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Salivary inflammatory biomarkers during initial orthodontic tooth movement

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Boston University
BOSTON UNIVERSITY
HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

THESIS

SALIVARY INFLAMMATORY BIOMARKERS DURING INITIAL ORTHODONTIC TOOTH MOVEMENT

by

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SALIVARY INFLAMMATORY BIOMARKERS DURING INITIAL ORTHODONTIC TOOTH MOVEMENT

ANA CRISTINA SERRENHO

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ABSTRACT

**Background:** Orthodontic tooth movement is a complex process involving a number of inflammatory and anabolic/catabolic pathways. Further, successful treatment depends on proper timing of orthodontic measures with regard to patient’s growth spurt. Saliva analysis has emerged as a non-invasive collection method to track biomarkers relevant to both tooth movement biology and growth prediction. The aim of the present study was to analyze changes in tooth movement and assess relationships with salivary levels of alkaline phosphatase, IGF-1, TGF-β1, testosterone, BMP-2, BMP-4, and BMP-9t.

**Methods:** Twenty seven healthy patients (17 females, 10 males) with a mean age of 13.3 years, undergoing orthodontic treatment with a non-extraction treatment plan were selected for the study. Height, weight, and other demographic features were recorded; maxillary and mandibular alginate impressions were obtained, and 10 mL saliva samples were taken at the initial visit (T0), after 4 weeks (T1), 3 months (T2), and then at 3-month intervals (T3, T4, T5). Casts were made from alginate impressions. Little’s Irregularity Index, intercanine widths, intermolar widths, arch lengths, and tooth widths were measured. Saliva samples were analyzed for the concentrations of alkaline phosphatase,
IGF-1, TGF-β1, testosterone, BMP-2, BMP-4, and BMP-9 by multiplex immunoassay and/or ELISA. Data were analyzed using correlation coefficients and ANOVA.

**Results**: Over the six timepoints, there were statistically significant changes in the level of testosterone (p < 0.05) and Little’s Irregularity Index (p < 0.01), with the most significant changes occurring at the beginning of treatment (T0 to T1). Statistically significant correlations (p < 0.05) were observed between testosterone and height/weight, intercanine width and intermolar width, BMP-4 and intercanine/intermolar width, alkaline phosphatase and testosterone, alkaline phosphatase and TGF-β1, and among the BMPs.

**Conclusion**: The changes in Little’s Irregularity Index reflects successful progress of the orthodontic treatment. The correlations observed among the various salivary concentrations suggest a number of further directions for study.
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BACKGROUND AND LITERATURE REVIEW

Biological Foundations of Tooth Movement

Tooth movement induced by orthodontic forces is a complex process involving bone remodeling. The most widely accepted model for the biology of tooth movement is the pressure-tension theory\(^1\). The foundation of the pressure-tension theory was laid primarily by Sandstedt, Oppenheim, and Schwarz\(^2\). When a tooth is subjected to a continuous force, the opposing sides of the surrounding periodontal ligament (PDL) undergo distinct changes: on the leading or “pressure” side, bone is resorbed, while on the trailing or “tension” side, new bone is formed\(^3\). Since Sandstedt’s work in 1904, other theories have been suggested as alternatives or supplements to the pressure-tension theory, specifically the hydrostatic theory and bone-bending theory. Despite the number of theories, the consensus is that tooth movement is likely a product of multiple biological pathways acting simultaneously.

Histological observations indicate that tooth movement occurs in multiple phases\(^4\). Application of a loading force leads to initial tooth movement until compression on the leading side of the tooth reduces localized blood flow, leading to necrosis and the creation of a cell-free area termed a hyalinization zone. Further tooth movement cannot occur until the hyalinized tissue is destroyed and cells re-populate the space. After cells migrate to the zone, tooth movement resumes, with direct resorption of bone along the pressure side and bone deposition on the tension side. Burstone classified this process
into three phases: an initial phase of tooth movement lasting 1-2 days, a lag phase of 4-20 days, and a post-lag phase of increasing tooth movement after hyalinized tissue has been removed\textsuperscript{5}.

Molecular regulation of the processes of tooth movement is complex and a large number of signaling molecules have been implicated\textsuperscript{6}. Of particular interest are the processes involving the regulation of osteoclastic and osteoblastic activities. Osteoclasts are multinucleated cells derived from fusion of macrophages\textsuperscript{7}. Osteoclast differentiation and activation are thought to be regulated by cytokines (extracellular signaling proteins), particularly the receptor activator of nuclear factor kappa-b (RANK) system\textsuperscript{8}. There are 3 key elements to the RANK system: the receptor (RANK), the ligand (RANKL) and the inhibitor osteoprotegerin (OPG). RANKL production is up-regulated in response to orthodontic forces and RANKL binding to RANK is critical for osteoclast activation\textsuperscript{9}. OPG acts as a competitive inhibitor of RANKL; the RANKL/OPG ratio is important for maintaining a balance to the level of osteoclastic activity. Salivary levels of soluble RANKL and OPG have been measured in an attempt to identify the different phases of tooth movement\textsuperscript{10}. Roberts \textit{et al} found that the bone resorption by osteoclasts is the rate-limiting step in tooth movement\textsuperscript{11}.

Osteoblast activity is regulated by a number of signaling molecules, including various bone morphogenetic proteins (BMPs), and transforming growth factor-beta (TGF-β)\textsuperscript{12}. Osteoblasts are derived from mesenchymal stem cells (MSCs); the fact that MSCs can
differentiate into numerous tissue types such as adipose and bone explains some of the intricacy and redundancy of the factors involved in osteoblast differentiation\textsuperscript{13}.

**Orthodontic measurements**

Dental cast measurement is an important tool for orthodontic record keeping and tracking of treatment outcomes. A variety of different measurement techniques and standards have been used to assess changes due to treatment, including Little’s Irregularity Index, intercanine and intermolar width, and space analysis\textsuperscript{14}.

*Little's Irregularity Index.* Little’s Irregularity Index (LII) is a scoring method devised by Little in 1975 to provide a quantitative method for evaluation of incisor crowding\textsuperscript{15}. LII has been used as an indicator of crowding in studies measuring the comparative success of various orthodontic treatments as well as post-treatment relapse\textsuperscript{16,17}. LII can be a less time-consuming method for estimating crowding relative to some systems for measuring arch length discrepancy, making it an attractive tool for larger scale epidemiological studies\textsuperscript{18}.

*Intercanine/intermolar width.* Intercanine width (ICW) can be measured as the distance between cusp tips or the gingival margins or the canines, while intermolar width can be measured between mesiolingual or distolingual cusps, or at the gingival level\textsuperscript{19}. Reliability studies have shown that ICW and IMW measurements are highly reproducible and clinically acceptable\textsuperscript{20}. Digital analysis of casts indicate a strong correlation between
ICW measurements and arch length, while only a weak correlation exists between IMW and arch length\textsuperscript{21}. ICW and IMW measurements exhibit statistically significant gender-based differences, making them a potentially useful tool in sex determination in the field of forensics\textsuperscript{22}. However, other studies have shown that related metrics, such as mandibular canine index (the ratio of mesiodistal dimensions of lower canines to the ICW) do not show statistically significant gender differences. This contradiction may be due to differences in the observed populations among the various studies, as well as the way that the ratio is calculated\textsuperscript{23}. In an orthodontic context, ICW and IMW are used to track whether variations in treatment (for example, extraction vs. non-extraction) result in differences in arch form\textsuperscript{24,25}.

*Space Analysis.* Space analysis can be defined as the discrepancy between the space needed for the properly aligned teeth and the space available in the dental arch, and therefore serves as a measure of dental crowding\textsuperscript{26,27}.

**Salivary biomarkers of growth and tooth movement**

In recent years, salivary diagnostic tools have emerged that provide a noninvasive alternative to drawing blood\textsuperscript{28}. The applications of saliva-based diagnostics include the diagnosis of endocrine and immune disorders, identification of infectious agents, screening for cancer, and evaluation of psychiatric issues such as anxiety and stress\textsuperscript{29,30}. 

4
In addition to predicting disease states, salivary diagnostics allow for the measurement of hormones and other factors that play key roles in growth and dental development. These factors include:

*Insulin-like growth factor-1 (IGF-1).* IGF-1 has been shown to play important roles in development. Along with growth hormone (GH), IGF-1 regulates the pubertal growth spurt\(^\text{31}\). Circulating levels of IGF-1 promote bone growth and bone density\(^\text{32}\). IGF-1 has also been shown to stimulate growth of the mandibular condyle in rats\(^\text{33,34}\). Salivary levels of IGF-1 can be measured, but accuracy may be an issue due to low levels relative to the level of serum IGF-1\(^\text{35}\). In contrast, IGF-1 levels measured by blood spot have been shown to be reliable\(^\text{36}\). In 1985, Massoud *et al* used blood spot analysis showed that IGF-1 levels in rabbits increase progressively through puberty, then fall to pre-puberty levels\(^\text{37}\). Blood spot analysis has also been used to establish ranges for both males and females through age 18: mean IGF-1 levels peak at age 13 in females, and age 15 in males\(^\text{38}\). IGF-1 levels have been shown to correlate positively with maturation stages determined by hand-wrist radiographic analysis and with changes in mandibular length\(^\text{39}\). Jain *et al* found statistically significant differences in serum levels of IGF-1 between cervical maturation stages CS-4 and CS-5, and suggested a minimum serum level of 310 ng/mL as a key factor in whether orthodontic treatment should be considered\(^\text{40}\). In 2015, Masoud *et al* concluded that longitudinal changes in IGF-1 levels correlate with vertical facial growth\(^\text{41}\). However, the authors found no significant relationship between single (non-longitudinal) IGF-1 measurements and facial growth. The authors reasoned that
individual measurements are uninformative, given that low IGF-1 could indicate both pre- and post-pubescent state. In addition to its use as a marker of maturation, IGF-1 levels may change in response to orthodontic treatment. IGF-1, in concert with GH, may play a role in regulating root resorption: rats given subcutaneous injections of recombinant GH showed an increase in IGF-1 levels in cells and a corresponding decrease in indexes of root resorption\(^42\). Several studies have suggested IGF-1 plays a role in repair during tooth movement\(^43\); recently, Proff et al showed an up-regulation in the IGF-1 genes of PDL cells in response to a mechanical stressor\(^44\).

*Testosterone*. Testosterone has been shown to promote growth by stimulating GH secretion both directly and through its aromatization to estrogen\(^45\). Evidence from GH-deficient patients suggests that testosterone can independently trigger a substantial pubertal growth spurt\(^46\). This direct mechanism is supported by the presence of androgen receptors in hypertrophic chondrocytes and bone cells at sites of endochondral ossification\(^47\). Testosterone affects both cell differentiation (through an IGF-mediated process) and cell proliferation\(^48\). Salivary testosterone levels have been shown to be highly correlated with free testosterone levels in blood, and have been used in studies across a spectrum of disciplines, from development to athletic performance to psychology\(^49,50,51\). In relation to oral biology, testosterone has been implicated in a number of roles. For instance, rats deficient in testosterone were found to have increased rates of tooth movement and reduced resorption\(^52\). Steffens et al found an association between high levels of serum testosterone and increased prevalence and severity of
periodontitis, although the mechanism by which testosterone mediates periodontitis is unclear\textsuperscript{53}. Testosterone has also been administered to improve orthodontic outcomes: Birlik \textit{et al} increased the rate of bone formation in the mid-palatal suture of rats for rapid maxillary expansion\textsuperscript{54}.

\textit{Transforming growth factor-beta}. The transforming growth factor-beta superfamily of signaling proteins includes TGF-\(\beta\)s and bone morphogenetic proteins (BMPs)\textsuperscript{55}. These proteins play various roles in growth regulation and development. For example, TGF-\(\beta\)3 is necessary for proper secondary palate fusion, while BMPs and related growth and differentiation factors (GDFs) regulate the development of skeletal structures\textsuperscript{56,57}. TGF-\(\beta\) consists of three isoforms (\(\beta1, \beta2, \beta3\)) and has been detected in the saliva\textsuperscript{58}. The TGF-\(\beta\)1 isoform is important in all stages of skeletal development and bone growth, with the most widely accepted view that TGF-\(\beta\)1 act by stimulating proliferation and differentiation of osteoblasts while inhibiting osteoclast activity\textsuperscript{59}. However, other studies have suggested that TGF-\(\beta\)1 plays a role in increasing osteoclast activity and bone resorption\textsuperscript{60}. Karst \textit{et al} attribute the dual nature of TGF-\(\beta\)1 to a concentration-dependent mechanism: low levels of TGF-\(\beta\)1 stimulate osteoclast differentiation by maintaining a high RANKL/OPG ratio, while high levels repress differentiation through several pathways\textsuperscript{61}. Studies of tooth movement further illustrate the complex role of TGF-\(\beta\)1, with one study finding similar expression of TGF-\(\beta\)1 at both the compression and tension sides of teeth subjected to rapid maxillary expansion, and another study finding a higher concentration at the compression site, supporting TGF-\(\beta\)1’s role in bone resorption\textsuperscript{62,63}. 
BMPs. Bone morphogenetic proteins are a subset of the TGF-β superfamily. BMPs play essential roles in osteoblast differentiation through Smad-mediated signaling\textsuperscript{64}. Members of the BMP family exhibit similar yet distinct functions. For example, BMP-7 plays an important role in skeletal patterning and cartilage growth, while BMP-2 is critical for postnatal regeneration of bone\textsuperscript{65,66,67}. The role of BMPs in tooth movement was explored by Mitsui et al, who showed an increase in BMP-2 expression and concurrent decrease in BMP antagonists when human osteoblastic cells were subjected to a compressive force, with 1.0 g/cm\textsuperscript{2} as the optimal loading\textsuperscript{68}.

\textit{Alkaline Phosphatase.} Alkaline phosphatase has been observed to play a role in mineralization as early as 1924\textsuperscript{69}. Specifically, tissue-nonspecific alkaline phosphatase is thought to promote mineral formation by hydrolyzing pyrophosphate to inorganic phosphate\textsuperscript{70}. Alkaline phosphatase has been found in gingival crevicular fluid (GCF), with higher levels indicative of inflammation related to gingivitis and periodontitis\textsuperscript{71}. Alkaline phosphatase levels in GCF have also been identified as a tool for measuring the extent of bone modeling due to orthodontic tooth movement\textsuperscript{72,73,74}. In addition, Perinetti et al found a significant relationship between GCF alkaline phosphatase activity and CVM stages, with peaks levels occurring during the CS3 and CS4 stages of maturation\textsuperscript{75}. 
HYPOTHESIS

Null Hypothesis (H0): There is no correlation between orthodontically-induced tooth movement and salivary levels of the biomarkers alkaline phosphatase, IGF-1, TGF-β1, testosterone, BMP-2, BMP-4, and BMP-9.
MATERIALS AND METHODS

Subject Selection

Thirty-nine subjects were enrolled in the study, all orthodontic patients at the Boston University, Henry M. Goldman School of Dental Medicine, Department of Orthodontics and Dentofacial Orthopedics. Seven patients were terminated from the study for various reasons, and five patients had incomplete sample collections, leaving a total of twenty-seven subjects with complete data sets for all timepoints.

The inclusion criteria were:

- Males and females
- Ages 11 to 20
- Seeking orthodontic treatment
- Parents of minor patient willing to consent
- Patient under 18 years of age willing to assent, verbally if 11 years of age, or on the informed consent form if between the ages of 12 and 17
- English speaking
- Patient between the ages of 18 and 20 willing to consent for themselves
- A non-extraction orthodontic treatment plan

The exclusion criteria were:

- Any medical condition that could affect growth (i.e. acromegaly, dwarfism, hyper or hypothyroidism, achondroplasia, etc.) as noted in their medical history
- Any significant unstable health issue or condition per medical history review (i.e. cancer, hepatitis, TB, etc.)
- Any use of medication or condition that could affect saliva production (i.e. Ca channel blockers, beta-blockers, anti-depressants, etc.)
- Active orthodontic treatment
- Current smoking or tobacco use, which could alter tooth movement
- Chronic use of NSAID (more than three times per week), which could alter tooth movement
- Orthodontic treatment plan involving surgical correction
- Orthodontic treatment plan involving removable aligners for dental correction

The IRB – Institutional Review Board – of Boston University reviewed and approved the consent forms and study protocol. The consent forms for the study were comprised of two consent forms and two assent forms as follows:

- Consent form for subjects between the age of 18 and 20 years old
- Consent form for parents of subjects under the age of 18
- Assent form for subjects ages 12 through 17 years old
- Assent form for subjects 11 years old

Patients and parents were consented at the treatment planning orthodontic appointment. During this visit, the study was explained to potential subjects and parents if under the age of 18. The consent forms, the background and rationale for the study, the number of visits, the samples being collected and the procedures for such collections were explained in detail. It was explained to subject and parents that their participation was strictly
voluntary and that their participation in the study would not interfere with their orthodontic care. Subjects were encouraged to ask questions during this process. Subjects who desired to participate in the study, signed the appropriate consent forms, after which their medical history was reviewed and initial samples collected. There was no group assignment, no randomization, and no use of control subjects

**Data Collection**

Participating subjects had sample collection performed at six different time points designated as T0 through T5 over the course of approximately twelve months. All data collection was performed during the patient’s regular orthodontic treatment for the convenience of the patient – saving them from unnecessary appointments. T0 sample collection was recorded at the treatment planning orthodontic visit. T1 was collected four weeks after orthodontic appliances and initial wire were placed – initial wire being a 0.016 Nickel-Titanium, or a multi-stranded stainless steel wire. T1 represents the initial phase of tooth movement. The third time point collected during a regular orthodontic visit was T2 – this was performed at least two months after T1. T3, T4, and T5 sample collections were collected consecutively at intervals of approximately three months. The following data was collected at time point T0:

- Age, gender, height (in centimeters, cm), weight (in kilograms, kgs)
- 10 mL sample of saliva, collected in a Falcon™ 50 mL tube
- Upper and lower alginate impressions using Dentsply Jeltrate Plus Fast Set and PVS bite registration using Dentsply Regisil Super Fast Set
The following data was collected at each time point from T1 through T5:

- Height (in centimeters, cm), weight (in kilograms, kgs)
- 10 mL sample of saliva, collected in a Falcon™ 50 mL tube
- Upper and lower alginate impressions using Dentsply Jeltrate Plus Fast Set and PVS bite registration using Dentsply Regisil Super Fast Set

All subjects were de-identified and assigned a unique code – the data was organized in Excel spreadsheets and kept in a special drive accessible only by the orthodontic department. Each salivary sample and dental cast were labeled with the same unique subject code as well as the time point at which it was collected, i.e. LC22 T1, LC34 T0.

**Height and Weight measurements**

Height and weight was recorded at each visit T0 through T5 using a Detecto Scale Weigh Beam Eye-Level analog physician’s scale. Height was recorded in centimeters and weight was recorded in kilograms. All measurements were recorded in an Excel spreadsheet.

**Cast/tooth measurements**

The tooth movement was measured on all casts, from the six time points - T0 through T5. All casts were poured in Type 3 dental stone for durability. The casts of twenty-seven subjects at the six different time points were used to measure Little’s Irregularity index, intercanine width, intermolar width, and space analysis\(^7,8,7\). All measurements were taken by the same investigator (A.C.S.) using a digital caliper (Machine DRO 200mm digital
caliper with fine pointed jaws, ME-CAL-FP-200). All measurements were recorded on an Excel spreadsheet. Little’s Irregularity Index (LII) was used to quantify the mandibular anterior alignment from cuspid to cuspid. This involves measuring the linear displacement between adjacent teeth at their contact points. A total of five measurements between the mesial of the mandibular cuspids were recorded and summed, yielding the irregularity index. The mandibular intercanine width (ICW) was measured from cusp tip to cusp tip and recorded in millimeters. The mandibular intermolar width (IMW) was measured from the cusp tip of the mesiobuccal cusp to the cusp tip of the mesiobuccal cusp of the permanent first molars and recorded in millimeters. The space analysis was conducted using the methodology described by Bishara et al.\textsuperscript{90} The space analysis was computed by dividing and measuring the mandibular cast into four straight line segments as follows: mesial of lower left mandibular first molar to mesial of lower left canine, mesial of lower left canine to mesial of lower left central, mesial of lower left central to mesial of lower right canine, mesial of lower right canine to mesial of lower right mandibular first molar. The mesiodistal width of each individual tooth from the lower left 2nd premolar to the lower right 2nd premolar were then measured and totaled. The sum of the tooth widths was then subtracted from the sum of the four arch segments. A positive difference represents spacing in the arch, a negative difference represents crowding in the arch.
**Saliva collection**

Each subject was asked to rinse with water before collection. The patient was given a Falcon™ 50 mL tube and was asked to let the saliva pool in their mouth and occasionally tip their head forward and allow the saliva to drool into the tube. The subject was encouraged not to spit forcefully into the tube to prevent bubbles in the sample. If the subject was having difficult time producing saliva, they were given one or two small squares of odorless, tasteless, semi-transparent, moisture-resistant paraffin wax film (Parafilm M®) to chew on to help stimulating salivary secretion. The saliva samples were then stored at -80°C.

**Saliva Sample Analysis**

The number of saliva samples analyzed was one hundred and sixty-two (twenty-seven subjects with six collection time points each, T0 through T5). The samples were labeled with the de-identified subject number and the respective time point. Salivary samples were transported to the Forsyth Institute (Cambridge, Massachusetts) from Boston University Henry M. Goldman School of Dental Medicine (Boston, Massachusetts) in an insulated styrofoam cooler packed with dry ice (-78.5°C). Once samples arrived at Forsyth Institute, they were transferred to ice and organized by subject ID. Samples were thawed on ice (approx. 3 hours), vortexed 10 seconds each, and then centrifuged for 12 minutes at 1,500 g, at 4°C. Supernatants were aliquoted into four 1.5 mL sterile, labeled Eppendorf tubes per sample. Any remaining supernatant was distributed between the 4 tubes. All supernatant aliquots were re-frozen at -80°C. Assays performed at The Forsyth
Institute included: IGF-1, Testosterone, TGF-β1, BMP2, BMP4, BMP9, and Alkaline Phosphatase. Each assay had its own individual kit as follows:

1. R&D Systems (Minneapolis, MN), Quantikine ELISA Human IGF-1, cat# DG100;
   Sensitivity: range 0.007-0.056 ng/mL, mean 0.026 ng/mL

2. R&D Systems (Minneapolis, MN), Parameter Testosterone assay, cat# KGE010;
   Sensitivity: range 0.012-0.041 ng/mL, mean 0.030 ng/mL

3. R&D Systems (Minneapolis, MN), TGF-β1 Luminex kit, cat# LTGM100;
   Sensitivity: range 2.1-24.6 pg/mL, mean 11.1 pg/mL

4. R&D Systems (Minneapolis, MN), Screening Luminex panel (BMP-2, -4, -9) cat# LXSAHM-3; Sensitivity: BMP-2, 3.6 pg/mL; BMP-4, 5.0 pg/mL; BMP-9, 0.6 pg/mL

5. Abcam (Cambridge, MA) Colorimetric Alkaline Phosphatase Assay, cat# ab83369;
   Sensitivity: >10 µU
Instrumentation

Assays 3 and 4 above (TGF-β1 and BMP panel) were read on the Bio-Plex 200 instrument using Bio-Plex Manager version 6.0 software. Raw data included a graph of the standard curve of each analyte and the following:

**Table 1: Data collected on salivary samples**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Standard (S), Blank (B), Unknown (X)</td>
</tr>
<tr>
<td>Well</td>
<td>Well location on 96-well plate.</td>
</tr>
<tr>
<td>FI</td>
<td>Mean fluorescence intensity of analyte.</td>
</tr>
<tr>
<td>Obs Conc</td>
<td>Observed concentration of analyte, calculated from its FI and the standard curve. Note that obs. conc of standards are back-calculated from their FI and standard curve.</td>
</tr>
<tr>
<td>Std Dev</td>
<td>Standard deviation of a replicate group of wells.</td>
</tr>
<tr>
<td>Obs Conc %CV</td>
<td>Observed concentration of analyte with coefficient of variation of a replicate group of wells.</td>
</tr>
<tr>
<td>Exp Conc</td>
<td>Expected concentration (of standards).</td>
</tr>
<tr>
<td>Obs/Exp*100</td>
<td>Recovery rate for the observed vs. expected concentration of standards or controls.</td>
</tr>
</tbody>
</table>

Bio-Rad Classification/Verification kits are used on a weekly basis to verify quality control and classification parameters. Preventive maintenance of the instrument is performed on a yearly basis by Bio-Rad field specialists. In order to prevent cross-contamination between plates, a wash and sanitize step is performed between each plate. Sonication of the instrument’s probe is also performed between plates.

Assays 1, 2 and 5 above (IGF-1, Testosterone, Alkaline Phosphatase), were read on a SpectraMax 340PC spectrophotometer using SOFTmax Pro version 4.3 software. Quality control and calibration of the instrument are performed every six months. Generated
results are interpreted in the SOFTmax Pro software before being exported as an excel file. Raw data for the cytokine analysis were analyzed to optimize the standard curves using Bio-Plex Manager v6.0 software. Assays run on the SpectraMax were optimized for standard curves using the SOFTmax Pro v4.3 software. Only preliminary optimization of standard curves will be performed. All data were exported as Microsoft Excel files and saved in the following format: “BUortho_assay_date.xls”

The plate maps are as follows:

**Figure 1: Assay plate map**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
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<td>A</td>
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<td>S1</td>
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<td></td>
<td></td>
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<tr>
<td>B</td>
<td>S2</td>
<td>S2</td>
<td>B</td>
<td></td>
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<tr>
<td>C</td>
<td>S3</td>
<td>S3</td>
<td></td>
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<tr>
<td>D</td>
<td>S4</td>
<td>S4</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>S5</td>
<td>S5</td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>S6</td>
<td>S6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>S7</td>
<td>S7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>S8</td>
<td>S8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Assays were carried out per manufacturer instructions (for detailed assay protocols, see Appendix).
STATISTICS

Standard descriptive statistics including averages and standard deviations were computed for each salivary marker and cast measurement, at each time point T0 through T5. Changes in variables over time were evaluated using repeated measures ANOVA. Intra-examiner reliability was assessed using intraclass correlation tests using a mixed-effect, single measure model\(^9\). Statistical significance was determined by using an alpha level of 0.05.
RESULTS

Descriptive Statistics

Summary statistics for the sample studied are reported in Table 2.

Table 2: Descriptive Statistics

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age (Mean)</th>
<th>Age (S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>17</td>
<td>13.06</td>
<td>1.85</td>
</tr>
<tr>
<td>Males</td>
<td>10</td>
<td>13.60</td>
<td>1.96</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>13.26</td>
<td>1.87</td>
</tr>
</tbody>
</table>

The age range for study patients was 11 to 17. Height and weight were recorded at each time point for each patient. Summary statistics are shown in Tables 3 and 4.

Table 3: Mean Heights of Study Patients at each Timepoint (cm)

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>157.00</td>
<td>157.88</td>
<td>158.88</td>
<td>159.41</td>
<td>160.00</td>
<td>160.12</td>
</tr>
<tr>
<td>Males</td>
<td>171.30</td>
<td>172.00</td>
<td>172.40</td>
<td>173.50</td>
<td>174.30</td>
<td>175.10</td>
</tr>
<tr>
<td>Total</td>
<td>162.30</td>
<td>163.11</td>
<td>163.89</td>
<td>164.63</td>
<td>165.30</td>
<td>165.67</td>
</tr>
</tbody>
</table>

Table 4: Mean Weights of Study Patients at each Timepoint (kgs)

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>53.15</td>
<td>53.66</td>
<td>54.54</td>
<td>54.99</td>
<td>56.19</td>
<td>56.38</td>
</tr>
<tr>
<td>Males</td>
<td>70.58</td>
<td>70.17</td>
<td>70.67</td>
<td>72.57</td>
<td>74.80</td>
<td>75.89</td>
</tr>
<tr>
<td>Total</td>
<td>59.61</td>
<td>59.77</td>
<td>60.51</td>
<td>61.50</td>
<td>63.08</td>
<td>63.60</td>
</tr>
</tbody>
</table>

Over the course of the study, the average height for males increased by 2.2% and the average weight increased by 7.5%. Similar results were observed for females (height increase of 2% and weight increase of 6.1%). A statistically significant (p < 0.01), moderate, negative correlation was observed between age and the percent change in
height (r=-0.54) and weight (r=-0.59) between T0 and T5. The relationships between age and changes in height and weight are shown in Figure 2 and 3. Age of subjects was not strongly correlated with either height or weight changes over the course of the study.
Figure 2: Changes in subject weight vs. age

\[ R^2 = 0.3469 \]
Figure 3: Changes in subject height vs. age

![Graph showing changes in subject height vs. age with a trend line and R² value of 0.28368.](image-url)
Dental Cast Measurement Data

The mean and standard deviation for Little’s Irregularity Index (LII), intercanine width (ICW), intermolar width (IMW) and space analysis (SA) are summarized in Table 5 and Figures 4-7.

Table 5: Mean and standard deviation of cast metrics (mm)

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>S.D.</th>
<th>T1</th>
<th>S.D.</th>
<th>T2</th>
<th>S.D.</th>
<th>T3</th>
<th>S.D.</th>
<th>T4</th>
<th>S.D.</th>
<th>T5</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LII</td>
<td>3.00</td>
<td>2.09</td>
<td>1.65</td>
<td>1.73</td>
<td>0.86</td>
<td>1.11</td>
<td>0.58</td>
<td>0.69</td>
<td>0.42</td>
<td>0.44</td>
<td>0.41</td>
<td>0.49</td>
</tr>
<tr>
<td>ICW</td>
<td>27.04</td>
<td>3.34</td>
<td>27.16</td>
<td>3.12</td>
<td>27.46</td>
<td>2.88</td>
<td>27.24</td>
<td>2.48</td>
<td>26.92</td>
<td>2.07</td>
<td>26.79</td>
<td>1.97</td>
</tr>
<tr>
<td>IMW</td>
<td>45.23</td>
<td>3.66</td>
<td>44.97</td>
<td>3.51</td>
<td>45.62</td>
<td>3.43</td>
<td>45.60</td>
<td>3.22</td>
<td>45.47</td>
<td>3.11</td>
<td>45.18</td>
<td>3.21</td>
</tr>
<tr>
<td>SA</td>
<td>2.69</td>
<td>3.83</td>
<td>3.13</td>
<td>3.62</td>
<td>3.36</td>
<td>3.04</td>
<td>2.58</td>
<td>2.40</td>
<td>2.47</td>
<td>1.47</td>
<td>2.25</td>
<td>1.92</td>
</tr>
</tbody>
</table>

Results of ANOVA analysis show that changes in LII were statistically significant (p < 0.01), while the changes over time in ICW, IMW and SA were not statistically significant. For LII, pairwise comparisons using ANOVA showed significant differences (p < 0.05) between T0 and T1 with all other time points. Differences in LII between T2 and T5 were not statistically significant. Pairwise comparisons of the various time points for ICW, IMW, and SA failed to yield any significant variation. SA values for 21 of 27 patients were greater than zero at T0, reflecting the low frequency of crowding cases in the study. SA values correlated significantly with arch length measurements (r = 0.55, p < 0.01) but no significant correlation was observed between tooth measurements and SA.
To assess the reliability and reproducibility of dental cast measurements, casts for three patients (six casts each) were re-measured by the same examiner (ACS). Intraclass correlation coefficients for each category of measurement are listed in Table 6.

Table 6: Intra-examiner reliability measured by ICC

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Intraclass Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arch Segment</td>
<td>0.9985</td>
</tr>
<tr>
<td>Tooth Size</td>
<td>0.9517</td>
</tr>
<tr>
<td>LII</td>
<td>0.8531</td>
</tr>
<tr>
<td>ICW</td>
<td>0.9393</td>
</tr>
<tr>
<td>IMW</td>
<td>0.9189</td>
</tr>
</tbody>
</table>

Intra-examiner reliability was shown to be high for measurement types with the exception of LII, which was above the commonly accepted benchmark of 0.75 for clinical utility.
Figure 4: Mean value of Little’s Irregularity Index at each timepoint

**Little's Irregularity Index**

![Graph showing the mean Little's Irregularity Index over time, with timepoints 0 to 5 and mean values indicated with error bars for Total, Females, and Males.](image-url)

- **X-axis**: Timepoints
- **Y-axis**: Mean Little's Irregularity Index (mm)
Figure 5: Mean value of intercanine width at each timepoint
Figure 6: Mean value of intermolar width at each timepoint
Figure 7: Mean value of space analysis at each timepoint
Salivary Biomarker Data

The various antibody kits used to quantify salivary markers required minimum levels of the biomarker in order for a measurement to be made. The percentage of samples that met this requirement are shown in Table 7.

**Table 7: Percentage of samples meeting minimum biomarker sensitivity**

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>IGF-1</td>
<td>40%</td>
<td>48%</td>
<td>50%</td>
<td>41%</td>
<td>33%</td>
<td>30%</td>
</tr>
<tr>
<td>Testosterone</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>92%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>BMP-2</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>96%</td>
<td>93%</td>
<td>96%</td>
</tr>
<tr>
<td>BMP-4</td>
<td>20%</td>
<td>20%</td>
<td>19%</td>
<td>26%</td>
<td>26%</td>
<td>15%</td>
</tr>
<tr>
<td>BMP-9</td>
<td>72%</td>
<td>88%</td>
<td>85%</td>
<td>78%</td>
<td>85%</td>
<td>81%</td>
</tr>
</tbody>
</table>

IGF-1 and BMP-4 were the least successfully measured biomarkers, with only 40% and 21% of samples meeting the sensitivity requirements, respectively. The mean values and standard deviations for each biomarker at each time point are shown in Table 8.

**Table 8: Mean and standard deviation of biomarkers at each timepoint**

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP</td>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td></td>
<td>0.66</td>
<td>0.46</td>
<td>0.80</td>
<td>0.51</td>
<td>0.67</td>
<td>0.53</td>
</tr>
<tr>
<td>IGF-1</td>
<td>105.82</td>
<td>131.37</td>
<td>77.38</td>
<td>109.96</td>
<td>114.12</td>
<td>142.35</td>
</tr>
<tr>
<td>Testosterone</td>
<td>557.60</td>
<td>373.49</td>
<td>607.29</td>
<td>509.83</td>
<td>677.91</td>
<td>522.18</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>93.44</td>
<td>35.41</td>
<td>110.96</td>
<td>43.71</td>
<td>144.83</td>
<td>121.54</td>
</tr>
<tr>
<td>BMP-2</td>
<td>25.55</td>
<td>20.48</td>
<td>20.79</td>
<td>8.53</td>
<td>23.84</td>
<td>16.23</td>
</tr>
<tr>
<td>BMP-4</td>
<td>25.58</td>
<td>17.05</td>
<td>27.82</td>
<td>23.31</td>
<td>39.58</td>
<td>51.70</td>
</tr>
<tr>
<td>BMP-9</td>
<td>1.40</td>
<td>1.55</td>
<td>1.77</td>
<td>3.52</td>
<td>2.13</td>
<td>5.85</td>
</tr>
</tbody>
</table>

*ALP in units of U/mL, all others in units of pg/mL*
Changes in biomarker measurements by gender are shown in Figures 8-14. Of the T0 to T5 time series for each biomarker, only testosterone showed statistically significant variation (p = 0.046, power = 0.708). Pairwise comparisons between time points for testosterone showed highest significance was observed between T0 and subsequent time points.
Figure 8: Mean value of alkaline phosphatase at each timepoint
Figure 9: Mean value of IGF-1 at each timepoint
Figure 10: Mean value of testosterone at each timepoint
Figure 11: Mean value of TGF-β1 at each timepoint
Figure 12: Mean value of BMP-2 at each timepoint

![Graph showing mean BMP-2 concentration over time for males, females, and total population.](image-url)

**BMP-2**

- **X-axis**: Timepoints (0, 1, 2, 3, 4, 5)
- **Y-axis**: Mean BMP-2 Concentration (pg/mL)

- **Legend**:
  - Males
  - Females
  - Total

The graph illustrates the mean BMP-2 concentration at each timepoint for males, females, and the total population. The concentration varies over time, with notable peaks and troughs.
Figure 13: Mean value of BMP-4 at each timepoint
Figure 14: Mean value of BMP-9 at each timepoint

![Graph showing Mean Value of BMP-9 at Each Timepoint](image-url)
Correlations between independent variables

The correlations among the various dental cast measurements and salivary biomarkers were calculated. The resulting correlation matrix is shown in Table 9.

Table 9: Correlation between measured variables

<table>
<thead>
<tr>
<th></th>
<th>Ht.</th>
<th>Wt.</th>
<th>LII</th>
<th>ICW</th>
<th>IMW</th>
<th>SA</th>
<th>ALP</th>
<th>IGF-1</th>
<th>Test.</th>
<th>TGFβ1</th>
<th>BMP-2</th>
<th>BMP-4</th>
<th>BMP-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ht.</td>
<td></td>
<td>-0.66*</td>
<td>-0.09</td>
<td>0.32*</td>
<td>0.46*</td>
<td>-0.06</td>
<td>-0.14</td>
<td>-0.15</td>
<td>0.71*</td>
<td>0.06</td>
<td>-0.06</td>
<td>-0.60*</td>
<td>-0.16</td>
</tr>
<tr>
<td>Wt.</td>
<td>-0.22*</td>
<td></td>
<td>0.46*</td>
<td>0.55*</td>
<td>0.21*</td>
<td>0.10</td>
<td>-0.14</td>
<td>0.49*</td>
<td>0.04</td>
<td>0.05</td>
<td>-0.66*</td>
<td>-0.19*</td>
<td></td>
</tr>
<tr>
<td>LII</td>
<td>-0.10</td>
<td>-0.14</td>
<td>-0.27*</td>
<td>-0.07</td>
<td>-0.14</td>
<td>-0.01</td>
<td>-0.09</td>
<td>-0.03</td>
<td>-0.09</td>
<td>-0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICW</td>
<td>-</td>
<td>0.76*</td>
<td>0.49*</td>
<td>-0.04</td>
<td>-0.15</td>
<td>0.22*</td>
<td>0.16*</td>
<td>0.01</td>
<td>-0.51*</td>
<td>-0.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMW</td>
<td>-</td>
<td>0.36*</td>
<td>0.01</td>
<td>-0.18</td>
<td>0.42*</td>
<td>0.19*</td>
<td>-0.00</td>
<td>-0.52*</td>
<td>-0.14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SA</td>
<td></td>
<td>-</td>
<td>0.23*</td>
<td>-0.05</td>
<td>-0.11</td>
<td>0.14</td>
<td>0.03</td>
<td>-0.17</td>
<td>-0.08</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ALP</td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
<td>-0.18*</td>
<td>0.33*</td>
<td>-0.03</td>
<td>-0.28</td>
<td>-0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.19</td>
<td>-0.01</td>
<td>-0.09</td>
<td>0.09</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.16</td>
<td>-0.06</td>
<td>-0.40*</td>
<td>-0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-0.15</td>
<td>-0.31</td>
<td>-0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.73*</td>
<td>0.70*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.94*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMP-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
DISCUSSION

This study tracked several types of data over the course of orthodontic treatment for 27 subjects: biometric (height/weight), clinical (LII, ICW, IMW, and space analysis measurements from casts) and biological (salivary concentrations of ALP, IGF-1, TGF-β1, testosterone, BMP-2, BMP-4 and BMP-9). This combination of traditional orthodontic analytical tools with the comparatively new field of salivary diagnostics has the potential to provide new insights into the biology of tooth movement. The findings of the present study support the use of these new tools and suggest directions for future research.

Of the series of measurements taken on each dental cast, LII was found to exhibit significant changes over the course of the study. For all 27 patients examined, LII decreased between T0 and T5, indicating the success of orthodontic treatment in reducing mandibular anterior crowding. Improvement in crowding as measured by LII at each timepoint is shown in Table 10.

Table 10: Distribution of subjects by degree of crowding

<table>
<thead>
<tr>
<th># of subjects with:</th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor crowding (LII &lt; 3)</td>
<td>17</td>
<td>21</td>
<td>25</td>
<td>26</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>Moderate crowding (3 &lt; LII &lt; 6)</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Severe crowding (LII &gt; 6)</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

*Classifications based on Little irregularity scatter diagram*¹⁵
The predominance of minor crowding among patients is partly a consequence of the enrollment criteria: patients who were likely candidates for extraction were excluded. In addition, patients that began as non-extraction treatment but later required extractions were also excluded.

The Pearson correlation coefficients and intraclass correlation coefficients (ICC) were calculated to evaluate intra-examiner reliability. Strong correlations (>0.90) were observed for all measurements with the exception of LII, which at an ICC = 0.8531 was still above the benchmark of 0.75 for “good” reliability. This result is consistent with other research that showed relatively poor reproducibility of the contact point displacement measurements used to calculate LII.

Cumulative measurements of tooth size and arch lengths were found to be highly correlated. A correlation of 0.55 was found between arch length measurements and the calculated value for crowding/spacing, while no correlation was observed between tooth size and crowding/spacing. This observation is consistent with studies that found no significant relationship between tooth size and crowding, as well as studies indicating that arch length is a strong predictor of degree of crowding. However, similar studies have reached conflicting conclusions, suggesting a complex relationship between tooth width, arch length, and crowding.
The inclusion criteria of the study also played some role in the correlations observed: only non-extraction cases were considered, so no teeth were removed and therefore the sum of the tooth widths would remain constant. A possible exception would be if interproximal reduction was used, but this study did not look at the different treatment mechanic/modalities among the sample. However, evidence of interproximal reduction was visually observed on 2-3 patients, but was not taken into consideration.

This study illustrates the potential for salivary analysis in reflecting orthodontic tooth movement. Moderate correlations were observed between ICW/IMW and the biomarkers testosterone, TGF-β1, and BMP-4. While these results suggest a relationship between orthodontic treatment and biomarkers, confounding factors make it difficult to interpret these results. For example, ICW was found to correlate weakly with testosterone levels, but this may be due to males in general having both larger ICW and higher testosterone than females\(^\text{105}\). The weak negative correlation observed between BMP-4 concentration and ICW/IMW (r = -0.51, p <= 0.01) was not expected, as BMP-4 is thought to assist in the process of osteoblast differentiation\(^\text{106}\). Wescott et al found that BMP-4 gene expression was down-regulated in human PDL cells subjected to tensile strain, but were unsure how to interpret the functional significance of this finding\(^\text{107}\).

A number of correlations were observed among the various salivary makers. The strongest correlations were observed among the BMPs. This finding is consistent with the synergistic effects that the various BMPs are thought to have with each other\(^\text{108}\). A
weak negative correlation was also observed between ALP and testosterone. This relationship is consistent with that observed by Steffens et al, who found that in an inflammatory environment, high testosterone levels were associated with decreased ALP levels\textsuperscript{109}. Steffens proposed that testosterone acts as an immunosuppressant, dampening the inflammatory response to bone remodeling. Seifi et al showed that in castrated rats with orthodontic appliances, tooth movement was increased relative to non-castrated rats with normal testosterone levels\textsuperscript{52}. Similarly, the results of the present study may reflect the impact of testosterone on localized inflammation during orthodontic movement\textsuperscript{110}. This linkage would also explain the negative correlation observed between testosterone levels and BMP-4. Finally, a positive correlation was observed between ALP and TGF-β1. As discussed previously, TGF-β can promote either osteoblastic or osteoclastic activity, depending on the TGF-β concentration\textsuperscript{61}. The positive correlation with ALP suggests that TGF-β1 is promoting osteoblast differentiation at the concentrations found in our saliva samples.

In terms of study limitations, the high number of out-of-range salivary concentrations was a considerable issue, particularly for IGF-1 and BMP-4. The incompleteness of the data could result from issues with saliva sample handling and storage. For example, Antonelli et al found that saliva specimens stored at 4°C or 25°C for 24 hours had significantly reduced levels of free IGF-1 relative to specimens stored immediately at -80°C\textsuperscript{111}. Further, they found that samples retested after 9 months showed 28% of original IGF-1 levels unless a preservative solution had been initially added to the
sample. These findings may have implications for the present study, as sample collection was done in a clinical setting after which samples were transported to the research laboratory. In addition, samples were tested at the end of the longitudinal study period, and early samples may have partially degraded due to re-testing and the thawing/refreezing steps involved in that process; IGF-1 levels in later samples (T4, T5) were on average three times higher than earlier samples (Table 8). Similar issues with sample handling may have affected BMP-4 levels, although no literature was found regarding BMP-4 in saliva.

The correlations observed are likely due to a variety of causes not fully measured or covered by the data collected. In particular, changes in salivary biomarkers are due to some combination of physiological maturation (systemic factors) and orthodontic treatment (local factors), but it is difficult to isolate the impact of each. Future studies should include a control group of similar age, race, and gender mix to the treatment group, but who are not undergoing orthodontic treatment. Salivary measurements of this control group would provide a baseline that may make it easier to observe changes in biomarkers resulting from the orthodontic treatment.

In addition, salivary flow rates vary within and between subjects, and flow rate differences can affect biomarker levels, especially those at low concentrations\textsuperscript{112}. Specifically, some studies have shown that females have lower whole saliva flow rates and decreased protein content relative to males\textsuperscript{113,114}. The current study only tracked 10
male and 17 female subjects; future studies should explore the impact of gender on biomarker and orthodontic movement correlations by increasing sample size to allow for higher power analysis within genders.

Advances in technology have led to increased scanning of casts into 3-dimensional models that can be used with software for measuring the relevant parameters. Early studies showed that manual measurement provided better accuracy and reproducibility but, with computer methods and technology becoming more sophisticated, digital models can now offer similar reliability and reproducibility to manual measurements of plaster casts\textsuperscript{115,116,117,118}. In some cases, particularly with LII, the use of digital models has been shown to provide superior reproducibility to digital calipers\textsuperscript{119}. Future investigation of the hypotheses of the present study could incorporate computer-aided analysis of dental measurements to automate the process and potentially allow for a larger scale study.
CONCLUSIONS

The study used multiple observation methods in a longitudinal and cross-sectional analysis of patients undergoing orthodontic treatment: biometric data collection, dental cast measurements, and salivary concentration analysis. Statistically significant changes over time were observed for Little’s Irregularity Index, with the largest changes occurring between T0 and T1, and T1 and T2. We also observed significant longitudinal changes in testosterone, with most of the increase driven by the male subjects. A number of correlations among the various measurements were observed. Among salivary concentrations, the three BMPs measured were highly positively correlated. We also observed a negative correlation between alkaline phosphatase and testosterone, while alkaline phosphatase was positively correlated with TGF-β1.


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APPENDIX

Assay Protocols

1. R&D Systems (Minneapolis, MN) Quantikine ELISA Human IGF-1, cat#DG100.
   Remove kit from 4°C and allow components to warm to RT (room temperature),
   EXCEPT conjugate which should remain on ice.

Prepare reagents:
1. Wash buffer: Add 20 mL concentrate to 480 mL distilled water.
2. Substrate solution: Color reagents A and B should be mixed together in equal
   volumes within 15 minutes of use. Protect from light. 200 µL per well.
3. IGF-1 standard: Reconstitute standard with distilled water (according to vial
   label). Produces stock of 60 ng/mL. Leave at RT for 15 minutes.
4. Label tubes S1-S8.
5. Standard curve serial dilution: Add 360 µL calibrator diluent RD5-22 into S1
   tube. Add 200 µL RD5-22 into S2-S8. Add 40 µL stock solution to S1, mix. Add
   200 µL S1 to S2, mix. Add 200 µL S2 to S3, mix. Continue to S7. S8 is RD5-22
   only for blank.
6. Standards are: 6000 pg/mL; 3000 pg/mL; 1500 pg/mL; 750 pg/mL; 375 pg/mL;
   188 pg/mL; 94 pg/mL; 47 pg/mL; 0 pg/mL

Assay procedure:
1. Remove excess microplate strips from plate and return to foil pouch for later use.
2. Add 150 µL assay diluent RD1-53 to each well.
3. Add 50 µL standards and samples to appropriate wells. Incubate 2 hours at 4°C.
4. Aspirate all wells. Wash 4 x 400 µL wash buffer.
5. Add 200 µL cold IGF-1 conjugate to each well. Cover and incubate for 1 hour at
   4°C.
6. Wash 4 x 400 µL wash buffer
7. Add 200 µL substrate solution to each well. Incubate for 30 minutes at RT.
   Protect from light.
8. Add 50 µL Stop solution to each well. Color will change from blue to yellow.
9. Read within 30 minutes at 450 nm with wavelength correction at 540 nm.
2. R&D Systems, Parameter Testosterone assay, cat# KGE010
Bring all reagents to room temperature before use.

Prepare reagents:
1. Wash buffer: Add 20 mL concentrate to 480 mL distilled water.
2. Substrate solution: Color reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL per well.
3. Testosterone Std: Reconstitute with 1 mL deionized water to produce a 100 ng/mL stock. Mix completely and let stand for 15 min with gentle agitation.
4. Label tubes S1-S8.
5. Standard curve serial dilution: Add 900 µL calibrator diluent RD5-48 into S1 tube. Add 200 µL RD5-48 into S2-S8. Add 100 µL stock solution to S1, mix. Add 400 µL S1 to S2, mix. Add 200 µL S2 to S3, mix. Continue to S7. S8 is RD5-22 only for blank.
6. Standards are: 10,000 pg/mL; 3,333 pg/mL; 1,111 pg/mL; 370 pg/mL; 123 pg/mL; 41 pg/mL; 13.7 pg/mL; 4.5 pg/mL; 0pg/mL

Assay procedure:
1. Remove excess microplate strips from plate and return to foil pouch for later use.
2. Add 50 µL Primary antibody solution to each well, excluding the non-specific binding wells. All wells except NSB should be blue now.
3. Incubate for 1 hour at RT on shaker.
4. Wash 4 x 300 µL wash buffer
5. Add 100 µL Calibrator diluent RD5-48 to NSB wells and to Blank wells.
6. Add 100 µL standard and samples
7. Add 50 µL Testosterone Conjugate to all wells.
8. Incubate 3 hours at RT on shaker
9. Wash 4 x 400 µL wash buffer
10. Add 200 µL substrate solution to each well. Incubate for 30 minutes at RT. Protect from light.
11. Add 50 µL Stop solution to each well. Color will change from blue to yellow.
12. Read within 30 minutes at 450 nm with wavelength correction at 540 nm.
3. R&D Systems, TGF-β 1 Luminex kit, cat# LTGM00

Activation/Neutralization of samples:
1. Prepare 1N HCl: slowly add 8.33 mL 12N HCl to 91.67 mL deionized water. Mix well.
2. Prepare 1.2N NaOH/0.5M HEPES: slowly add 12 mL 10N NaOH to 75 mL deionized water. Mix well. Add 11.9g HEPES. Mix well and bring final volume to 100 mL with deionized water.
3. Add 20 µL 1N HCl to 100 µL of sample. Mix well and incubate for 10 min at RT.
4. Add 20 µL 1.2N NaOH/0.5M HEPES. Mix well.

Reagent preparation:
1. Wash buffer: Add 20 mL concentrate to 480 mL distilled water.
2. Reconstitute std cocktail with RD5-49 (1 mL) allow to sit for 15 min at RT.
3. Label tubes S1-S8.
4. Standard curve serial dilution: Add 500 µL stock cocktail to S1. Add 200 µL calibrator diluent RD5-49 into S2-S8. Add 100 µL S1 to S2, mix. Add 100 µL S2 to S3, mix. Continue to S7.
5. Diluted microparticle preparation: vortex each bead vial for 1 min. Combine 50 µL bead concentrate with 5 mL bead diluent. Protect from light. Vortex 1 min before adding to plate.
7. Streptavidin-PE preparation: Combine 55 µL strep-PE concentrate with 5.5 mL wash buffer. Mix well.

Assay Procedure:
1. Bring all reagents to RT before use.
2. Prepare all reagents, working standards, and samples as directed.
3. Add 50 µL standard or sample per well.
4. Add 50 µL beads per well.
5. Incubate 2 hours at RT on shaker.
6. Wash 3 x 100 µL wash buffer.
7. Add 50 µL biotin antibody cocktail to each well.
8. Incubate 1 hour at RT on shaker.
9. Wash 3 x 100 µL wash buffer.
10. Add 50 µL Strep-PE to each well.
11. Incubate 30 min at RT on shaker.
12. Wash 3 x 100 µL wash buffer.
13. Resuspend beads in 100 µL sheath fluid and incubate for 5 min on shaker.
4. R&D Systems, Screening Luminex panel (BMP-2, -4, -9)

Reagent preparation:
1. Wash buffer: Add 20 mL concentrate to 480 mL distilled water.
2. Reconstitute std cocktail with RD6-52 (250 µL) allow to sit for 15min at RT.
3. In tube labeled S1: Add 900 µL RD6-52 to 100 µL stock standard produce 1X standard.
4. Label tubes S2-S8.
5. Standard curve serial dilution: Add 200 µL calibrator diluent RD6-52 into S2-S8. Add 100 µL S1 to S2, mix. Add 100 µL S2 to S3, mix. Continue to S7.
6. Diluted microparticle preparation: vortex each bead vial for 1 min. Combine 500 µL bead concentrate with 5 mL bead diluent. Protect from light. Vortex 1 min before adding to plate.
7. Diluted biotin antibody cocktail preparation: Add 500 µL antibody concentrate to biotin antibody diluent. Mix well.

Assay Procedure:
1. Bring all reagents to RT before use.
2. Prepare all reagents, working standards, and samples as directed.
3. Add 50 µL standard or sample per well.
4. Add 50 µL beads per well.
5. Incubate 2 hours at RT on shaker
6. Wash 3 x 100 µL wash buffer
7. Add 50 µL biotin antibody cocktail to each well
8. Incubate 1 hour at RT on shaker
9. Wash 3 x 100 µL wash buffer
10. Add 50 µL Strep-PE to each well
11. Incubate 30 min at RT on shaker
12. Wash 3 x 100 µL wash buffer
13. Resuspend beads in 100 µL sheath fluid and incubate for 5 min on shaker.
5. Abcam (Cambridge, MA) Alkaline Phosphatase Assay kit (colorimetric) cat#ab83369
Remove from -20°C and bring to RT. Keep samples, ALP enzyme and pNPP solution on ice during the assay.
Prepare reagents:
1. pNPP solution: dissolve 1 tablet of pNPP in 2.7 mL assay buffer to make a 5 mM working solution. (Never touch tablets with bare hands). This is stable on ice for 12 hours.
2. ALP enzyme: reconstitute ALP enzyme with 1 mL assay buffer. Do not freeze. Enzymes are stable for up to 2 months at 4°C after reconstituting.

Standard preparation:
1. Prepare 1 mM pNPP standard by diluting 40 μL 5 mM pNPP in 160 μL assay buffer.
2. Prepare standard curve as follows:

<table>
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<tr>
<th>Standard #</th>
<th>Vol. 1 mM pNPP (μL)</th>
<th>Assay buffer (μL)</th>
<th>Conc. pNPP (nmol/well)</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
<td>7</td>
<td>60</td>
<td>300</td>
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</table>

3. Add 120 μL standard to standard wells
4. Add 80 μL sample to sample wells
5. Add 80 μL assay buffer to blank wells
6. Add 50 μL 5 mM pNPP solution to sample and blank wells.
7. Add 10 μL of ALP enzyme solution to each well of standard curve, mix well.
8. Incubate plate for 1 hour at RT, protect from light.
9. Stop all reactions by adding 20uL Stop Solution to each well, gently shake plate.
10. Measure OD at 405 nm.
VITA

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