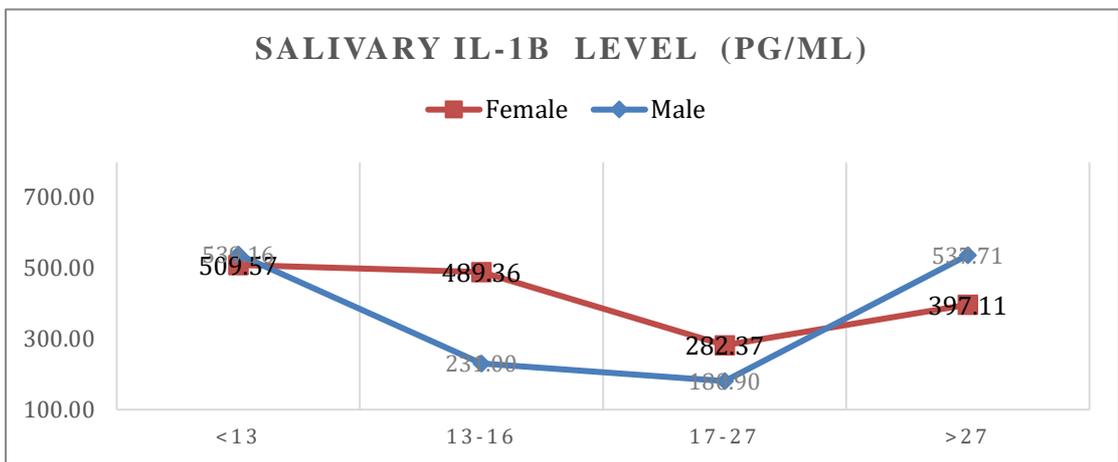
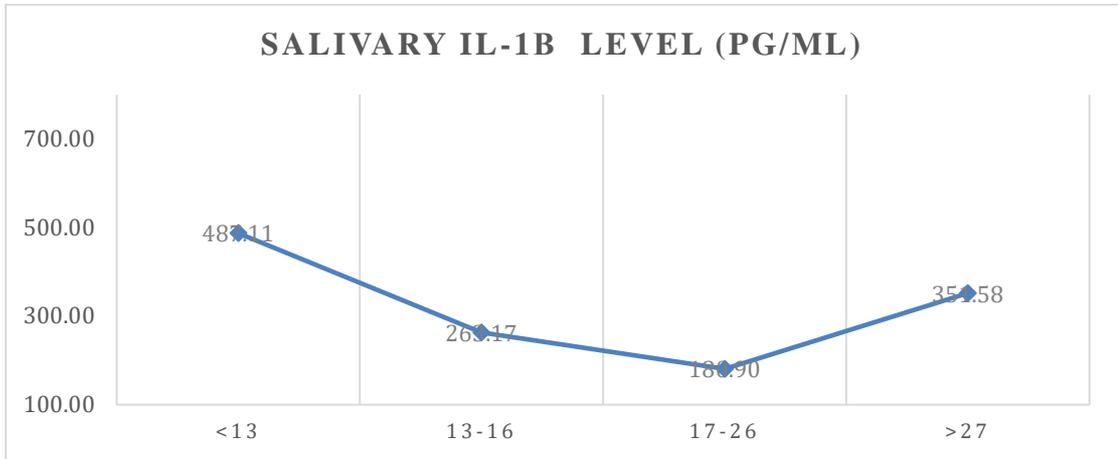


Figure 11: Median IL-1 β levels among different age groups (top), and among females and males separately (bottom)

Table 45: Multiple comparison analysis of IL-1 β levels between the sample age groups.

(Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method)			
IL-1β			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	0.8842
Q1 vs. Q3	-2.8297	4.0018	0.0358*
Q1 vs. Q4	-0.8596	1.2157	0.8842
Q2 vs. Q3	-2.2369	3.1634	0.1562
Q2 vs. Q4	-0.6900	0.9758	0.9774
Q3 vs. Q4	1.4620	2.0676	0.0458*
Age groups based on quartiles: Q1: (<13 years old) , Q2: (13-16 years old) , Q3: (17-27 years old) , Q4: (<27 years old)			
*: Significant at P<0.05			

IL-2: Descriptive measures for IL-2 in the study population are presented in **Table 46**. There was an overall increase with age, with a peak in Q3. Levels decreased between Q1 and Q2, increased in Q3 then decreased in Q4. The difference across age groups was not statistically significant ($p < 0.05$) [**Table 48**] [**Figure 12**]. Gender difference across sample groups was not significant ($p < 0.05$) [**Table 47**]. Across age groups, females showed higher values in Q2 and Q3, while males showed higher values in Q4 [**Figure 12**].

Table 46: IL-2 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
IL-2	40.15 \pm 33.34	30.69	24.99

Table 47: IL-2 among gender groups

IL-2	N	Mean	SD	Median	IQ Range
Female	n=44	42.74	36.57	30.56	29.98
Male	n=30	36.35	28.10	31.44	20.00
P-value	0.70				

Table 48: IL-2 among different age groups

IL-2	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	31.42 \pm 13.68	30.81	8.24
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	39.77 \pm 36.75	25.06	29.26
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	43.17 \pm 35.61	33.56	25.48
4th Quartile (Q4) (Age: >27 years old)	n=20	44.79 \pm 39.25	27.56	41.71
P-value	0.88			

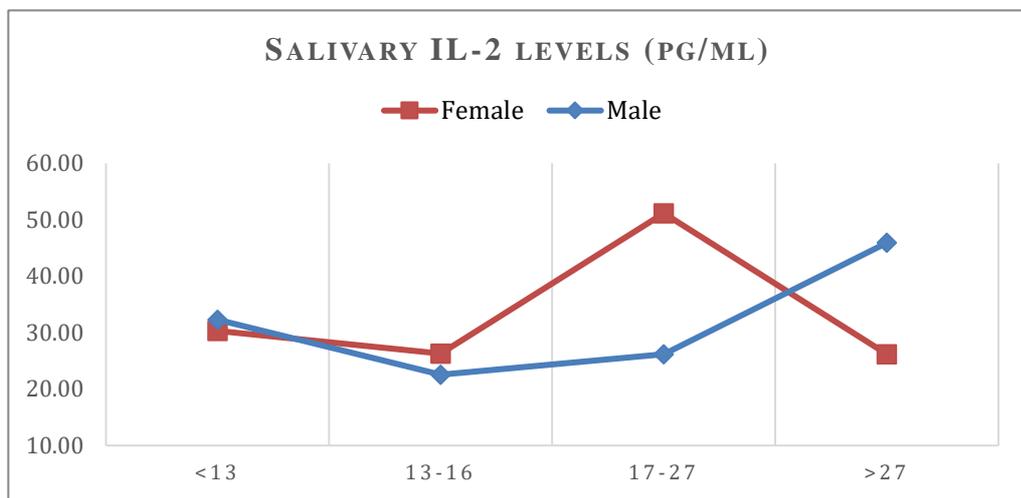
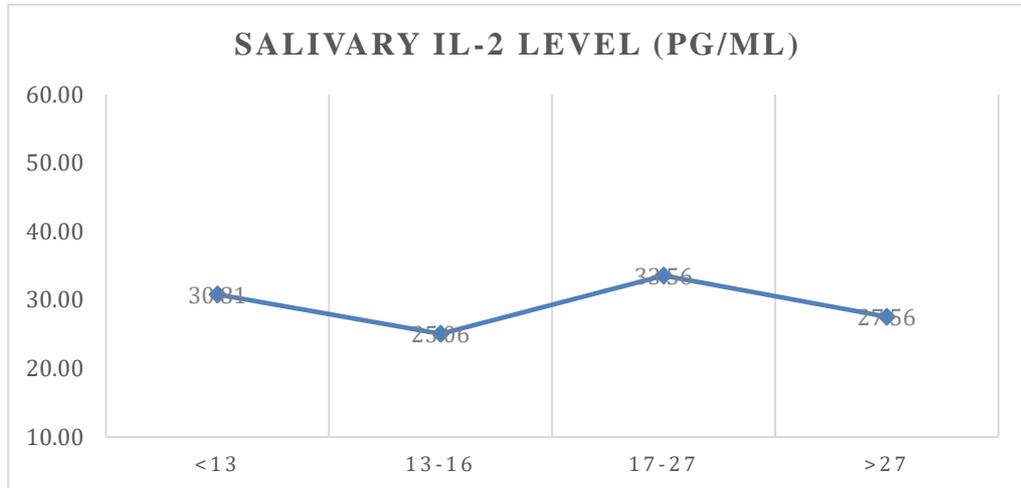


Figure 12: Median IL-2 levels among different age groups (top), and among females and males separately (bottom).

IL-6: Descriptive measures for IL-6 in the study population are presented in **Table 49**. There was a slight change in IL-6 with age which was not statistically significant at $p < 0.05$. Levels appeared to be unchanged between Q1, Q2 and Q3, then a mild change was observed in Q4 [**Table 51, Figure 13**]. No statistical significance was found between males and females at $p < 0.05$ [**Table 50**]. Males showed a slight decrease in Q2 and Q3, with a marked increase in Q4, while female values slightly gradual increase across age groups.

Table 49: IL-6 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
IL-6	24.26 \pm 55.25	10.33	10.97

Table 50: IL-6 levels among gender groups

IL-6	N	Mean	SD	Median	IQ Range
Female	n=44	15.72	13.77	11.25	8.38
Male	n=30	36.79	84.45	8.79	14.01
P-value	0.25				

Table 51: IL-6 among different age groups

IL-6	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	20.15 \pm 35.71	9.56	5.91
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	19.69 \pm 31.78	10.63	14.27
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	12.49 \pm 13.00	9.01	7.73
4th Quartile (Q4) (Age: >27 years old)	n=20	42.72 \pm 95.06	13.11	26.14
P-value	0.1095			

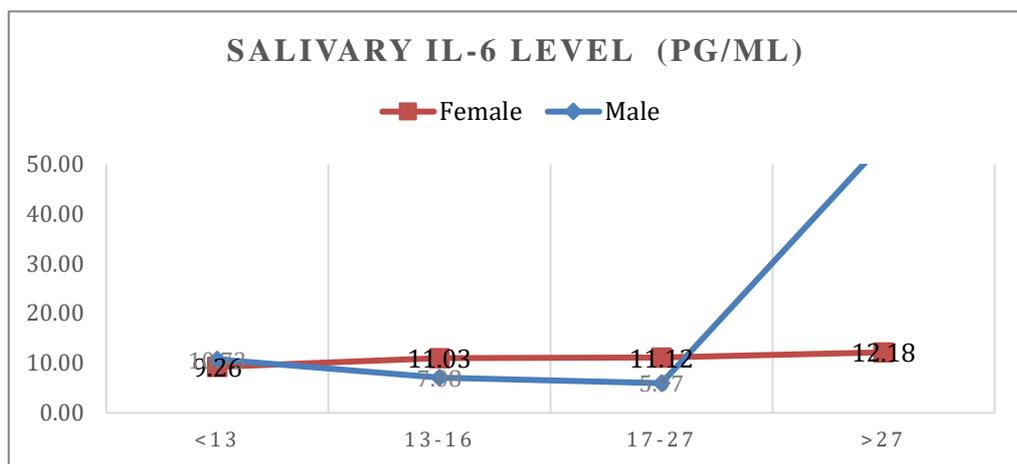
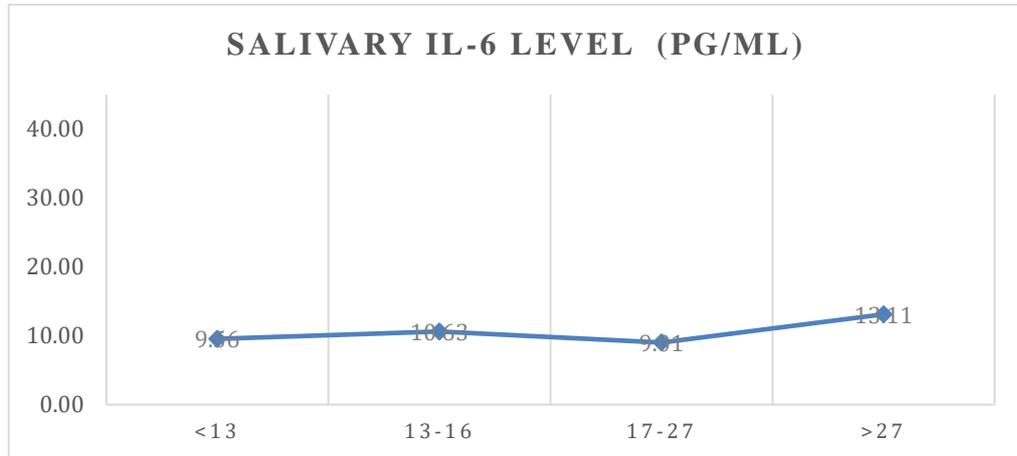


Figure 13: Median IL-6 levels among different age groups (top), and among females and males separately (bottom).

IL-8: Descriptive measures for IL-8 in the study population are presented in **Table 52**. IL-8 showed an overall increase with age which was not found statistically significant $p < 0.05$ [**Table 54, Figure 14**]. Levels increased from Q1 to Q2, and then decreased in Q3, followed by an increase in Q4. Females showed higher values than males, which was not significant at $p < 0.05$ [**Table 53**]. Comparing genders across age groups, males showed high values in Q1 that decreased with age, until a slight increase occurred in Q4. Females showed higher values than males in Q4 [**Figure 14**].

Table 52: IL-8 levels in the study population

Variable	Mean \pm SD.	Median	IQ Range
IL-8	965.54 \pm 975.70	700.68	564.30

Table 53: IL-8 among gender groups

IL-8	N	Mean	SD	Median	IQ Range
Female	n=44	1007.60	1154.86	635.54	813.60
Male	n=30	904.97	654.61	713.16	440.38
P-value	0.58				

Table 54: IL-8 among different age groups

IL-8	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	1272.32 \pm 1720.42	594.29	725.84
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	922.62 \pm 623.34	723.07	1019.79
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	690.94 \pm 590.17	583.04	421.28
4th Quartile (Q4) (Age: >27 years old)	n=20	1072.09 \pm 730.04	871.12	1069.47
P-value	0.38			

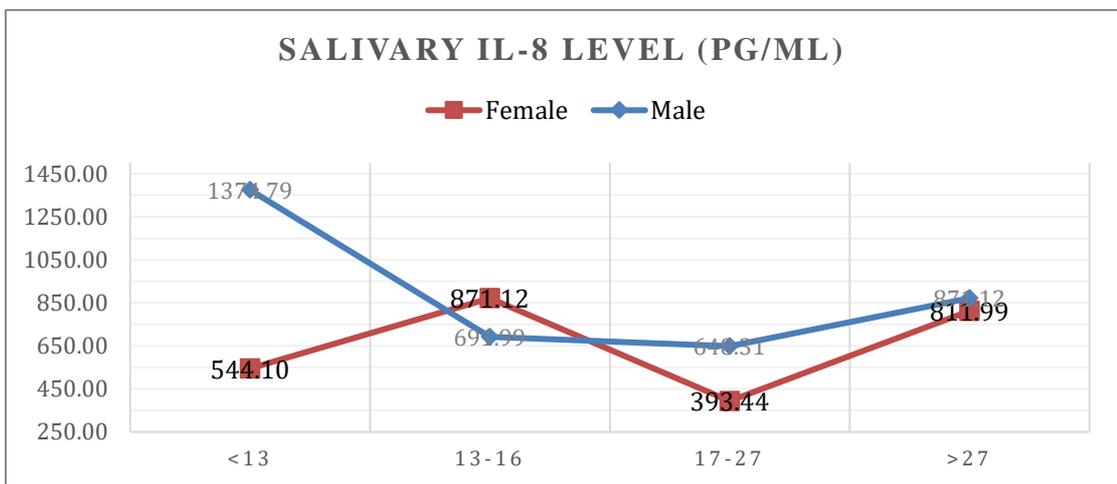
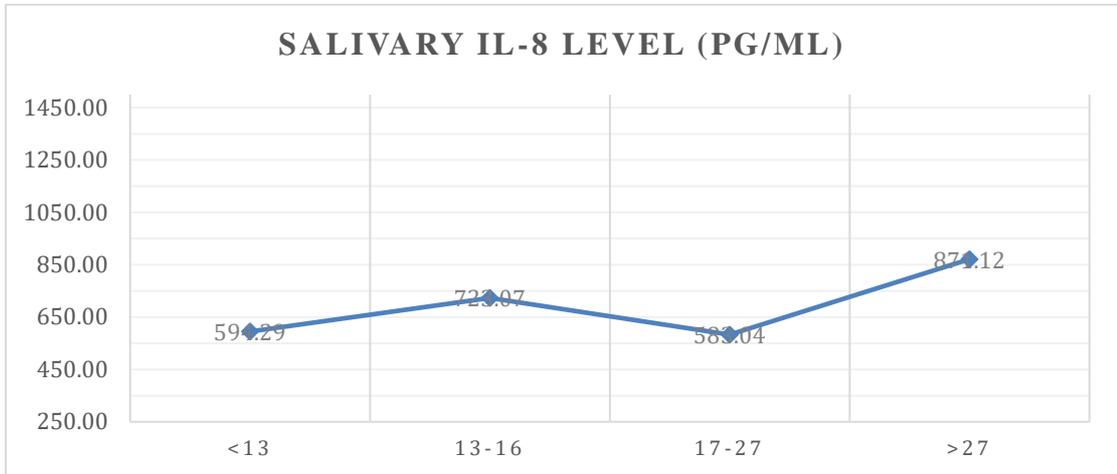


Figure 14 Median IL-8 levels among different age groups (top), and among females and males separately (bottom).

TNF- α : Descriptive measures for TNF- α in the study population are presented in **Table 55**. Values decreased from Q1 to Q2, increased in Q3, then decreased again in Q4. Difference between age groups was not statistically significant ($p < 0.05$) [**Table 57**] [**Figure 15**]. Females showed higher values than males but this was not significant. [**Table 56**]. Highest values among females were in Q3, while males had higher values in Q4 [**Figure 15**].

Table 55: TNF- α levels in the study population

Variable	Mean \pm SD	Median	IQ Range
TNF-α	105.20 \pm 114.48	73.12	151.82

Table 56: TNF- α among gender groups

TNF-α	N	Mean	SD	Median	IQ Range
Female	n=44	109.22	113.90	78.97	153.62
Male	n=30	99.31	117.02	65.89	125.50
P-value	0.44				

Table 57: TNF- α among different age groups

TNF-α	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	92.38 \pm 103.05	76.55	92.67
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	95.34 \pm 135.85	12.90	130.21
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	124.96 \pm 103.39	84.11	104.54
4th Quartile (Q4) (Age: >27 years old)	n=20	107.54 \pm 115.48	45.58	167.14
P-value	0.36			

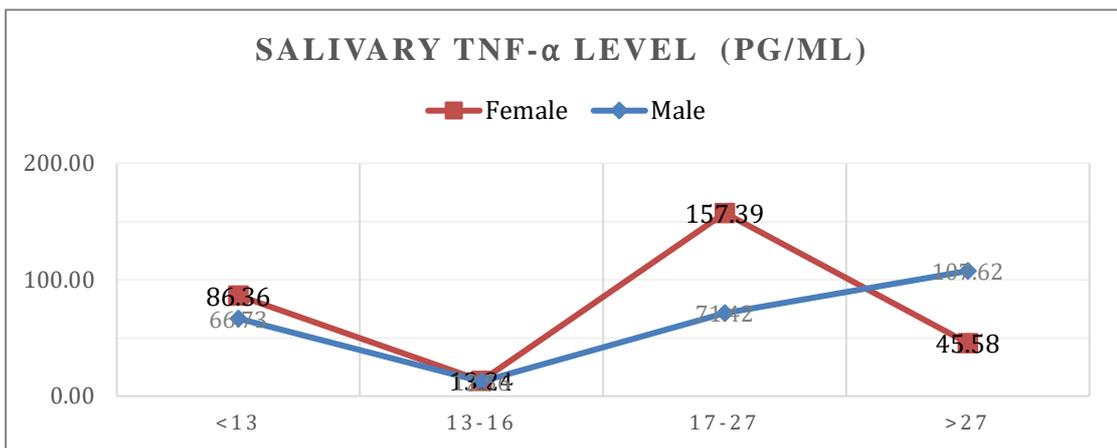
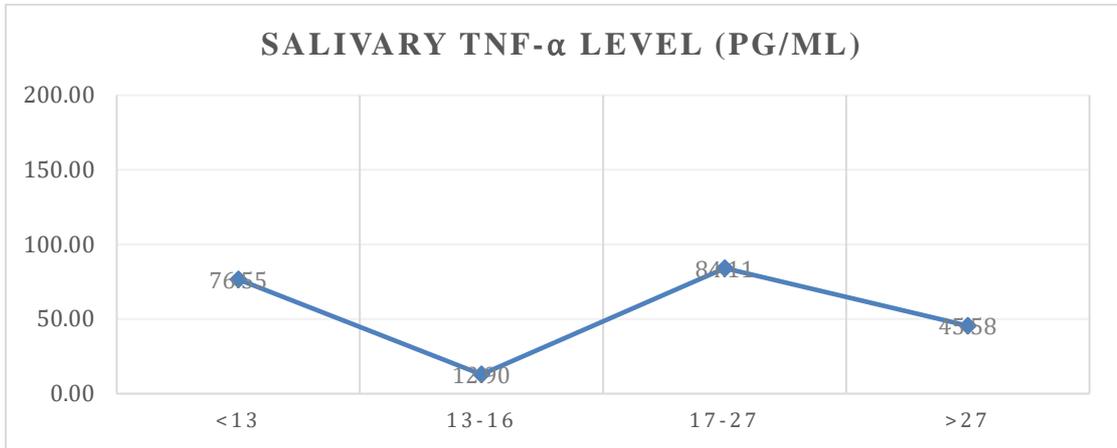


Figure 15: Median TNF- α levels among different age groups (Top), and among females and males separately (Bottom).

Lactoferrin: Descriptive measures for lactoferrin in the study population are presented in **Table 58**. There was a decrease in lactoferrin with age after Q2, with lowest values in Q3 (statistically significant at $p < 0.05$). A marked increase was noticed between Q4 and Q3, but levels were still lower than Q1 and Q2 (not statistically significant at $p < 0.05$) [**Tables 60 and 61**] [**Figure 16**]. There was no significant difference between males and females [**Table 59**]. Among age groups, males had lower values than females in Q3 and higher values in Q4. Other age groups showed similar values in both genders [**Figure 16**].

Table 58: Lactoferrin levels in the study population

Variable	Mean \pm SD	Median	IQ Range
Lactoferrin	23029.41 \pm 2278.10	23787.80	677.50

Table 59: Lactoferrin among gender groups

Lactoferrin	N	Mean	SD	Median	IQ Range
Female	n=44	23211.66	1827.43	23724.30	767.50
Male	n=30	22762.10	2826.27	23818.80	671.00
P-value	0.51				

Table 60: Lactoferrin among different age groups

Lactoferrin	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	23744.96 \pm 311.53	23821.55	401.50
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	23847.47 \pm 310.39	23962.30	233.25
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	21110.02 \pm 3993.30	23137.80	2779.00
4th Quartile (Q4) (Age: >27 years old)	n=20	23366.35 \pm 867.95	23801.80	848.00
P-value	0.0002*			

(*) Statistically significant at $p < 0.05$

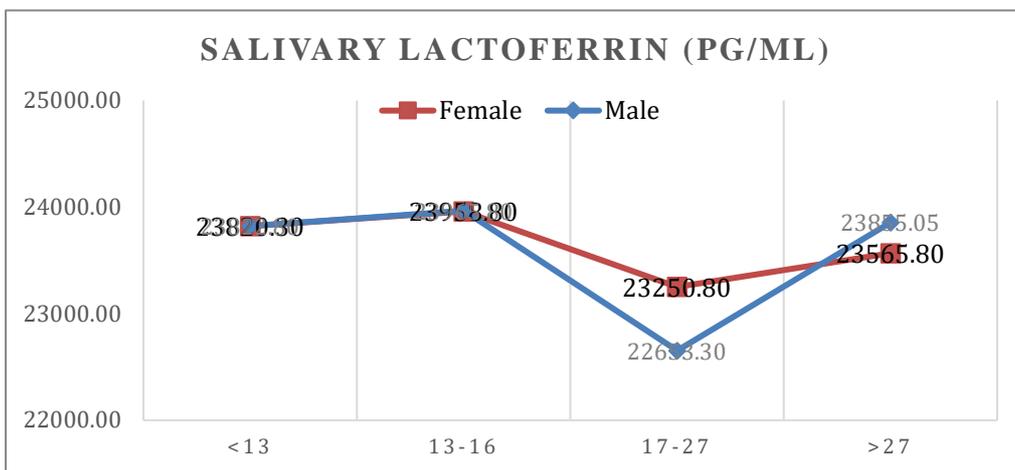
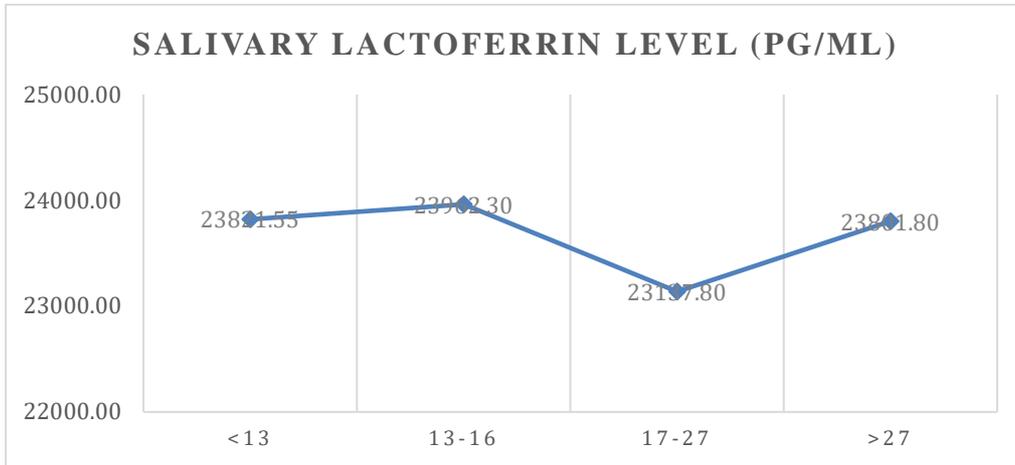


Figure 16: Median Lactoferrin levels among different age groups (top), and among females and males separately (bottom).

Table 61: Multiple comparison analysis of Lactoferrin levels between the sample age groups.

(Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method)			
Lactoferrin			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	0.4397
Q1 vs. Q3	-2.8297	4.0018	0.0092*
Q1 vs. Q4	-0.8596	1.2157	0.8906
Q2 vs. Q3	-2.2369	3.1634	0.0003*
Q2 vs. Q4	-0.6900	0.9758	0.1257
Q3 vs. Q4	1.4620	2.0676	0.0894

Age groups based on quartiles: Q1: (<13 years old) , Q2: (13-16 years old) , Q3: (17-27 years old) , Q4: (<27 years old)

*: Significant at P<0.05

MMP-1 Descriptive measures for MMP-1 in the study population are presented in [Table 62]. There was a decrease from Q1 to Q2, then values increased in Q3 and Q4. Difference across age groups was not statistically significant at $p < 0.05$ [Table 64]. Difference among genders was not statistically significant [Table 63]. Females showed higher values than males in all age groups except in Q4 [Figure 17].

Table 62: MMP-1 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
MMP-1	147.53 \pm 461.09	34.81	65.36

Table 63: MMP-1 among gender groups

MMP-1	N	Mean	SD	Median	IQ Range
Female	n=44	159.39	564.60	38.80	64.34
Male	n=30	130.14	248.98	30.16	99.53
P-value	0.73				

Table 64: MMP-1 among different age groups

MMP-1	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	144.18 \pm 211.85	45.37	105.95
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	322.93 \pm 851.11	27.96	117.92
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	60.49 \pm 79.95	32.35	26.86
4th Quartile (Q4) (Age: >27 years old)	n=20	53.14 \pm 48.79	36.99	59.74
P-value	0.81			

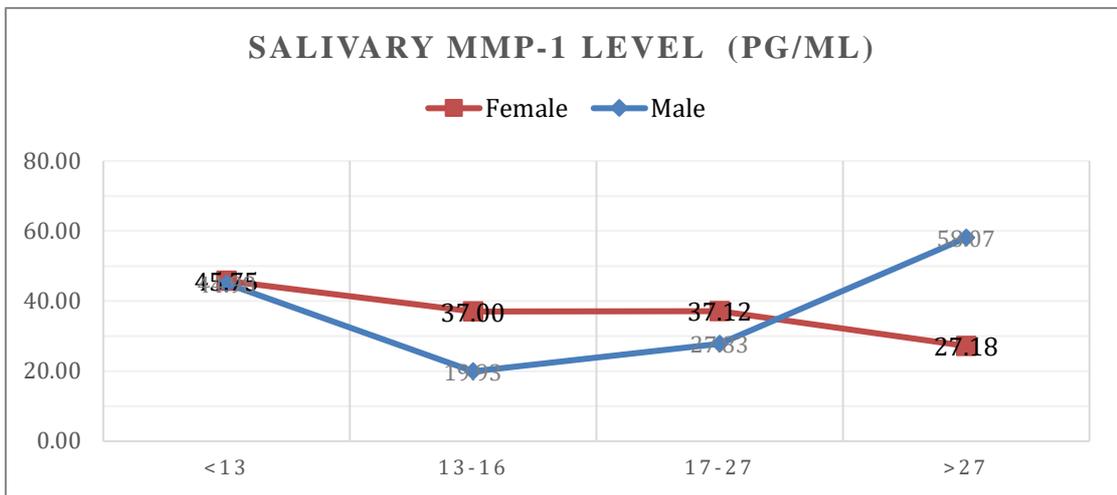
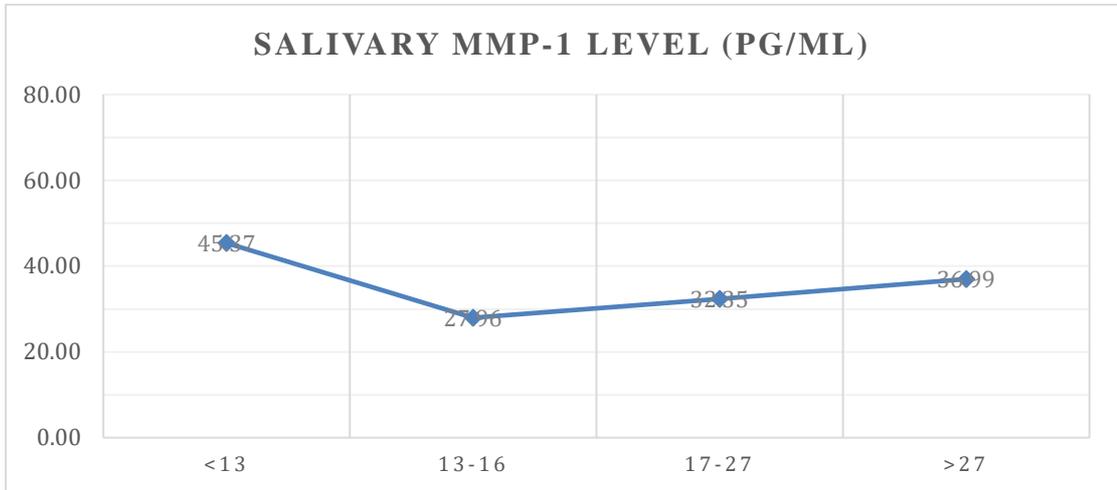


Figure 17: Median MMP-1 levels among different age groups (Top), and among females and males separately (Bottom).

MMP-2: Descriptive measures for MMP-2 in the study population are presented in **Table 65**. There was an overall decrease in MMP-2 level across age groups. The lowest values were in Q3, which was marginally significant compared to Q1 at $p < 0.05$. MMP-2 levels increased in Q4 but did not exceed Q1 and Q2 values [**Tables 67 and 68**] [**Figure 18**]. The difference between genders was not statistically significant at $p < 0.05$ [**Table 66**]. Females showed higher values than males in Q1, Q2 and Q3, while males showed higher values in Q4 [**Figure 18**].

Table 65: MMP-2 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
MMP-2	2496.48 \pm 954.84	2340.01	1495.34

Table 66: MMP-2 among gender groups

MMP-2	N	Mean	SD	Median	IQ Range
Female	n=44	2658.27	988.52	2611.58	1333.73
Male	n=30	2259.18	864.95	2069.10	1264.68
P-value	0.08				

Table 67: MMP-2 among different age groups

MMP-2	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	2753.07 \pm 1003.32	2803.08	1615.15
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	2628.72 \pm 973.44	2604.51	1917.38
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	1933.92 \pm 589.20	1950.32	916.40
4th Quartile (Q4) (Age: >27 years old)	n=20	2665.27 \pm 1022.91	2497.48	1310.21
P-value	0.037*			

(*) Statistically significant at $p < 0.05$

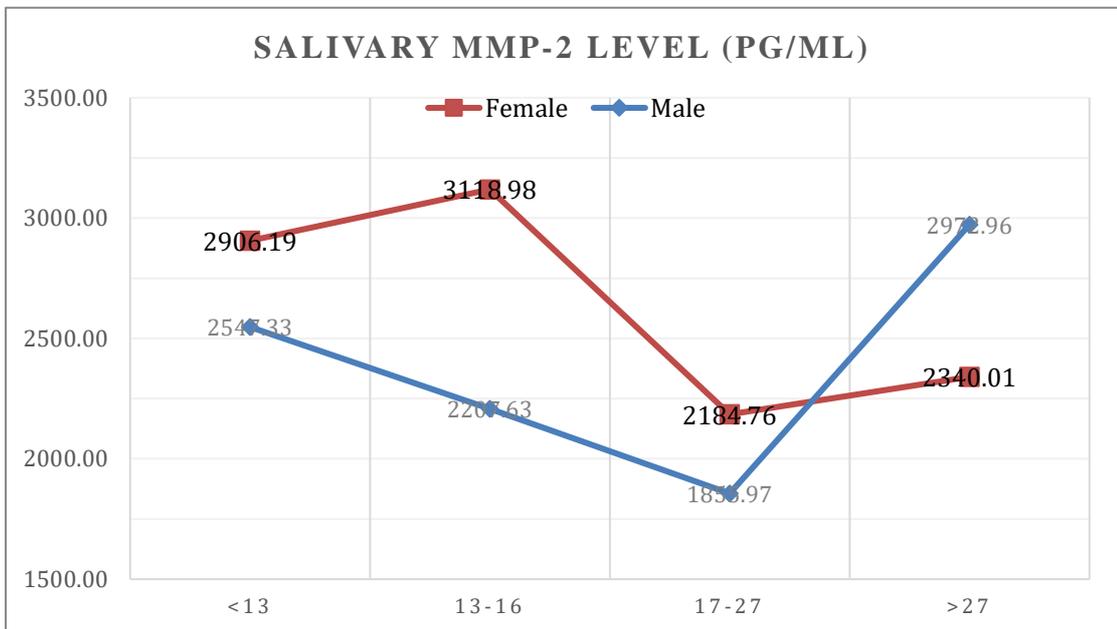
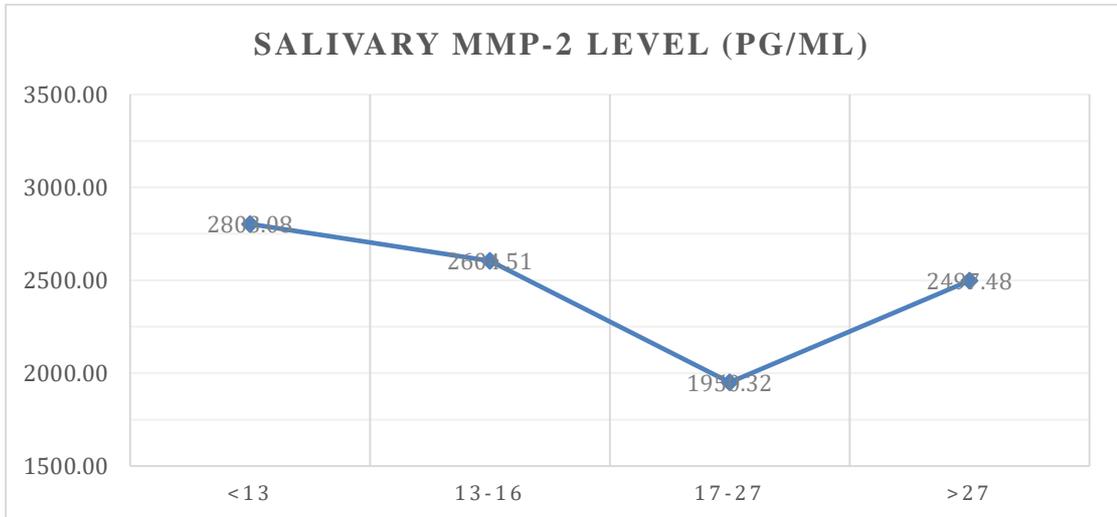


Figure 18: Median MMP-2 levels among different age groups (top), and among females and males separately (bottom).

Table 68: Multiple comparison analysis of MMP-2 levels between the sample age groups.

(Pairwise Two-Sided Multiple Comparison Analysis; Dwass, Steel, Critchlow-Fligner Method)			
MMP-2			
Age groups	Wilcoxon Z	DSCF Value	Pr > DSCF (P-Value)
Q1 vs. Q2	-0.0318	0.0450	0.9853
Q1 vs. Q3	-2.8297	4.0018	0.0624
Q1 vs. Q4	-0.8596	1.2157	0.9853
Q2 vs. Q3	-2.2369	3.1634	0.1252
Q2 vs. Q4	-0.6900	0.9758	0.9998
Q3 vs. Q4	1.4620	2.0676	0.0776

Age groups based on quartiles: Q1: (<13 years old) , Q2: (13-16 years old) , Q3: (17-27 years old) , Q4: (<27 years old)

*: Significant at P<0.05

MMP-13: Descriptive measures for MMP-13 in the study population are presented in **Table 69**. Data showed that MMP-13 levels increased with age, with levels being highest in Q3 [**Figure 19**]. The difference among age groups was not statistically significant at $p < 0.05$ [**Table 71**]. Difference between genders was not significant at $p < 0.05$ [**Table 70**]. Across age groups, females had higher values in Q1, Q2 and Q4 [**Figure 19**].

Table 69: MMP-13 levels in the study population

Variable	Mean \pm SD	Median	IQ Range
MMP-13	294.33 \pm 215.55	255.74	229.96

Table 70: MMP-13 among gender groups

MMP-13	N	Mean	SD	Median	IQ Range
Female	n=44	289.87	214.78	235.60	219.80
Male	n=30	301.94	221.28	263.57	253.53
P-value	0.97				

Table 71: MMP-13 among different age groups

MMP-13	N	Mean \pmSD	Median	IQ Range
1st Quartile (Q1) (Age: <13 years old)	n=16	255.41 \pm 222.61	190.35	291.95
2nd Quartile (Q2) (Age: 13 -16 years old)	n=20	296.91 \pm 195.97	259.51	168.74
3rd Quartile (Q3) (Age: 17 -27 years old)	n=18	319.41 \pm 229.91	272.16	226.97
4th Quartile (Q4) (Age: >27 years old)	n=20	304.91 \pm 227.09	271.40	232.55
P-value	0.83			

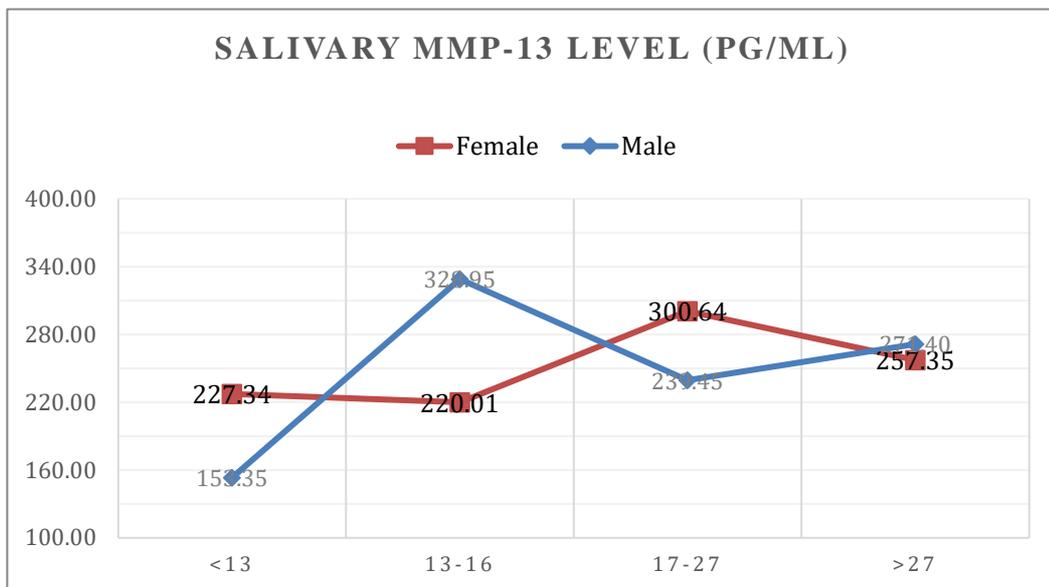
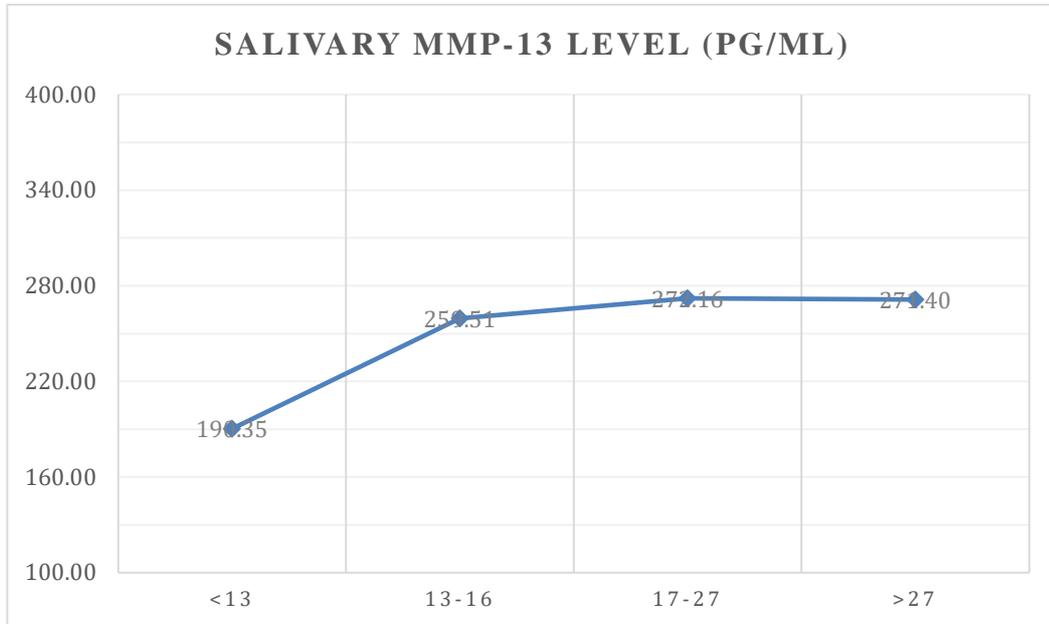


Figure 19: Median MMP-13 levels among different age groups (top), and among females and males, separately (bottom).

DISCUSSION

Aging is characterized by multiple cellular and tissue level changes. This includes the aging of salivary glands and therefore impacting the saliva composition. Salivary gland function and saliva flow rate are known to decrease with aging (Nam et al., 2019). Comparisons of saliva between young and old subjects showed differences in salivary mineral content, cell populations, levels of inflammatory cytokines and proteomic profile (Jenkinson et al., 1999). In this study we investigated the changes in inflammatory and senescence-related markers in saliva with age.

BMP-4

BMP-4 is one of the tissue forming proteins. It has an important function in signaling for cartilage and bone formation. It also plays a role in tooth development, limb formation and fracture repair (Chen et al., 2004; Grzibovskis et al., 2010). BMP-4 has been investigated in cancer research for its effect on tumor growth suppression. It was reported to induce replicative senescence to malignant lung cells *in vitro* (Katakura et al., 1999; Buckley et al., 2004).

BMP-4 signaling is also known to inhibit hippocampus neurogenesis. Meyers et al. showed that there is an age-related increase in BMP-4 expression that is proportional to the decline in neurogenesis and cognition (Meyers et al., 2016). It is reported that inhibition of BMP-4 signaling in the brain caused an increase in neural stem cell proliferation, increased neurogenesis and improved cognitive function (Yousef et al., 2015; Meyers et al., 2016).

BMP-4 serum levels are also associated with obesity and metabolic syndrome, as it is thought to be involved in adipocyte differentiation and insulin resistance (Son et al., 2011).

Albilgia *et al.* reported that high serum level of BMP-4 are linked to poor prognosis of degenerative joint disease of the hip and TMJ. It was also correlated with patients' need for surgical arthroplasty (Albilgia *et al.*, 2013). Salles *et al.* reported that BMP-4 expression in saliva was linked to tendinitis among athletes (Salles *et al.*, 2015).

In salivary glands, BMP-4 was reported to have an inhibitory effect on tissue proliferation, reduced salivary gland duct branching and end bud number (Hoffman *et al.*, 2002; Mattingly *et al.*, 2015). Similar effects of BMP-4 were found in other glandular organs such as kidney and prostate (Lamm *et al.*, 2001; Piscione *et al.*, 2001).

Our results showed an age-related increase in BMP-4 in saliva, particularly during the first three age quartiles. This increase might be associated with the reported decline in salivary gland function with age. Our study results also agree with findings by Meyers *et al.*, who found that BMP-4 increased with age. However, that does not explain the minimal change in Q4 compared to Q1 and Q2. Given the non-normal distribution of the data, the median value was a better measure to describe the results; however, the mean of BMP-4 level showed more increase in Q4 (Mean 93.73, median 58.79). To have better conclusions about the change among older individuals, the sample size might need to be increased to allow for better chance in data normality.

No published research was found about changes in saliva levels of BMP-4 with age. Since the local effect of BMP-4 on salivary tissue has been reported (Hoffman *et al.*, 2002; Mattingly *et al.*, 2015). Further investigation that can correlate between BMP-4 and other parameters of salivary gland functions might be of important value for future studies.

Fibronectin

Fibronectin is one of the extra-cellular matrix glycoproteins that has multiple functions in cell adhesion, motility and growth (Kumazaki et al., 1997; Li-Korotky et al., 2007). It has a role in wound healing and tissue remodeling by regulating extracellular matrix components. Fibronectin regulates and is regulated by multiple proteases and enzymes in the extracellular matrix. It also participates in modulating the inflammatory response in cells (Kumazaki et al., 1997; Kaufman et al., 2000; Bhat et al., 2019).

In addition to its presence in serum, fibronectin has been investigated in multiple body fluids such as saliva, tears, breast milk and urine (Kanehisa et al., 1991; M. Fukuda et al., 1996; Dong et al., 2018).

One of the main roles of fibronectin in the oral cavity is regulating host colonization by bacteria. Soluble fibronectin has an inhibitory effect on the adherence of *Streptococcus mutans* to teeth (Babu et al., 1986). It also inhibits the effect of *Porphyromonas gingivalis* on periodontal tissues (Murakami et al., 1998). Low levels of fibronectin in saliva were found related to periodontal disease and susceptibility to dental caries (Llena-Puy et al., 2000; Laputková et al., 2018).

Conflicting results were found about age-related changes in circulating fibronectin levels. One report by Ignjatovic *et al.* measured plasma fibronectin levels in a groups of children and adults. The study age range included neonates from 0-3 days old, children from 1-16 years old and adults from 21-43 years old. The report did not show quantitative values; however, it showed that fibronectin was higher in neonates and children compared to adults (Ignjatovic et al., 2011).

In contrast, Labat-Robert *et al.* reported that plasma fibronectin increased with age among healthy subjects (Labat-Robert et al., 1981). However, when the same

comparison was done in a sample of breast cancer and diabetic patients, plasma fibronectin showed no correlation with age (Labat-Robert et al., 1984, 1985, 1988). This was explained by the authors as a result of increased tissue permeability in diabetes, leading to the accumulation of fibronectin in tissues. Similarly, in cancer, cells are thought to lose their ability to retain fibronectin and it is usually trapped in the tumor stroma, leading to decreases plasma fibronectin levels (Labat-Robert et al., 1981, 1984, 1985, 1988).

Another report by McCafferty *et al.* measured serum fibronectin in healthy children from age 0-15 years old. They reported that it increased from infancy to one year of age, then stayed constant from 1-15 years of age (McCafferty et al., 1983). On cellular level, increased expression of fibronectin was reported in aged endothelial cells (Kumazaki et al., 1997; Bhatwadekar et al., 2008).

No reported studies were found in the literature about change in saliva fibronectin with aging. Our results indicated an overall decrease in fibronectin with age. This decrease was consistent among the first three quartiles; (Q1, Q2, and Q3). Fourth quartile levels showed an increase compared to Q3, but were still lower than Q1. Findings by Labat-Robert *et al.* are in contrast with the decrease found from Q1 to Q3, and in agreement with the increase found from Q3 to Q4 in our sample group. In regards to the reports by McCafferty *et al.*, comparison with our results cannot be accurate as their sample age range was only from 0 to 15 years old. However, this age range could be most closely comparable to the first and second quartiles of age (Q1: 8 to 12 vs Q2: 13-16 years old). Our results reported a decrease from Q1 to Q2 with was opposite to the increase they found from age 0-15 years old (McCafferty et al., 1983).

In support of our findings, Ignjatovic *et al.* showed a higher level in children (0-16 years old) compared to adults (21-43 years old). This could be similar to the higher

level we found in Q1 and Q2 (<16 years old), compared to Q3 and Q4 (>17 years old) (Ignjatovic et al., 2011). However, these comparisons cannot be applied on the increase seen between Q3 and Q4.

Until today, there is not enough information available to accurately compare circulating fibronectin with salivary fibronectin. A number of factors might suggest an explanation for the age-related decrease seen in this study. First, changes in salivary fibronectin might be related to changes in cellular composition of saliva with age. Theda *et al.* reported that there was a change in cells population found in saliva between children and adults. The most predominant cell type in children's saliva was epithelial cells, while in adults, it was leukocytes (Theda et al., 2018). Fibronectin is reported to be produced by epithelial cells in addition to multiple other cell types (Sakai et al., 2003; Larsen et al., 2006). However, it is not reported to be produced by leukocytes. This might explain why higher levels were observed in saliva from children than adults.

Second, current findings might be related to the function fibronectin has in saliva. Fibronectin has anti-microbial functions by preventing bacterial adhesion. It also contributes in modulating inflammatory response in oral tissues. Change in fibronectin levels might be a function of changes that happen in immune system with aging. This would contribute to the nature of the increased inflammation and infection risk among older adults.

VEGF

Vascular endothelial growth factor (VEGF) is a one of the markers of angiogenesis. It has an important role in tissue remodeling and vascularization. It is also thought to have a role in bone formation and resorption (Di Domenico et al., 2012). VEGF has a regenerative effect in multiple tissues including eyes, bone, salivary

glands, and skin. This effect happens through its ability to enhance vascularization and tissue repair (Nam et al., 2019). In a mouse model, VEGF was found to have a regenerative effect on salivary gland tissues and improve wound healing (Nam et al., 2019).

VEGF has been reported to have multiple changes with age and age-related pathologies. Villar *et al.* reported that VEGF mRNA expression in brain of rats was decreased with aging (Villar-Cheda et al., 2009).

Aging is known to be accompanied with changes vascularity in the brain and other tissues (H. Wang et al., 2004; Iemitsu et al., 2006). Since VEGF vitally contributes to angiogenesis, it is thought to have an association with age-related changes in vascular tissues. (H. Wang et al., 2004; Iemitsu et al., 2006). VEGF levels show changes with Parkinson's, as an indication of altered brain vascularization (Faucheux et al., 1999; Barcia et al., 2005). Mernerros *et al.* reported that cardiac hypertrophy was associated with chronic increase in VEGF (Marneros, 2018).

VEGF is also associated with obstructive sleep apnea (OSA). OSA causes hypoxia, which turns on the body's physiological response to release VEGF and stimulates angiogenesis (Peled et al., 2006). De Oliveira *et al.* investigated VEGF expression in tissue samples from salivary gland biopsies. They reported no difference was found in VEGF with age (De Oliveira et al., 2002). This study results showed an overall decrease in VEGF with age. This is in contrast findings by De Oliveira *et al.* (De Oliveira et al., 2002), and might be contributed to the reduced regenerative function in salivary tissue with age. From our findings, we conclude that saliva levels of VEGF do not follow the trend observed in serum. More research is needed to understand changes in salivary VEGF with age.

IGFBP-2

IGFBP-2 is secreted by vascular smooth muscle cells, and has been reported to have a role in tissue turnover, metabolism and insulin balance. It plays a role in insulin sensitivity and low levels are associated with obesity and metabolic dysfunction. Blum *et al.* reported that low IGFBP-2 serum levels were observed with untreated insulin-dependent diabetes mellitus (Blum et al., 1993; Zhang et al., 2019).

IGFBP-2 has an effect on bone metabolism. Amin *et al.* reported that IGFBP-2 was positively correlated to osteocalcin and bone tissue turnover. Increased serum IGFBP-2 was linked to decreased bone mineral density (Amin et al., 2004, 2007). Increased IGFBP-2 serum level was also linked to chronic renal failure.

IGFBP-2 plays a role in cancer development. It is thought to contribute to cancer cell invasion, metastasis and tissue resistance to chemotherapy (Yau et al., 2015). Increased IGFBP-2 has been reported with multiple tumors including breast cancer, esophageal adenocarcinoma, lung cancer, ovarian cancer, pancreatic tumor and leukemia (Blum et al., 1993; Yau et al., 2015).

IGFBP-2 was detectable in saliva from patients with salivary adenoid cystic carcinoma, and showed a positive correlation with cancer severity and poor prognosis (Yau et al., 2015).

IGFBP-2 is known to be highly expressed in embryonic tissue. It then decreases after birth until age of puberty. Little change in IGFBP-2 is observed during puberty. After puberty, it starts to increase gradually with a marked increase shown after 50-60 years of age (Clemmons et al., 1991; Juul et al., 1995; Amin et al., 2004, 2007; Van Den Beld et al., 2019).

Our data showed an overall decrease in salivary IGFBP-2 with age. This is contrast with data found about serum IGFBP-2. However, an increase was noted in the

fourth quartile Q4, which might indicate a similar pattern of the shown increase in IGFBP-2 in older adults from other reports (Van Den Beld et al., 2019). This might also be related to decreased bone density among older adults (Amin et al., 2004, 2007; Van Den Beld et al., 2019).

There is a difference in the pattern of IGFBP-2 age-related changes in saliva and serum. This might be related to differences in the local effect of IGFBP-2 on salivary tissue.

IGFBP-4

IGFBP-4 is a protein that has been reported to have a role in multiple tissue turnover and metabolism, including bone tissue. Mohan *et al.* mentioned that IGFBP-4 levels in serum were associated with reduced bone formation, decreased osteoblasts activity and bone formation (Mohan et al., 1997). They also reported that IGFBP-4 increased with age, comparing subjects 24-40 years old with subjects 61-87 years old.

Karasik *et al.* showed an increase in IGFBP-4 levels among elderly men from a similar age group (60-87 years old). They associated IGFBP-4 levels with changed bone mineral density in that age group (Karasik et al., 2002). This was in agreement with other reports indicating higher serum IGFBP-4 levels among aged individuals (Jehle et al., 2003).

However, other reports indicated no change was found in circulating IGFBP-4 with age. Van Doorn *et al.* measured serum levels in a group of individuals ranging from 0-78 years of age. They found no age-related change was seen in males. In their sample, females showed slightly higher levels related to age that was not statistically significant. Gender comparison in their group revealed no significant difference either (Van Doorn et al., 2001).

IGFBP-4 showed an inhibitory effect to cell proliferation and differentiation. Wu *et al.* reported that IGFBP-4 overexpression impaired the osteogenic differentiation of stem cells in rats (J. Wu et al., 2017). It also been reported to have a senescence effect on cells. Severino *et al.* reported IGFBP-4 was among the senescence-associated secretome of mesenchymal stem cells (MSC) *in vitro*. It also was reported to induce senescence in adjacent young MSC cells, contributing to premature aging in mesenchymal tissue (V. Severino et al., 2013).

No studies mentioned the change in IGFBP-4 in saliva with aging. Van Doorn *et al.* mentioned that IGFBP-4 was detectable in saliva from their sample group, with less quantity than in serum. However, they did not report any correlation between IGFBP-4 levels in saliva and serum. They also did not mention if any age-related comparison were done for salivary IGFBP-4 in their sample (Van Doorn et al., 2001).

This study demonstrated an overall increase in saliva IGFBP-4 levels with age. Levels increased gradually with age until it reached a peak in the third quartile (Q3; 17-27 years old). Then, a decline was observed in the fourth quartile of age (Q4; <27 years old). The overall increase with age is consistent with reports about circulating IGFBP-4 levels. However, this does not explain the decline in the fourth quartile, (not statistically significant at $p < 0.05$), where most of the reports indicated an increase in serum IGFBP-4. The levels in Q4 at this study were still slightly higher than levels in Q1, but lower than Q2. The overall age-related increase in IGFBP-4 presented in this study might be related to changes in bone turnover with age.

IGFBP-6:

Similar to IGFBP-4, IGFBP-6 has an inhibitory effect of osteoblast differentiation, contributing to progression of osteoporosis (Strohbach et al., 2008).

They are both increased in serum of osteoporosis patients. Serum IGFBP-6 showed 2.1 folds increase in adults with osteoporosis (Koh et al., 2011). Moreover, IGFBP-6 showed higher expression in nonunion fractures and contributed to delayed bone healing in fracture sites (Koh et al., 2011; Clemmons, 2018). IGFBP-6 showed a local senescence effect on cells *in vitro* and *in vivo*, therefore is thought to have a tumor suppressive effect in tissues (Bach, 1999). High IGFBP-6 levels were reported in type I diabetic patients, and were correlated with disease complications (Lu et al., 2012). High IGFBP-6 serum levels were also reported in patients with chronic renal failure (Powell et al., 1997; I. Fukuda et al., 1998). Van door *et al.* studied age-related changes in IGFBP-6 in plasma of 847 healthy male and females, ranging from 0 to 75 years of age. They showed that IGFBP-6 levels gradually elevated up to 2 folds with age (Van Doorn et al., 1999). This is largely in agreement with this study's findings. Our data show an overall increase in IGFBP-6 with age from Q1 to Q2 to Q4, which is consistent with findings from plasma (Van Doorn et al., 1999). However, our data showed a significant decrease in the third quartile Q3 (subjects 17-27 years old). There was no published data showing similar findings.

IGFBP-7

IGFBP-7 is considered one of the recently appraised components of senescence-related phenotype. Its activity increases in response to cell injury and tissue damage, leading to cell cycle arrest and inhibition of cell proliferation as a protective mechanism in the cell (Januzzi et al., 2018). IGFBP-7 is considered as a biomarker of myocardial aging, and was correlated with increased risk of heart failure in obese elderly (Januzzi et al., 2018).

Wajapeyee *et al.* mentioned similar findings about IGFBP-7 role in cellular senescence. They reported that IGFBP-7 had a tumor suppressive effect on human melanoma cells. They indicated that loss of IGFBP-7 expression in human skin contributes to pathogenicity of melanoma and skin cancer (Wajapeyee *et al.*, 2008). Succeeding work by the same group indicated that IGFBP-7 had an important role in treatment of multiple malignancies including skin and colorectal cancer (Wajapeyee *et al.*, 2008). Another report mentioned similar senescence effect of IGFBP-7 on mesenchymal stem cells culture (V. Severino *et al.*, 2013). This affects the body's ability for regeneration and repair, and ultimately contributes to aging. IGFBP-7 was also to be found to increase with obesity and increased body mass. IGFBP-7 has a high affinity for insulin, therefore can interfere with insulin action on tissues. High IGFBP-7 in serum was associated with increased insulin resistance and the development of diabetes and diabetes related complications (Gu *et al.*, 2013). There were no reports found about changes in circulating IGFBP-7 with age. No reports were found either about levels of salivary IGFBP-7 in healthy subjects. Our results indicated an overall decrease of IGFBP-7 in saliva with age that was significantly prominent between adolescents (Q2) and young adults (Q3), which might be an interesting area of investigation in circulatory levels of IGFBP-7 in the future.

Our data also showed a non-statistically significant increase in older adults (Q4). This can be consistent with the senescence-inducing effect of IGFBP-7; however more data is needed to conclude such findings. Also, this doesn't explain the overall decrease in saliva IGFBP-7, given how Q1 levels are higher than Q4. This might suggest more research is needed on the effect of IGFBP-7 on saliva and possibly salivary tissues.

IL-1 β :

Interleukin 1 *beta* is a polypeptide cytokine secreted by multiple cell types, mainly: macrophages and neutrophils (Madej et al., 2017). IL-1 β plays an important role in generating the pro-inflammatory reaction in the body. It also regulates the production of other inflammatory cytokines such as IL-6. The over-expression of IL-1 β was linked with aging, cellular senescence and multiple age-related pathologies such as: diabetes, arthritis, and neurodegenerative diseases (Chung et al., 2009; Rathnayake et al., 2013). It was also reported to be increased in saliva of subjects with periodontal disease (Tobón-Arroyave et al., 2008; Y. C. Wu et al., 2018). Our data showed a decrease in IL-1 β levels between children and adults that was significant comparing first and third quartiles of age ($p < 0.05$). There were no findings in the literature (in English language) comparing healthy children with adults, in parallel to our age ranges. Riis *et al.* studied levels of salivary IL-1 β among healthy adolescent girls 11-17 years old compared to serum and showed high correlation between IL-1 β in saliva and serum; however it didn't show a comparison based on age (Riis et al., 2014). Sirit *et al.* studied IL-1 β in serum and saliva in healthy children and adolescent. Their age range was 4-17 years old. Only the study abstract was in English, it showed high correlation in IL-1 β levels between saliva and serum (Sirit et al., 2011). Nam *et al.* measured oxidative stress markers in healthy adults (mean age 27 years old), and they found no correlation between serum and saliva levels of IL-1 β in their sample groups. However, their results did not include any age comparison (Nam et al., 2019). Results from this study indicated an increase in IL-1 β between young adults and older adults (Q3 and Q4 respectively). This agrees with reported literature about the increase in IL-1 β in similar age ranges. Fagiolo *et al.* showed age increase from 30 years old to 70 years old in healthy subjects (Fagiolo et al., 1993). Alvarez-Rodriguez *et al.* showed an increase in

inflammatory cytokines level in saliva with age (Álvarez-Rodríguez et al., 2012). Rathnayake *et al.* showed a similar increase in IL-1 β with age (Rathnayake et al., 2013).

This age increase in IL-1 β was also reported in animal models. Yamakawa *et al.* showed increase in IL1 β with age in acinar parotid gland cells in healthy mice (Yamakawa et al., 2000). Similar findings were reported by Robinson *et al.*, who reported increased IL-1 β expression in submandibular glands of mice with age, comparing 8-weeks old mice to 12-week old (Robinson et al., 1998) while Ambatipudi *et al.* showed a non-significant difference in IL-1 β from parotid salivary glands in healthy adult females between age 20-30 and 50-65 (Ambatipudi et al., 2009).

Lactoferrin

Lactoferrin has anti-microbial properties, it plays an important role in fighting against *Streptococcus mutans* in the primary dentition (Fine et al., 2013; Moslemi et al., 2015; Hemadi et al., 2017).

Lactoferrin holds an important function in diagnosis of age-related neurodegenerative diseases. Decreased salivary lactoferrin levels were found in patients with Alzheimer's disease (Carro et al., 2017; François et al., 2018). While with Parkinson's disease, patients showed higher lactoferrin levels than normal controls. In both disease, there was a strong correlation in lactoferrin levels between saliva and cerebrospinal fluid. These findings are thought to hold high importance in diagnosis of early development of Alzheimer's disease, as it shows to be specific to Alzheimer's disease than other types of dementia and neurodegenerative conditions (Carro et al., 2017; François et al., 2018; Glerup et al., 2019).

Reports mentioned conflicting results about lactoferrin changes with age in healthy adults. Some studies reported an age-related increase in salivary lactoferrin.

Fox *et al.* mentioned statistically significant slight increase in whole saliva lactoferrin levels with age among subjects from 22 -81 years old (Fox et al., 1987). Also, Nahri *et al.* reported higher levels of lactoferrin among elderly aged 76– 86 years compared to young adults (Närhi et al., 1994). Similarly, Ambatipudi *et al.*, reported increased abundance of lactoferrin in saliva from subjects 50-65 years old compared to 20-30 years old (Ambatipudi et al., 2009).

Other reports mentioned no change in lactoferrin levels with age. Wu *et al.* reported no significant change in lactoferrin levels among a group of healthy individuals ranging between 24-74 years old (A. J. Wu et al., 1993). Another report showed similar results among a sample of young and old adults (21-50 vs 79-89), and showed no significant difference was found (Shugars et al., 2001)

In contrast, others mentioned a decrease in lactoferrin levels in whole saliva of healthy subjects with age (Denny et al., 1991; Baum et al., 1993; Salvolini et al., 2000; Tanida et al., 2001; Nagler et al., 2005; W. I. Chang et al., 2011; Xu et al., 2019). Our results show an overall decrease with age that varied across age groups. Levels increased slightly from Q1 to Q2, the decreased to lowest values in the third quartile of age (Q3), followed by an increase in Q4. This increase was still found lower than the younger subjects in Q1 and Q2. This cumulative decrease with age might appear to be in agreement with previous reports (Denny et al., 1991; Baum et al., 1993; Salvolini et al., 2000; Tanida et al., 2001; Nagler et al., 2005; W. I. Chang et al., 2011; Xu et al., 2019). However, analyzing these reports age groups indicated that their samples did not include younger subject. In the report by Chang *et al.*, the sample groups were: young subjects (mean age, 26.4 ± 2.4 years) and elderly (mean age, 71.1 ± 4.6 years) (W. I. Chang et al., 2011) In Denny *et al.* study, comparison included young (18-35 years of age) and aged subjects (65-83 years of age). In report by Nagler and Hershkovich, the

age range was 18-90 years old (Nagler et al., 2005) . None of these groups included subjects younger than 18 years old that would be parallel to our Q1 and Q2. Therefore, it would be more suitable to compare their findings with the change seen between Q3 and Q4. In that case, our findings are opposite to these previous reports. There was an increase in salivary lactoferrin with age in subjects older than 17 years old in this study (Q3, and Q4). This increase then was found in agreement with reports by Fox *et al.*, and Ambatipudi *et al.*. These reports' age groups ranged from 20 to 87 years old, and showed an increase in lactoferrin with age.

In regards to the decrease we found between Q1 (children < 13 years old) and Q4 (older than 27 years old), one report had similar age comparison. Cole *et al.* 1981 compared lactoferrin in plaque and saliva of children (7-12 years old) with older subjects (51-75 years old). In contrast to our findings, they reported higher levels of lactoferrin in the older group. Few reports have investigated the age-related change of salivary lactoferrin in young subjects. Most of those reports described lactoferrin relationship with caries among children, with few age comparisons (Hao et al., 2009; Moslemi et al., 2015; Hemadi et al., 2017). One of these studies is by Moslemi *et al.*, who investigated saliva lactoferrin levels among young children in relation to early childhood caries. They reported decreased levels with age in children from 37-71 months (Moslemi et al., 2015). This might be an indication of possible change in lactoferrin function with age with a possible shift of pathogenicity and prevalence in the oral cavity from more risk of caries in young age to risk of periodontal disease in old age.

MMP-2

Matrix metalloproteinase-2 (MMP-2) or collagenase type IV is among the proteolytic enzymes responsible for connective tissue remodeling and turnover (Freitas-Rodríguez et al., 2017). This enzyme has multiple substrates that form the extracellular matrix including Collagen (IV, IV, V, VII, X, XI), elastin, gelatin and fibronectin. It also has an effect on multiple inflammatory molecules and chemokines (Nagase et al., 1992). Among other MMPs, MMP-2 release was also reported to regulate IL-1 β activity and therefore can contribute to the state of inflammation in the body (Ito et al., 1996). MMP-2 plays a role in modulating the vascular balance in tissues (Mäkälä et al., 1994; Medley et al., 2003). It also plays an important role in skeletal maturation, and regulating bone development and mineralization (H. P. H. Liang et al., 2016).

MMP-2 release is considered among the secretory cell phenotype of multiple cells. Increased MMP-2 expression is one of the hallmarks of age-related macular degradation that can lead to blindness (Liutkeviciene et al., 2017). It has been linked to tenocytes aging leading to tendon's age-related pathologies (T. Y. Yu et al., 2013). MMP-2 also showed to be increased in senescent periodontal ligament cells along, with decreased collagen I expression (Benatti et al., 2008; Konstantonis et al., 2013). In aged mice, salivary glands showed higher MMP-2 activity than in young mice (Tumer et al., 2018). Also, salivary MMP-2 was reported to increase with periodontal disease and to decrease after periodontal treatment (Goncalves et al., 2009). In this study, there was a non-significant decrease across age quartiles. This decrease was marginally significant comparing Q3 to Q1 ($p=0.062$). In the literature, there were no reports of direct comparison about the changes in MMP-2 with age in healthy children compared to adults. Two studies reported that MMP-2 deficiency in children was associated with

osteolytic syndromes and reduced bone tissue formation. They suggested that against the general consensus that MMP-2 increase is associated with bone loss and degeneration, MMP-2 gene mutation and decreased serum levels showed defects in bone formation, in humans and animal models. They concluded that the MMP-2 balance was crucial for bone health and development (Al Aqeel et al., 2000; Mosig et al., 2007).

The relationship between MMP-2 and bone formation might be related to changes in skeletal growth throughout life. It is known that skeletal growth reaches its maximum level at age 17 for girls and 18 for boys (Rogol et al., 2000). Therefore, the decrease found in this study by the third quartile of age might be proportional to the end of most of the skeletal growth in the subjects.

A longitudinal observation of MMP-2 levels in healthy children might give a better understanding about the effect of MMP-2 in normal skeletal development through life. Also, it might be interesting to investigate if any relationship is present between salivary MMP-2 and parameters of skeletal growth that are collected in routine orthodontic diagnostic imaging. This includes radiographic analyses of Skeletal Maturity Index (SMI) and Cervical Vertebrae Maturation Index (CVM).

Overall, the markers tested in this study showed several differences based on subjects' age. Comparison of our findings with reported circulatory levels of these markers showed there are differences in their saliva and serum expression in the body. This might indicate that the reported markers might have local functions in saliva and oral tissue that varies with advancing in age. More studies are needed about the consistency between circulating level of these biological markers and their presence in saliva.

Limitations and future directions:

One of the limitations of this study was the ratio of males to females in each age group, particularly in the fourth age quartile. Including more balanced number of males to females in each age group might reduce confounding factors in the study. Although there was no overall significant difference in the markers of interest among different genders, there was a noted gender difference in the sample subgroups. Males and females showed a clear difference in expression of markers in certain age groups. These differences can be analyzed further in future work.

Another limitation was the small number of subjects older than 40 years old. This, along with the higher number of females than males in the study sample, might be due to the fact that the sample group was recruited from an orthodontic clinic. Lastly, most of patients attending orthodontic clinic are children and young adults, with more females than males. This might explain why the sample group included less males and older subjects. Since the samples in this study were baseline (pre-treatment) saliva samples, one suggestion is to include subjects from other dental clinics, while maintaining the same inclusion criteria such as healthy periodontal status and medical condition. Expanding the sample group to include more male subjects and older individuals might help provide more conclusive findings.

CONCLUSIONS

Aging is accompanied by multiple changes in the human body. These changes occur gradually throughout the individual's life on cellular and molecular levels. These lead to altered pattern of tissue morphology and biological function. There is a reported increase in the body's susceptibility to environmental and pathological insults, accompanied by a reduced capacity for repair and resolution. This contributes to the decline in function and more vulnerability to disease with age.

Saliva provides an important milieu to study biological changes with aging. It provides a simple and non-invasive way to analyze multiple important markers involved in the aging process. From this study we concluded that:

- Saliva showed a different profile of markers between different age groups.
- The age-related change was not always linear across the age groups.
- Multiple changes occurred significantly at the third quartile of age (Q3; 17-27 years old), indicating that aging might be preceded by changes that occur at an earlier time point in life.
- Comparing the overall sample group, no difference was found between females and males.

PART II: SENESCENCE IN PERIODONTAL LIGAMENT FIBROBLASTS

INTRODUCTION

Periodontal ligament

Periodontal ligament is the fibrous connective-tissue layer covering the cementum of a tooth and holding it in place in the alveolar bone. The periodontal ligament serves multiple important functions, these include the following:

- Periodontal ligament supports the teeth by attaching them to the surrounding alveolar bone. This is achieved primarily by the principal fibers of the periodontal ligament that form a strong fibrous connection between the cementum and the bone. In addition, the periodontal ligament serves as a shock-absorber by mechanisms that provide resistance to a whole range of forces. Light and moderate forces are cushioned by intravascular fluid that is forced out of the blood vessels, while heavier forces are mitigated by the principal fibers (M. G. Newman et al., 2011).
- The periodontal ligament provides a sensory function. Periodontal ligament tissue is rich with nerve endings that are primary receptors for pain and pressure and play an important role in proprioception (Palumbo A 2011; Newman et al. 2011; Moxham and Evans 1995).
- The periodontal ligament has a nutritive function. It provides blood supply to the cells of the periodontium and the surrounding structure such as cementum and alveolar bone (Sinha, 1997; Mashtan, 2010).
- Another function of the periodontal ligament is remodeling. This happens through large population of cells that are able to both form and resorb tissues that make up the attachment apparatus around teeth, including: bone, cementum and periodontium (Feller et al. 2015). This includes cells with catabolic function

such as osteoclasts and odontoclasts, along with undifferentiated mesenchymal cells that can differentiate into osteoblasts, cementoblasts, and fibroblasts to help form bone, cementum and periodontal tissue (Jia et al. 2018). This function is of a particular importance to orthodontic tooth movement. The basis of orthodontic tooth movement depends on a coordinated changes in the surrounding bone and periodontium (Feller et al. 2015). When teeth move, multiple cellular and molecular changes occur in the periodontium, activating an inflammatory response that leads to tissue resorption in areas of compressive forces and deposition in areas of tensile forces. This process of “aseptic inflammation” needs to be well-balanced and orchestrated during the tooth movement. Any imbalance in this system could lead to adverse effects such as root resorption or periodontal pathologies (Feller, Khammissa, Schechter, Moodley, et al., 2015; Y. Li et al., 2018).

- Lastly, an important characteristic of the periodontal ligament structure is its regenerative function, as it contains stem cells that responsible for differentiating into multiple cell types based on the need of the periodontal apparatus (Jia et al. 2018).

Principal fibers of the periodontal ligament

Fibers of the periodontal ligament are mainly composed of bundles of type I collagen fibrils. They have been classified into several groups on the basis of their anatomic location; alveolar crest, horizontal, oblique, periapical and interradicular fibers (M. G. Newman et al., 2011).

The so-called “Sharpey's fibers” are the mineralized portion connecting the collagen fibers with the mineralized surfaces of teeth, i.e.: bone and cementum. These

fibers are wider in diameter on the bone side than the cementum side. As they originate from bone or cementum, they join up with smaller adjacent fibers, forming a meshwork of interconnected fibers that connects both structures rather than a singular connection of fibers (Sinha, 1997; Mashtan, 2010).

There are wide variety of cell types in the periodontal ligament that are responsible of generating and maintaining the periodontium, alveolar bone and cementum. The cells of the periodontium include fibroblasts, undifferentiated stem cells, macrophages, osteoblasts, osteoclasts, cementoblasts, cementoclasts and epithelial cell rests of Malassez (Huttner et al., 2009; M. G. Newman et al., 2011).

The periodontal ligament also contains a large part of ground substance, filling up the extracellular spaces between fibers and cells. It consists of 70% water and has two main components: glycosaminoglycans, such as hyaluronic acid and proteoglycans, and glycoproteins, such as fibronectin and laminin. The cell surface proteoglycans participate in several biologic functions, including cell adhesion, cell-cell and cell-matrix interactions, binding to various growth factors as co-receptors, and cell repair (Worapamorn et al., 2000; M. G. Newman et al., 2011).

Physiology and function of periodontal ligament fibroblasts

Periodontal ligament fibroblasts are mesenchymal cells with a spindle-like shape and are the most predominant cells in the periodontal connective tissue. Along with their function as one of the main structural cells of the periodontium, they carry an important role in development, remodeling and regeneration of the periodontal ligament and their surrounding tissues (M. G. Newman et al., 2011).

Periodontal ligament fibroblasts are responsible for secreting the elastic fibers, collagen, glycoproteins and extracellular matrix proteins. They also have the ability to

degrade collagen by phagocytosis, thus controlling collagen homeostasis in the periodontal tissue (M. G. Newman et al., 2011).

Periodontal ligament fibroblasts participate in bone metabolism and turnover. They secrete alkaline phosphatase, which is an important enzyme in bone mineralization. They also show osteogenic potential in the presence of the supporting media by releasing bone forming proteins and producing mineralizing nodules *in vitro* (Basdra et al., 1997).

Another function of periodontal ligament fibroblasts is their ability to incite inflammatory response to pathogens or stressful stimuli, aiding in regulation of the immunological process in the periodontium (Sundar et al. 2016). This role creates a special importance to studying this type of cells, as in addition to their predominance in the periodontal tissue, they have the ability to act as a source of inflammation upon contact with stresses that could frequently occur in the oral cavity such as presence of pathogens or mechanical insults. This importance increases particularly with arising scientific interests in the effect of local inflammation (such as periodontitis) on the systemic immune system and other inflammatory diseases such as Alzheimer's disease, Diabetes and cardiovascular disease (Hasturk et al. 2007; Morimoto-Yamashita et al. 2012; Nassar et al. 2014; Shoemark and Allen 2015; Wu, Xiao, and Graves 2015).

Aging and senescence-associated changes in cells

Cellular senescence is one of the hallmarks of aging, and is characterized by a decrease in overall function of cells including their ability to proliferate & replicate (Tigges et al. 2014). Cellular senescence is usually accompanied by multiple changes in the cell and nuclear level, including: telomere shortening, DNA damage, release of secretory senescence phenotype, change in cell morphology, reduced mitochondrial

activity and reduce proliferative potential (Lee et al. 2006; Boskey and Coleman 2010; López-Otín et al. 2013; Tigges et al. 2014).

Cellular senescence is also thought to be one of the physiological fate cells can face when they grow old. In contrast to cancer cells, normal cells are mortal and have a definite life span. This life span might differ depending on tissue type. However, as time passes, all normal cells show a decline in biological functions and eventually lose the ability to replicate and regenerate. This phenomenon goes in parallel with what we define as “aging” on the organ level (Campisi et al., 2007).

Senescence is divided into two types: replicative senescence, and premature-induced senescence. Induced senescence could occur due to multiple reasons such as: oncogene attack, chemical or physical stimuli (Campisi et al., 2007; de Magalhães et al., 2018). In case of an oncogene stimulus, adjacent cells respond by preventing the tumor cells from replicating by cell cycle arrest, going into senescence as a form of defense (Ohtani et al., 2009). Reports showed that cells exposure to high levels of physical stimuli such as ionizing radiation and thermal energy sources could result in premature cell senescence (Papadopoulou and Kletsas 2011; Despars et al. 2013). Similarly, chemicals such as Hydrogen Peroxide (H_2O_2) and D-Galactose showed to induce senescence in cells both in animals and in cell culture models (Campisi et al., 2007; de Magalhães et al., 2018).

The suggested explanation behind H_2O_2 and D-Galactose senescence models is due to cells response to oxidative damage. It is thought that these chemicals can cause an accumulation of reactive oxygen species beyond the cells' ability to neutralize. This results in DNA damage and mitochondrial exhaustion causing a decline in cell function and the expression of inflammatory phenotype (Kiyoshima et al. 2012; CHEN 2006;

Frippiat et al. 2001; Severino et al. 2000; Sun et al. 2015; Rahimi, Askari, and Mousavi 2018).

Senescence-associated changes in the periodontal ligament

The loss of periodontal attachment and alveolar bone are known physiological changes that occur with age (Burt, 1994). However, reports indicate these changes alone have little clinical significance on the development of periodontal disease. (Lim et al. 2014).

Aging is associated with a generalized decline in the body's immunological function that is defined as immunosenescence. The immune system capacity decreases and the body becomes more susceptible to infections and stresses (Huttner et al. 2009).

Since the immune system works by firing the body's inflammatory response to defend an insult (either stress or a pathogen), with immunosenescence, it is thought that immune cells have a decreased efficiency in fighting the stressor, causing a persistent inflammatory status, unrelieved (Ebersole et al., 2016, 2018; Bandaranayake et al., 2016). This applies to the periodontal tissue as well. With aging, periodontal immune cells become less efficient in dealing with the inflammatory insult, creating a continuous state of a low-grade chronic inflammation, a phenomenon known as inflammaging, in the background (Ebersole et al., 2016, 2018). This explains why older individuals can show higher severity of disease compared to their younger counterparts, in response to similar pathogen stimuli (Huttner et al., 2009; Ebersole et al., 2016, 2018).

It has been reported that with advancing in age, multiple functional and morphological changes happen to the periodontal ligaments. Their thickness decreases and they become less cellular (Huttner et al. 2009; Krieger, Hornikel, and Wehrbein

2013; Lim et al. 2014). Periodontal vascularity also decreases, leading to reduced tissue ability for wound healing and regeneration (Lim et al. 2014). There is also a change in periodontal ligaments physiological functions such as reduced collagen formation (Moxham et al., 1995) and increased collagen degradation (Lim et al. 2014). There is also an overall decrease in bone formation potential and increased osteoclastic function (Huttner et al., 2009; Lim et al., 2014).

Senescence-associated changes in periodontal ligament fibroblasts:

With aging, it has been reported that periodontal ligament fibroblasts show different morphology from small spindle-like shape in young individuals to widely sparse and diverse shape (Sawa et al. 2000).

Reports indicated that periodontal ligament fibroblasts reach replicative senescence in *vitro* around passage 22-25 (Sawa et al. 2000). Aged periodontal ligament fibroblasts showed markers of senescence in *vitro* including increased expression of p53 and senescence-associated beta galactosidase (SA- β -Gal) activity, compared to younger cells (Sawa et al. 2000). Periodontal ligament fibroblasts also showed lower proliferative rate, indicated by decreased expression of the proliferation marker c-fos (Huttner et al. 2009; Asahara et al. 2008). Others reported that periodontal ligament fibroblasts also exhibited lower chemotaxis and less motility with aging (Nishimura et al. 2008; Huttner et al. 2009).

Changes in periodontal ligament fibroblasts functions with age is reported, such as reduced osteocalcin and alkaline phosphatase release (Benatti et al. 2008; Goseki et al. 1996; Sawa et al. 2000), reduced collagen formation, and increased collagen degradation (Moxham and Evans 1995; Nishimura et al. 2008).

In addition to collagen degradation, senescent periodontal ligament fibroblasts were reported to release other tissue degrading factors such as plasminogen activator factor, MMPs and cathepsin (Miura, Yamaguchi, and Shimizu 1999; Goseki et al. 1996). These enzymes cause extracellular matrix and protein degradation and have an overall catabolic effect on periodontal tissue (Miura, Yamaguchi, and Shimizu 1999; Goseki et al. 1996).

Periodontal ligament fibroblasts also show an age-related increased release of inflammatory markers such as IL-1 β , IL-6 and PGE₂, which have been reported as part of the secretory senescent phenotype of fibroblast. (Shimizu 1997; Bae et al. 2018; Mayahara et al. 2007). Both IL-6 and IL-1 β are associated with increased osteolytic and tissue-degrading activity (Bae et al. 2018).

D-Galactose-induced aging

D-Galactose is a simple monosaccharide that serves as an energy source and as an essential component of glycolipids and glycoproteins. Administration of high levels of D-Galactose has been used in experimental research as a way to induce premature aging (Tang et al., 2013).

In high levels, D-Galactose leads to accumulation of advanced glycation end-products (AGEs). When AGE's accumulate, they activate their receptor on the cells (receptor for advanced glycation End-products RAGE), which in turn up-regulate a cascade of signals that lead to formation of reactive free radicals and the release of pro-inflammatory cytokines [**Figure 20**](Ali et al., 2015).

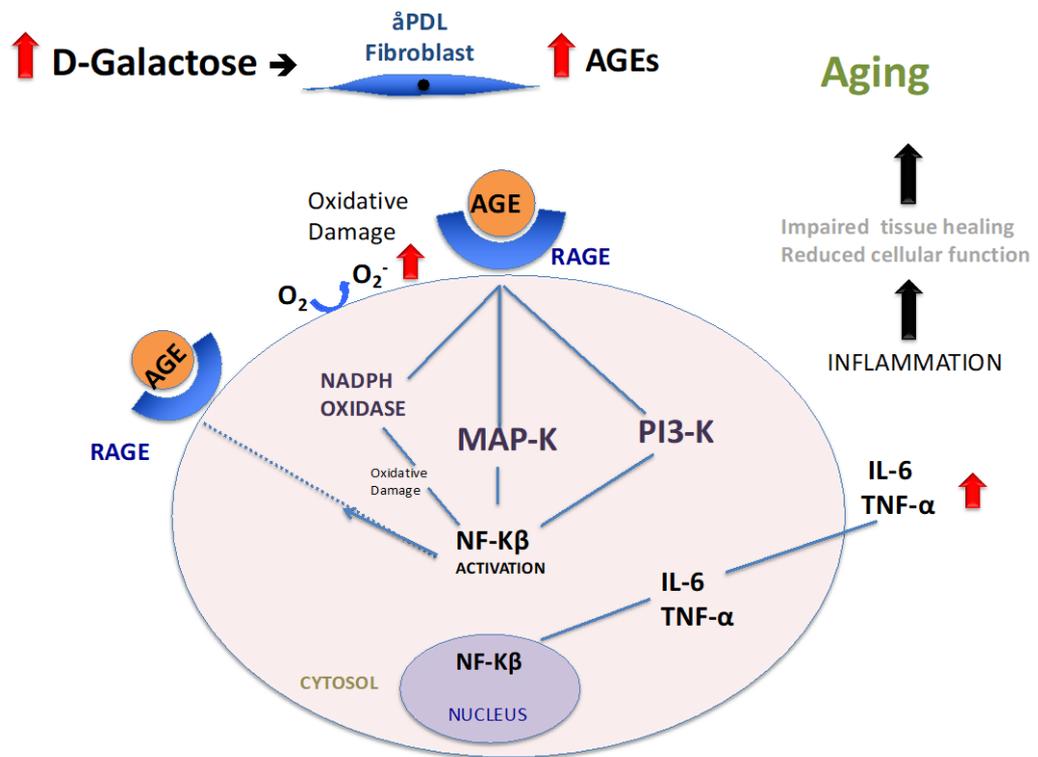


Figure 20: AGE/RAGE Pathway

It has been reported that elevated levels of AGEs *in vivo* contribute to acceleration of the aging process in animals and humans (Y. Y. Liu et al., 2013). AGEs deleterious effect on different tissues is attributed to their chemical, oxidant, and inflammatory actions (Deora et al., 1998; Senatus et al., 2017).

Chronic administration of D-Galactose to animals was reported to show symptoms of aging such as reduced motor and cognitive abilities, and have been widely used in aging and anti-aging pharmacology research (Ho et al., 2003; Mao et al., 2012; Y. Y. Liu et al., 2013).

D-Galactose effect on cells *in vitro* is not as widely studied, and the exact mechanism how it contributes to cell senescence is still not entirely understood (Shen et al., 2014; Bo-Htay et al., 2018). Until now, here is no published research on the effect of D- Galactose on periodontal ligament fibroblasts.

Senescence-Associated Beta Galactosidase

Senescence Associated Beta Galactosidase (SA- β -Gal) is a hydrolase enzyme that catalyzes the hydrolysis activity in senescent cells. Its expression is not required for senescence; however, it remains one of the most widely used biomarkers for senescent and aging cells, because it is easy to detect and reliable both *in situ* and *in vitro* (Dimri et al., 1995). With microscopic imaging it appears as a peri-nuclear blue stain [**Figure 21**]. It's expressed by a number of different cell types. High intensity was observed in late passages of replicative senescence and cells from donors with premature aging conditions (Dimri et al., 1995), and it was found to increase in expression with age in multiple types of tissues (Macieira-Coelho, 2000).

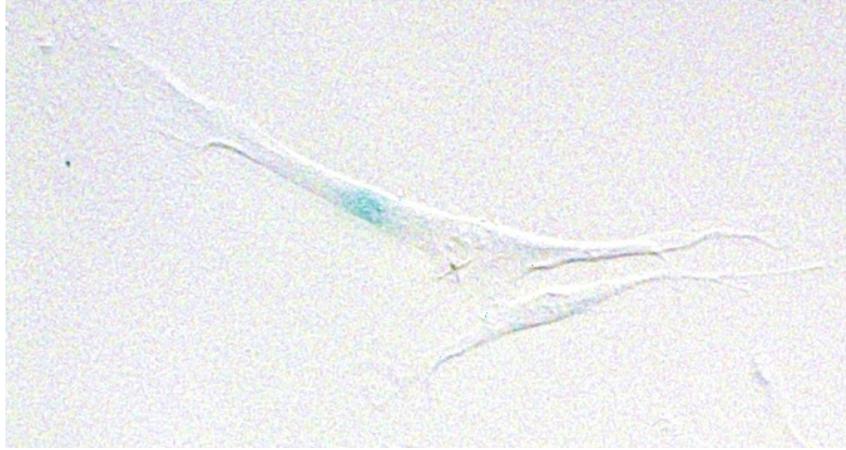


Figure 21: Senescence-Associated Beta Galactosidase staining in periodontal ligament fibroblasts.

HYPOTHESIS

- **Null Hypothesis:** D-Galactose does not induce senescence in periodontal ligament fibroblasts.
- **Alternative Hypothesis:** D-Galactose induces senescence in periodontal ligament fibroblasts.

Overall Aim

- Investigate the effect of D-Galactose in *vitro* senescence model on periodontal ligament fibroblasts.

Specific Aims

- Investigate which passages of periodontal ligament fibroblasts can be used as control in aging studies.
- Investigate if D-Galactose concentration is correlated to the expression of senescence in periodontal ligament fibroblasts.
- Investigate the underlying mechanism for D-Galactose effect on periodontal ligament fibroblasts in *vitro*.
- Investigate the difference in phenotype of D-Galactose treated periodontal ligament fibroblasts compared to control cells.

MATERIALS AND METHODS

Reagents

DMEM (Dulbecco's Modified Eagle Medium) with Glucose, L-glutamine, phenol red and sodium pyruvate (Gibco™, #11995-065), Fetal Bovine Serum (FBS) (Atlanta biologicals, #S11595), Penicillin/Streptomycin (10,000 U/mL) (Gibco™, #15140122), Trypsin/EDT (0.025%) (Gibco™, #25200056), PBS (Gibco™, #10010023), Dimethylsulfoxide (DMSO) (Sigma, #D8418), D-Galactose powder (Sigma, #G0625), SA-β-Gal staining kit (Sigma, #CS0030), Trizol (Invitrogen, #15596026), (Cell Lytic^{MT} supplemented with protease and phosphatase inhibitors; Sigma #C3228, PPC1010), BCA Protein Assay Kit (Pierce™), blotting buffer (25 mM Trizma base, 192 mM glycine, 20% 79 methanol, blocking solution (5% (w/v) milk in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 + 5% milk (TBS-T)), IgG-horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology, Inc. Danvers, MA, #7074), Antibodies: RAGE, phospho-p44/42 MAPK (ERK1/2) (Thr²⁰²/Tyr²⁰⁴), p44/42 MAPK (ERK1/2), Phospho-NF-κB (p65) (Ser536), NF-κB (p65), Phospho-PI3 Kinase p85 (Tyr⁴⁵⁸)/p55 (Tyr¹⁹⁹) and PI3 Kinase p85 (Cell Signaling Technology, Inc. Danvers, MA, catalog numbers: 4679, 4089, 4695, 3033, 8242, 4228, 4292) and NADPH oxidase 4 (Abcam, #154244)

Cell culture

Human periodontal ligament fibroblasts were purchased from Lonza (Cat. #: CC7049). Cells arrived cryopreserved and stored in liquid nitrogen until ready to use. Prior to use, cells were thawed for 60-90 seconds in 37°C sterile water bath, cell vial was wiped with alcohol and opened in a sterile culture hood. Cells were resuspended in already prepared and room temperature media: DMEM from Gibco (Cat. # 11995-

065) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S). Cells were seeded following manufacturer instructions (seeding density 3500 per cm²) and placed in a sterile incubator (37°C, with H₂O and CO₂). Media was changed the day after seeding and every 2-3 days thereafter (5-7.5 ml³ per cm², depending on cell confluency status). Cells were passaged to next passage at 85% confluence to ensure they were in the log phase for optimum growth. For sub-culture procedures, cells were first gently washed with PBS. Then, Trypsin/EDTA (T/E) and 2x volume of media (to neutralize T/E) were used to detach and collect cells. Cells were centrifuged for 5 minutes at 500xg. Supernatant was removed and cells were resuspended in fresh media, counted by hemocytometer using Trypan-Blue and seeded in new flasks according to manufacturer's instructions. By the end of each passage, a group of cells were placed in labeled cryovials with cell culture media supplemented by 10% DMSO and placed in -80° C freezer for future use. Cells were usually passaged at least one passage in culture prior to be used in an experiment to avoid using cells from frozen.

D-Galactose preparation

D-Galactose was prepared by dissolving D-Galactose powder (Sigma Catalog #G0625) in ultrapure distilled water. Given D-Galactose powder molecular weight (180.16 g/mol) and maximum solubility threshold in water (100 mg/ml), the starting stock solution was set to have a 100mM concentration. Required amount of water was always placed first then weighted powder was added. Solution was mixed by mix-pipetting and vortex for 2 minutes until all powder was fully dissolved. Culture media was used to be mixed with 100mM D-Galactose to make different concentrations of D-Galactose-supplemented media: (50μM, 100μM, 1mM, 10mM, 50mM). New stock of

100mM D-Galactose was prepared the same day of each experiment according to the needed volume.

Senescence-Associated Beta-Galactosidase (SA- β -Gal) activity across untreated cells from passages 1-10

Cells were seeded and passaged continuously from P1 to P10 with same culture conditions. From every other passage (P3, P5, P7, P9), cells were seeded in a 24-well plate (S.D. 7×10^3 /well) and left to attach for 24 hours, then SA- β -Gal staining (Sigma CS0030) was done (Appendix: protocol 1). After Staining, images were taken by microscope (Zeiss 780) at 10x. Images were analyzed by (Zen) software and cell counting was done.

Number of (SA- β -Gal)-positive cells were quantified as follows: top left and bottom right corners of each well were always counted. Middle of the well was always excluded to avoid higher cell concentrations and possible counting errors due to confluency. Each well had six duplicates, all were counted, and their average was calculated. Quantification was done by Microsoft Excel software.

Optimization of D-Galactose concentration and incubation time

Cells from passage 3-6 were used for experiments. When cells reached 85% confluence, they were plated in 24-well plate (7×10^3 /well). Cells were left to attach for 24 hours. Then, media was changed:

- Control group: fresh media to ensure media change was done to all cells, control and treated.
- D-Galactose groups: D-Galactose supplemented media in different concentrations and labeled as such:
 - a. 50 μ M
 - b. 100 μ M
 - c. 1 mM
 - d. 10 mM
 - e. 50 mM

Cells were left to incubate for two time points (24 hours and 48 hours), then they were stained by the end of their time points with (SA- β -Gal) staining. Images were taken by microscope and cells were counted as previously mentioned.

Total protein isolation and quantification

Same cell culture and D-Galactose administration conditions were followed with seeding cells at 1×10^5 /well in 6-well plates. By the end of experiments' time points, cells were washed with ice-cold DPBS. Appropriate volume of cell lysis buffer was added to the cells (Cell Lytic ^{MT} supplemented with protease and phosphatase inhibitors; Sigma: C3228, PPC1010). Cells were scraped and protein was isolated according to cell lysis protocol (Appendix; protocol 2). Cell lysates were preserved at -80°C until ready to use while minimizing thawing cycles to 1-2.

To measure protein concentration in cell lysates for western blot experiments, bicinchoninic acid assay (BCA) was used. A standard curve was prepared using serial dilutions of bovine serum albumin (BSA) at 0, 5, 25, 50, 125, and 250 $\mu\text{g/ml}$. 200 μl BCA reagent (Pierce™ BCA Protein Assay Kit) were added to 10 μl sample and standards and assayed in duplicates. Absorbance was measured at 562 nm (Spectramax 340PC384 with SoftMax 4.3LS, Molecular Devices). Background absorbance of de-ionized water was subtracted from all measurements. Sample concentrations were extrapolated from the standard curve. Full BCA protocol is presented in Appendix; protocol 3.

Western blotting

Proteins were prepared by mixing 5 μg of lysate with 4x sample loading buffer with 2.5% volume β -ME. The lysates were denatured by heating to 95°C for 8 min. The proteins were separated by electrophoresis in 12% polyacrylamide gels in running buffer (25 mM Trizma base, 192 mM glycine, 0.1% sodium dodecyl sulfate (SDS)) at 100 V for 1.5 hours. Molecular weight standards were added in a separate well in the gel. A polyvinylidene difluoride (PVDF) membrane was prepared by 1 min incubation in methanol followed by 10 min incubation in blotting buffer (25 mM Trizma base, 192 mM glycine, 20% 79 methanol). Proteins were transferred to the prepared PVDF membrane at 66 mA overnight at 4°C. The next day, the membrane was incubated in blocking solution (5% (w/v) BSA in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 + 5% BSA (TBS-T)) for 1 hr and then incubated with the targeted primary antibodies overnight at 4°C in 5% BSA in TBS-T on a shaker. The targets were explored using antibodies to RAGE, phospho-p44/42 MAPK (ERK1/2) (Thr²⁰²/Tyr²⁰⁴), p44/42 MAPK (ERK1/2), Phospho-NF- κ B (p65) (Ser536), NF- κ B (p65), Phospho-PI3 Kinase

p85 (Tyr⁴⁵⁸)/p55 (Tyr¹⁹⁹) and PI3 Kinase p85 (Cell Signaling Technology, Inc. Danvers, MA.) and NADPH oxidase 4 (Abcam). The next day, the membrane was washed 3 times with TBS-T for 10 min and incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology, Inc. Danvers, MA) in blocking solution (5% (w/v) milk in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 + 5% milk (TBS-T)) for 1 hr at room temperature. The membrane was then washed 3 times for 10 min in TBS-T. Then, an image was taken using chemiluminescence imaging system (G:Box, Syngene) and processed with (ImageJ, NIH). Results of phosphorylated antibodies were normalized to the expression of β -actin and the total protein. To assay for additional targets, the membrane was incubated for 20 min in a stripping buffer and washed 3 times in TBS-T before incubation with the next primary antibody. Full Western blotting protocol is presented in Appendix; protocol 4.

Supernatant analysis

Supernatants were collected and stored at -80°C until ready for analysis. Thawing cycles were limited to 1-2 times to avoid sample changes. Custom Multiplex kit was purchased from R&D® including: IL-1 β , IL-8, Fibronectin, IL-6, MMP-1, TNF- α and cathepsin S. Luminex 200® was used to analyze the samples following the protocol described in Part I Methodology section page (17) .

Statistical Analysis

All experiments were performed 3-5 times and the average values were used for statistical analyses, performed by SAS® software [9.4] (SAS Institute Inc., Cary, NC, USA) and Microsoft Excel 2018 software. Depending on samples normality (tested by Shapiro-Wilk test), parametric or non-parametric statistical tests were used, including: t-test, one-way ANOVA, Kruskal-Wallis and Tukey Post-hoc analysis as needed. The level of significance was noted at $p < 0.05$ for all analyzed data.

RESULTS:

Senescence -Associated -Beta-Galactosidase (SA- β -Gal) Staining:

- **Comparison of (SA- β -Gal) expression between control (untreated) periodontal ligament fibroblasts in P3, P5, P7 and P9:**

The average percentage of (SA- β -Gal)-positive cells was (27.20 %, SD 12.19) in passage 3, (35.88 %, SD 8.58) in passage 5, (25.90 % \pm SD 11.11) in passage 7 and (23.64 %, SD 9.79) in passage 9 [Table 72] [Figure 22]. One-way ANOVA was used to investigate the difference in SA- β -Gal expression between the different passages. The difference was found to be statistically non-significant at $p < 0.05$ ($p = 0.2887$).

Table 72: Proportion of (SA- β -Gal)-positive cells in control periodontal ligament fibroblasts from Passage 3 (P3) - Passage 9 (P9).

	Mean (%)	SD
P3	27.20	12.19
P5	35.88	8.58
P7	25.90	11.10
P9	23.64	9.78

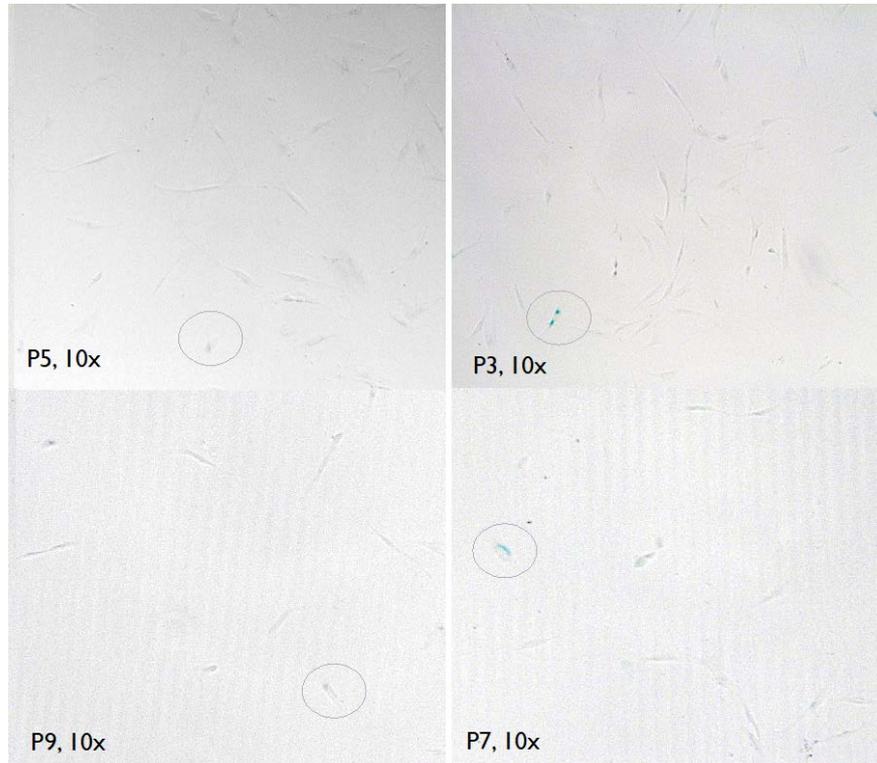
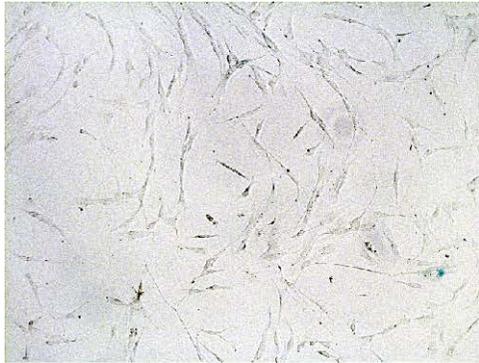


Figure 22: (SA- β -Gal) staining for untreated periodontal ligament fibroblasts P3, P5, P7 and P9 (10x).

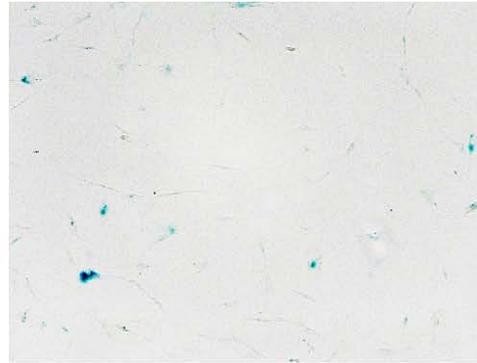
- **Comparison of (SA- β -Gal) expression between control and (D-Galactose)-treated periodontal ligament fibroblasts:**

The percentage of (SA- β -Gal)-positive cells was calculated for control cells and cells treated with different concentrations of D-Galactose at two time-points: 24 hours and 48 hours [Tables 73, and 74 respectively] [Figure 23]. There was a significant difference in (SA- β -Gal)-positive cells found between control cells and D-Galactose-treated cells in both 24 hours and 48 hours at $p < 0.05$ (One Way ANOVA indicated $p = 0.0035$). At 24 hours, the difference between percentage of (SA- β -Gal)-positive cells in variable concentrations of D-Galactose cells was found to be statistically non-significant at $p < 0.05$ (One-Way ANOVA, $p = 0.149$). While at 48 hours, significant difference was found between the five D-Galactose groups (One- Way ANOVA $p = 0.0001$). Post-hoc Tukey analysis of the D-Galactose groups at 48 hours was done for multiple comparison. It showed a significant difference between the following groups: 10mM compared to 50 μ M, and 50mM compared to all other concentrations ($p < 0.05$) [Table 75].

Among all experiments, number of (SA- β -Gal)-positive cells showed a gradual increase proportional to the concentration of D-Galactose, except for 100 μ M D-Galactose group at the 24 hours' time point [Figure 24].



P3.10x
0 μ M, 26% (Control)



P3.10x
50 μ M, 47%



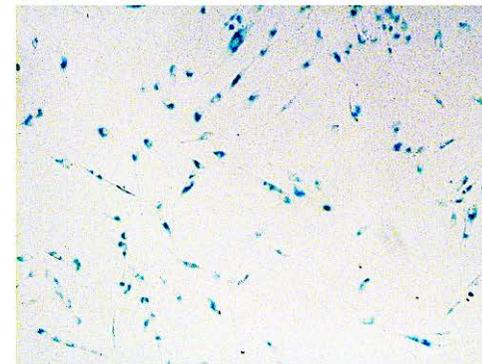
P3.10x
100 μ M, 54%



P3.10x
1 mM, 55%



P3.10x
10 mM, 72%



P3.10x
50 mM, 97%

Figure 23: (SA- β -Gal) staining for (P3) periodontal ligament fibroblasts with different concentrations of D-Galactose

Table 73: Proportion of (SA- β -Gal)-positive periodontal ligament fibroblasts in control and (D-Galactose)-treated groups at 24 hours. All cells are from Passage 3.

D-Galactose Concentration	Mean (%)	SD
0.05 mM (50 μM)	81.16	18.90
0.10 mM (100 μM)	77.80	11.56
1 mM	93.08	3.95
10 mM	95.42	1.26
50 mM	96.62	3.83

Table 74: Proportion of (SA- β -Gal)-positive periodontal ligament fibroblasts in control and (D-Galactose)-treated groups at 48 hours. All cells are from passage 3.

D-Galactose Concentration	Mean (%)	SD
0.05 mM (50 μM)	46.80	6.89
0.10 mM (100 μM)	53.80	9.96
1 mM	55.20	7.26
10 mM	71.84	7.07
50 mM	97.44	1.44

Table 75: Post-hoc Tukey test of (SA- β -Gal)-positive cells in control and (D-Galactose)-treated periodontal ligament fibroblasts at 48 hours.

	0 uM	50 uM	100 uM	1 mM	10 mM	50 mM
0 μM	-	-14.19	-21.19	-22.58	-39.23*	-64.83*
50 μM	14.19	-	-7.0	-8.39	-25.04*	-50.64*
100 μM	21.19	7.0	-	-1.39	-18.05	-43.64*
1 mM	22.58	8.39	1.39	-	-16.65	-42.25*
10 mM	39.23*	25.04*	18.05	16.65	-	-25.6*
50 mM	64.83*	50.64*	43.64*	42.25*	25.6*	-

All cells are from Passage 3.

(*) Statistically significant at $p < 0.05$

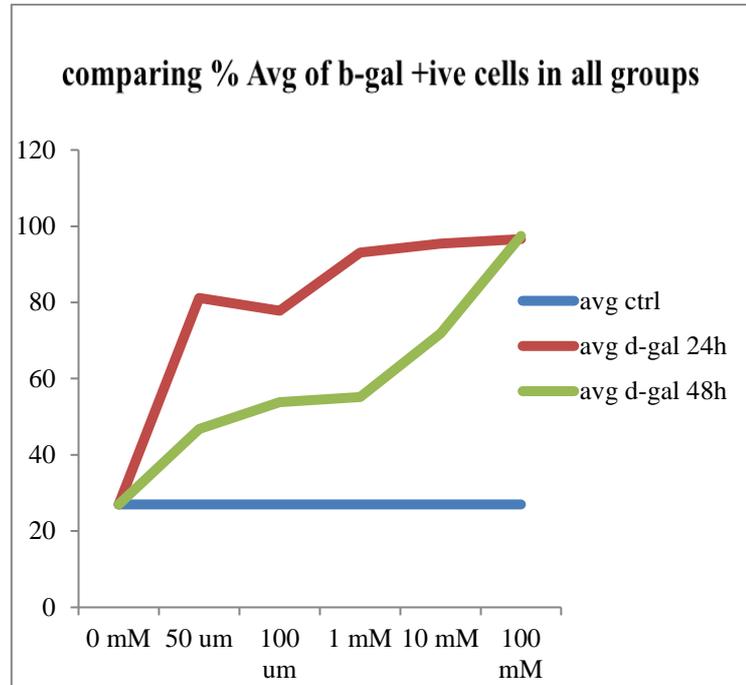


Figure 24: Average percentage of (SA-β-Gal)-positive cells between time points: 24 hours and 48 hours.

Western-blot Analysis

Five different proteins (PI3K, MAPK (ERK.1/2), NF- κ B, NADPH, RAGE) were investigated for their expression among the experiment groups at two different time points: 24 hours and 48 hours. Results were calculated after normalizing for indigenous control (β -Actin) and total antibody for each protein.

PI3K: Using PI3K antibodies (cell signaling #9655 PI3K sampler kit), PI3K was not detectable in the experiment groups in three repeated experiments.

MAPK (ERK1/2): Using MAPK ERK1/2 antibodies (cell signaling #9102, #3510), the expression of phosphorylated MAPK ERK1/2 (pMAPK) was measured and the data from two separate experiments were analyzed. Experiment groups were all compared to control cells at 24 hours. Average of two experiment findings is displayed in [Figure 25] and [Table 76]. At 24 hours: (D-Galactose)-treated cells showed an average of 2.5-fold increase in phosphorylated MAPK ERK1/2 expression, compared to their control counterparts. At 48 hours, control cells showed an average of 5.42-fold increase in pMAPK expression, while (D-Galactose)-treated cells showed an average of 9.63-fold increase.

Although the difference in pMAPK ERK/12 expression was noticeable between experiment groups and in each time points, it was not statistically significant at $p < 0.05$. Statistical analysis (student t-test) comparing each time point experiment groups separately showed no statistical significance [Tables 77, 78]. Similarly, in all groups comparison, one-way ANOVA showed the difference to be statistically non-significant at $p < 0.05$ ($P=0.47$) [Tables 79].

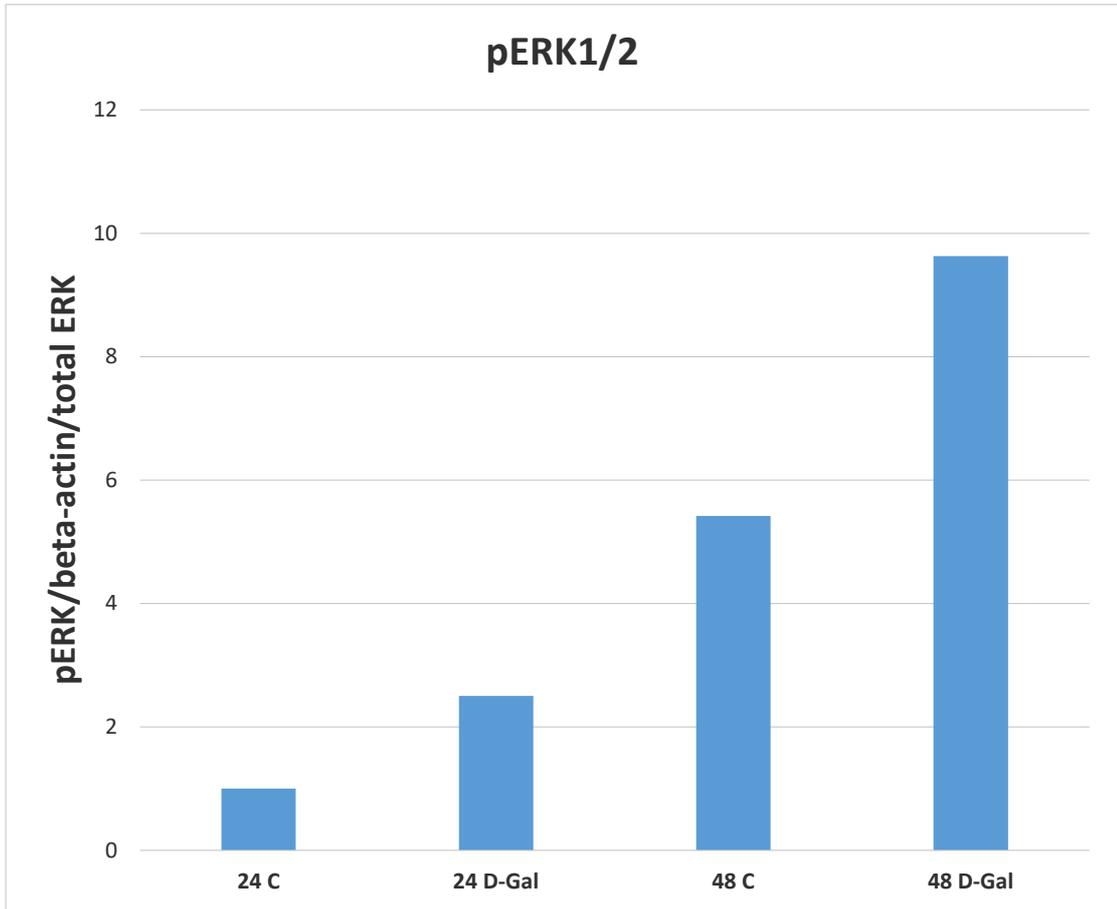


Figure 25: The difference in phosphorylated MAPK (ERK1/2) expression among experiment groups compared to control cells at T24 hours. Values averaged from two experiments

Table 76: Fold increase in MAPK (ERK1/2) expression in each experiment groups compared to control cells at 24 hours

Amount of fold change in MAPK (ERK1/2)				
	1st Experiment	2nd Experiment	Mean	SD
24 hours Control	1	1	1	0
24 hours D-Galactose	3.32	1.69	2.50	1.32
48 hours Control	8.16	2.67	5.42	15.18
48 hours D-Galactose	16.58	2.68	9.63	96.70

Table 77: T-test Comparing MAPK (ERK1/2) expression. at 24 hrs. control and 24 hrs.

	24c	24 D-Gal
Mean	1	2.50
Variance	0	1.32
Observations	2	2.00
Hypothesized Mean Difference	0	
Df	0	
t Stat	0	
P(T<=t) one-tail	0	
t Critical one-tail	0	
P(T<=t) two-tail	0	
t Critical two-tail	0	
P-Value	0.32	

Table 78: t-test Comparing MAPK (ERK1/2) expression at 48 hrs. control and 48 hrs. D-Galactose treated cells.

	48 c	48 D-Gal
	5.4	
Mean	2	9.63
	15.	
Variance	18	96.70
	2.0	
Observations	0	2.00
	0.0	
Hypothesized Mean Difference	0	
	1.0	
Df	0	
	-	
	0.5	
t Stat	6	
	0.3	
P(T<=t) one-tail	4	
	6.3	
t Critical one-tail	1	
	0.6	
P(T<=t) two-tail	7	

	12.
t Critical two-tail	71
<hr/>	
	0.6
P-value	5

Table 79: ANOVA test to compare all four experiment groups for MAPK (ERK1/2) expression.

SUMMARY		mean	SD
Groups	Sum	Average	Variance
24 hrs. Controls	2.00	1.00	0.00
24 hrs. D-Galactose	5.00	2.50	1.32
24 hrs. Controls	10.84	5.42	15.18
48 hrs. D-Galactose	19.26	9.63	96.70

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	86.68	3	28.89	1.02	0.47	6.59
Within Groups	113.20	4	28.30			
Total	199.88	7				

NADPH: Using NADPH antibodies (Abcam #1554245), NADPH was not detectable in the experiment groups in three repeated experiments.

NF- κ B: Using NF- κ B p65 antibodies (cell signaling #3033, #8242), NF- κ B was not detectable in the experiment groups in three repeated experiments.

RAGE: Using RAGE antibodies (cell signaling #4679), RAGE was not detectable in the experiment groups in three repeated experiments.

Membranes from both experiments displayed in [**Figure 26, 27**].

1st Experiment

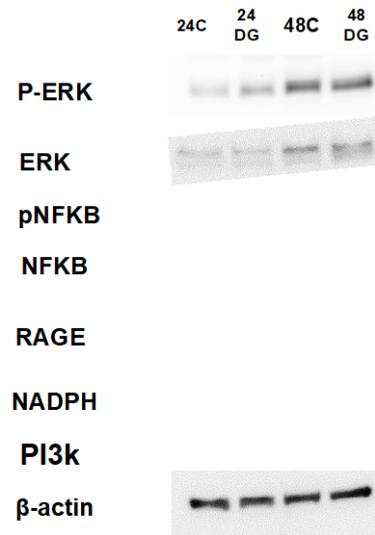


Figure 26: Polyvinylidene difluoride (PVDF) membrane from the first Western blot experiment. The figure shows protein expression of tested antibodies across all experiment groups.

24 C: Control group at 24 hours, 24 DG: D-Galactose group at 24 hours., 48 C: Control group at 48 hours.. 48 DG: D-Galactose group at 48 hours

2nd Experiment



Figure 27: Polyvinylidene difluoride (PVDF) membrane from the second Western blot experiment. The figure shows protein expression of tested antibodies across all experiment groups.

24 C: Control group at 24 hours, 24 DG: D-Galactose group at 24 hours., 48 C: Control group at 48 hours.. 48 DG: D-Galactose group at 48 hours

Supernatant Analysis

A total of six markers were tested for the experiment groups at two time points, 24 and 48 hours. The average values of five repeated experiments were analyzed. Analysis started by normality testing of the sample distribution [**Table 80**].

Table 80: Distribution Normality of study sample measures among experiment groups (after averaging all five experiments)

	IL-1β	1L-8	Fibronectin	IL-6	MMP-1	TNF-α
24 hrs. Control	Normal	Normal	Normal	Normal	Normal	Normal
24 hrs. D-Galactose	Not applicable	Normal	Normal	Normal	Not normally distributed	Not normally distributed
48 hrs. Control	Not normally distributed	Normal	Normal	Normal	Not normally distributed	Normal
48 hrs. D-Galactose	Not normally distributed	Normal	Not normal	Normal	Normal	Not normally distributed
Statistical test used	Kruskal- Wallis	ANOVA	Kruskal- Wallis	ANOVA	Kruskal- Wallis	Kruskal- Wallis
24 hrs comparison	Wilcoxon	T-test	T-test	T-test	Wilcoxon	Wilcoxon
48 hrs comparison	Wilcoxon	T-test	Wilcoxon	T-test	Wilcoxon	Wilcoxon
Descriptive measure used	Median	Mean	Median	Mean	Median	Median

IL-1 β : Results of Interleukin-1 β expression among all the experiment groups are displayed in **Table 81**. At 24 hours, supernatant from D-Galactose group showed a decrease in IL-1 β compared to supernatant from controls (Medians 0.45 pg/ml and 1.18 pg/ml, respectively) [**Figure 28**], this difference was found statistically not significant at $p < 0.05$ (Wilcoxon test $p = 0.40$). While at 48 hours, control group and D-Galactose group both showed the same level of IL-1 β [**Figure 29**]. Comparing all experiment groups, the difference in IL-1 β level was found statistically not significant at $p < 0.05$ (Kruskal Wallis $p = 0.143$).

Table 81: Descriptive statistics of IL-1 β among all experiment groups (pg/ul).

Interleukin -1β				
Time	N	Mean	SD	Median
24c	5	1.40	1.08	1.18
24dg	5	0.45	0.00	0.45
48c	5	0.63	0.37	0.45
48dg	5	0.32	0.23	0.45

IL-1b - 24 hours (median)

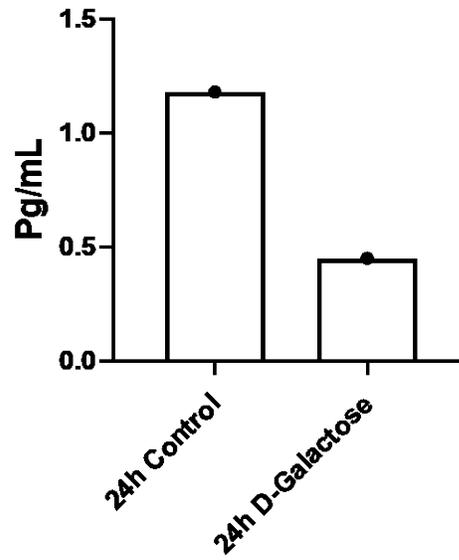


Figure 28: Levels of IL-1 β among 24 hours' time point.

IL-1b - 48 hrs (median)

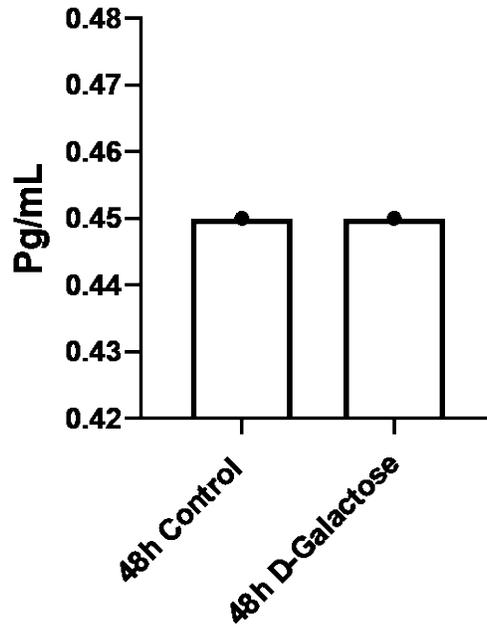


Figure 29: Levels of IL-1 β among 48 hours' time point.

IL-6: results of Interleukin-6 expression among all the experiment groups are displayed in **Table 82**. At 24 hours, supernatant from D-Galactose group showed a slight decrease of IL-6 compared to supernatant from controls (means 0.93 pg/ml and 1.03 pg/ml, respectively) [**Figure 30**], this difference was found statistically not significant at $p < 0.05$ (T-test $p = 0.70$). Similarly, at 48 hours, D-Galactose group showed a slight decrease in IL-6 level compared to their control counterpart (means 1.42 pg/ml and 1.67 pg/ml) [**Figure 31**], this difference was found statistically not significant $p < 0.05$ (T-test $p = 0.47$). Comparing all experiment groups, the difference in IL-6 level found statistically not significant at $p < 0.05$ (ANOVA $p = 0.88$).

Table 82: Descriptive statistics of IL-6 levels among all experiment groups. (pg./ml)

Interleukin 6				
Time	N	Mean	SD	Median
24c	5	1.03	0.46	0.96
24dg	5	0.93	0.38	0.79
48c	5	1.67	0.42	1.85
48dg	5	1.42	0.62	1.49

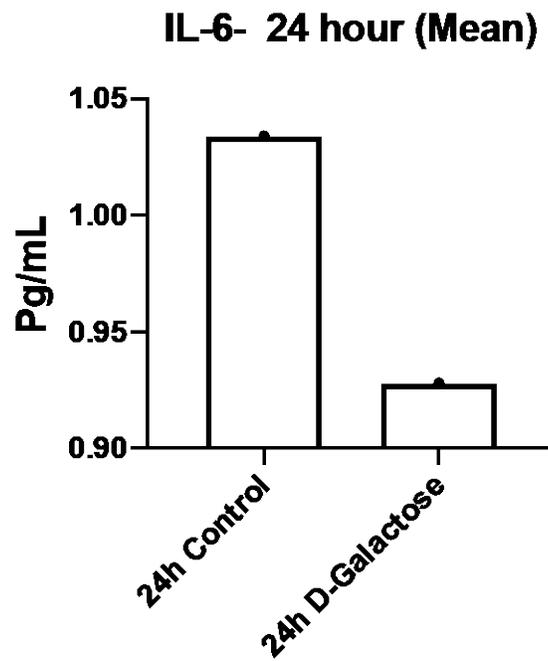


Figure 30: Levels of IL-6 among 24 hours' time point.

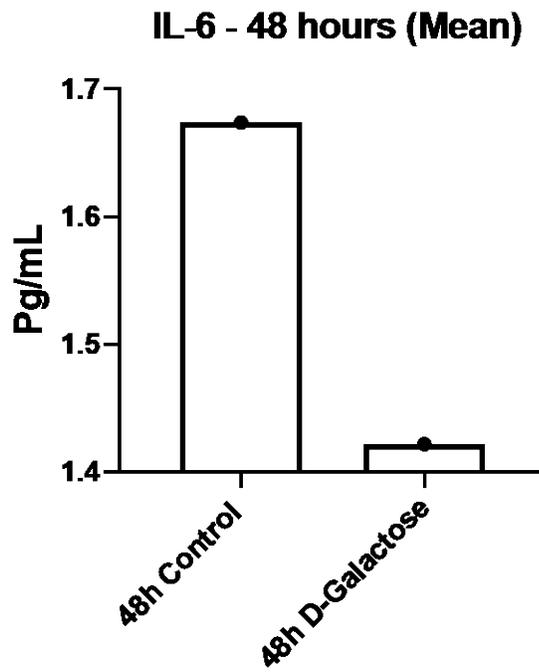


Figure 31: Levels of IL-6 among 48 hours' time point.

IL-8: results for Interleukin-8 expression among all the experiment groups are displayed in **Table 83**. At 24 hours, supernatant from D-Galactose group showed a mild decrease of IL-8 compared to supernatant from controls (means 11.27 pg/ml and 11.61 pg/ml, respectively) [**Figure 32**], this difference was found statistically not significant at $p < 0.05$ (t-test $p < 0.93$). Similarly, at 48 hours, D-Galactose group showed a decrease in IL-8 levels compared to their control counterpart (means 11.71 pg/ml and 13.36 pg/ μ ml [**Figure 33**], this difference was found statistically not significant $p < 0.05$ (t-test $p < 0.69$). Comparing all experiment groups, the difference in IL-8 level was found statistically not significant at $p < 0.05$ (ANOVA $p = 0.95$).

Table 83: Descriptive statistics of IL-8 levels among all experiment groups. (pg./ml)

Interleukin-8				
Time	N	Mean	SD	Median
24c	5	11.61	7.27	11.46
24dg	5	11.27	3.99	9.88
48c	5	13.36	7.64	15.24
48dg	5	11.71	4.30	11.02

IL-8 - 24 hours (Mean)

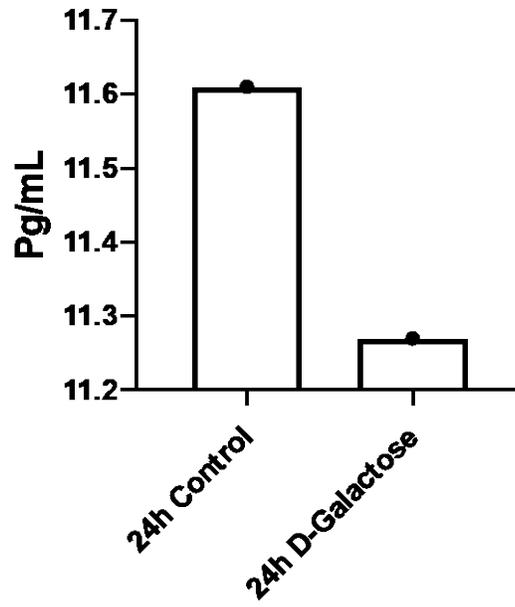


Figure 32: Levels of IL-8 among 24 hours' time point.

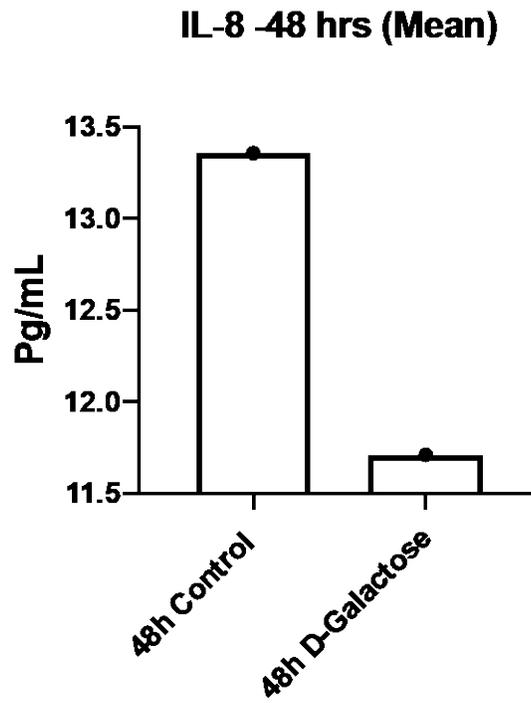


Figure 33: Levels of IL-8 among 48 hours' time point.

MMP-1: Results of MMP-1 expression among all the experiment groups are displayed in **Table 84**. At 24 hours, supernatant from D-Galactose group showed a marked reduction in level of MMP-1 compared to supernatant from controls (medians 50.98 pg/ml and 61.25 pg/ml, respectively) [**Figure 34**], this difference was found statistically not significant at $p < 0.05$ (Wilcoxon test $p = 0.41$). Similarly, at 48 hours, D-Galactose group showed a marked decrease in level of MMP-1 compared to their control counterpart (mean 86.73 pg/ml and 102.67 pg/ml) [**Figure 35**]; however, this difference was found statistically not significant $p < 0.05$ (Wilcoxon test $p = 0.51$). Comparison of MMP-1 level among all experiment groups showed the difference to be statistically not significant at $p < 0.05$.

Table 84: Descriptive statistics of MMP-1 levels among all experiment groups. (pg./ml)

<hr/>				
MMP-1				
Time	N	Mean	SD	Median
<hr/>				
24c	5	75.31	34.07	61.25
24dg	5	62.24	27.60	50.98
48c	5	102.67	40.73	85.51
48dg	5	86.73	31.74	81.29
<hr/>				

MMP-1- 24 hrs (median)

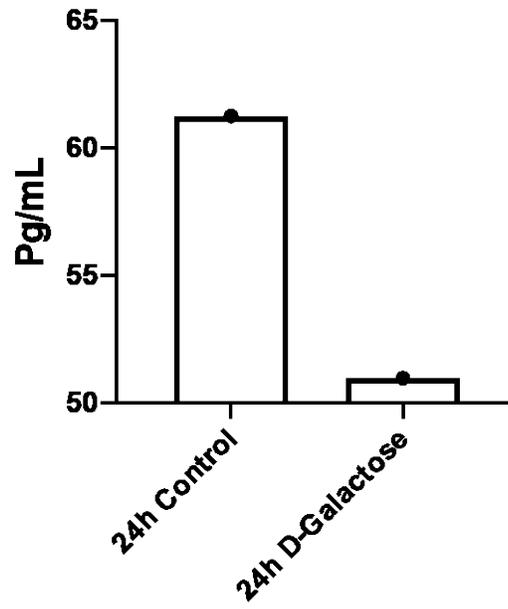


Figure 34: Levels of MMP-1 in 24 hours' time point

MMP-1- 48 hrs (median)

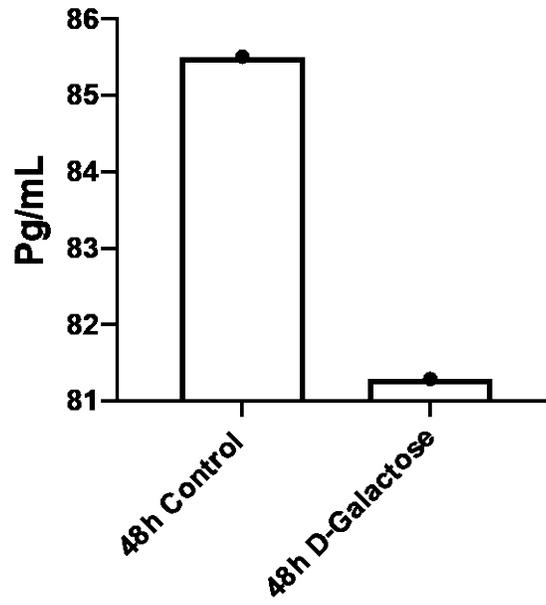


Figure 35: Levels of MMP-1 among 48 hours' time point

Fibronectin: Results of Fibronectin expression among all the experiment groups are displayed in **Table 85**. At 24 hours, supernatant from D-Galactose group showed a significant decrease in fibronectin levels compared to supernatant from controls (means 208838.85 pg/ml and 372528.64 pg/ml, respectively) [**Figure 36**], this difference was found statistically significant (t-test $p=0.0001$). At 48 hours, D-Galactose group showed a significant decreased in fibronectin levels compared to their control counterpart (medians 385245.73 and 533355.24 pg/ml; respectively) [**Figure 37**], this difference was found statistically significant $p<0.05$ (Wilcoxon $p=0.036$). Difference in Fibronectin level among all groups was found statistically significant at $p<0.05$ (Kruskal Wallis $p=0.004$).

Table 85: Descriptive statistics of Fibronectin levels among all experiment groups. (pg/ml)

Fibronectin				
Time	N	Mean	SD	Median*
24c	5	372528.64	43499.66	371628.10
24dg	5	208838.85	25684.58	213259.01
48 c	5	603544.14	136755.47	533355.24
48dg	5	405807.80	63413.26	385245.73

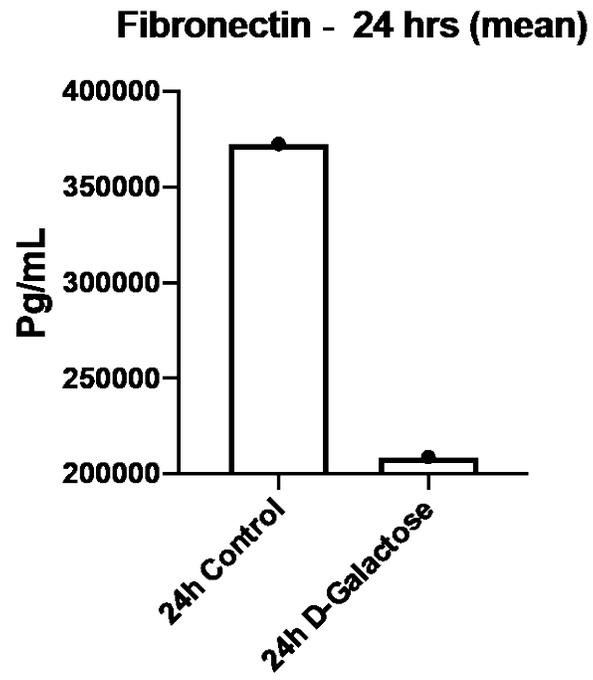


Figure 36: Levels of fibronectin in 24 hours' time point.

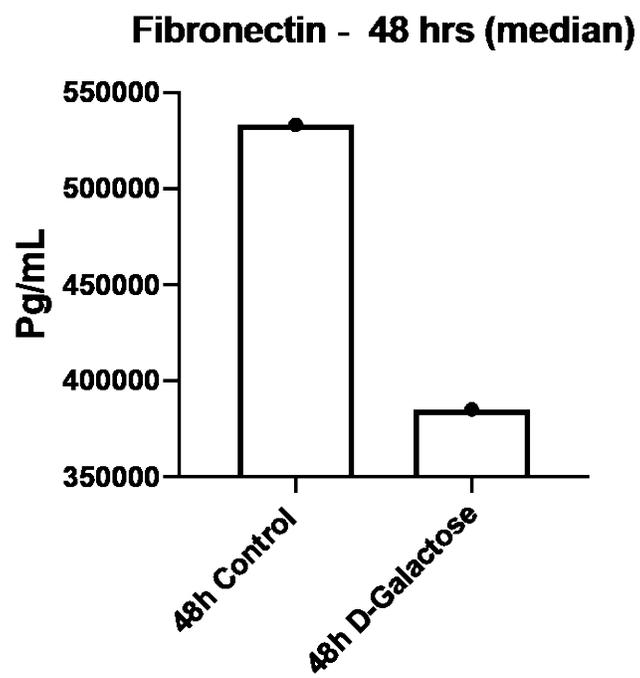


Figure 37: Levels of fibronectin in 48 hours' time point

TNF- α : Results of TNF- α expression among all the experiment groups are displayed in **Table 86**. At 24 hours, supernatant from D-Galactose group showed a slight increase in level of TNF- α compared to supernatant from controls (medians 1.37 pg/ml and 1.28 pg/ml, respectively) [**Figure 38**], this difference was found statistically not significant at $p < 0.05$ (Wilcoxon test $p = 0.90$). At 48 hours, D-Galactose group showed a decrease in level of TNF- α compared to their control counterpart (medians 0.95 pg/ml and 1.78 pg/ml) [**Figure 39**], this difference was found statistically not significant $p < 0.05$ (Wilcoxon test $p = 0.27$). Difference in TNF- α level among all groups was found statistically not significant at $p < 0.05$ (Kruskal Wallis $p = 0.57$).

Table 86: Descriptive statistics of TNF- α levels among all experiment groups (pg/ml)

<hr/>				
TNF-α				
Time	N	Mean	SD	Median
<hr/>				
24c	5	1.28	0.48	1.16
24dg	5	1.29	0.19	1.37
48c	5	1.57	0.51	1.78
48dg	5	1.20	0.37	0.95
<hr/>				

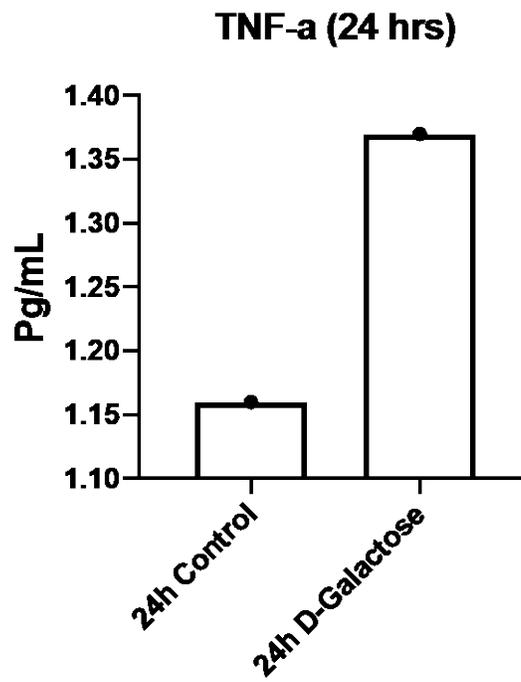


Figure 38: Levels of TNF- α (median) in 24 hours' time point

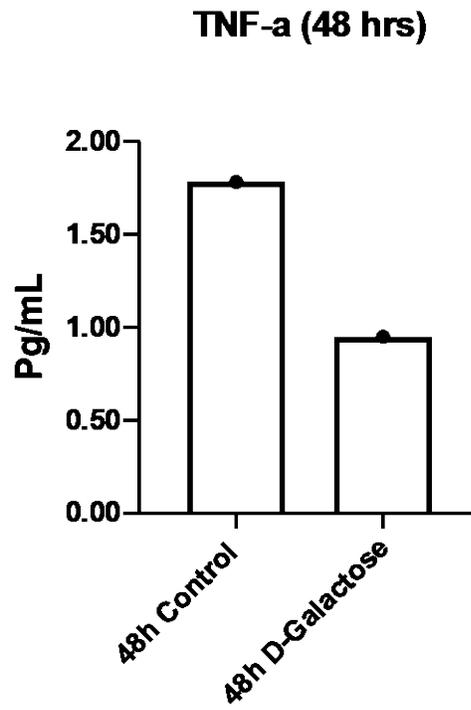


Figure 39: Levels of TNF- α (median) in 48 hours' time point

DISCUSSION

In this study, we tested the hypothesis that aging is characterized by molecular-level mechanisms that lead to inflammation and tissue damage. This is the second part of our work that focused on the impact of aging on saliva markers and the mechanism of senescence-induced changes in periodontal ligament fibroblasts. The premise is that orthodontic treatment at varying ages in humans may show different patterns and treatment outcomes. Therefore, our overall goal was to understand potential differences between individuals at varying ages and how periodontal ligament fibroblast phenotype changes. In this part, we cultured periodontal fibroblasts at multiple passages and tested for the presence of senescence phenotype in the cells. Then, we used D-Galactose to induce premature senescence to periodontal ligament fibroblasts. Our protocol included investigating senescence in periodontal ligament fibroblasts on multiple levels: First, senescence-associated changes in cells' proliferation. Second, molecular level changes in protein expression. Third, changes in periodontal ligament fibroblast secretory phenotype with senescence.

Senescence-induced periodontal ligament fibroblast proliferation

Senescence-Associated beta-Galactosidase (SA- β -Gal) is one of the most widely used markers for cellular senescence. Multiple reports indicated that the expression of SA- β -Gal increases with age in tissues (Konstantonis et al., 2013; Bae et al., 2018). SA- β -Gal is expressed in periodontal ligament fibroblasts. It has been reported to increase in periodontal ligament fibroblasts with replicative cell senescence and with premature senescence models such as exposure to H₂O₂ (Konstantonis et al., 2013; Bae et al., 2018).

One of the aims of our study was to investigate how SA- β -Gal expression varied across passages from cell culture of periodontal ligament fibroblasts to assess its stability without any induction of senescence. As SA- β -Gal expression showed no significant difference across passages 3-9, it was concluded that no considerable replicative senescence occurred in periodontal ligament fibroblasts during these passages. This is consistent with findings of previous studies that mentioned that periodontal ligament fibroblasts reach replicative senescence around passages from P22-P25 (Sawa et al., 2000). This finding indicates that passages from the P1-P10 range can be used as control cells for aging studies in the future. Passage 5 showed slightly higher numbers of (SA- β -Gal) positive cells, this can be due to intra-passage variability; however this finding was not statistically significant when compared to other passages.

Multiple studies reported the effect of D-Galactose induced aging on animals (Mao et al., 2012; Tang et al., 2013; Ali et al., 2015; Y. Yu et al., 2015). However, fewer studies were done to investigate the effect of D-Galactose on cells *in vitro* (Y. Y. Liu et al., 2013; Shen et al., 2014; Rahimi et al., 2018). Until the date of this work, no literature was found on the effect of D-Galactose on periodontal cells. D-Galactose is proposed to induce the senescence effect on tissues via creating oxidative damage (Zheng et al., 2009; Ali et al., 2015; Y. Yu et al., 2015). When D-Galactose accumulates in high concentrations, advanced glycation end-products (AGE) form. AGEs activate their receptor on the cells (RAGE) to start a series of signaling mechanisms that include activation of phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase).

This leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and ultimately to the cellular production of oxidative free radicals and the release of pro-inflammatory cytokines (see Introduction, **Figure 20**) (Deora et al., 1998; Hofmann et al., 1999; Wautier et al., 2001; Ding et al., 2007). The deleterious effect of advanced glycation end products is known to have a strong link with aging and multiple age-related diseases such as Alzheimer's disease, diabetes, kidney failure and cardiovascular diseases (A. Ajith et al., 2016). Advanced glycation end-products are also largely linked to periodontal disease and destruction (Katz et al., 2005; H. Nassar et al., 2007; Ren et al., 2009; Zizzi et al., 2013).

One of this study aims was to investigate the effect of D-Galactose on periodontal ligament fibroblasts. Multiple concentrations of D-Galactose were used to incubate the cells in culture for different time points. Staining showed a significant increase in the expression of SA- β -Gal in D-Galactose treated cells compared to controls. This finding was in agreement with previous literature that used SA- β -Gal to test the senescence effect of D-Galactose on cells (Shen et al., 2014; Rahimi et al., 2018). The effect of D-Galactose on SA- β -Gal expression showed a dose-dependent pattern in both experiment time points. In the 24 hours group, the effect of D-Galactose was higher, presented by higher percentages of SA- β -Gal positive cells, which increased gradually throughout all different D-Galactose concentrations. At 48 hours, the levels of SA- β -Gal expression increased gradually across the first four D-Galactose concentrations: 50 μ M, 100 μ M, 1mM and 10mM. This increase was lower than seen in 24 hours. With 50mM D-Galactose, the percentage of SA- β -Gal expression in the cells remained the same at both time points. This

might indicate that the D-Galactose effect was partially decreased by the 48-hour time point. This could be due to cells replication and presence of new cells that might have increased the total cell population, therefore decreased the percentage of SA- β -Gal-positive cells in proportion. Also, with the increase in cell number, the amount of D-Galactose in the cell culture media would be diluted when divided on a larger number of cells, reducing the D-Galactose effect. Given that the media (for both control and D-Galactose treated cells) was changed only once during the experiments, the amount of D-Galactose in media was distributed on a larger number of cells over the 48 hours.

Previous models of D-Galactose aging had longer duration of D-Galactose administration to provide a chronic effect. In animal models, D-Galactose was injected for a period of 6-10 weeks (Mao et al., 2012; Tang et al., 2013; Banji et al., 2014; Ali et al., 2015; C. yan Liang et al., 2017), and in cell culture, continuous exposure lasted for 7-14 days (Xie et al., 2012; Shen et al., 2014). In our cell senescence model, we had to change the experiment timing to shorter durations. After deciding the proper seeding density for cells to grow healthy in culture, periodontal ligament fibroblasts were found to reach confluence at a maximum of three days. This study initially included a 72-hour time point; however, we excluded this time point due to multiple reasons. Firstly, as cells reached confluency, they are at more risk of contact inhibition, which happens when cells are in dense populations in culture (Gos et al., 2005). High confluency affects cells growth, and function in culture (Nelson et al., 2002), leading to inaccurate study design, as the outcome might not be entirely due to senescence. Secondly, SA- β -Gal has been reported to be increased with confluency (N. C. Yang et al., 2005), therefore this might create false-positive information about the level of senescence expression in the cells. Although longer

cell culture duration might give a more resemblance to chronic administration of D-Galactose in animal models; however, the findings of this experiment might shed light on short-term effect D-Galactose on cells; particularly as it shows that the effect of D-Galactose starts directly at 24 hours. Modification in this study design by including an earlier time points could help in the understanding the nature of any “acute” changes that happen to cells after D-Galactose exposure.

II. Molecular mechanisms of senescence-induced changes in periodontal ligament fibroblasts

For phosphorylated MAPK (ERK1/2) expression, there was a marked increase with D-Galactose at both time points. This is in agreement with previous research that investigated the expression of MAPK (ERK1/2) with D-Galactose aging models (Y. M. Chang et al., 2017; Bo-Htay et al., 2018). This also comes in agreement with reports indicating the increase of MAPK (ERK1/2) with AGE’s-induced oxidative damage in periodontal and gingival fibroblasts (Kanzaki et al., 2017; Nonaka et al., 2018; Elenkova et al., 2019). Comparing each D-Galactose group to its control group in each time point, the change in phosphorylated MAPK (ERK1/2) with D-Galactose treatment at 24 hours appear to be higher compared to the change in the D-Galactose group on 48 hours (3.3-fold and 2-fold changes respectively, compared to their control counterparts). This shows a similar pattern to the staining results, indicating that a large part of the D-Galactose effect takes part as early as 24 hours. Another finding was the change in MAPK (ERK1/2) expression among control cells at 48 hours compared to the 24 hours groups. There is an apparent increase in MAPK (ERK1/2) levels which might be due to the difference in cells’

age, as higher expression of MAPK (ERK1/2) was reported in senescent periodontal ligament cells (Konstantonis et al., 2013; Jia et al., 2018). This change was not found in SA- β -Gal expression comparing control cells at 24 hours with 48 hours group. This might be an indication that the increase in MAPK (ERK1/2) protein is one of the earlier signs of D-Galactose induced senescence in periodontal ligament fibroblasts *in vitro*, whereas changes in (SA- β -Gal) expression need more time to occur.

Given the limitations of this study, we cannot conclude findings about the expression of other markers including RAGE, NADPH oxidase, PI3K, NF- κ B. There is a possibility that D-Galactose effect on periodontal ligament fibroblasts involves signaling through RAGE then MAPK (ERK1/2) rather than through NADPH oxidase and PI3K. However, this doesn't explain why NF- κ B and RAGE were not detected. Antibodies effectiveness might be a reason for the lack of detection. Repeating the experiment with antibodies from manufacturers that provide positive controls can be beneficial to clarify such findings in the future.

Fibronectin is a glycoprotein released from periodontal ligament fibroblasts and creates a part of their extracellular matrix. Fibronectin plays an essential role in cell growth, migration and proliferation, in addition to cell-cell and cell-matrix communication (Benatti et al., 2008). Changes in fibronectin levels have been associated with multiple age-related pathologies and are known to compromise wound repair and alter tissue integrity. Fibronectin degradation in presence of (AGEs) has been reported. It is thought to contribute to impaired wound healing in diabetic foot and periodontal disease associated with diabetes (Murillo et al., 2008; Stanley et al., 2008; J. qiang Liu et al., 2011). It has also been reported that oxidative radical changes in fibronectin can affect osteoblasts activity and bone

formation potential, which might be related to decreased bone formation during aging (Abiko et al., 1998; Huttner et al., 2009).

In this study, analyzing the periodontal ligament fibroblast supernatants showed a significant decrease in fibronectin levels with D-Galactose treatment. This decrease was observed at both time points of the experiment. This finding is in agreement with another study where they showed that activation of AGE/RAGE pathway in periodontal ligament fibroblasts *in vitro* caused decreased levels of supernatant fibronectin (Zhan et al., 2018). However, contrasting results were found about fibronectin changes in D-Galactose aging models on different cell types. D-Galactose aging model in rats' kidney showed an increase in fibronectin levels in senescent glomerular mesangial cells and tubular epithelial cells (Z. Li et al., 2004). Similar results were found in a rats' retina D-Galactose aging model, where increased fibronectin levels caused basement membrane thickening of the retinal capillaries (Roy et al., 2003). This might be attributed to the different function of fibronectin in different tissue types. As aging is associated with an imbalance in extracellular matrix components and function, it is expected this imbalance would affect body organs and tissues differently (Labat-Robert, 2003). Fibronectin plays an important role in periodontal ligament attachment to gingival fibroblasts, as degraded fibronectin contributes to poor wound healing and tissue integrity (Ohshima et al., 2003). It has been reported that fibronectin fragments lead to activation of matrix metalloproteases and extracellular matrix degradation in chronic inflammatory conditions of bone loss, such as osteoarthritis and periodontitis (Kapila, and Johnson 1996). However, excess levels of fibronectin deposition in the extracellular matrix can cause conditions like fibrosis, retinopathy and stiffening of vascular tissues (To et al., 2011; Harvey et al., 2016; Valiente-Alandi et al., 2018).

Therefore, fibronectin regulation is important for tissue health. Part of this regulation happens by extracellular matrix proteases, which their release in the tissue is regulated by fibronectin. With age, there is multiple alterations in extracellular components, which leads to different changes in tissues' function and physiology, and consequently, age-associated pathologies.

From our findings we observed that D-Galactose caused senescence in periodontal ligament fibroblasts. The expression of senescence-associated marker: Beta-Galactosidase increased according to D-Galactose concentration in a dose-dependent pattern. D-Galactose acted on cells through RAGE/AGE pathway via activating MAPK signaling in the cell. Along with increased SA- β -Gal expression, senescent periodontal ligament fibroblast showed a decreased release of fibronectin.

Limitations and future directions:

As previously mentioned, the use of primary antibodies with positive control for western-blotting could help provide more information about protein expression in the cells. Repeating this experiment with positive control could provide more information about the expression of RAGE, PI3K, NF- κ B, and NADPH oxidase.

The supernatant analysis showed that some markers in the supernatant were lower than the detectable range. This might suggest that supernatant was too dilute. The culture media volume was chosen following the cells' manufacturer (Lonza) instruction, and to ensure cells healthy growth conditions. However, optimizing the protocol to improve the supernatant protein yields might be beneficial. For example, it might be possible to perform the experiment using the same cell seeding density in smaller culture vessels and using lower media volume.

CONCLUSIONS

- No senescence-related changes were observed in periodontal ligament fibroblasts from passage 1-passage 10 *in vitro*.
- Treatment with 50mM D-Galactose for 24 and 48 hours induced senescence in periodontal ligament fibroblasts.
- (D-Galactose)-induced senescence in periodontal fibroblasts is thought to be due to upregulation of MAPK (ERK1/2) through AGE/RAGE pathway.
- Senescent periodontal ligament fibroblasts showed a decrease in released fibronectin after treatment with D-Galactose for 24 hours and 48 hours.

APPENDIX

Protocol (1)

Senescence-Associated B-Galactosidase staining: (Sigma Cat. # CS0030)

1. Aspirate the growth medium from the cells.
 2. Wash the cells twice with 1 ml of 1× PBS per well/plate. Carefully remove the entire wash solution by aspiration, so the cells do not detach.
 3. Add 1.5 ml per well of 1× Fixation Buffer and incubate the plate for 6–7 minutes at room temperature.
 4. During the fixation process prepare the Staining Mixture as described in the Preparation Instructions.
 5. Rinse the cells 3 times with 1 ml of 1× PBS per well/plate.
 6. Add 1 ml of the Staining Mixture per well.
 7. Incubate at 37 °C without CO₂ until the cells are stained blue (2 hours to overnight). Seal the plate with Parafilm to prevent it from drying out. The exact incubation time must be optimized.
- Note: The staining of senescent cells is pH dependent. Therefore, the cells cannot be incubated in a CO₂ enriched atmosphere during the staining step.*
8. Observe the cells under a microscope. Count the blue-stained cells and the total number of cells. Calculate the percentage of cells expressing b-galactosidase (senescent cells).
 9. After staining, if required, the Staining Mixture may be replaced with 1xPBS.
 10. For long-term storage of the stained plate, aspirate the staining mixture, overlay the cells with a 70%, glycerol solution, and store at 2–8 °C.

* Staining Mixture - (Prepare just prior to use):

Mix the following for preparation of 10 ml of the Staining Mixture:

- 1 ml of Staining Solution 10x (Catalog Number S5818)
- 125 ml of Reagent B (Catalog Number R5272)
- 125 ml of Reagent C (Catalog Number R5147)
- 0.25 ml of X-gal Solution (Catalog Number X3753)
- 8.50 ml of ultrapure water

Source:

<https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/cs0030bul.pdf>

Protocol (2)

Cell lysis by Cell Lytic M (Sigma Cat. #C2978)

- 1- Remove the growth medium from the cells to be assayed.
- 2- Rinse the cells once with DPBS being careful not to dislodge any of the cells and discard DPBS.
- 3- Add appropriate volume of cell lytic cocktail (prepared by adding 1:100 Protease/ inhibitor (Sigma #C3228) to cell Lytic TM (Sigma))
- 4- Incubate the cells for 15 minutes on a shaker (preferably on ice). Meanwhile, prepare chilled tubes.
- 5- Centrifuge the lysed cells for 15 min at 12,000 -20,000 x g to pellet the cellular debris
- 6- Remove the protein-containing supernatant to a chilled test tube

Note: Lysate preservation requires low temperatures. Therefore, for long term storage it is recommended to store lysate at -70⁰C.

Source:

<https://www.sigmaaldrich.com/life-science/proteomics/recombinant-protein-expression/cell-lysis/mammalian-cell-lysis/cellytic-mt.html>

Protocol (3)

BCA protein concentration (Thermofisher Cat. #23225)

- 1- Pipette 25µl of each standard or protein sample replicate into individual wells of a 96-well plate
- 2- Add 200µl BCA working reagent to each well.
- 3- Mix on plate shaker for 30 seconds.
- 4- Cover plate.

Standard assay: incubate reaction at 37⁰C for 30 min or at room temperature for 2-16 hrs.

Enhanced assay: incubate reactions at 60⁰C for 15 min 4- Cool plate to room temperature

- 5- Measure absorbance at 562 nm on a plate reader

Note: if the plate reader does not have a 562 nm filter, wavelengths from 540-590 nm have been used successfully

- 6- To obtain corrected absorbance, subtract the absorbance of the blank standard from the absorbance measurement of all other standard and protein samples
- 7- Plot the corrected absorbance versus the known mass of the BSA standards to generate the standard curve.

Note: if a curve fitting algorithm associated with the microplate reader is used for preparing the standard curve, a four parametric (quadratic) or best-fit curve will provide the most accurate results.

8. Using the standard curve, interpolate the recorded corrected absorbance reading for the samples assayed which fall within the linear range of the standard curve.
9. Calculate the amount of protein present in the original sample by correcting for dilution and sample volume.

Source:

TVD-Lab protocol: Danielle Stephens, Daniel Nguyen.

<https://www.thermofisher.com/order/catalog/product/23250#/23250>

Protocol (4)

Western Blot

Sample preparation

1. Wash cells 1x with DPBS.
2. Collect cells and follow Lysis buffer specific protocol, centrifuge and use supernatant.
3. Perform BCA or Bradford assay to determine protein concentration. Freeze samples at -20 or -80°C for later use or continue.
4. Thaw samples completely and mix well. Centrifuge at 10,000 x g for 8 min to pellet debris.
5. Calculate amount/volume of sample needed. Always load equal amounts of sample, volumes may vary or bring to equal volumes with PBS.
6. Add LDS buffer to calculation.
7. Boil for 5-8min at 100°C in boiling water bath.
8. Let cool and centrifuge at 10,000 x g for 1 min.
9. Load all sample to gel.
10. Remove green casting frame, place gels in inner electrophoresis chamber, wells facing in- ward. Squeeze gel molds together and lock into place. Put inner chamber into running tank.
11. Fill inner chamber with running buffer to just overflow it.
12. Pour out some buffer from inner chamber until level is about halfway filling the gel wells. 13- Use 1ml pipette to clean each well and remove excess Acrylamide, using 800ul buffer.

13. Refill inner chamber with running buffer (if leaky dump out all running buffer, remove inner chamber, re-lock the gels into place, and try again).
14. Load samples into wells (always load 10ul of ladder at one or both ends of the gel).
15. Add more running buffer in outer chamber to be around 2 inches from the bottom.
16. Put lid in place, red-red and black-black.
17. Run gel at 80-85V for 2 hours or 100V for 1 hour.

Transfer process

18. Soak two filter papers and two foam pads in blotting buffer (BB) for 10 min.
19. Fill one tray with methanol to activate membrane (1min).
20. Fill second tray with BB to equilibrate gel.
21. Gently open glass gel frame and remove top glass pane.
22. Cut off gel comb and any excess gel. Gently rock the gel off the glass plate into BB to equilibrate (5-10 min).
23. Dump methanol from membrane and pour in BB (it will float so keep it wet).
24. Dump gel's BB into the transfer apparatus and refill with new BB.
25. Slide filter paper under the gel in the tray in BB, center the gel on top of filter paper.
26. On the BLACK side of the clamp case, layer as follows:
 - a. Bottom: 1 foam pad
 - b. Filter paper with gel on top
 - c. PVDF membrane (cut with razor to just cover the gel, ensure no bubbles and keep wet with BB)
 - d. Filter paper (do NOT move once laid down)
 - e. Top: 1 foam pad

27. Close case, squeeze and keep pressure in the centre of the case, slide lock over.
28. Insert into inner chamber with black side to black side.
29. Fill outer tank with BB (do not pour over clamp/case) until level with top edge of outer tank.

Running the gel:

30. Run at 4°C at constant mAmps at 66mA overnight.
31. Remove membrane from transfer apparatus. Ensure all of the visible ladder has been transferred from the gel to the membrane. Handle the membrane with forceps gently.
32. Discard blotting buffer in hazardous waste container.

Blocking

33. Use milk or BSA depending on the primary antibody incubation buffer recommendation.
34. Prepare 10ml block buffer per membrane (5% milk or BSA): 20ml TBS-T + 1g dried milk or BSA.
35. Remove membrane, place in tray with TBS-T for 5 min then replace with blocking buffer for 1-2 hours at room temperature.

Primary Antibody incubation

36. Prepare 10 ml of 5% milk or BSA per membrane depending on antibody manufacturer's datasheet with appropriate concentration of antibody.
37. Make pouches for membrane, seal three sides with heat sealer.
38. Add antibody/buffer (10ml per pouch), seal and shake overnight at 4°C or 2 hr at RT.

Secondary Antibody incubation

39. Remove from pouches and wash 3 x 10 min in TBS-T.

40. Make 10ml 5% milk per membrane with appropriate concentration of secondary antibody.

41. Replace TBS-T with antibody/buffer and shake for 1-2 hours at room temperature.

Developing

Follow enhanced chemiluminescence ECL disclosing kit instructions (ThermoFisher scientific Cat #32106)

Stripping/Re-probing

42. Rinse membranes in TBS-T for 3 x 5min

43. Place in pouch and add 10ml Strip Buffer and incubate at 37°C in shaker for 20 min.

44. Discard strip buffer in hazardous waste container.

45. Rinse 3 x 10 min in TBS-T.

46. Re-block for 1 hour at room temperature in 5% milk or BSA (antibody dependent

47. Continue with new target antibody as above.

Membrane Preservation

- After transferring, wash membranes in ultrapure water 3 x 10 min .
- Dry membrane completely on filter paper and place in a pouch in 4°C
- When ready to use activate membrane in methanol for 2 min
- Wash in ultrapure water 3 x 10min.
- Block in 5% milk or BSA for 1-2 hrs at room temperature
- Continue with WB procedures.

Source:

TVD-Lab protocol: Danielle Stephens

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CURRICULUM VITAE

