Tufts UNIVERSITY | School of Dental Medicine

Salivary Parathyroid Hormone-related Protein (PTHrP) and Alkaline Phosphatase (ALP) as biomarkers for skeletal maturity

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Abstract

Background

Skeletal maturity assessment is critical to determine the amount of remaining facial growth and contributes in the diagnosis and treatment of patients under orthodontic therapy. Since most of the skeletal maturation indicators are invasive and require radiographic exposure, new non-invasive biomarkers have been suggested to assess skeletal maturity. Alkaline phosphatase (ALP) in serum has been reported to be correlated with mandibular growth spurt and to assist in skeletal maturity prediction. In addition, parathyroid hormone-related protein (PTHrP) in serum has been associated with skeletal maturation. The goal of this study was to investigate the relationship between salivary ALP and PTHrP levels and skeletal maturation in growing orthodontic patients as non-invasive biomarkers for skeletal maturity assessment.

Materials & methods

This cross-sectional study included 79 subjects (48 females, 31 males; aged 7 to 23 years) categorized into 5 cervical vertebral maturation stages (CVMS) based on an evaluation of lateral cephalograms by three different examiners. Unstimulated whole saliva samples were collected from all subjects, centrifuged and stored at -80°C until use. Total protein levels, ALP activity and PTHrP in unstimulated whole saliva samples were analyzed. The statistical analysis included Cohen's kappa statistics for inter-examiner reliability and Kruskal-Wallis test, with Dunn's test and Bonferroni correction used for post-hoc comparison. The Mann-Whitney U test was used to compare ALP activity between genders.

The associations between age and CVMS and between age and ALP activity were assessed via Spearman's correlation. Five multinomial logistic regression models were utilized to predict CVMS.

Results

ALP activity in CVMS I was significantly higher than CVMS II ($P < 0.001$) and CVMS V ($P = 0.0014$). Total protein levels in CVMS I were significantly lower than CVMS III ($P = 0.005$). The chronological age was positively correlated with CVMS ($r_s = 0.763$, $p < 0.001$), but weakly correlated with ALP activity ($r_s = 0.108$, $p > 0.05$). The combination of chronological age ($p < 0.001$) and ALP activity ($p = 0.002$) were significant in predicting the CVMS with model's overall correct classification rate of 53.2%. PTHrP was not detected in unstimulated whole saliva in our study.

Conclusions

Salivary ALP activity was higher at early pubertal stage and then declined with a statistically significant difference between CVMS I and CVMS II and between CVMS I and CVMS V. Therefore, salivary ALP may to be a promising diagnostic tool for pre-pubertal growth prediction. The combination of salivary ALP activity and chronological age showed the best prediction for CVMS compared to the other models. However, salivary ALP alone presented modest contribution to CVMS prediction. Further longitudinal studies with a larger sample size are warranted in order to validate the potential use of salivary ALP activity as a non-invasive biomarker for skeletal maturity.

“Embrace each challenge in your life as an opportunity for self-transformation.”

- Bernie S. Siegel

I would like to dedicate this work to my parents “Nawal and Zaid”, my sister “Reem”, my brothers “Fahad, Khalid and Ahmed” and my friends, for their unconditional love and support, throughout my journey

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List of Abbreviation

ALP	Alkaline phosphatase
BSA	Bovine serum albumin
CVMS	Cervical vertebrae maturation stages
ELISA	Enzyme-linked immunosorbent assay
GCF	Gingival crevicular fluid
HRP	Avidin-Horseradish Peroxidase
PTHrP	Parathyroid hormone related protein
RCF	Relative centrifugal force
ROC	Receiver operator curve
RPM	Revolution per minute
TUSDM	Tufts University School of Dental Medicine
OD	Optical density

List of Symbols

nm: nanometer

µl: microliter

µU: microunits

mU: milliunits

Mm: millimolar

mg: milligram

ml: milliliter

°C: the degree Celsius

pg: picogram

Salivary Parathyroid Hormone-related Protein (PTHrP) and Alkaline Phosphatase (ALP) as biomarkers for skeletal maturity

Introduction

Craniofacial growth assessment and prediction in orthodontic therapy:

Skeletal maturity assessment plays a significant role in orthodontic diagnosis, treatment planning and stability of orthodontic treatment [1]. Skeletal age has been used to determine the amount of remaining facial growth that impacts the decisions of orthodontic treatment onset and optimal orthodontic treatment [2]. In addition, assessment of skeletal growth spurt affects the efficiency and effectiveness of orthodontic treatment and diagnosis such as the decision of extraction versus non-extraction orthodontic therapy [2,3]. Moreover, accurate prediction of growth spurt is critical for modifying the growth of jaw bones [4]. For example, functional appliances are used in planning orthopedic treatment in class II growing individuals to modify mandibular growth [5]. Furthermore, maxillary expansion will benefit from determining the maturation stage of an individual [5]. Also, the end of individual's growth spurt determines the performance and outcomes of some orthognathic surgeries [5]. Knowing the end of skeletal maturation will avoid unwanted effects such as ongoing growth after mandibular setback surgery [6]. Furthermore, the end of skeletal vertical growth determines the optimal timing for dental implant therapy in young growing patients [6].

Craniofacial growth assessment methods:

Craniofacial growth prediction methods have been proposed such as craniometry, anthropometry and cephalometry. In addition, experimental procedures such as vital staining, implant markers, natural markers, comparative anatomy have been proposed for growth prediction [6]. Moreover, Baccetti et al. have proposed four features that should characterize an ideal skeletal maturation indicator [6]. First, a certain stage of the indicator should be efficient in detecting the peak of mandibular growth [2]. Second, it should be consistent between the examiners in predicting the growth stage [2]. In addition, it should be simple in recording the stages without any additional X-ray exposure [2]. Finally, the growth assessment method should include a phase that predicts the onset of pubertal growth stage that occurs before the peak of mandibular growth stage [7].

Besides the above mentioned craniofacial growth indicators, several craniofacial assessment methods have been also proposed such as chronological age and physiological age [6]. Patel et al defined chorological age as the amount of time an individual lived after birth and physiological growth as the biological maturity of an individual [5,8]. There have been some controversial data regarding the validity of chronological age for skeletal maturation assessment. Several studies have reported that chronological age is not a reliable indicator for growth maturation due to growth spurt variation among individuals [9]. In addition, the use of chronological age to assess the maturity state of a child was questionable [9]. On the other hand, Safavi et al have reported a positive association between chronological age and growth stages in his sample study [10]. Besides chronological age, the physiological age can be

predicted by somatic, sexual, dental and skeletal maturation [9]. The somatic maturity is impacted by the incremental increase in height and weight [11]. Studies have reported that increase in height is the most valid representation of growth peak [9]. It has been shown that the onset of growth spurt occurs two years before the height peak which is approximately 12 years in girls and 14 years in boys [2]. In addition, sexual maturity is the stage when an individual reaches adulthood and acquires the ability to reproduce [12]. Secondary sexual characteristics are important in identifying the pubertal stage, that is characterized by changes in voice and growth of facial hair in males and menarche in females [12].

Besides somatic and sexual maturation, dental maturation has been introduced as a biological indicator for craniofacial growth maturation [12]. It has been reported that calcification of second mandibular molar, second premolar, canine and third molar can be used as indicators for growth stages [12]. Studies have shown a correlation between maturation of canine, second premolar and second molar with the onset of pubertal growth spurt [12]. On the other hand, the end of growth spurt is associated with mandibular third molar maturation [13]. Skeletal maturity is characterized by ossification of bones and is influenced by genetic and environmental factors [13,14]. The orthodontic treatment onset is mainly based on skeletal age [15]. Compared to the chronological age, skeletal and dental maturation have been reported though as better indicators for growth maturation [16].

The radiographic analysis such as lateral cephalometric and hand and wrist radiographic examination are also common methods to predict skeletal maturation [17]. Radiographic evaluation of hand and wrist has been proposed to be the most reliable method

of skeletal maturity assessment due to multiple ossification centers that ossify at different maturation stages [13]. By determining the onset of ossification of the adductor sesamoid of the thumb, pubertal growth spurt can be predicted [14]. Besides that, Fishman and Bowden have described specific hand and wrist indicators related to skeletal maturation [5]. However, this method is accompanied by several limitations such as the extra-radiation exposure from hand and wrist X-ray. Moreover, the polymorphism and agenesis of some hand and wrist bones limit the identification of skeletal maturation indicators. Also, this method does not indicate the onset of pubertal growth, but it predicts the peak of growth spurt and end of pubertal growth [18].

Besides hand and wrist radiographs, lateral cephalometric radiography has been used to predict the growth stage by using the Lamparski method of cervical vertebrae maturation (CVM) [18]. This method overcomes the limitations of hand and wrist radiograph by including cervical vertebral maturation stage I (CVMS I) that occurs before the peak of the mandibular growth and impacts the right timing for dentofacial orthopedics [19-22]. Studies have shown that the CVM method can be correlated with hand wrist method in determining growth spurt [19-22]. In 1972, Lamparski based the skeletal maturation assessment on the morphological changes of the six cervical vertebrae; C1-C6 [19-22]. Hassel and Farman suggested limiting the number of vertebral bodies into five (C1-C5), because the sixth cervical vertebrae can be obscured by the protective radiation collar [23]. In addition, Franchi et al. provided six CVMS for skeletal maturation assessment [13]. After that, Baccetti et al. have introduced the modified version of CVM method that consists of five stages (CVMS I to V) [14]. This modified version of CVM has been also included by the American Board of Orthodontics [6]. According to the CVM

method, the peak of growth occurs between CVMS II and CVMS III. The five CVM stages were defined as following:

CVMS I: The lower border of second vertebrae (C2) is flat or concave and the third and fourth vertebrae (C3, C4) are flat. The bodies of C3 and C4 are trapezoid in shape. This stage occurs one or two years before the peak of mandibular growth.

CVMS II: The lower borders of C2 and C3 are concave and the bodies of both C3 and C4 are trapezoid or rectangular horizontal in shape. The peak of the mandibular growth will occur during the year of this stage.

CVMS III: The lower borders of C2, C3 and C4 are concave and the bodies of C3 and C4 are rectangular horizontal in shape. The peak of mandibular growth has occurred 1 or 2 years before this stage.

CVMS IV: The lower borders of C2, C3 and C4 are concave. At least one of the bodies of C3 and C4 is square in shape. The peak of mandibular growth has ended 1 year before this stage.

CVMS V: The lower border of C2, C3 and C4 are present. At least one of the bodies of C3 and C4 is rectangular vertical in shape. The peak of the mandibular growth has ended 2 years before this stage.

Since CVM method is associated with low intra-examiner reproducibility, the validity of using CVM to predict skeletal age has been questionable [13]. Compared to CVM method, serial lateral cephalometric radiograph super-impositions have been proposed to monitor

facial growth and orthodontic treatment results and have been considered more accurate to predict skeletal change [24]. In addition to the CVM method, studies have suggested the use of the frontal sinus growth or enlargement to predict skeletal maturation [folly25].

Biomarkers and skeletal maturation assessment:

A biomarker refers to a "biological marker" that has been used as diagnostic tool in clinical and basic research [folly25]. In 1998, the National Institute of Health Biomarkers defined biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [26,27]. The World Health Organization defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [24]. Biomarkers are noninvasive, affordable diagnostic tools and can be repeated as many as possible [9,24,28]. Biomarkers are classified into proteins byproducts after synthesis of new bone and by products generated after bone resorption [12]. During bone formation and resorption, the biomarkers are released into the circulation [5]. Studies have reported a correlation between circulating biomarkers and tissue volume of biomarkers [28].

Unlike most of these biological methods mentioned above that are invasive, require X-ray exposure and long observation periods, new non-invasive mediators such as growth factors have been introduced to assess the skeletal maturity [29]. Systemic and local growth factors are closely associated with craniofacial growth and mandibular condylar growth [30]. Systemic growth factors include insulin like growth factor 1 (IGF-1), thyroid hormones,

parathyroid hormone related protein (PTHrP), growth hormone, estrogen, glucocorticoids and androgens [10]. Studies have reported an association between growth spurt and elevation in biomarkers of bone metabolism [31]. For instance, serum IGF-1, stimulated by growth hormone, increase during pubertal growth spurt [24].

Salivary biomarkers:

Saliva as an oral diagnostic fluid has been described as "mirror of the body" since it could be used to monitor the general health and the onset of several oral and systemic diseases [32]. Human saliva is secreted from pairs of major salivary glands; parotid, submandibular and sublingual glands as well as minor salivary glands in the oral cavity [14]. Whole saliva contains water, cells, proteins, nucleic acid, steroid hormones, lipids, mucins and electrolytes [33]. Humans produce a range of 1 to 1.5 liters of saliva per day [33]. Saliva plays an important role in the maintenance of oral health [33]. It aids in food tasting and digestion, lubricating and hydrolyzing oral surfaces, buffering pH, and in tooth remineralization and oral defense mechanisms [34]. It has been suggested that diagnostic biomarkers in the blood are absorbed by the salivary gland and then secreted into the oral cavity [34]. Therefore, saliva can be used as a diagnostic tool monitoring the health status since it contains of several biomarkers [35]. For instance, salivary biomarkers have been utilized in cancer diagnosis, such as breast and ovarian cancers [35]. Besides malignancy detection, salivary biomarkers have been proposed as indicators for skeletal growth and remodeling [36]. Salivary exosomes (micro RNA), and cytokines (e.g. tumor necrosis factor, interleukins) are examples of salivary biomarkers [37,38].

Salivary biomarkers versus serum biomarkers:

Serum and saliva are the most commonly used body fluids for clinical diagnostic procedures [39]. Salivary collection procedures are preferable compared to serum because it is painless and noninvasive [35]. In addition, it is possible to collect multiple times from the same individual [40]. Moreover, saliva collection does not require personal training and it is easier to manipulate and store [41,42]. However, immunoglobulins and albumins exist in higher concentration in serum compared to saliva such as IgA and IgG levels [43].

Salivary biomarkers and skeletal maturation assessment:

In normal or healthy individuals, salivary biomarkers are telltale molecules that could be used to monitor health status, disease diagnosis and treatment response and outcome [43]. Several studies have investigated the use of salivary biomarkers for bone growth and remodeling [44]. Salivary Alkaline phosphatase (ALP) and salivary insulin-like growth factor-1 (IGF-1) have been investigated as potential biomarkers for assessing skeletal maturity [43]. Masoud et al. investigated the relationship between blood-spot IGF-1 and hand and wrist assessment of skeletal maturity [43]. He found that higher levels of IGF-1 mirrored the mandibular growth spurt [44].

PTHrP and its role in bone metabolism:

Parathyroid hormone is an endocrine hormone that play major role in regulating plasma concentration of calcium and phosphate in kidneys and bone [44]. On the other hand, parathyroid hormone-related protein (PTHrP) is known as a cancer related hormone and was discovered during the investigation of hypercalcemia of malignancy [42]. It shares the same receptor as parathyroid hormone, but it has different structure and function [45]. PTHrP plays a major role in bone and cartilage development and relaxation of smooth muscle [42]. In addition, it has been reported that PTHrP regulates growth plates of long bones [42]. Studies have also reported an association between PTHrP and mandibular condylar growth [42,46].

Hussain et al. studied serum PTHrP levels and correlated the values with the six cervical maturation stages [42,46]. Although serum PTHrP levels had a positive correlation with cervical vertebral maturation stages from the pre-pubertal to the late pubertal stages, that study concluded that the peak of serum PTHrP was not correlated with the early pubertal stages questioning the validity of serum PTHrP to predict the growth spurt [44]. However, a correlation between the peak serum PTHrP and late pubertal stage has been reported [44]. Dua et al. measured salivary and plasma levels of PTHrP in normal adult sheep after intravenous infusion [41]. It was concluded that there was a higher concentration of salivary PTHrP compared to PTHrP in plasma after intravenous infusion [45]. These findings suggest further exploration of salivary PTHrP for assessment of skeletal maturation [47,48]. It has been reported that PTHrP is also synthesized in the salivary glands which might explain the higher salivary concentration of PTHrP than that in plasma observed in sheep [40].

Alkaline phosphatase and bone metabolism:

Alkaline phosphatase (ALP) is a natural enzyme located in the plasma cell membrane that is found in liver, intestine and placenta and is produced by osteoblasts [40,49]. ALP is also formed by mucosal salivary glands [50]. The ALPL gene encodes for ALP family of proteins [50]. ALP comes in different variations called tissue-specific isoenzymes [50,51]. Among these isoenzymes, a bone-specific isoenzyme is associated with bone growth [52]. It hydrolyzes inorganic pyrophosphate, which in turn affects osteoblast function and bone mineralization [53,54].

Tobium et al. reportd serum bone-specific ALP as a biomarker for bone health [52]. The study reported increased levels of bone-specific ALP during infancy and puberty in which bone metabolism occurs [52]. In addition, Tuchman et al. reported serum ALP as a biomarker for bone formation that was correlated with growth spurt [52]. Also, Christenson et al. stated increase bone-specific ALP during puberty [49]. Moreover, ALP was previously investigated in the gingival crevicular fluid (GCF) as a biomarker for skeletal maturation [49]. It was shown that the levels of ALP in GCF were increased in relation to mandibular growth spurt [49]. Also, Tarvade et al. have investigated the use of salivary ALP for growth prediction [49]. It was concluded that higher levels of salivary ALP were associated with pubertal growth spurt [34,55]. In addition to skeletal growth, bone specific ALP has been associated with medical conditions such as Paget's disease of bone, osteoporosis and osteosarcoma [56].

Specific Aims and Hypothesis

The primary aim of the study was to investigate the relationship between salivary ALP and PTHrP levels with skeletal maturation in growing orthodontic patients to be used as non-invasive biomarkers for skeletal maturation assessment.

The secondary aim was to evaluate salivary protein concentration and chronological age as non-invasive biomarkers for skeletal maturation assessment.

Our hypothesis was that salivary ALP activity and PTHrP levels may be candidates as non-invasive biomarkers for skeletal maturity assessment.

Study Design

This cross-sectional clinical study design investigated the use of salivary ALP activity and PTHrP as biomarkers for skeletal maturation in growing subjects (n=79) categorized into 5 groups according to their cervical vertebral maturation stage assessed by lateral cephalographs by three different examiners in the department of Orthodontics at Tufts University School of Dental Medicine (TUSDM), Boston, MA. Salivary ALP activity and PTHrP levels in unstimulated whole saliva samples were measured.

Materials and Methods

Forty-eight females and thirty-one males aged between 7 to 23 years old were included in the study. They either proceeded to begin orthodontic therapy or were under current orthodontic treatment in the department of Orthodontics at TUSDM. For all subjects lateral cephalographs existed within the last 6 months in their records.

The study protocol was approved by Tufts Institutional Review Board (#11986). For subjects who were under 18 years of age, informed consent from legal guardian and informed assent form were obtained. However, for subjects who were 18 years of age or older, informed consent form from subjects were obtained. Subjects were given ample time to read the consent form carefully and to have any questions answered prior to signing. Then, they were instructed to sign the informed consent or the assent form and copies were given to both the subject and their guardian.

After subjects' agreement to participate in the study, they were asked to complete demographic information and their medical history. Then, oral exam was completed by the examiner (N. A.) using a mouth mirror and dental explorer. After that, eligibility for participation were evaluated by reviewing the following inclusion and exclusion criteria (visit 1-screening visit):

Inclusion criteria:

Patients aged from 7 to 23 years old who either proceeded to begin orthodontic therapy or were under current orthodontic treatment in the department of Orthodontics at TUSDM and for whom lateral cephalographs within the last 6 months existed in their records were included in this study. The lateral cephalometric radiographs are routinely obtained during comprehensive orthodontic examination in order to aid in the diagnosis and orthodontic treatment plan and are also taken every 6 months for those patients that are under active orthodontic therapy in order to monitor changes.

Exclusion criteria:

1. Any subject diagnosed with medical condition, systemic disease or taking medication that affects the growth and/or bone metabolism, including the following:

- Growth abnormality such as cleft lip and palate patients.
- Syndromes like Turner's syndrome, Cushing's syndrome, Down syndrome and achonroplesia.

- Endocrine disturbance such as hypothyroidism, hyperparathyroidism, hyperthyroidism, Cushing's syndrome, thyrotoxicosis, growth hormone deficiency and uncontrolled diabetes type 1.
- Autoimmune disorders such as rheumatoid arthritis, juvenile arthritis, lupus and multiple sclerosis.
- Malignancy.
- Heart disease such as cyanotic heart disease or congenital heart disease.
- Lung disease such as severe asthma with a history of hospitalization.
- Digestive and gastrointestinal disorders such as celiac disease, inflammatory bowel disease, ulcerative colitis, weight loss surgery, gastrectomy and gastrointestinal bypass procedure.
- Blood disorders such as leukemia, multiple myeloma, sickle cell disease, iron deficiency anemia, hypophosphatemia, blood and bone marrow disorders and thalassemia.
- Neurological disorders such as Parkinson's disease, stroke, multiple sclerosis and previous head and neck injury.
- Mental illnesses such as depression, eating disorder and constant malnutrition.
- Chronic kidney disease such as chronic kidney disease
- Chronic liver disease such as chronic liver disease
- Current diagnosis of salivary gland disorder such as salivary stones or current salivary gland bacterial or viral infection such as mumps. Salivary gland cysts or tumors as pleomorphic adenoma or Warthin's tumor.
- Medications that affect bone metabolism such as heparin, warfarin, cyclosporine,

glucocorticoids, medroxyprogesterone acetate, chemotherapy, radiation therapy and thyroid hormone. In addition, systemic and high doses of inhaled corticosteroids for asthmatic patients and vitamin C and D that affect bone growth. Also, medications that affect alkaline phosphatase secretion like interferon beta 1 A or Verapamil.

2. Patients who self-reported pregnancy or lactation (studies have reported increase alkaline phosphatase serum levels during pregnancy especially during the third trimester and early lactation period) [57].

3. Non-English speakers.

4. Patients with current smoking habits.

5. Patients who require antibiotic premedication for routine dental procedures according to 2015 American Dental Association (ADA) guidelines.

6. Patients with an acute intra-oral infection (e.g., herpetic gingivostomatitis, herpangina, abscesses).

Subjects who were eligible to participate were scheduled for visit 2. They were instructed verbally and given the following written instructions to ensure high quality of salivary sample collection for analysis:

- No alcohol 24 hours prior to sample collection.
- No tooth brushing 1.5 hours prior to sample collection.
- No drinking 1.5 hours prior to sample collection (water is acceptable up to hour prior to collection).

- No eating 1.5 hours prior to collection.
- No eating or drinking during collection period.
- No gum chewing and consumption of candy 1.5 hours prior to the collection.

In case that the subject fulfilled the above instructions and confirmed that they have refrained from the above as described, screening visit and visit 2 could be combined as shown below in Table 1.

All tables are shown in Appendix A and all figures are shown in Appendix B as well.

Table 1: Subject timeline

Appointment procedures	Visit 1 (Screening visit)	Visit 2
Informed consent/assent form	X	
Demographics	X	
Medical/dental history	X	X
Intra-oral examination	X	X
Evaluate eligibility and withdrawal criteria	X	X
Sample collection sample		X

Saliva Sample collection procedure:

During visit 2 (or screening visit if the subject fulfilled the saliva collection instructions), unstimulated whole saliva was collected by asking the subject to sit quietly and drool saliva into the tube for 5 minutes after swallowing. A sample volume of 1-5 ml was collected from the subjects (or as much as they could manage in 5 minutes). After that, saliva samples were immediately sealed in Eppendorf tubes that were pre-weighted using and labeled stating only a subject ID (subjects' initials with a serial number), and placed immediately on ice. The Eppendorf tubes' weight (g) was also measured using Ohaus Adventurer SL Precision Balance (AS-153) after collecting the sample. The times for the start of collection was recorded so that we can test all samples at specific time periods after collection and for the sake of accuracy (when we make comparisons from sample to sample and batch to batch). In the present study, most of the saliva samples were collected at the same time period, from 9:00 am-12:00 pm. Then, the samples were transported to the laboratory at TUSDM. The laboratory analyzer (N. A.) was blinded to the subject and group that the sample is from.

Pre-treatment of Saliva Samples:

The sample's weight (g) was measured and then aliquoted into 1 ml solutions from the whole saliva from individual patients. Samples were then centrifuged in de-identified tubes, maintaining the temperature at 4°C for 20 min at a force of 12,000 relative centrifugal forces (RCF) using an Eppendorf

centrifuge. The supernatant and pellet were separated and stored at -80°C until the analysis.

Cervical vertebral maturation (CVM) staging:

Cervical vertebral maturation staging of the subjects were evaluated independently by three blinded orthodontists (C. T., G. K. and B. C.) using lateral cephalographs independently in order to distribute the subjects into 5 groups. The examiners were blinded to the subject's personal information (name, age, ethnicity, etc.) and they examined the cephalometric radiographs that were labeled stating only a subjects' ID [10]. In case that there was a disagreement between the examiners in staging some of the subjects' lateral cephalographs, the examiners met, discussed the cases and reached a consensus for staging those subjects.

Laboratory analysis:

Biochemical assays: all the biochemical assays were performed by a single blinded operator (N. A.). The biochemical assays include protein assay (Bio-Rad), ALP assay and ELISA for PTHrP.

Protein analysis:

Principle: Protein analysis was performed using Bio-Rad (Bradford) protein assay (Kit II, catalog number 500-0002) for all the subjects' samples. Bio-Rad protein assay is a dye-binding assay in which different concentrations of protein causes differential color change of

the dye [34,55]. The absorbance for an acidic solution of Coomassie brilliant blue G-250 dye when binding to protein was measured at 590 nm [58]. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine [59].

Standard curve preparation: Dilutions of protein standard were performed as shown in Table 2 [58]. Protein standard solutions were assayed in triplicate [58]. Ten µL of each standard were pipetted into separate microtiter plate wells (Eppendorf assay, VIS 96/F, 00-3073-0020) [35].

Table 2: Standard curve preparation for protein assay [56]

Standard	Concentration (mg/ml)	Protein (mg)	Water (ml)
Dilution 1 (D1)	500	100 (from stock*)	300
Dilution 2 (D2)	400	80 (from stock*)	320
Dilution 3 (D3)	300	60 (from stock*)	340
Dilution 4 (D4)	250	200 (from D1)	200
Dilution 5 (D5)	125	200 (from D4)	200
Dilution 6 (D6)	62.5	200 (from D5)	200
Dilution 7 (D7)	32.250	200 (from D6)	200
Negative control	0	0	400
* Stock solution: 1part Dye Reagent Concentrate with 4 parts distilled deionized water			

Sample preparation: The samples were allowed to thaw on ice for the analysis. After that, the samples were diluted to 1:5. Twenty µL of distilled water was added to each 5 µL of the samples and then were mixed using Fisher Vortex Genie 2 mixer speed of 3200 rpm for 2-3

seconds. Thereafter, each sample was assayed in duplicates and 10µL of each sample was pipetted into a separate microtiter plate wells.

According to the Bradford protein assay manual, BSA was prepared in 1:5; the dye reagent was prepared by diluting 1part dye reagent concentrate with 4 parts distilled water. After that, 200µL of diluted dye reagent was added to each standard and sample well. Afterwards, the microtiter plate wells were incubated at room temperature for at least 5 minutes and then the absorbance was measured at spectrum of 590 nm. [36]

Alkaline phosphatase analysis:

Principle: Alkaline phosphatase assay kit (abcam, Colorimetric) (ab83369) was used in this study [36]. The ALP assay is simple, sensitive and can be used multiple times to measure ALP activity in biological samples such as saliva. It uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate which turns yellow when dephosphorylated by ALP. The detection range is 10-250 µU of ALP activity [47].

Standard preparation: 5 mM of pNPP solution was prepared by reconstituting two pNPP tablets in 5.4 ml assay buffer. Then, 1mM pNPP standard was prepared by diluting 40 µL pNPP 5 mM standard in 320 µL of Assay Buffer. The standard curve dilution was prepared as described in Table 3.

Table 3: Standard curve preparation for ALP assay [47]

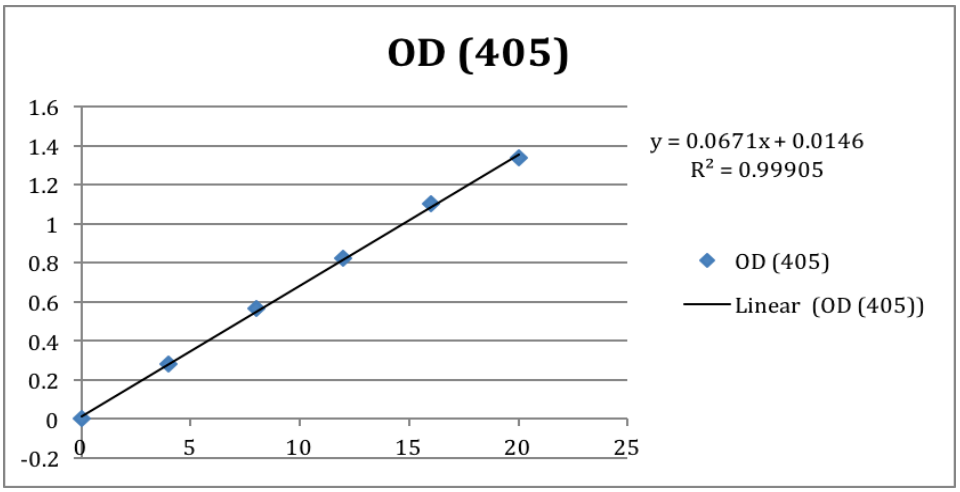
Standard	Concentration (unit/ml)	Volume of pNPP * (microliter)	Assay buffer ** (microliter)
D1	0	0	396
D2	4	13	377
D3	8	25	350
D4	12	38	342
D5	16	50	325
D6	20	62	310

* pNPP standard was prepared by diluting 40µL pNPP 5mM Standard in 320 µL of Assay Buffer.
** Assay buffer was provided from the manufacturer.

The standard dilutions were set up in triplicate. Then, 120 µL of each standard were pipetted into separate microtiter plate wells [60].

Figure 1 depicts the ALP standard curve.

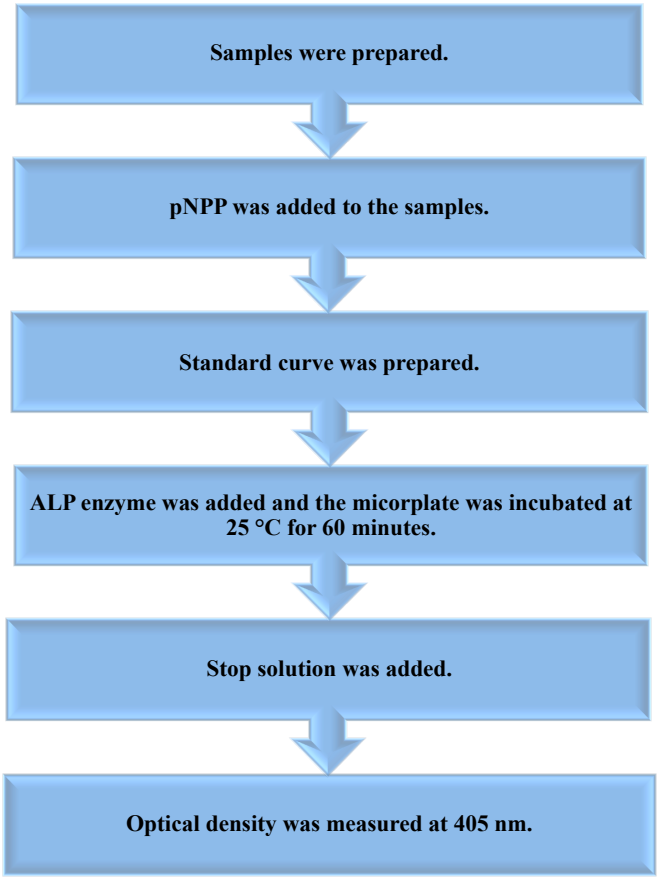
Figure 1: Alkaline phosphatase standard curve



Sample preparation: First, all the samples (n=79) were placed at room temperature to allow them thaw on ice for the analysis. Each sample was loaded with (80 µL) was loaded in duplicate into the wells of the microplate (Eppendorf assay, VIS 96/F, 00-3073-0020) [61,62].

ALP assay steps: Fifty µL of 5 mM pNPP was added to each standard and sample wells. After that, 10 µL of ALP enzyme were added to the standard well. Then, the plates were incubated at 25 °C for 60 minutes protected from light. Next, 20 µL of stop solution was added to each well and then the output was measured on a microplate reader at OD 405. Figure 2 summarizes the ALP assay steps [10].

Figure 2: Alkaline phosphatase assay summary [63]



ALP calculations: First, the triplicate for standards and duplicate for samples were averaged. Then, the sample background was subtracted from the sample readings. After that, the absorbance values were plotted for each standard. The ALP activity in the test samples was measured using the following formula: P activity (U/mL) = (A/V)/T

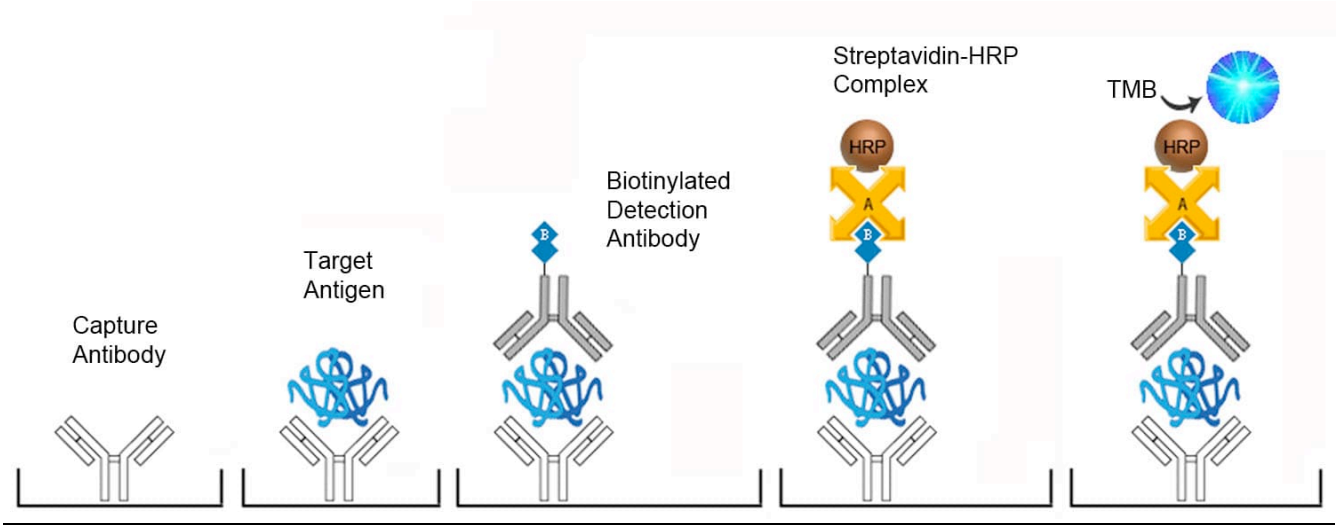
(A = amount of pNP generated in samples calculated from standard curve (μmol), V = volume of sample added in the assay well (80 mL) and T = reaction time; 60 minutes). Lastly, the ALP activity was normalized by dividing the ALP activity by the protein concentration of each sample [63].

ELISA analysis:

Principle: The human PTHrP ELISA kit (LSBio, sandwich ELISA) (LS-F 22967) was used in this study [63]. Each well of the supplied microtiter plate has been pre-coated with target specific capture antibody [63]. The samples were added to the wells, incubated for 180 minutes at 37°C and then unbound standard or sample were washed away. After that, a biotin-conjugated detection antibody has been added which binds to the capture antigen and incubated for 1 hour at 37°C [63]. Thereafter, it was washed away [63]. An Avidin-Horseradish Peroxidase (HRP) is added which binds to the biotin, incubated for 30 minutes at 37°C and then it was washed away 5 times [63]. Next, TMB substrate was added to react with the HRP enzyme leading to color change and incubated for 15 minutes at 37°C protected from light. Lastly, a sulfuric acid stop solution is added and then measured at a wavelength of 450 nm.

The detection range for this kit is 15.625-1000 pg/ml [63]. The ELISA principle is described in Figure 3.

Figure 3: ELISA (Sandwich technique) principle [64]

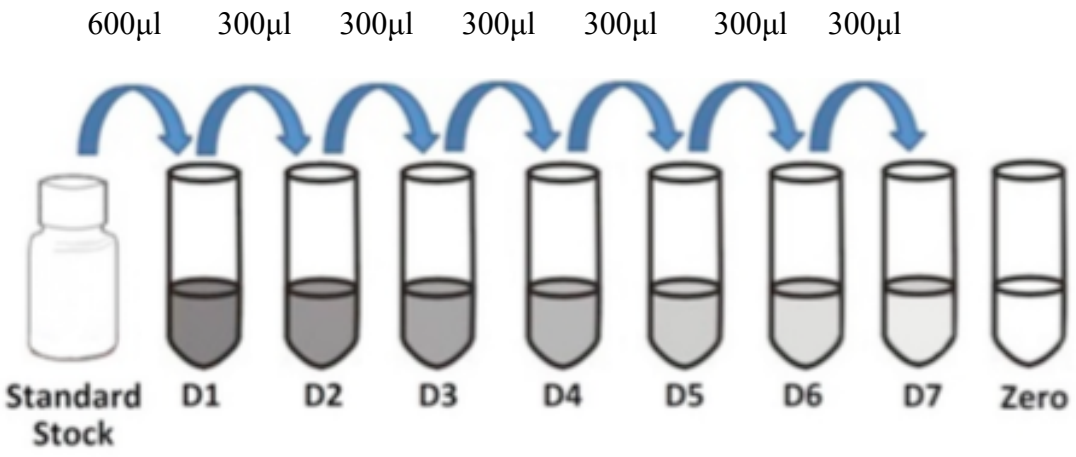


Standard preparation: According to the kit instructions, the standard preparation is from the standard stock solution (1000 pg/m). The standard curve was prepared from the dilution standards as shown in Table 4 and Figure 4 [64]. The standards were assayed in triplicate.

Table 4: Standard curve preparation for ELISA assay [64]

Standard	Concentration (pg/ml)	Standard solution (microliter)	Sample Diluent ** (ml)
D1	1000	600 (from stock *)	0
D2	500	350 (from D1)	350
D3	250	350 (from D2)	350
D4	125	350 (from D3)	350
D5	62.5	350 (from D4)	350
D6	31.25	350 (from D5)	350
D7	15.63	350 (from D6)	350
Zero standard	0	0	600
* Stock solution: 1 tube of lyophilized standard with 1.0 ml of Sample Diluent ** Sample Diluent provided from the manufacture			

Figure 4: ELISA standard curve preparation [64]



Reagent preparation:

Wash Buffer: 750 ml of Working Wash Buffer was prepared by diluting the supplied 30 ml of 25x Wash Buffer Concentrate with 720 ml of deionized or distilled water [64].

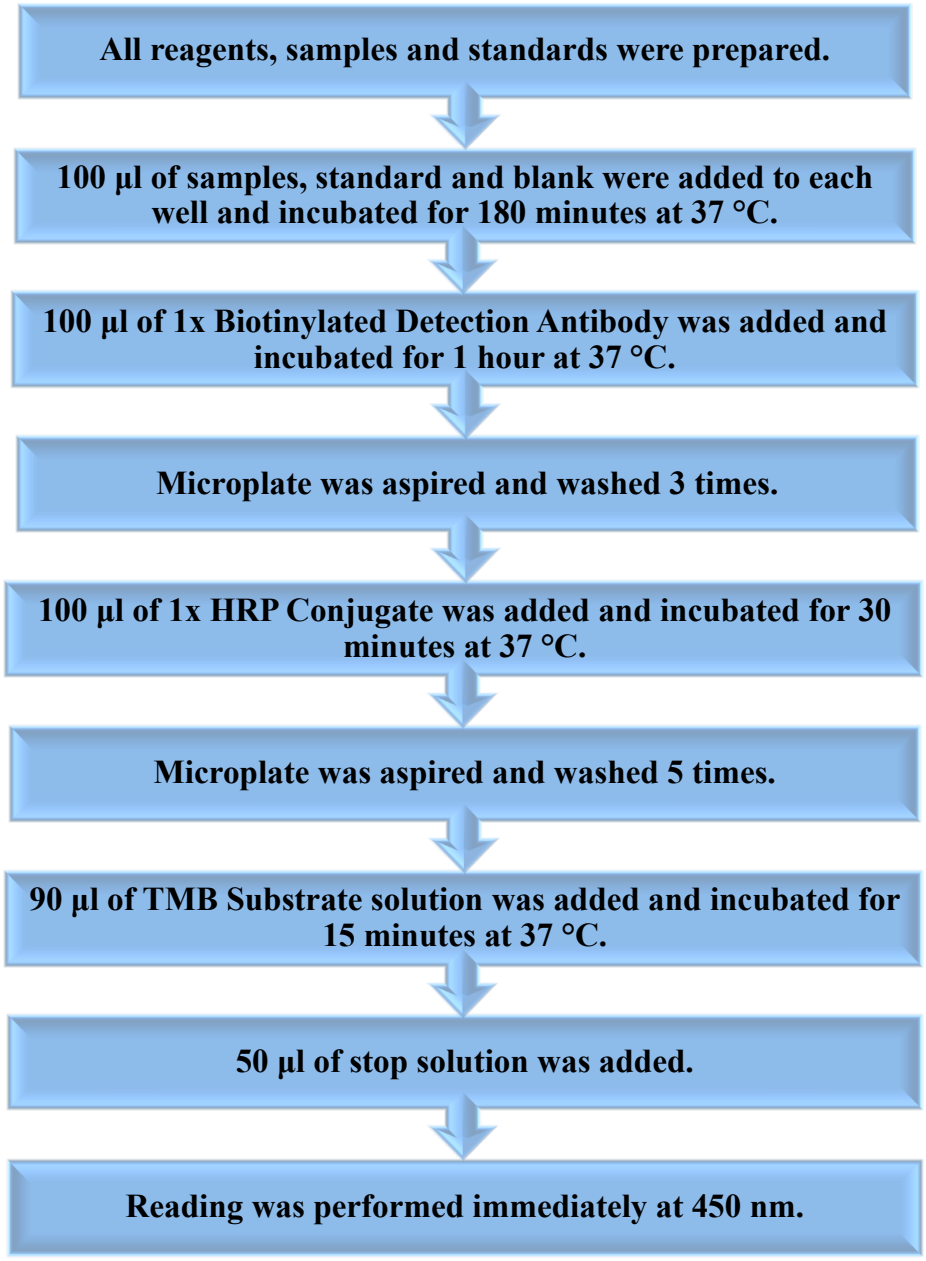
TMB Substrate Solution: the needed volume of TMB Substrate solution was used according to the number of wells and the unused TMB Substrate solution was disposed according to the manufacturer's instructions [64].

1x Biotinylated Detection Antibody: The required amount needed was calculated before beginning the experiment (100µl/well) and included a 200µl excess. The stock tube was centrifuged before we started the experiment. The concentrated Biotinylated Detection Antibody was diluted to the working concentration using the Biotinylated Detection Antibody Diluent (1:100); 92 µL from concentrated BDA was diluted in 900.108 ml of the wash buffer [64].

1x HRP Conjugate: The required amount needed was calculated before beginning the experiment (100µl/well) and included a 200µL excess. The HRP Conjugate was diluted to the working concentration using the HRP Conjugate Diluent (1:100); 92 µL from concentrated HRP Conjugate was diluted in 900.108 ml of the wash buffer [64].

Sample preparation: The study samples were placed at room temperature to allow them thaw on ice for the analysis. Each sample was diluted 1:10; 20µl of the sample diluted in 180µl of the dilution buffer. In addition, each sample was loaded in duplicate; 80µL in each well. After that, the ELISA steps were completed as shown below in Figure 5 [65].

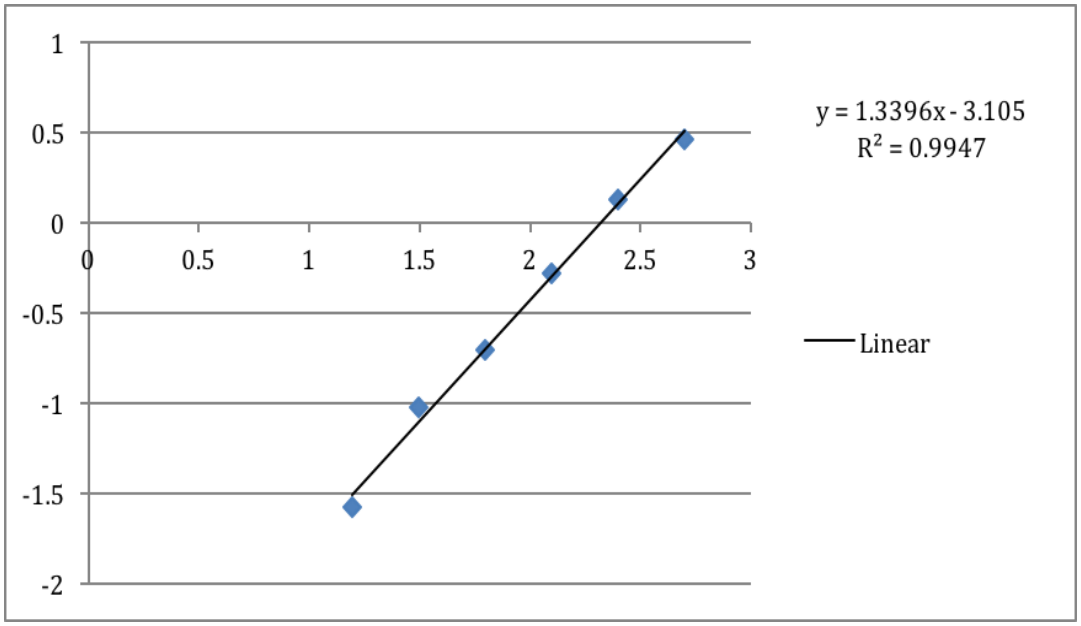
Figure 5: ELISA assay summary [65]



Calculations: The average of the triplicate standards and duplicate samples were calculated. After that, the averages were subtracted from zero standard optical density. Then, the standard curve was created as shown in Figure 6. After that, the data was linearized by

plotting the log of the target antigen concentrations versus the log of the OD and the best fit was determined by regression analysis [65].

Figure 6: ELISA standard curve



Re-concentration of 10 random samples:

Sample preparation: 10 samples were selected randomly and were placed at room temperature to allow them thaw on ice for the analysis. They were concentrated using the Amicon Ultra- 0.5 centrifugal filter devices. First, the Amicon ultra 0.5 device was inserted into one of the provided microcentrifuge tubes. Then, 500µL of samples were added to the

Amicon Ultra filter device and covered. Lastly, the devices were placed in the Eppendorf centrifuge 5415 D machine and span 14 rpm for 15 minutes.

Protein assay preparation for the re-concentrated samples: Following the same steps mentioned in protein assay analysis section. Table 5 demonstrates the protein concentration for 10 random samples after dilution with distilled water.

Table 5: Re-concentration of 10 random samples

Samples	Dilution ratio	Initial Protein levels (mg/ml)	Concentrated Protein levels (mg/ml)	Factor increase (initial/concentrated)
Sample 1 (S1)	1:30	2.45	0.88	2.78
Sample 2 (S2)	1:40	2.96	1.14	2.59
Sample 3 (S3)	1:50	1.9	1.6	1.18
Sample 4 (S4)	1.50	3.91	1.67	2.34
Sample 5 (S5)	1.50	3.09	1.5	2.06
Sample 6 (S6)	1.50	2.12	1.56	1.35
Sample 7 (S7)	1:50	0.833	1.42	0.58
Sample 8 (S8)	1:50	0.8	1.78	0.44
Sample 9 (S9)	1:30	2.13	0.92	2.31
Sample 10 (S10)	1:70	1.97	2.48	0.79

ELISA analysis after re-concentration of the samples:

Standard preparation: as previously described in the ELISA analysis procedure section.

Reagent preparation:

Wash Buffer: 750 ml of Working Wash Buffer was prepared by diluting the supplied 30 ml of 25x Wash Buffer Concentrate with 720 ml of deionized or distilled water [65].

TMB Substrate Solution: the needed volume of TMB Substrate solution was used according to the number of wells and the unused TMB Substrate solution was disposed according to the manufacture instructions [65].

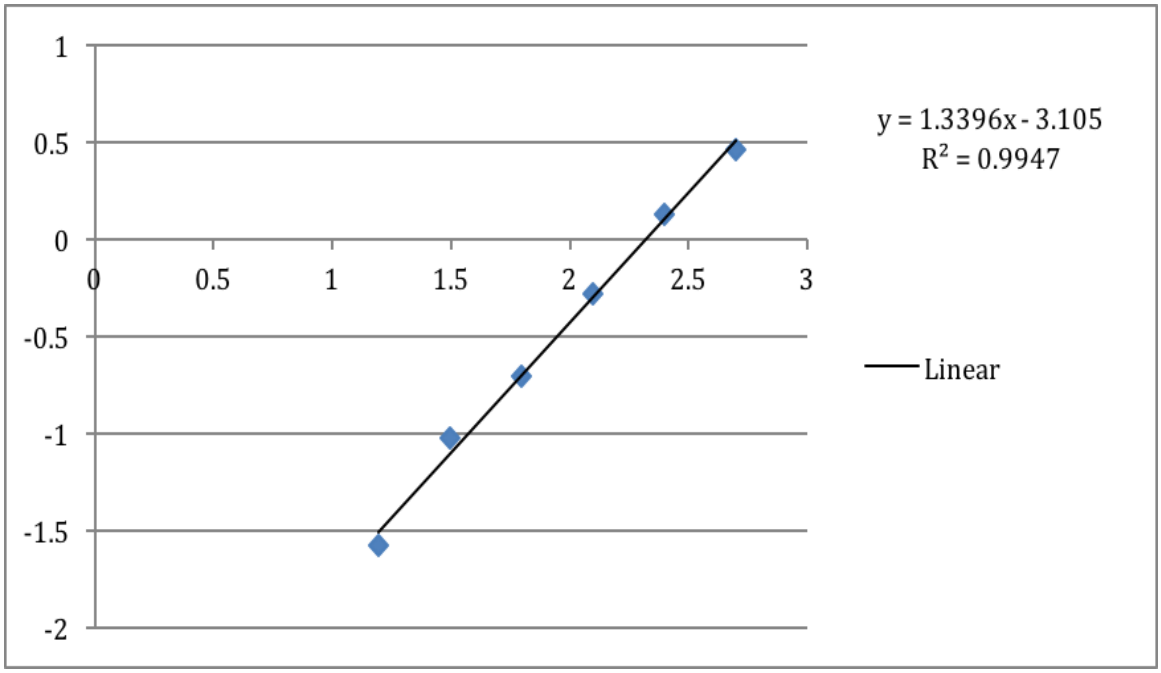
1x Biotinylated Detection Antibody: The required amount needed was calculated before beginning the experiment (100µl/well) and included a 200µl excess. The stock tube was centrifuged before we started the experiment. The concentrated Biotinylated Detection Antibody was diluted to the working concentration using the Biotinylated Detection Antibody Diluent (1:100); 46 µL from concentrated BDA was diluted in 4554 ml of the wash buffer.

1x HRP Conjugate: The required amount needed was calculated before beginning the experiment (100µl/well) and included a 200µL excess. The HRP Conjugate was diluted to the working concentration using the HRP Conjugate Diluent (1:100); 46 µL from concentrated HRP Conjugate was diluted in 4554 ml of the wash buffer. The ELISA steps were summarized in Figure 3.

Calculations: The average of the triplicate standards and duplicate samples were calculated. After that, the averages were subtracted from zero standard optical density. Then, the standard curve was created as shown in Figure 7. After that, the data was linearized by

plotting the log of the target antigen concentrations versus the log of the OD and the best fit was determined by regression analysis.

Figure 7: ELISA standard curve for the re-concentrated samples



Sample size calculation

A power calculation was conducted for the primary analysis using nQuery Advisor (version 7.0), based on the effect size reported in Table III of Hussain et al.[65]. Based on that calculation, a sample size of n=10 per group was determined to be adequate to obtain a Type I error rate of 5% and a power greater than 99% for the comparison of ALP and PTHrP levels between the cervical stages. A total of 79 subjects (at least 10 in each group) were finally included in this study.

Statistical analysis:

Descriptive statistics (counts and percentages for categorical variables; means, standard deviations, medians and interquartile ranges for continuous variables) were calculated. Cohen's kappa was used to assess inter-examiner reliability. The normality assumption for ALP activity and protein concentration was tested by the Shapiro-Wilk test. Due to non-normality of the data, inter-group comparisons of ALP (normalized; ALP activity divided by the protein content for each sample) and protein concentration were conducted via non-parametric tests (the Kruskal-Wallis test), with Dunn's test and Bonferroni correction used for post hoc comparisons. The Mann-Whitney U test was used to compare males and females in terms of ALP levels. The Spearman correlation was used to measure the association between chronological age and CVMS and between chronological age and ALP activity. Additionally, five multinomial logistic regression models were utilized to predict CVM stage. In the first model, the independent variable was the ALP (mU/mg x 10);

multiplication by 10 was done in order to place the values on a larger scale and thereby facilitate the interpretation of the analysis. In the second model, the independent variable was protein concentration. The independent variable was chronological age in the third model. The fourth model had two independent variables: ALP (mU/mg x 10) and age. Protein concentration and age were the independent variables in the last model. For each multinomial logistic regression model, p-values were calculated along with McFadden's pseudo R^2 and the model's correct classification rate. The latter statistic represents the percentage of subjects for whom the actual CVM stage matched the stage predicted by the model. P-values less than 0.05 were considered statistically significant, with the exception of tests in which the Bonferroni correction was used ($p \leq 0.005$). Data were analyzed using SPSS software version 24.

Results:

Descriptive statistics:

Descriptive statistics are presented in Table 6, Table 7 and Table 8. The study sample was comprised of 79 subjects: 48 females (60.8%) and 31 males (39.2%) as shown in Table 6. The age and race distribution of the study sample are shown in Table 7 and Table 8.

Table 6: Distribution of study sample

CVM stages	N	Percentage	Female	Male
CVMS I	20	25.3%	9	11
CVMS II	19	24.1%	15	4
CVMS III	17	21.5%	11	6
CVMS IV	13	16.5%	10	3
CVMS V	10	12.7%	3	7
Total/percentage	79	100.0%	48 (60.8%)	31 (39.2%)

Table 7: Age distribution (years) of the study sample

	CVMS I	CVMS II	CVMS III	CVMS IV	CVMS V
Mean	9.90	11.05	14.11	15.08	18.30
SD	1.80	1.35	2.44	3.99	3.23
Median	10.00	11.00	13.00	14.00	17.50
Min-Max	7-13	8-14	10-19	10-23	14-23

Table 8: Race distribution of the study sample

Race	CVMS I	CVMS II	CVMS III	CVMS IV	CVMS V	N	Percentage
Asian	4	2	4	1	3	14	17.72 %
Black	3	3	2	1	1	10	12.66 %
White	11	11	10	8	4	44	55.70 %
Hispanic	0	2	0	3	2	8	10.12 %
Multi-Racial	1	1	0	0	0	2	2.53 %
Unknown	1	0	1	0	0	1	1.27 %

Inter-examiner agreement:

Cohen's kappa coefficient was used in this study to measure the inter-examiner agreement for CVM staging for all subjects based on their lateral cephalographic radiographs' evaluation. According to Landis and Koch's guidelines, values below 0 indicate no agreement. Additionally, values between 0 and 0.20 indicate slight agreement and fair agreement is indicated for values between 0.21 and 0.40. Moreover, values between 0.41 and 0.60 are considered as moderate agreement, values between 0.61 are 0.80 as substantial and values between 0.81 and 1 as almost perfect agreement [66]. In the present study, the agreement was moderate between rater 1 and rater 3 (0.43) and between rater 2 and rater 3 (0.49). However, the agreement between rater 1 and 2 was fair (0.33).

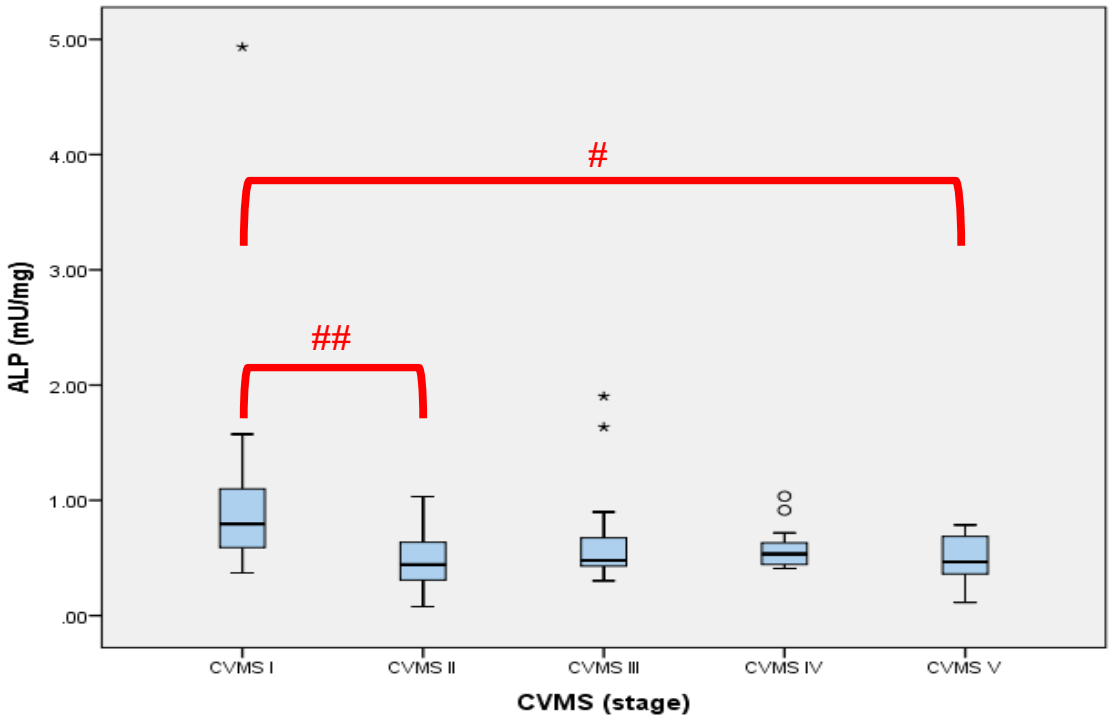
Comparisons of ALP activity and protein concentration by CVM stage:

The ALP median was high in CVMS I compared to the other stages. The medians for ALP activity were 0.80 mU/mg in CVMS I, 0.44 mU/mg in CVMS II, 0.48 mU/mg in CVMS III, 0.53 mU/mg in CVMS IV and 0.47 mU/mg in CVMS V as shown in Table 9. The Kruskal-Wallis test showed a significant difference in ALP activity distribution among the CVMS ($p = 0.002$). The ALP levels were significantly different between CVMS I and CVMS II ($p < 0.001$) and between CVMS I and CVMS V ($p = 0.004$) as shown in Figure 8. Despite all troubleshooting that was attempted with ELISA for analyzing PTHrP levels in samples of saliva, including concentrating the saliva samples, PTHrP was not detected at any sample.

Table 9: Descriptive statistics of the ALP activity in the 5 cervical stages and post-hoc comparisons using Dunn's test and Bonferroni correction

Alkaline phosphatase activity (mU/mg)			
CVMS	Median	IQR	N
CVMS I	0.80	0.54	20
CVMS II	0.44 [a]	0.40	19
CVMS III	0.48	0.36	17
CVMS IV	0.53	0.23	13
CVMS V	0.47 [b]	0.38	10
Significant differences in levels of ALP activity ($p = 0.002$) were found between the following groups: [a] CVMS I vs CVMS II ($p < 0.001$) [b] CVMS I vs CVMS V ($p = 0.004$)			

Figure 8: Distribution of ALP activity by CVMS



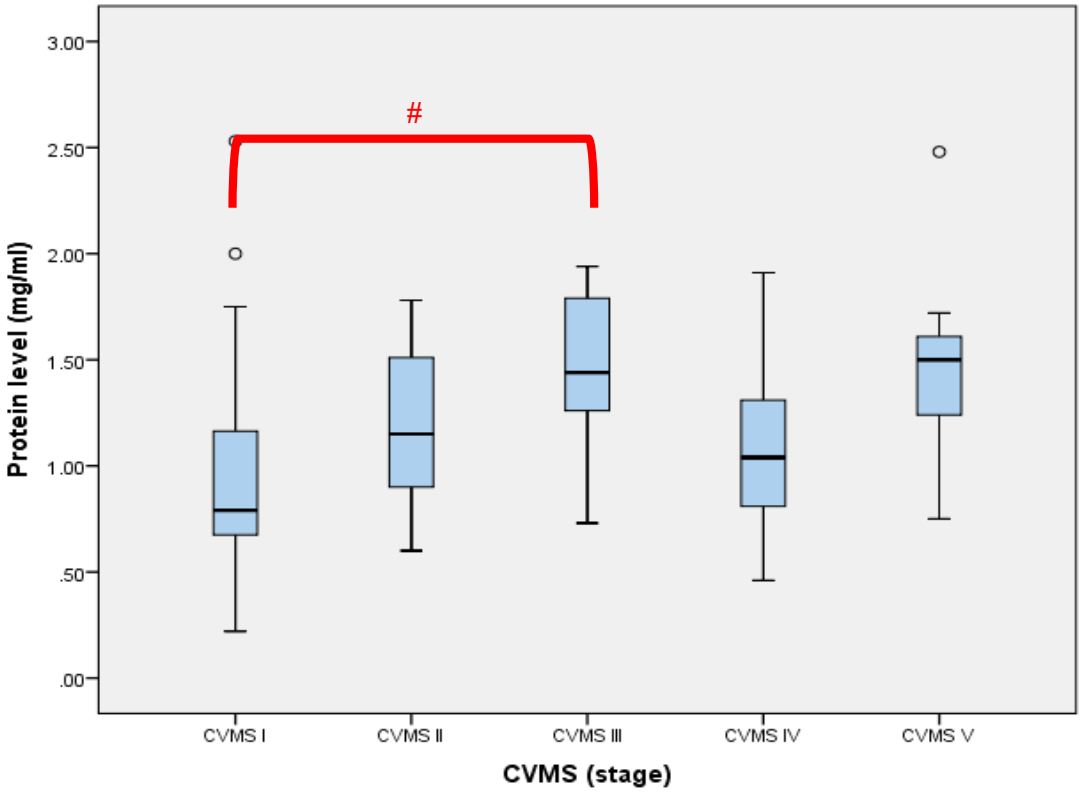
 # P = 0.004, ## P < 0.001

Regarding to protein concentration, our results showed higher median protein values in CVMS III and CVMS V. The medians for protein concentration were 0.79 mg/ml in CVMS I, 1.15 mg/ml in CVMS II, 1.44 mg/ml in CVMS III, 1.04 mg/ml in CVMS IV and 1.50 mg/ml in CVMS V as shown in Table 10. The Kruskal-Wallis test comparing protein concentration distribution between different stages was significantly significant (p = 0.014). The protein concentration was significantly different between CVMS I and CVMS III (p = 0.005) as shown in Figure 9.

Table 10: Descriptive statistics of the protein concentration in the 5 cervical stages and post-hoc comparisons using Dunn's test and Bonferroni correction

Protein concentration (mg/ml)			
CVMS	Median	IQR	N
CVMS I	0.79	0.52	20
CVMS II	1.15	0.64	19
CVMS III	1.44 [a]	0.65	17
CVMS IV	1.04	0.60	13
CVMS V	1.50	0.46	10
Significant difference in levels of protein activity ($p = 0.014$) was found between the following groups: [a] CVMS I vs CVMS III ($p = 0.005$)			

Figure 9: Distribution of protein concentration by CVM stage



 # P = 0.005

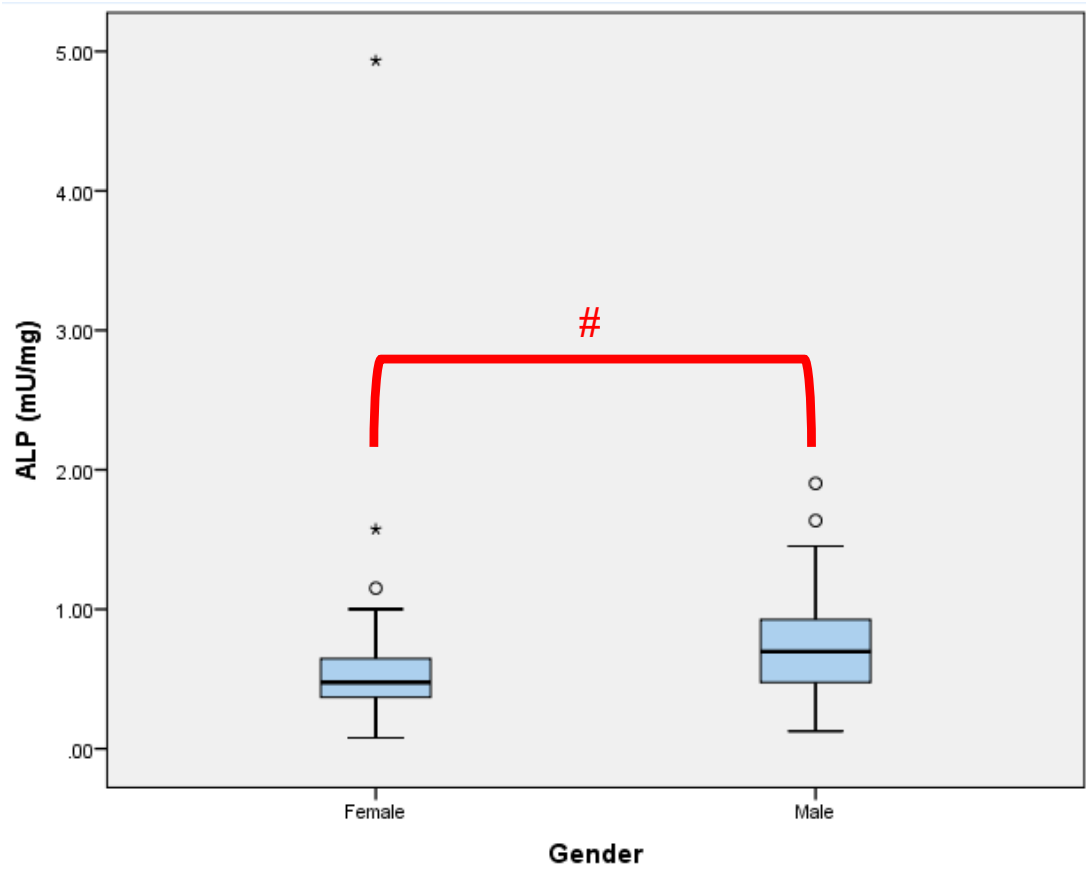
Comparison of males and females in terms of ALP activity:

Due to non-normality of the ALP activity data, the Mann-Whitney U test was used to compare ALP activity of males and females. The median ALP for females was 0.48 mU/mg and the interquartile range (IQR) was 0.29 mU/mg. The median ALP was higher in males (the median was 0.70 mU/mg, with an IQR of 0.54 mU/mg). The difference was statistically significant (p = 0.007). See Table 11 and Figure 10.

Table 11: Descriptive statistics of ALP activity by gender (p = 0.007)

ALP activity (mU/mg)		
Gender	Median	IQR
Female	0.48	0.29
Male	0.70	0.54

Figure 10: Impact of gender on ALP activity



 # P = 0.007

Spearman correlations:

The Spearman correlation was used to assess the association between two sets of variables in our study: age and CVMS, and age and ALP. As shown in Figure 11, there was a significant positive correlation between chronological age and CVMS (r_s= 0.763, p < 0.001).

Figure 11: The association between chronological age and CVMS

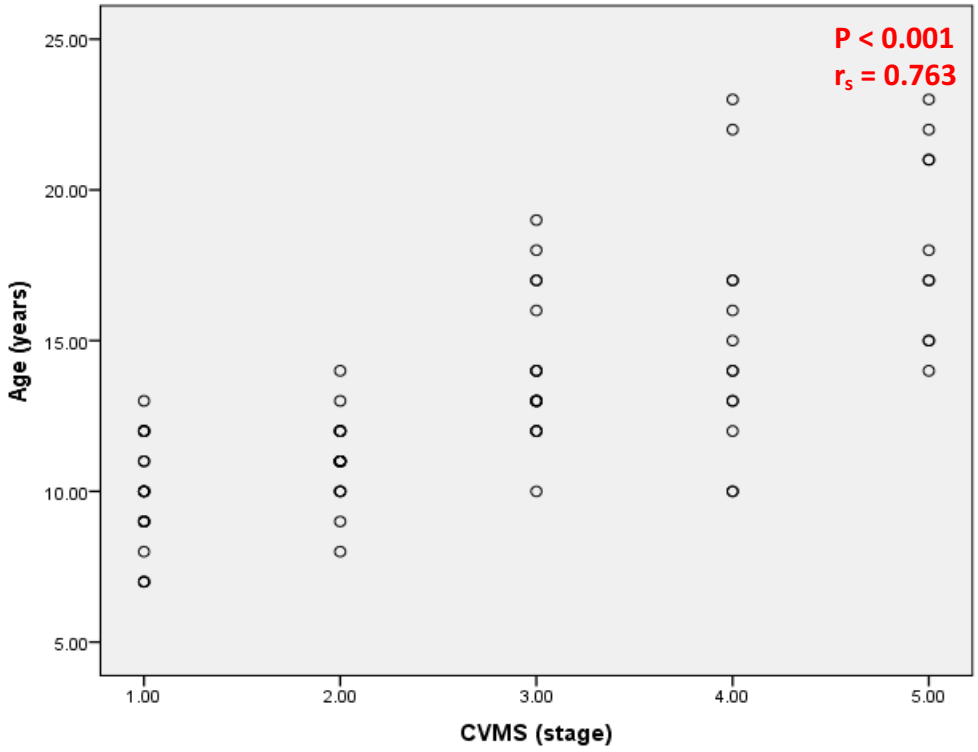
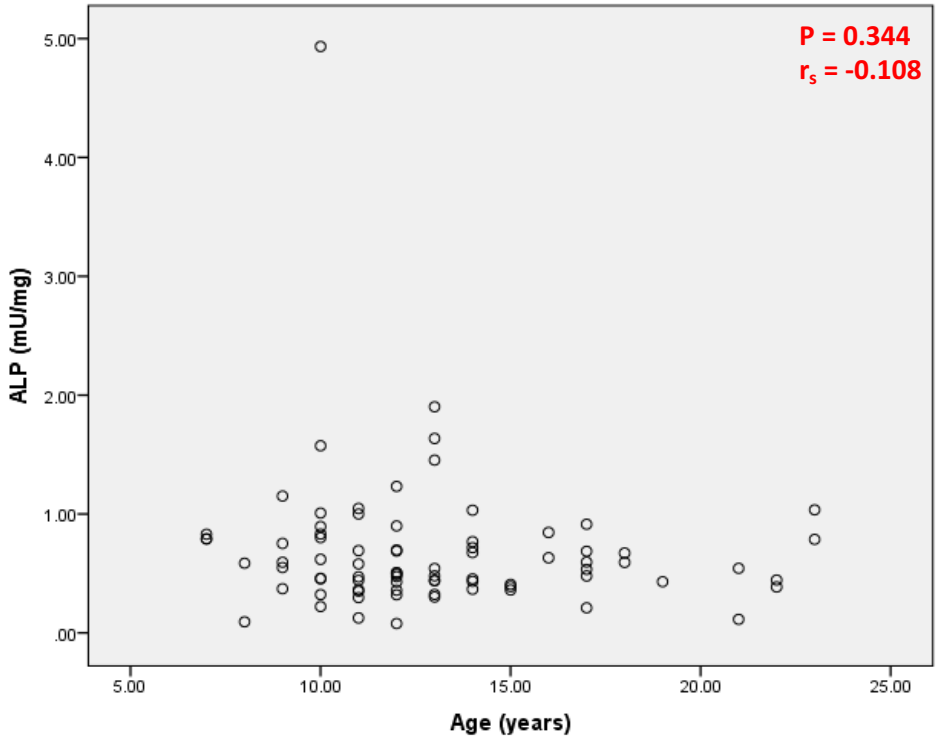


Figure 12 presents a scatterplot of chronological age and ALP activity. There was a weak negative correlation between these two variables that was not statistically significant (r_s= -0.108, p = 0.344).

Figure 12: The association between chronological age and ALP activity



Multinomial logistic regression models to predict CVM stage:

In the first multinomial logistic regression model (in which CVM stage was predicted from ALP), the latter variable was a statistically significant predictor of the former ($p = 0.002$).

From the multinomial logistic regression models, odds ratios can be computed (adjusting for the other predictors, when present). For instance, in Table 12, odds ratios corresponding to the first model are provided. The reference category was CVMS I, and the odds ratio for CVMS II was 0.675. This indicates that when we increase the ALP level by 1, the odds of being in CVMS II relative to CVMS I decrease (the odds are multiplied by 0.675). Also, when we increase the ALP level by 1, the odds of being in CVMS III compared to CVMS I decrease (are multiplied by 0.881). Relative to CVMS I, when we increase the ALP by 1, the odds of being in CVMS IV decrease (are multiplied by 0.814). Additionally, when we increase the ALP by 1, the odds of being in CVMS V compared to CVMS I decrease (are multiplied by 0.688). In the regression analysis, when we used the model to predict CVMS from the ALP, the model's overall correct classification rate was 35.4%.

Table 12: Multinomial logistic regression predicting CVMS from ALP activity (p = 0.002)

ALP activity*		
CVMS	Odds ratio	95% Confidence Interval
CVMS II	0.675	(0.518, 0.881)
CVMS III	0.881	(0.735, 1.056)
CVMS IV	0.814	(0.645, 1.028)
CVMS V	0.688	(0.499, 0.948)
- The reference category was CMVS I *ALP (mU/mg) x 10		

In the second model, the independent variable was protein concentration. The protein concentration was statistically significant (p = 0.021) in predicting the CVMS. The McFadden's pseudo R^2 was 0.046. Table 13 presents odds ratios with CVMS I as the reference category. Our data showed that if we increase the protein concentration by 1, the odds of being in CVMS II, CVMS III, CVMS IV and CVMS V increase. When using the model to predict CVMS from the protein concentration, the model's overall correct classification rate was 32.9%.

Table 13: Multinomial logistic regression predicting CVMS from protein concentration (p = 0.021)

Protein concentration (mg/ml)		
CVMS	Odds ratio	95% Confidence Interval
CVMS II	2.858	(0.609, 13.406)
CVMS III	7.659	(1.501, 39.071)
CVMS IV	1.337	(0.237, 7.539)
CVMS V	11.619	(1.778, 75.906)
- The reference category was CVMS I		

In the third model, the independent variable was chronological age. The chronological age was statistically significant ($p < 0.001$) in predicting the CVMS. The McFadden's pseudo R^2 was 0.282. Table 14 presents odds ratios with CVMS I as the reference category. Our results showed that if we increase the age by 1, the odds of being in CVMS II, CVMS III, CVMS IV and CVMS V increase. When using the model to predict CVMS from the age, the model's overall correct classification rate was 51.9%.

Table 14: Multinomial logistic regression predicting CVMS from Chronological age ($p < 0.001$)

Chronological age (years)		
CVMS	Odds ratio	95% Confidence Interval
CVMS II	1.541	(1.005, 2.363)
CVMS III	3.590	(1.937, 6.655)
CVMS IV	4.034	(2.135, 7.621)
CVMS V	5.285	(2.723, 10.259)
- The reference category was CVMS I		

In the fourth model, the independent variables were ALP activity and age. Both ALP activity ($p = 0.002$) and chronological age ($p < 0.001$) were significant in predicting the CVMS. The McFadden's pseudo R^2 was 0.348. Odds ratios are presented in Table 15, with CVMS I as the reference category. If we adjust for age and we increase the ALP by 1, the odds of being in CVMS II, CVMS III, CVMS IV and CVMS V decrease. On the other hand, if we adjust for ALP and we increase the age by 1, the odds of being in CVMS II, CVMS III, CVMS IV and CVMS V increase. When using the model to predict CVMS from the combination of ALP activity and age, the model's overall correct classification rate was 53.2%.

Table 15: Multinomial logistic regression predicting CVMS from ALP activity (p = 0.002) and chronological age (p < 0.002)

CVMS	Independent variable	Odds ratio	95% Confidence Interval
CVMS II	ALP*	0.683	(0.527, 0.885)
	Age**	1.698	(1.003, 2.873)
CVMS III	ALP*	0.821	(0.659, 1.023)
	Age**	3.977	(1.985, 7.968)
CVMS IV	ALP*	0.758	(0.574, 1.000)
	Age**	4.477	(2.197, 9.126)
CVMS V	ALP*	0.569	(0.353, 0.917)
	Age**	6.085	(2.888, 12.821)

- The reference category was CVMS I

* ALP (mU/mg) x 10

** Age (years)

In the fifth model, the independent variables were protein concentration and age. Protein concentration was not statistically significant (p = 0.072) in predicting the CVMS, whereas chronological age was significant in predicting the CVMS (p < 0.001). McFadden's pseudo R^2 was 0.317. Table 16 shows odds ratios, with CVMS I as the reference category. If we adjust for age and we increase the protein concentration by 1, the odds of being in CVMS II, CVMS III, CVM IV and CVM V increase. When using the model to predict CVMS from the combination of protein concentration and age, the model's overall correct classification rate was 49.4%.

Table 16: Multinomial logistic regression predicting CVMS from protein concentration ($p = 0.072$) and chronological age ($p < 0.001$)

CVMS	Independent variable	Odds ratio	95% Confidence Interval
CVMS II	Protein conc.*	2.460	(0.532, 11.373)
	Age**	1.553	(0.992, 2.431)
CVMS III	Protein conc.*	10.072	(1.205, 84.176)
	Age**	3.637	(1.944, 6.805)
CVMS IV	Protein conc.*	1.221	(0.108, 13.777)
	Age**	4.122	(2.159, 7.869)
CVMS V	Protein conc.*	8.713	(0.579, 131.090)
	Age**	5.276	(2.700, 10.307)
- The reference category was CMVS I * Protein conc. (mg/ml) ** Age (years)			

Table 17 presents the multinomial logistic regression models with McFadden's pseudo R^2 and the model's overall correct classification rates among all the five models.

Table 17: Multinomial logistic regression models with McFadden's pseudo R^2 and correct classification rate by model.

Models for multinomial logistic regression	McFadden's pseudo R^2	Correct classification rate
Model 1 **Independent variable: ALP activity**	0.069	35.4%
Model 2 **Independent variable: protein conc.**	0.046	32.9%
Model 3 **Independent variable: chronological age**	0.282	51.9%
Model 4 **Independent variables: ALP and age**	0.348	53.2%
Model 5 **Independent variables: protein conc. and age**	0.317	49.4%

Discussion

The assessment of skeletal age plays an important role in optimal orthodontic diagnosis and treatment planning [65]. Since most of the craniofacial assessment methods are invasive, require radiographic exposure and long observation period, new non-invasive biomarkers have been proposed to assess skeletal maturation [65,65]. These biomarkers are secreted into the body fluid such as blood and saliva [65]. Thus, saliva has been described as "mirror of the body" and can be used as a diagnostic tool for monitoring the health status [65,65]. In the present study we investigated the correlation of salivary ALP activity, protein concentration and PTHrP levels with skeletal age since they have been associated with bone metabolism, cells structure and function, and cartilage development, respectively [65,65].

In the present study, all subjects were given instructions to refrain from drinks, food and tooth brushing a certain time before the saliva collection procedure in order to standardize the sample's collection and prevent confounding factors that could affect biomarker analysis. For the sake of accuracy and to control the diurnal fluctuations in saliva flow, most of the saliva samples were collected at the same time period; 9 am-12 pm. This time frame was selected in order to standardize the samples' collection. In addition, studies have reported higher concentration of pubertal hormones in the morning such as testosterone and cortisol [65,65]. Studies have reported that the normal range of protein concentration in saliva ranged between 0.72 mg/ml to 2.45 mg/ml [65]. Our data showed slightly wider range of the salivary protein concentration that ranged between 0.22 mg/ml to 2.53 mg/ml. Also, Perinetti et al. found that the total protein concentration in GCF were between 0.2 mg/ml to 2.4 mg/ml [65].

Lee et al. and Wang et al. reported that salivary proteome can be used as diagnostic biomarker for the identification of diseases such as periodontitis, caries, breast cancer and diabetes [52,66]. Previous data reported that total protein concentration in GCF were not statistically significant among the different skeletal maturation stages and protein concentration could not serve as a biomarker for skeletal maturity assessment [7]. However, to date, no published studies have investigated the prediction of skeletal maturation from salivary proteins. The present data demonstrated different protein concentration among the CVMS with a statistically significant difference between CVMS I and CVMS III and that the highest level of protein in saliva was noted at the pubertal growth stage. Similar to our results, Cabras et al. have reported elevated levels of proline-rich proteins in whole saliva during the age of adolescence [67]. The increased salivary protein levels during puberty could be correlated with hormonal and growth maturation that influence salivary glands function [67]. This association could also explain the increased salivary protein concentration during puberty that were observed in our population. Based on our findings, salivary protein concentration was statistically significant in predicting CVMS. However, the ability of predicting CVMS correctly (model's overall correct classification rate) was 32.9%; lower than ALP activity (35.4%) and the combination of ALP activity and age (53.2%). Therefore, protein concentration as an indicator for skeletal maturation assessment is questionable.

Interestingly, salivary ALP activity was higher at early pubertal stage (CVMS I) and then declined with a statistically significant difference between CVMS I and CVMS II and between CVMS I and CVMS V. Our results are different from those of Perinetti et al. who reported that the peak of ALP activity in the GCF was during the pubertal growth spurt [13]. In

addition, Tobiume et al. and Christenson et al. stated that serum bone ALP activity peak occurred during infancy and puberty indicating high bone metabolism during these periods while we found the salivary ALP activity peak in pre-pubertal period [44,44]. However, it is important to mention that, in contrast to our study, in these studies ALP activity was not normalized to the total protein concentration in saliva (ALP activity divided by the protein concentration of each sample) as we did for our samples. We consider this approach very important in fluid analysis in order to standardize the samples' analysis and comparisons between different patients.

According to Perinette et al., ALP activity in GCF ranged between 1.35 mU/mg to 121.60 mU/mg [41,42]. However, our data presented a range of 0.07 mU/mg to 2.91 mU/mg of ALP activity in saliva. Our results showed that the skeletal growth prediction from salivary ALP activity was statistically significant ($p = 0.002$). Additionally, the model's overall correct classification rate to predict the CVMS from ALP activity was 35.4%. This figure was higher than the protein model's overall correct classification rate (32.9%), but lower than chronological age (51.9%) and the combination of ALP activity and age (53.2%). In sum, ALP activity had a modest factor in predicting CVMS when combined with age. Compared to CVMS I, if we increased the ALP by 1, the odds of being in CVMS II, CVMS III, CVMS IV and CVMS V decreased. To date, no published studies have investigated the prediction of skeletal maturation from salivary ALP activity and this study provides some preliminary data about the value of salivary ALP activity in skeletal maturity assessment.

Tarvade et al. compared salivary ALP activity in growing subjects to their hand and wrist radiographs as a biomarker for skeletal maturation assessment [53,54]. He found that highest levels of salivary ALP activity were correlated with the growth spurt of the study sample. However, there are several drawbacks in that paper. First, although the sample size was large (n=120) in that study, the age range was limited (10 to 15 years), questioning if it included all skeletal maturation stages [35,68]. Moreover, the sample size for each group was not reported. Finally, they examined ALP activity without normalization, so the different protein levels between the subjects' samples might act as confounding factor in their data analysis [69].

The literature reports controversies regarding the use of chronological age for skeletal maturation assessment as well. Alkhal et al. have questioned the use of chronological age to assess the maturity state of a child and found a weak correlation between age and skeletal maturity in southern Chinese population [70]. Also, Ramos et al. found a weak correlation between skeletal age and chronological age [71]. Although previous studies have reported that chronological age is not associated with skeletal maturation, our data showed positive correlation between chronological age and skeletal growth [36]. Our findings are in accordance with Litsas and Lucchese who reported that skeletal growth is positively associated with chronological age and dental age [72]. Furthermore, Safavi et al. have reported a positive correlation between chronological age and growth stages in Iranian females [73]. Moreover, a retrospective study about the correlation between chronological age and skeletal age among children aged 9-14 years reported a strong positive correlation between the two variables [15]. To date, no published studies have investigated the CVMS prediction from chronological age, the combination of chronological age and salivary ALP and the combination of

chronological age and protein concentration. Our data showed that chronological age was statistically significant in predicting the CVMS ($P<0.001$). The combination of ALP activity and chronological age showed the highest correct classification rate and McFadden's pseudo R^2 values among the models used in this study. The McFadden's pseudo R^2 was described in the logistic regression for assessing the predictive strength of the model.[74] According to Mokhtarian, a pseudo R^2 of 0.3 was considered a good fit model. In our results, the combination of ALP activity and age have the highest McFadden's pseudo R^2 value (0.348) compared to the other models [15]. On the other hand, the lowest McFadden's pseudo R^2 value (0.046) was in the model in which protein concentration was an independent variable. Therefore, the combination of chronological age and ALP could provide the best CVMS prediction compared to the other models.

To date, no published studies have investigated the relationship between chronological age and ALP activity in saliva. Our data showed a negative weak correlation between salivary ALP activity and chronological age. This association means that ALP activity was higher at early age group and then declined during growth. Therefore, the combination of increased salivary ALP activity and young age can help clinicians identifying the pre-pubertal stage. The weak correlation between salivary ALP activity and chronological age might be due to individual variation of ALP activity peak onset since there was a significant overlap between the age groups. Increase in height among adolescents as mentioned previously has been reported as the most valid representation of skeletal maturation [2]. However, height was not measured in the sample study, because it was a cross-sectional study and longitudinal observation of height changes are needed to use height as a valuable predictor of growth assessment. In contrast to our results, Fleisher et al. investigated

the association between serum ALP activity and chronological age [58]. He reported that there was no statistically significant difference between serum ALP activity and age in children younger than 10 years. However, the serum ALP activity significantly increased after 10 years of age during the pubertal growth spurt which then declined in adulthood [56].

According to age distribution of the study sample as shown in Table 2, there was a significant overlap between the age groups. This could be as a result of unreliable CVM method or it might be due to the individual variation of the sample study. The overlap between the age groups could affect our findings on correlation between CVMS and ALP activity. Perinetti et al. reported similar overlap between the groups in relation to chronological age as in our studied population [36]. Regarding gender differences, our findings showed a statistical significant difference of salivary ALP activity between males and females. The median in males' ALP activity (0.697 mU/mg) was higher compared to females' ALP activity (0.476 mU/mg) in this study sample. Males have more growth potential and long growth spurt duration compared to females, and that could explain the increased enzymatic activity in males [75,76]. Likewise, Fleisher et al. who studied the relationship between ALP activity in plasma of children and adolescents reported that males' serum ALP activity in adolescents (1100 U/L) was higher than females' ALP activity (900 U/L) [47]. Also, he found that the pubertal growth peak occurred in females earlier (11-12 years) than in males (13-14 years) according to the serum ALP activity [47]. However, in this study in order to identify the occurrence of salivary ALP peak in females compared to males, no conclusion can be drawn based on our data, since more patients with equal distribution between gender should be included in a future study.

Since PTHrP is associated with condylar cartilage development in the mandible, Hussain et al. investigated the use of PTHrP as a biomarker for skeletal maturation assessment [47]. Hussain et al. found that serum PTHrP levels increased during post-pubertal stage [77]. Therefore, the use of serum PTHrP in predicting growth stages was questionable, because it was not correlated with early pubertal stages. To date, no published studies have further investigated the prediction of skeletal maturation from serum or salivary PTHrP levels. However, our data showed that PTHrP levels in saliva were outside of the detection range of the ELISA kit used in this study (15.625-1000 pg/ml) [78]. We conducted several methods of troubleshooting in order to overcome this challenge. Re-concentration of random samples using Amicon Ultra-0.5 centrifugal filter devices was performed in order to improve the identification of the PTHrP in saliva samples as presented in Table 5. Although the samples were re-concentrated, the results of the ELISA assay were lower than the detection range of the kit that was not expected. To date, no previous studies have investigated the correlation between salivary PTHrP and skeletal maturation stages and this is a first attempt to investigate the levels of PTHrP in saliva. The only previous attempt to investigate PTHrP in saliva happened in sheep without any previous data on human [14]. The low readings in ELISA results could be due to several factors. First, the PTHrP might not be naturally present in saliva. Second, it could be secreted in saliva, but hydrolyzed by oral enzymes and not collected enough to be detected during ELISA. One additional explanation could be that PTHrP levels in saliva were not detected since PTHrP levels could be different than the detection range of the ELISA kit. In our study we cannot draw any conclusion about the value of PTHrP as a biomarker for skeletal growth predication and more pilot studies are

needed in order to draw a final conclusion about the detection and the role of PTHrP in saliva.

Translational/Clinical application of our results:

Our data can potentially help the clinicians identify the pre-pubertal growth stage in growing patients, a critical phase in determining the optimal timing of orthodontic treatment for a successful orthodontic outcome. The combination of high levels of salivary ALP activity and chronological age could provide the clinicians with additional insight about proper timing of orthopedic treatment and modifying the growth of jaw bones [79]. For example, functional appliances such as twin block, Frankel II and bionator are used in the treatment of class II growing individuals to modify mandibular growth. The effectiveness of these appliances are minimal if it occurs after the pubertal growth spurt [16]. Moreover, if the orthopedic appliances placed for post-pubertal patients, only dento-alveolar changes will occur [80]. Regarding headgear appliance onset, Baccetti et al. reported that the significant favorable dento-skeletal corrections of class II treatment are best achieved if the treatment occurs before or during the pubertal growth spurt [81]. Therefore, the headgear appliances are best used in pre-pubertal patients to restrict maxillary growth [82].

In addition to orthopedic appliances, Baccetti et al. investigated the short-term and the long-term effect of rapid maxillary expansion in subjects with constricted maxillary arch before the pubertal growth spurt, during the peak of growth and after puberty [83]. He found

that treatment of maxillary expansion in pre-pubertal subjects produced significant and stable skeletal changes than the subjects who were treated during or after the pubertal growth peak [83]. Therefore, maxillary expansion will have a more effective and efficient results if it takes place during the pre-pubertal stage that is critical to be identified as accurately as possible from the clinicians.

Limitations:

One of the main limitations of this study was the inter-examiner reliability for CVM staging based on lateral cephalometric radiographs evaluation. According to Landis and Koch guidelines [15], the highest agreement was moderate between rater 1 and rater 3 and between rater 2 and rater 3. However, the agreement between rater 1 and 2 was fair. Based on these findings, the CVM method to predict the skeletal age was questionable in our study. In the literature, there was controversy regarding the validity of CVM method in predicting skeletal maturation. Therefore, it is recommended to establish first a calibration between the examiners in the CVM method which did not happen in our project.

Another limitation is the questionable correlation of CVMS and skeletal age prediction. In agreement with our results, Gray et al. questioned the use of morphological changes in the cervical vertebrae to predict the mandibular growth spurt [84]. They concluded that CVMS method was subjective, poorly reproducible and invalid in predicting mandibular growth spurt [84]. On the other hand, Alkhal et al. and Cericato et al. found that CVM method is a valid indicator for skeletal growth [83,52].

An additional limitation in our study was the two-dimensional representation of three-dimensional object of lateral cephalometric radiographs that were used in our CVM evaluation. As a result, this will influence the identification of the morphological changes of the cervical vertebrae. Also, the dental development that was obvious in lateral cephalometric radiographs could affect the decision of CVMS. However, clinicians routinely examine the dental maturation with the morphological changes of cervical vertebrae of lateral cephalometric radiograph during skeletal maturation assessment, inducing some bias in their decisions [85]. Lastly, the ethnic majority of the sample study was White as shown in Table 8. In the present study, non-English speakers were excluded and that could make White as the racial majority. Increasing ethnic diversity is preferable in order to have better representation of the population.

Future studies:

Our data warrant a future longitudinal study or larger sample size in cross-sectional study to investigate the potential use of salivary ALP activity as a non-invasive biomarker for skeletal maturity. Also, a further study which compare between salivary ALP activity and serum ALP activity is suggested. Moreover, it is recommended to compare between the combination of chronological age and ALP activity versus the CVM method in skeletal maturation assessment. In addition, further studies are recommended to investigate the use of PTHrP as a biomarker for skeletal maturity. Another suggestion is to perform the saliva collection procedure directly from the salivary gland duct to prevent the break-down of the PTHrP. However, this proposed method would have significant difficulties in its application

compared to the collection of unstimulated saliva that was performed in our study. Also, comparison between serum PTHrP and salivary PTHrP is recommended to investigate further the detection of this protein in saliva.

Conclusions:

Based on our data's findings, the following conclusions can be drawn:

i. Our study demonstrated that salivary protein concentration was higher at growth spurt with a statistically significant difference between CVMS I and CVMS III. However, the salivary protein concentration has a weak contribution to skeletal growth prediction.

ii. Salivary ALP activity was higher at early pubertal stage (CVMS I) and then declined with a statistically significant difference between CVMS I and CVMS II and between CVMS I and CVMS V.

iii. In our study sample, salivary ALP activity was higher in males compared to females.

iv. Our data presented a strong positive association between chronological age and CVMS.

v. In the present study, the PTHrP could not be detected in saliva. Thus, the ability of PTHrP to predict skeletal maturity was questionable and further studies are suggested to investigate the use of PTHrP biomarker for skeletal maturity assessment.

vi. A weak negative correlation was found between chronological age and salivary ALP activity.

vii. Salivary ALP activity may be a promising diagnostic aid for prediction of pre-pubertal growth phase.

viii. Overall, salivary ALP activity has a modest contribution to CVMS prediction. On the other hand, the combination of chronological age and ALP activity prediction could provide the best CVMS prediction compared to the other models.

ix. Further cross-sectional or longitudinal studies with a larger sample size are suggested in order to validate the potential use of salivary ALP activity as a non-invasive biomarker for skeletal maturity.

x. Further studies are needed to discover novel biomarkers in saliva that could aid the clinicians assess the skeletal age of their patients.

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Appendices

Appendix A: Tables 1-17

Appendix B: Figures 1-12

Appendix A: Tables

Table 1: Subject timeline

Appointment procedures	Visit 1 (Screening visit)	Visit 2
Informed consent/assent form	X	
Demographics	X	
Medical/dental history	X	X
Intra-oral examination	X	X
Evaluate eligibility and withdrawal criteria	X	X
Sample collection sample		X

Table 2: Standard curve preparation for protein assay [52]

Standard	Concentration (mg/ml)	Protein (mg)	Water (ml)
Dilution 1 (D1)	500	100 (from stock*)	300
Dilution 2 (D2)	400	80 (from stock*)	320
Dilution 3 (D3)	300	60 (from stock*)	340
Dilution 4 (D4)	250	200 (from D1)	200
Dilution 5 (D5)	125	200 (from D4)	200
Dilution 6 (D6)	62.5	200 (from D5)	200
Dilution 7 (D7)	32.250	200 (from D6)	200
Negative control	0	0	400
* Stock solution: 1part Dye Reagent Concentrate with 4 parts distilled, deionized water			

Table 3: Standard curve preparation for ALP assay [65]

Standard	Concentration (unit/ml)	Volume of pNPP * (microliter)	Assay buffer ** (microliter)
D1	0	0	396
D2	4	13	377
D3	8	25	350
D4	12	38	342
D5	16	50	325
D6	20	62	310
* pNPP standard was prepared by diluting 40µL pNPP 5mM Standard in 320 µL of Assay Buffer. ** Assay buffer was provided from the manufacturer.			

Table 4: Standard curve preparation for ELISA assay [49]

Standard	Concentration (pg/ml)	Standard solution (microliter)	Sample Diluent ** (ml)
D1	1000	600 (from stock *)	0
D2	500	350 (from D1)	350
D3	250	350 (from D2)	350
D4	125	350 (from D3)	350
D5	62.5	350 (from D4)	350
D6	31.25	350 (from D5)	350
D7	15.63	350 (from D6)	350
Zero standard	0	0	600
* Stock solution: 1 tube of lyophilized standard with 1.0 ml of Sample Diluent ** Sample Diluent provided from the manufacture			

Table 5: Re-concentration of 10 random samples

Samples	Dilution ratio	Initial Protein levels (mg/ml)	Concentrated Protein levels (mg/ml)	Factor increase (initial/concentrated)
Sample 1 (S1)	1:30	2.45	0.88	2.78
Sample 2 (S2)	1:40	2.96	1.14	2.59
Sample 3 (S3)	1:50	1.9	1.6	1.18
Sample 4 (S4)	1.50	3.91	1.67	2.34
Sample 5 (S5)	1.50	3.09	1.5	2.06
Sample 6 (S6)	1.50	2.12	1.56	1.35
Sample 7 (S7)	1:50	0.833	1.42	0.58
Sample 8 (S8)	1:50	0.8	1.78	0.44
Sample 9 (S9)	1:30	2.13	0.92	2.31
Sample 10 (S10)	1:70	1.97	2.48	0.79

Table 6: Distribution of study sample

CVM stages	N	Percentage	Female	Male
CVMS I	20	25.3%	9	11
CVMS II	19	24.1%	15	4
CVMS III	17	21.5%	11	6
CVMS IV	13	16.5%	10	3
CVMS V	10	12.7%	3	7
Total/percentage	79	100.0%	48 (60.8%)	31 (39.2%)

Table 7: Age distribution (years) of the study sample

	CVMS I	CVMS II	CVMS III	CVMS IV	CVMS V
Mean	9.90	11.05	14.11	15.08	18.30
SD	1.80	1.35	2.44	3.99	3.23
Median	10.00	11.00	13.00	14.00	17.50
Min-Max	7-13	8-14	10-19	10-23	14-23

Table 8: Race distribution of the study sample

Race	CVMS I	CVMS II	CVMS III	CVMS IV	CVMS V	N	Percentage
Asian	4	2	4	1	3	14	17.72 %
Black	3	3	2	1	1	10	12.66 %
White	11	11	10	8	4	44	55.70 %
Hispanic	0	2	0	3	2	8	10.12 %
Multi-Racial	1	1	0	0	0	2	2.53 %
Unknown	1	0	1	0	0	1	1.27 %

Table 9: Descriptive statistics of the ALP activity in the 5 cervical stages and post-hoc comparisons using Dunn's test and Bonferroni correction

Alkaline phosphatase activity (mU/mg)			
CVMS	Median	IQR	N
CVMS I	0.80	0.540	20
CVMS II	0.44 [a]	0.400	19
CVMS III	0.48	0.360	17
CVMS IV	0.53	0.230	13
CVMS V	0.47 [b]	0.380	10
Significant differences in levels of ALP activity ($p = 0.002$) were found between the following groups: [a] CVMS I vs CVMS II ($p < 0.001$) [b] CVMS I vs CVMS V ($p = 0.004$)			

Table 10: Descriptive statistics of the protein concentration in the 5 cervical stages and post-hoc comparisons using Dunn's test and Bonferroni correction

Protein concentration (mg/ml)			
CVMS	Median	IQR	N
CVMS I	0.79	0.52	20
CVMS II	1.15	0.64	19
CVMS III	1.44 [a]	0.65	17
CVMS IV	1.04	0.60	13
CVMS V	1.50	0.46	10
Significant difference in levels of protein concentration (p = 0.014) was found between the following groups: [a] CVMS I vs CVMS III (p = 0.005)			

Table 11: Descriptive statistics of ALP activity by gender (p = 0.007)

ALP activity (mU/mg)		
Gender	Median	IQR
Female	0.48	0.290
Male	0.70	0.540

Table 12: Multinomial logistic regression predicting CVMS from ALP activity (p = 0.002)

ALP activity*		
CVMS	Odds ratio	95% Confidence Interval
CVMS II	0.675	(0.518, 0.881)
CVMS III	0.881	(0.735, 1.056)
CVMS IV	0.814	(0.645, 1.028)
CVMS V	0.688	(0.499, 0.948)
- The reference category was CMVS I *ALP (mU/mg) x 10		

Table 13: Multinomial logistic regression predicting CVMS from protein concentration (p = 0.021)

Protein concentration (mg/ml)		
CVMS	Odds ratio	95% Confidence Interval
CVMS II	2.858	(0.609, 13.406)
CVMS III	7.659	(1.501, 39.071)
CVMS IV	1.337	(0.237, 7.539)
CVMS V	11.619	(1.778, 75.906)
- The reference category was CVMS I		

Table 14: Multinomial logistic regression predicting CVMS from Chronological age (p < 0.001)

Chronological age (years)		
CVMS	Odds ratio	95% Confidence Interval
CVMS II	1.541	(1.005, 2.363)
CVMS III	3.590	(1.937, 6.655)
CVMS IV	4.034	(2.135, 7.621)
CVMS V	5.285	(2.723, 10.259)
- The reference category was CVMS I		

Table 15: Multinomial logistic regression predicting CVMS from ALP activity (p = 0.002) and chronological age (p < 0.002)

CVMS	Independent variable	Odds ratio	95% Confidence Interval
CVMS II	ALP*	0.683	(0.527, 0.885)
	Age**	1.698	(1.003, 2.873)
CVMS III	ALP*	0.821	(0.659, 1.023)
	Age**	3.977	(1.985, 7.968)
CVMS IV	ALP*	0.758	(0.574, 1.000)
	Age**	4.477	(2.197, 9.126)
CVMS V	ALP*	0.569	(0.353, 0.917)
	Age**	6.085	(2.888, 12.821)

- The reference category was CVMS I

* ALP (mU/mg) x 10

** Age (years)

Table 16: Multinomial logistic regression predicting CVMS from protein concentration ($p = 0.072$) and chronological age ($p < 0.001$)

CVMS	Independent variable	Odds ratio	95% Confidence Interval
CVMS II	Protein conc.*	2.460	(0.532, 11.373)
	Age**	1.553	(0.992, 2.431)
CVMS III	Protein conc.*	10.072	(1.205, 84.176)
	Age**	3.637	(1.944, 6.805)
CVMS IV	Protein conc.*	1.221	(0.108, 13.777)
	Age**	4.122	(2.159, 7.869)
CVMS V	Protein conc.*	8.713	(0.579, 131.090)
	Age**	5.276	(2.700, 10.307)

- The reference category was CMVS I

* Protein conc. (mg/ml)

** Age (years)

Table 17: Multinomial logistic regression models with McFadden's pseudo R^2 and correct classification rate by model.

Models for multinomial logistic regression	McFadden's pseudo R^2	Correct classification rate
Model 1 **Independent variable: ALP activity**	0.069	35.4%
Model 2 **Independent variable: protein conc.**	0.046	32.9%
Model 3 **Independent variable: chronological age**	0.282	51.9%
Model 4 **Independent variables: ALP and age**	0.348	53.2%
Model 5 **Independent variables: protein level and age)**	0.317	49.4%

Appendix B: Figures

Figure 1: Alkaline phosphatase standard curve

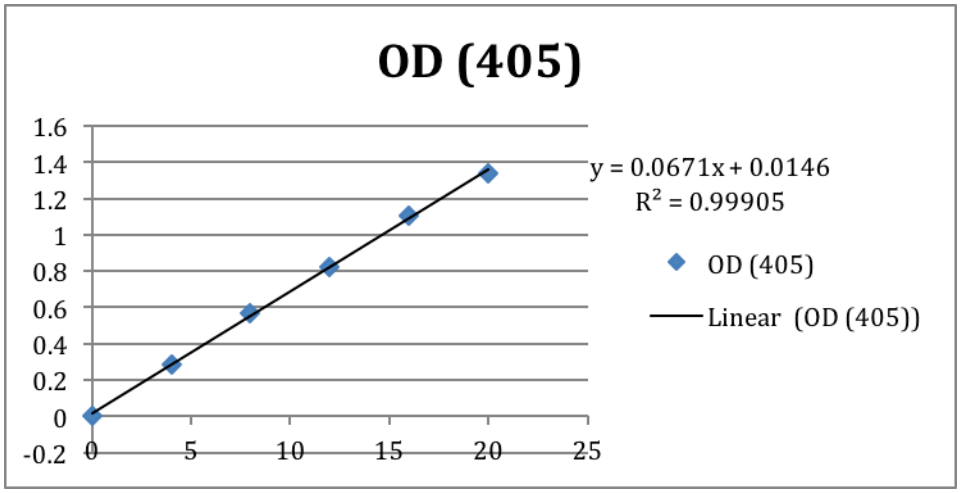


Figure 2: Alkaline phosphatase assay summary [9]

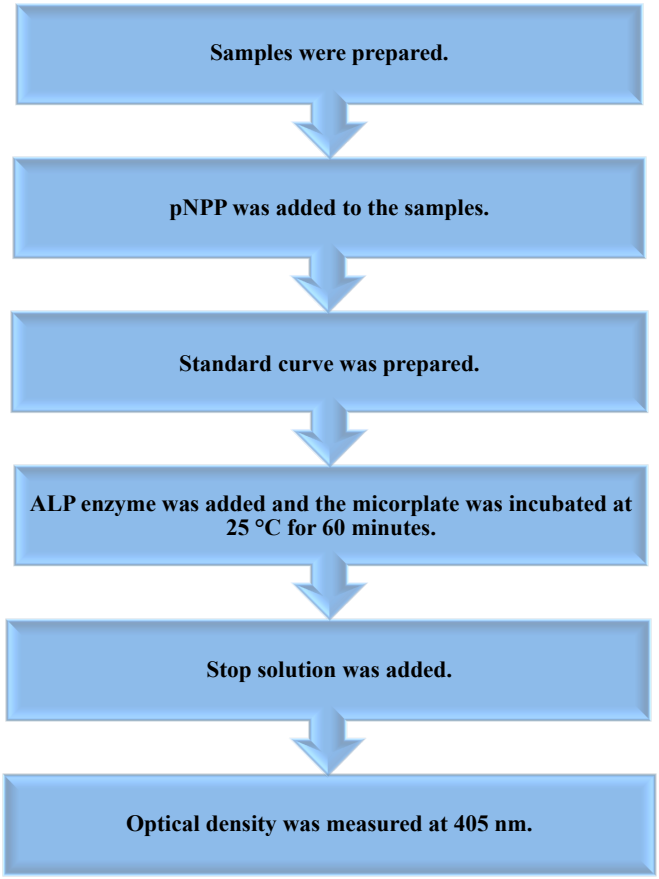


Figure 3: ELISA (Sandwich technique) principle [86]

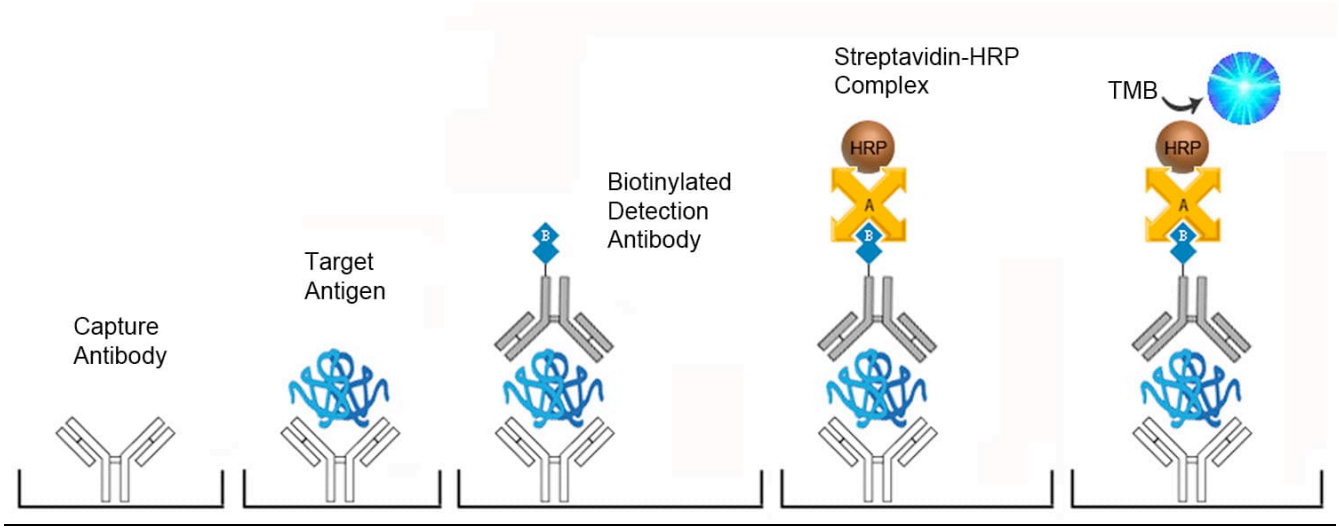


Figure 4: ELISA standard curve preparation [87]

Transfer volume

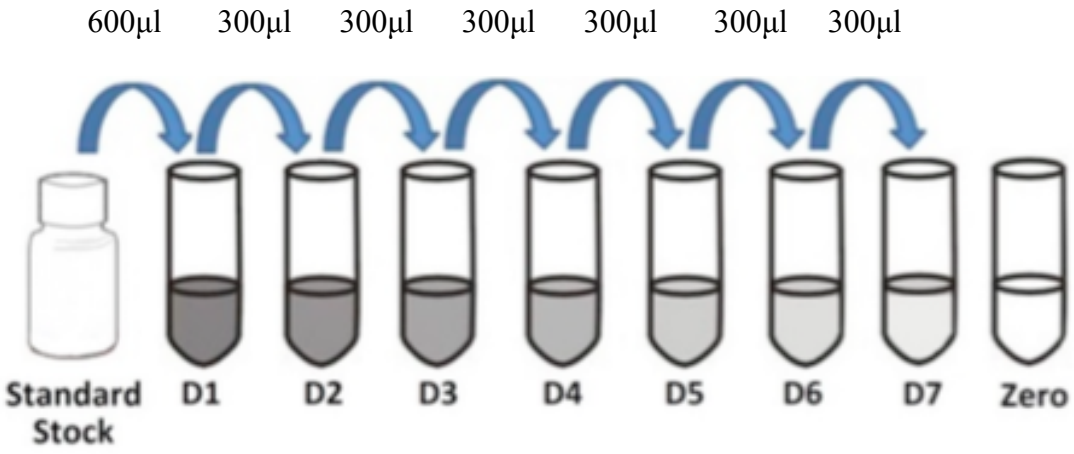


Figure 5: ELISA assay summary [87]

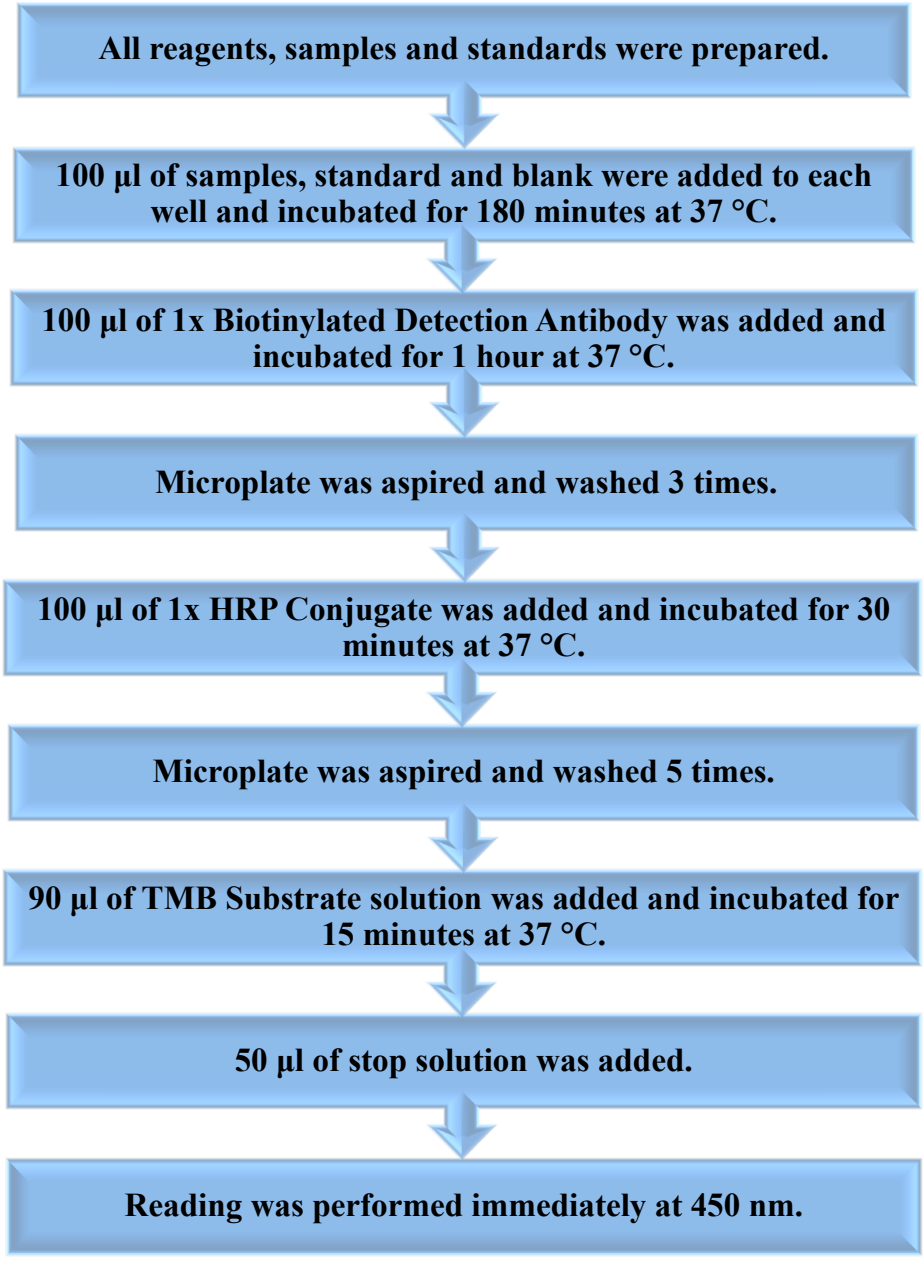


Figure 6: ELISA standard curve

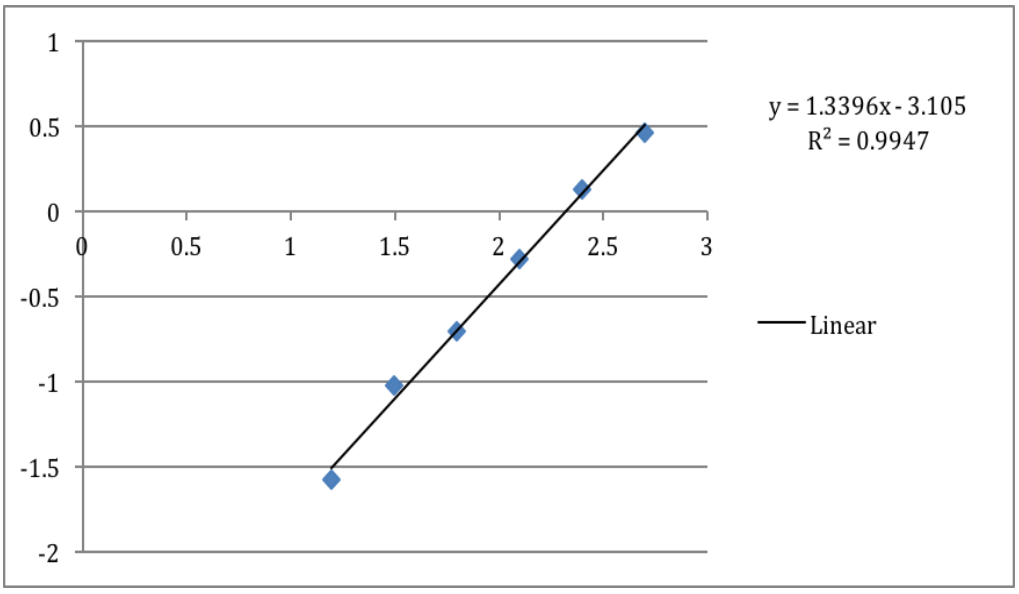


Figure 7: ELISA standard curve for the re-concentrated samples

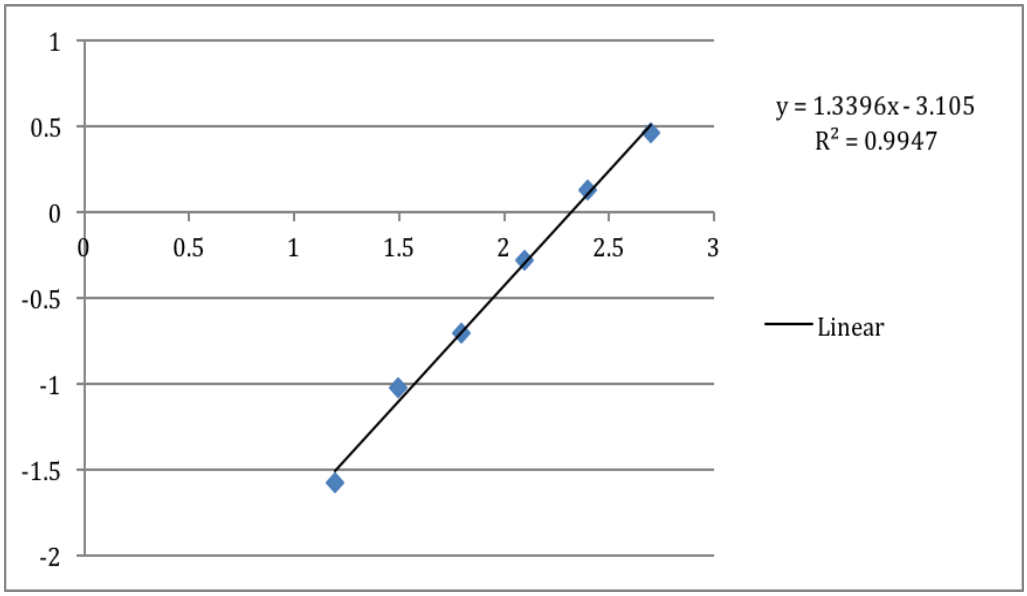
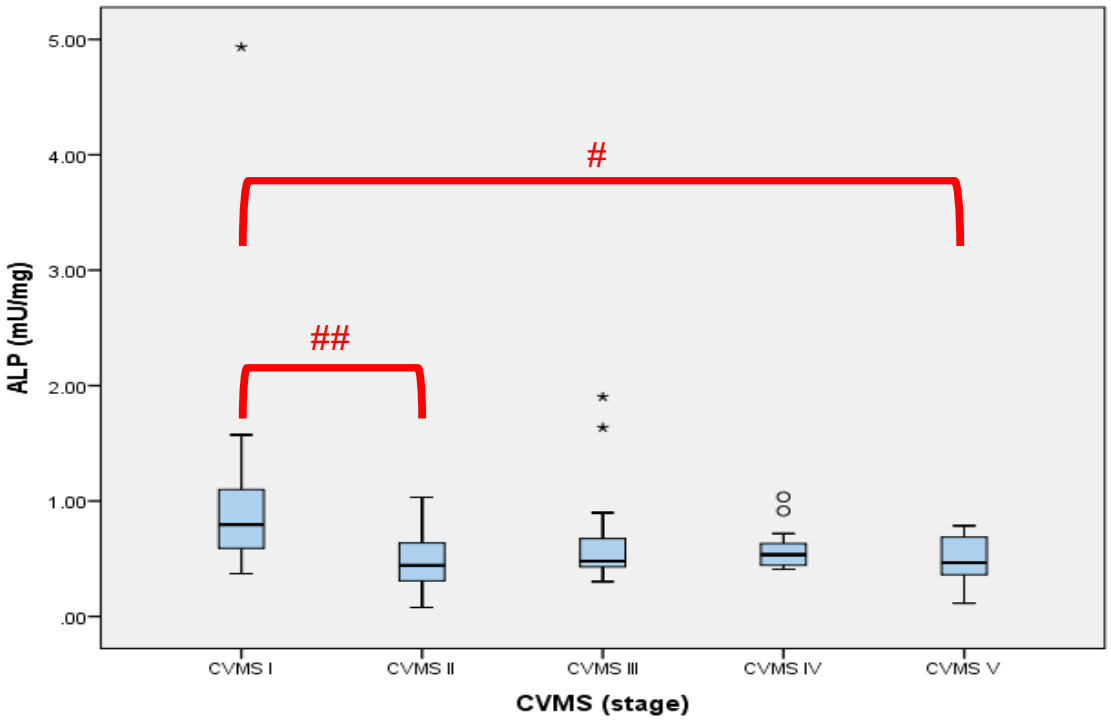


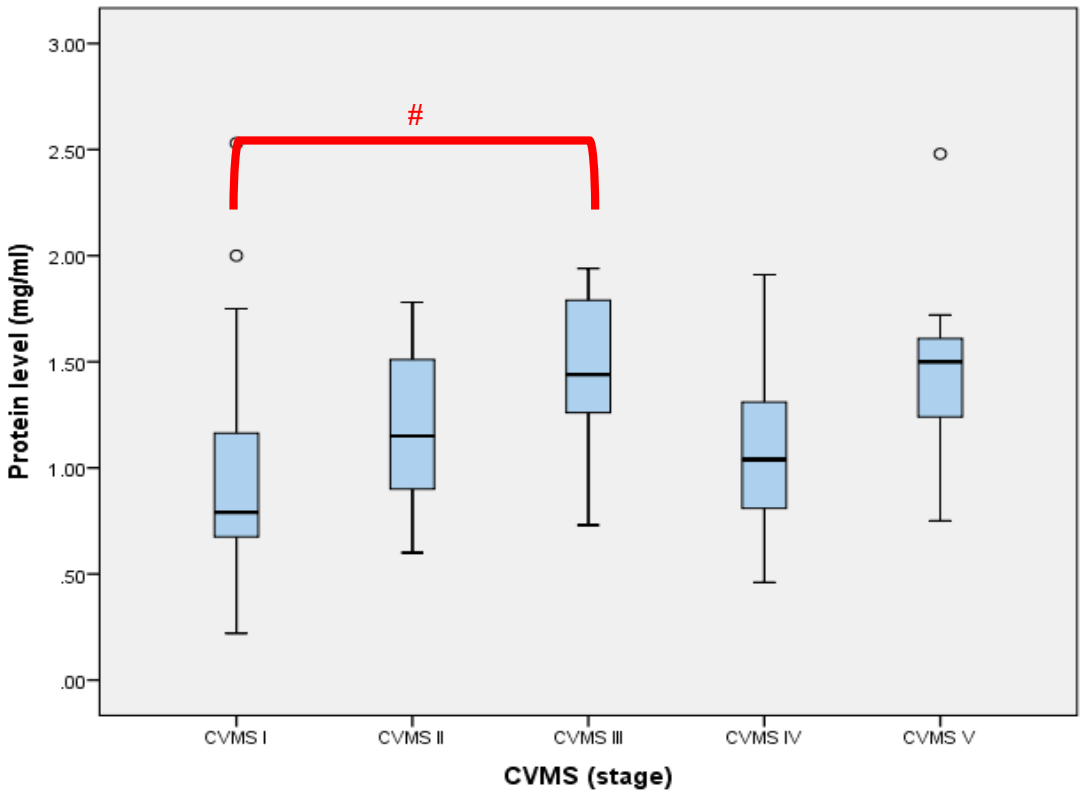
Figure 8: Distribution of ALP activity by CVMS



 # P = 0.004

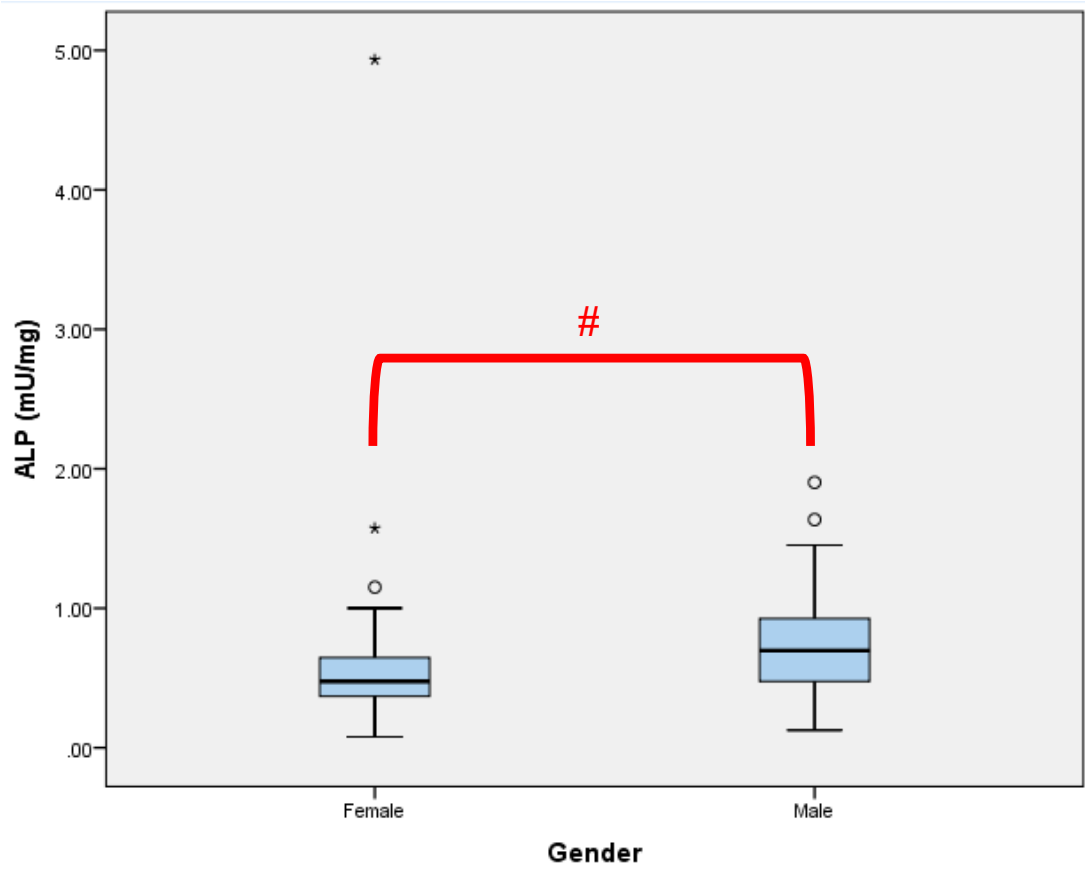
P < 0.001

Figure 9: Distribution of protein level by CVMS



 # P = 0.005

Figure 10: Impact of gender on ALP activity



 # P = 0.007

Figure 11: The association between chronological age and CVMS

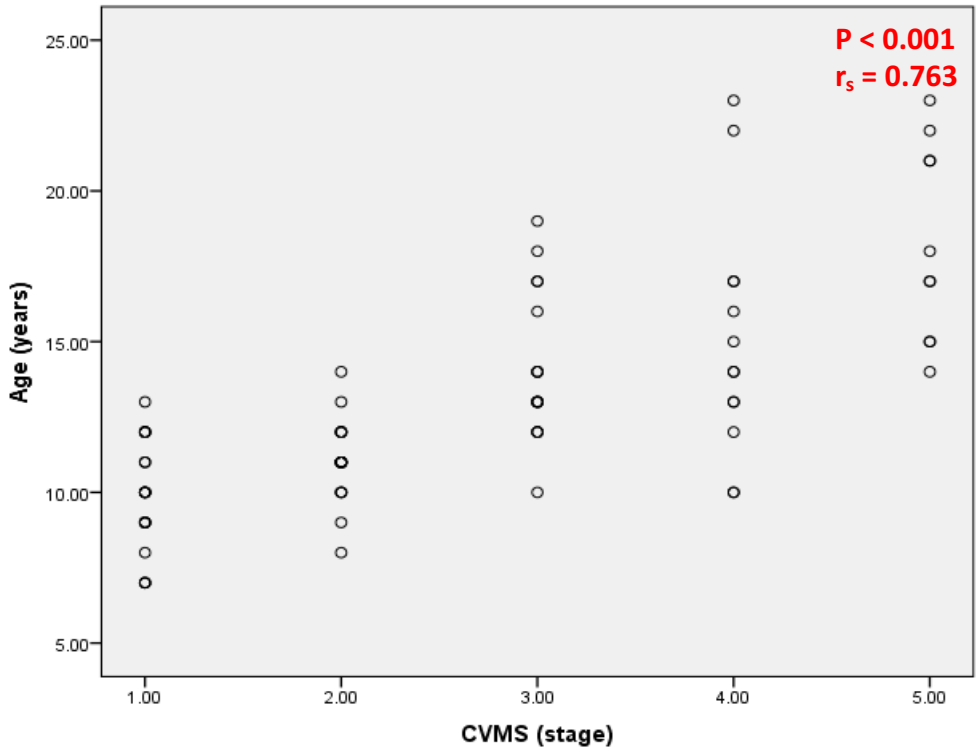


Figure 12: The association between chronological age and ALP activity