Article

The Role of Bone Alkaline Phosphatase and Osteocalcin in Saliva as Indicators of Skeletal Maturity in Children

Georgios Kouvelis, Sotiria Davidopoulou, Olga-Elpis Kolokitha, Moschos A. Papadopoulos and Athina Chatzigianni

Abstract: The aim of this study was to investigate the levels of bone alkaline phosphatase (BALP) and osteocalcin (OC) in the saliva of growing patients of different maturation levels. The sample consisted of 55 patients (34 females and 21 males of 7–16 years old). Two milliliters of saliva were collected and BALP and OC levels were assessed. Skeletal age was estimated using the cervical vertebral maturation method (CVM). The relationship between the biomarkers’ concentration in saliva and skeletal age was examined with the Spearman’s coefficient “ρ” (rho). Correlations between skeletal age groups and BALP and OC concentrations were assessed with the Kruskal–Wallis or the Mann–Whitney tests. No statistically significant differences in the levels of BALP (p = 0.568) and OC (p = 0.996) in saliva were identified according to the patient’s skeletal age. The use of BALP and OC levels in saliva seems to be dubious for skeletal growth assessment. However, slightly differentiated levels of those biomarkers, especially of BALP, through the different maturation stages, with higher concentrations at the pubertal phase, have been noticed. More studies are needed to clarify the exact potential role of these biomarkers as predictors of pubertal onset.

Keywords: saliva; bone alkaline phosphatase; osteocalcin; cervical vertebral maturation (CVM); growth phase

1. Introduction

A growth spurt is defined as an occurrence of growing quickly and suddenly in a short period of time and is directly connected with orthodontic treatment. Many methods of growth assessment have been established; however, skeletal age assessment was found to be more accurate with radiological methods, such as hand–wrist radiograph use, which was found to be a valid index for skeletal maturation and growth spurt assessment in children [1]. Another more recent radiological method of skeletal age assessment, which avoids the additional radiation of undertaking a hand–wrist radiograph, introduced by Hassel and Farman in 1995, is the Cervical Vertebrae Method (CVM) [2]. This is based on three vertebrae, while a further modified and refined version of the CVM method was proposed by Baccetti et al. in 2005, who used six consecutive annual cephalometric radiographs of patients, in order to observe the differences in vertebrae morphology during adolescence by examining the changes in the rate of increase in mandibular length instead of comparing with hand–wrist skeletal age [3].

Apart from the above methods, body fluid biomarkers have been investigated as potential molecules to predict the stage of maturation of a child. Among biofluids, oral biofluids, such as saliva, are a valuable source of biomarkers with many advantages, which may have the potential to predict stages of maturation [4]. Saliva contains a plethora of proteins, each with a different biological function. Although the protein content of saliva is only 30% of the blood content [5], saliva is being investigated as an abundant source of...
protein biomarkers [6]. In general, biomarkers can exist in various forms, such as DNA, RNA, metabolites, lipids, and proteins. Alterations in structure, function, or concentration can be linked with the onset of a disease or with the progression or regression of a disorder. Evaluating and understanding the importance of an individual’s biomarkers can be helpful in the definition of the presence, location, or even the potential of a disease.

Alkaline phosphatase is a membrane-bound glycoprotein [7], which has been found to be one of the primary biomarkers in the process of osteogenesis [8]. Bone alkaline phosphatase (BALP) is the bone-specific isoform of alkaline phosphatase and has been found to be a bone metabolism marker with high reliability [9]. BALP levels or activity have been linked with saliva, suggesting that salivary BALP could be considered a valuable diagnostic tool for growth spurt assessment, even though the results have been contradictory up until now [10,11].

Osteocalcin (OC) is a bone-specific protein synthesized by osteoblasts that can be found in dentin and bone [12] and plays a significant role in bone resorption and mineralization [13,14]. OC is a biomarker of late osteoblast differentiation [14]. The serum OC was found to increase in specific conditions, such as osteoporosis, which demands higher rhythms of bone turnover [15]. Moreover, OC levels have been linked with skeletal growth in puberty [16]. In saliva, OC levels have been examined as a biomarker of bone loss in periodontal studies [17], as well as in studies focusing on smoking [18]. OC levels have been assessed and compared with cervical vertebral maturation index stages by blood/serum examination [19].

To date, only a few studies with contradictory findings have investigated the levels of BALP in saliva [10,11,20–23], and the exact role of BALP is not well documented and needs to be addressed. Moreover, according to our knowledge, no published studies have investigated salivary OC levels in relation to skeletal growth. In light of the above, further research is required to detect if there is any relationship between BALP and OC levels in saliva and growth.

The aim of this study was to investigate the levels of BALP and OC in the saliva of growing patients and to find possible alterations in the levels of those biomarkers among different pubertal stages.

2. Materials and Methods

Following a power analysis for sample size calculation as described below, 55 patients (34 females (61.8%), 21 males (38.2%)), 7–16 years old, seeking orthodontic treatment at the Postgraduate Orthodontic Clinic of the Department of Orthodontics of the Faculty of Dentistry, School of Health Sciences, Aristotle University of Thessaloniki, Greece were included in this cross-sectional clinical study. The inclusion criteria included (1) mixed or permanent dentition with fully erupted upper permanent central incisors, (2) good general health without nutrition issues, (3) absence of use of anti-inflammatory or antibiotic drugs during the last month prior to the examination, (4) good oral hygiene and healthy periodontium with plaque index and bleeding index < 25%, and (5) absence of any kind of previous orthodontic treatment or orthodontic tooth movement. The exclusion criteria included (1) the presence of any chronic disease, hormonal disorder, or condition that may affect growth and (2) the intake of any medication that affects bone metabolism upon entering the study or in the past. Patients’ types of malocclusion or skeletal discrepancy were not taken into consideration during patient selection. The study protocol was approved by the Health Sciences Institutional Review Board (Ethical Committee of the Faculty of Dentistry, School of Health Sciences, Aristotle University of Thessaloniki, Greece) (#406/17.07.2019).

In addition, written informed consent was obtained from all participants included in the study and their legal guardians.

Patients enrolled in this study had professional supragingival scaling and polishing of their teeth 2 weeks before participating in the study. At that time, oral hygiene instructions and instructions to refrain from eating or drinking two hours prior to saliva collection were given to them.
Lateral cephalometric radiographs were available in the context of collecting all the initial diagnostic data prior to the orthodontic treatment and were used in order to examine the skeletal age of each patient by applying the CVM method on the cervical vertebrae. The lateral cephalometric radiographs were obtained with the same Panoramic X-ray Unit (Planmeca Proline XC, Planmeca OY 00880, Helsinki, Finland) and identical parameters were used (68 kV, 5 mA).

2.1. Clinical Procedure

A sample of unstimulated whole saliva was obtained by drooling into a sterile urine box from each child and immediately kept at \(-80\, ^\circ\mathrm{C}\) till analysis. A minimum of 2 mL of saliva was required from every patient. Sampling was performed at the same time of the day to avoid a possible diurnal variation in protein concentration, as observed with other saliva constituents [24]. All procedures were performed between 09.00 a.m. and 12.00 p.m. and were performed by one investigator.

2.2. Biochemical Analysis of Salivary Samples

The saliva samples were thawed and cleared by centrifugation at \(9700 \times g\) for 10 min. The concentration of BALP and OC in saliva was determined by enzyme-linked immunosorbent assays (ELISA), using commercially available analysis kits specific for BALP (Human BALP, Fine Biotech, Wuhan, Hubei, China) and OC (Gla-Type Osteocalcin, EIA Kit, Takara Bio, Kusatsu, Shiga, Japan), according to the manufacturer’s instructions. Each sample was analyzed in duplicate, and the mean value of the measurements was calculated.

2.3. Growth Classification

Determination of skeletal maturity was performed by the method of measuring the cervical vertebrae (Cervical Vertebral Maturation Method—CVM) of Baccetti et al. [3]. The stages CS1 to CS6 were applied to each cephalometric radiograph. The first author (GK), who was blinded to the chronological age and biochemical outcomes, assessed the CVM stages of all patients and repeated all evaluations two weeks after the first examination. The second author (AC), also blinded to the above-mentioned parameters, assessed the CVM stages, and in cases of disagreement, the examiners met and reached a consensus for staging the specific patient.

2.4. Subgroups

Patients were primarily divided into 6 subgroups according to their skeletal age (CS1–CS6) (Group 1). Moreover, further analysis was implemented and the study population was also divided into 3 subgroups according to the developmental phase: pre-adolescent (CS1 and CS2), adolescent (CS3 and CS4), and post-adolescent (CS5 and CS6) (Group 2). In order to examine whether a difference between premature and postmature patients existed, subjects were also divided into 2 subgroups, with one consisting of patients at pre-mature stages (CS1, CS2, and CS3) and the other consisting of patients at later maturation stages (CS4, CS5, and CS6) (Group 3). Moreover, separate analyses and gender stratification were implemented in order to observe whether there were any differences between growing boys and girls.

2.5. Statistical Analysis

The study aimed to correlate the skeletal age and the BALP and OC concentrations in saliva. Assuming a moderate association (of about 0.40) between the skeletal age and the two biomarkers’ concentrations, a minimum sample of 46 patients has enough power of 0.80 in order for the corresponding two-tailed t-test of significance to highlight the association as significant at the significance level \(a = 0.05\).

An a priori power analysis was conducted with the G*Power v.3.1.0 software [23]. Fifty-five patients were recruited in the current study to increase the sample size in case of bad quality radiographic data, saliva sample problems, or patients’ dropouts [25].
Intra-examiner and inter-examiner agreement of skeletal age stages were tested by computing and assessing the corresponding values of the Cohen’s kappa agreement coefficient. Data were summarized by estimating indices of central tendency (mean and median values) and measures of variability (minimum and maximum values, standard deviations).

The correlation between skeletal age and biomarker concentration in saliva was examined with the Spearman’s rank correlation coefficient (“ρ” rho). In addition, in order to get more insights into the nature of the possible correlation, the skeletal age groups were compared relative to their central tendency of the BALP and OC biomarker concentration. For the comparison of the three subgroups of skeletal age (groups 1, 2, and 3), non-parametric tests were used. The Kruskal–Wallis test was used in the case of comparing more than three groups and the Mann–Whitney test in the case of comparing two groups. Non-parametric statistical methods were preferred since the normality of biomarker (BALP and OC) concentration in saliva could not be confirmed or presumed. Moreover, the respective histograms of the parameters’ value distributions were visually inspected, and then the fit of the parameters’ values to the normal distribution was tested through the Kolmogorov–Smirnov test. Either way, the data did not appear to fit the normal distribution and, therefore, it was safer to use non-parametric statistical methods. In all hypothesis testing procedures, the observed significance level (p-value) was computed with the Monte-Carlo simulation method, utilizing 10,000 resampling circles [26]. This method leads to safe inferential conclusions even in cases where the methodological assumptions of the non-parametric tests are not fulfilled (e.g., random samples, independent measurements, symmetrical, distributions, large samples, and absence of “heavy” outliers). The significance level for all statistical tests was preset at α = 0.05 (p ≤ 0.05). Data were analyzed with the statistical software IBM SPSS Statistics (Version 27) enhanced with the module Exact Tests (for the implementation of the Monte-Carlo simulation method).

3. Results

Descriptive statistics of the sample’s skeletal age are shown in Table 1. The overall intra-observer agreement between the two measurements of the CVM method was very high (Cohen’s kappa = 0.912, p < 0.001) and the inter-examiner agreement was also very high (Cohen’s kappa = 0.864, p < 0.001).

<table>
<thead>
<tr>
<th>CS Stages</th>
<th>n</th>
<th>Age (Years) Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>9.78 (1.34)</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>10.13 (1.55)</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>11.07 (1.14)</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>13.02 (1.49)</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>13.76 (1.64)</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>14.35 (0.05)</td>
</tr>
</tbody>
</table>

CS: Cervical stage; SD: Standard deviation.

In group 1, no statistically significant association was found between the six skeletal age subgroups and the BALP (rho = 0.060, p = 0.690) and OC concentrations (rho = −0.049, p = 0.746).

Table 2 shows the mean levels of BALP and OC in the saliva among the six subgroups and between the three pubertal phases, respectively. The Kruskal–Wallis test showed no statistically significant differences between the mean values of the six skeletal age subgroups in the levels of BALP (p = 0.568) and OC (p = 0.996) in saliva. However, the highest concentration values of BALP were observed in CS3 (27.7 ng/mL), which corresponds to the growth peak, followed by CS4 (15.66 ng/mL). The lowest values of BALP were observed in stages CS6 and CS2, with 12.28 and 9.98 ng/mL, respectively. OC levels reached their peak in CS2 (1.70 ng/mL). The lowest values were observed in CS1 (1.36 ng/mL) and CS5 (1.31 ng/mL).
Table 2. BALP (ng/mL) and OC (ng/mL) in saliva among maturation stages (CS).

<table>
<thead>
<tr>
<th>CS Stages</th>
<th>n</th>
<th>Mean (SD)</th>
<th>Median (Min, Max)</th>
<th>p-Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>BALP (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>13.98 (10.7)</td>
<td>14.40 (1.04, 33.58)</td>
<td>0.568</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>9.98 (7.79)</td>
<td>7.32 (0.54, 24.26)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>27.7 (31.85)</td>
<td>17.98 (7.92, 98.44)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>15.66 (12.95)</td>
<td>14.40 (0.60, 47.08)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>12.84 (5.93)</td>
<td>11.31 (6.54, 23.12)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>12.28</td>
<td>12.28 (12.28, 12.28)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>15.13 (14.97)</td>
<td>12.28 (0.54, 98.44)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OC (ng/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>1.36 (1.30)</td>
<td>0.91 (0.12, 3.84)</td>
<td>0.996</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1.70 (1.77)</td>
<td>1.25 (0.14, 5.64)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>1.55 (1.69)</td>
<td>1.10 (0.06, 5.20)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>1.39 (1.58)</td>
<td>0.56 (0.04, 5.68)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>1.31 (1.52)</td>
<td>0.57 (0.14, 4.16)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>1.57 (1.96)</td>
<td>1.57 (0.18, 2.96)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>1.46 (1.51)</td>
<td>0.66 (0.04, 5.68)</td>
<td></td>
</tr>
</tbody>
</table>

* Kruskal–Wallis test; CS: Cervical stage; SD: Standard deviation; BALP: Bone alkaline phosphatase; OC: Osteocalcin.

In Group 2, where three maturation subgroups were created for further analysis, as shown in Tables 3 and 4, BALP values showed higher values in the pubertal stage (CS3–CS4) with mean values of 19.87 ng/mL, followed by the post-pubertal stage (CS5–CS6) with 12.79 ng/mL and the pre-pubertal stage (CS1–CS2) with 11.98 ng/mL. However, these differences were not statistically significant (p = 0.349). OC levels, on the other hand, showed higher values in the pre-pubertal stage with a mean value of 1.53 ng/mL, followed by the pubertal stage (1.45 ng/mL) and the post-pubertal stage (1.35 ng/mL). These differences, as shown in Table 5, were also not statistically significant (p = 0.878).

Table 3. Level of BALP (ng/mL) in saliva according to the pubertal growth phase.

<table>
<thead>
<tr>
<th>Pubertal Stages</th>
<th>n</th>
<th>Mean (SD)</th>
<th>Median (Min, Max)</th>
<th>p-Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-pubertal (CS1–CS2)</td>
<td>22</td>
<td>11.98 (9.37)</td>
<td>10.08 (0.54, 33.58)</td>
<td>0.349</td>
</tr>
<tr>
<td>Pubertal (CS3–CS4)</td>
<td>20</td>
<td>19.87 (21.47)</td>
<td>15.17 (0.60, 98.44)</td>
<td></td>
</tr>
<tr>
<td>Post-pubertal (CS5–CS6)</td>
<td>11</td>
<td>12.79 (5.63)</td>
<td>12.28 (6.54, 23.12)</td>
<td></td>
</tr>
</tbody>
</table>

* Kruskal–Wallis test; CS: Cervical stage; SD: Standard deviation; BALP: Bone alkaline phosphatase.

Table 4. Level of OC (ng/mL) in saliva according to the pubertal growth phase.

<table>
<thead>
<tr>
<th>Pubertal Stages</th>
<th>n</th>
<th>Mean (SD)</th>
<th>Median (Min, Max)</th>
<th>p-Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-pubertal (CS1–CS2)</td>
<td>20</td>
<td>1.53 (1.52)</td>
<td>0.91 (0.12, 5.64)</td>
<td>0.878</td>
</tr>
<tr>
<td>Pubertal (CS3–CS4)</td>
<td>21</td>
<td>1.45 (1.58)</td>
<td>0.56 (0.04, 5.68)</td>
<td></td>
</tr>
<tr>
<td>Post-pubertal (CS5–CS6)</td>
<td>12</td>
<td>1.35 (1.50)</td>
<td>0.57 (0.14, 4.16)</td>
<td></td>
</tr>
</tbody>
</table>

* Kruskal–Wallis test; CS: Cervical stage; SD: Standard deviation; OC: Osteocalcin.

Table 5. Level of BALP (ng/mL) in saliva according to the maturation stage.

<table>
<thead>
<tr>
<th>Pubertal Stages</th>
<th>n</th>
<th>Mean (SD)</th>
<th>Median (Min, Max)</th>
<th>p-Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-mature (CS1–CS2–CS3)</td>
<td>29</td>
<td>15.78 (18.17)</td>
<td>12.28 (0.54, 98.44)</td>
<td>0.849</td>
</tr>
<tr>
<td>Late mature (CS4–CS5–CS6)</td>
<td>24</td>
<td>14.34 (10.17)</td>
<td>12.36 (0.60, 47.08)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>15.13 (14.97)</td>
<td>12.28 (0.54, 98.44)</td>
<td></td>
</tr>
</tbody>
</table>

* Mann–Whitney test; CS: Cervical stage; SD: Standard deviation; BALP: Bone alkaline phosphatase.
In addition, the above comparisons were performed separately for boys and girls. However, as expected, no statistically significant differences were found between the three skeletal age subgroups relative to the biomarker concentrations \((p\)-values ranged from 0.316 to 0.775). Regarding boys, pubertal boys had a higher BALP concentration (28.57 ng/mL) than pre-pubertal boys (12.52 ng/mL); nevertheless, this difference was not statistically significant \((p = 0.316)\). OC values were also higher in pubertal boys (1.47 ng/mL) compared to pre-pubertal boys (1.08 ng/mL) but this difference was also not statistically significant \((p = 0.775)\). There were no boys at the post-pubertal stage in this research.

In girls, BALP concentration showed higher values at the pubertal stage (16.95 ng/mL), followed by the post-pubertal and pre-pubertal stages, but these differences were not statistically significant \((p = 0.448)\).

In Group 3, where the sample was categorized into only two maturation subgroups, the Mann–Whitney test showed that there were again no statistically significant differences concerning the concentration of these biomarkers in saliva. BALP values were almost on the same levels, as shown in Table 5. In the premature group, the mean value was 15.78 ng/mL, while in the late mature group, it was 14.34 ng/mL \((p = 0.849)\). OC values showed a similar pattern (Table 6). In the premature group, the mean OC level was 1.54 ng/mL and in the late mature group, it was 1.37 ng/mL. These differences were also not statistically significant \((p = 0.562)\). Separate statistical analyses were also implemented for boys and girls but as expected, no statistically significant results were found. Regarding BALP in boys, higher values were observed in the premature subgroup with 18.1 ng/mL compared to the late mature subgroup with 10.47 ng/mL \((p = 0.598)\). This outcome was expected since premature subgroup OC in boys showed a similar pattern, with higher values in the premature subgroup with 1.32 ng/mL \((p = 0.171)\). It is worth mentioning that in the late mature subgroup, there were only two boys since, as previously mentioned, no boys were categorized in skeletal subgroups CS5 and CS6. In skeletal maturation subgroup 3 (CS3), higher BALP values were primarily observed. In girls, BALP values were almost at the same levels in the premature (11.98 ng/mL) and late mature subgroups (14.70 ng/mL). This difference was not statistically significant \((p = 0.418)\). On the other hand, OC levels were higher in the premature subgroup (1.92 ng/mL) than in the late mature subgroup (1.47 ng/mL) \((p = 0.453)\).

### Table 6. Level of OC (ng/mL) in saliva according to maturation stage.

<table>
<thead>
<tr>
<th>Pubertal Stages</th>
<th>n</th>
<th>Mean (SD)</th>
<th>Median (Min, Max)</th>
<th>(p)-Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-mature (CS1–CS2–CS3)</td>
<td>28</td>
<td>1.54 (1.54)</td>
<td>0.91 (0.06, 5.64)</td>
<td>0.562</td>
</tr>
<tr>
<td>Late mature (CS4–CS5–CS6)</td>
<td>25</td>
<td>1.37 (1.51)</td>
<td>0.56 (0.04, 5.68)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>1.46 (1.51)</td>
<td>0.66 (0.04, 5.68)</td>
<td></td>
</tr>
</tbody>
</table>

\* Mann–Whitney test; CS: Cervical stage; SD: Standard deviation; OC: Osteocalcin.

In addition, the above comparisons were performed separately for boys and girls. However, as expected, no statistically significant differences were found between the three skeletal age groups relative to the biomarker concentrations between genders \((p\)-values ranged from 0.316 to 0.775). The same results were found also in the case of the two skeletal age groups \((p\)-values ranged from 0.171 to 0.598).

### 4. Discussion

The results of the present study show that there are no statistically significant differences in salivary BALP and OC levels among pre-pubertal, pubertal, and post-pubertal patients. The lack of statistical significance leads to the conclusion that the two biomarkers under investigation cannot be clinically used for the skeletal age estimation of young individuals at the moment.

However, despite the lack of statistical significance concerning BALP, peak levels seem to coincide with growth spurts (CS3), while reduced levels are seen in the previous and
following maturational stages. OC levels seem to follow a similar pattern to BALP, with higher values in CS2 and CS3 and lower in CS4 and CS5.

It is worth mentioning that BALP levels in the saliva have not been investigated thoroughly until now. Regarding the levels of salivary BALP, our findings agree with those of Wijaya et al. (2017) [20], who analyzed data from 136 growing patients and found no statistically significant differences among growing patients and BALP levels, with the use of the CVM method for skeletal age assessment of the sample. On the other hand, Irham et al. (2017) [21] found statistically significant differences among patients, with peak levels of ALP in the saliva, which coincided with the growth spurt as depicted in the CVM. Also, Tarvade et al. [10] and Hegde et al. [22] found a statistically significant correlation among patients between ALP and BALP levels in saliva and the growth spurt, respectively, by using hand–wrist radiographs for skeletal age estimation instead of the CVM. Alhazmi et al. (2019) [23], according to their conclusions, found that ALP activity in the saliva of 79 patients was correlated with the growth spurt. It is worth mentioning that inter-examiner reliability concerning the CVM method and categorization of the subjects according to vertebrae shape was poor in this study.

Nevertheless, in a recent systematic review by Khade et al. (2023), which aimed to assess the reliability of various salivary biomarkers as skeletal maturity indicators, the evidence suggested that up until now, salivary ALP could not be used as a diagnostic tool for skeletal maturation assessment, but only as an adjunct to other reliable methods, which is in accordance with the results of this study. The systematic review concluded that there is still a need for further research with longitudinal studies in this field [11].

Osteocalcin is a biomarker that has not been explored in saliva in relation to the pubertal growth phases before and evidence of a possible correlation between salivary OC and skeletal maturation is not available in the literature. Up until now, only one study has examined the potential role of osteocalcin as a growth biomarker in the blood serum of 150 patients and found that serum peak values were met in maturation stages CS5 in males and CS3 in females, but with insignificant differences between stages. The findings of the present research found insignificant differences in osteocalcin levels in the saliva among growing patients and agree with the abovementioned results in the serum [19].

The collection of saliva is a noninvasive method [27] and it can be easily collected and stored [4]. Drooling saliva passively was the chosen method for this study, since passive drooling is the ‘gold standard’ of saliva collection for obtaining many saliva components [28]. Moreover, the procedure took place between 09.00 and 12.00 a.m., since the amount of saliva secretion has been shown to change during the day [29]. As previously mentioned, the passive drooling saliva method obtains many advantages for biomarker assessment, especially compared to blood or serum collection, mostly regarding invasiveness. However, there is no scientific evidence robust enough to support the substitution of one collection method for another [30]. In a recent study where authors examined the potential correlation of the biomarker glutathione in saliva and blood, they found that there was no strong correlation between whole blood and salivary glutathione contents in healthy people [31], thus there is room for research in this field to determine if salivary and blood results obtain the same accuracy in biomarker detection.

Enzyme-linked immunosorbent assay (ELISA) is a labeled immunoassay that is considered the gold standard of immunoassays for the detection and quantification of substances such as antibodies, hormones, antigens, proteins, and glycoproteins. In this study, the ELISA method was used for the quantitative analysis of BALP and OC in saliva, since this method has high sensitivity, specificity, and efficiency, and the procedure is simple and cost-effective [32]. Concerning the method of Baccetti et al. for skeletal growth assessment, intra-examiner as well as inter-examiner reliability was high. It has been shown that this method has poor reproducibility [33], but in the present study, this was not confirmed. Regarding the validation of the CVM method for predicting skeletal maturation age, Santiago et al. (2012) implemented a systematic review to determine if the cervical vertebrae maturation method can determine peak pubertal growth reliably [34]. Six studies were of
sufficient quality to be included in the analysis. These studies found that the correlation between the CVM method and the hand–wrist method was statistically significant.

Cericato et al. (2014) [35] carried out a systematic review and meta-analysis on the validity of skeletal maturation assessment by analyzing cervical vertebrae in order to assess whether the technique can replace the use of hand–wrist radiographs to determine peak pubertal growth. This systematic review included nineteen articles that compared radiographs of the hand–wrist and cervical vertebral regions. Positive correlations were observed in all articles and the meta-analysis found a higher correlation in females. The authors concluded that cervical vertebrae maturation indexes show good reliability and can replace hand–wrist radiographs in determining peak pubertal growth. It can be concluded that orthodontic literature has sufficient documentation to prove both methods are almost similar in estimating growth, so the CVM method is a valid method for skeletal growth assessment.

The capacity of the ELISA kits to detect protein concentration in a wider range could be regarded as a limitation of the current study. In two patients, the values of BALP and OC were below the detectable range, and they could not be measured. Thus, they were excluded from statistical analyses. Other limitations of the current investigation include the small sample size due to the coronavirus pandemic, as well as the cross-sectional nature of the study. The strengths of the study are the protocol design and the examination of a new biomarker in saliva, which has not been investigated in this biofluid before.

In the future, a longitudinal sample to register the variations of the salivary biomarkers over time within the same subjects is needed. Furthermore, another maturation index, such as actual growth by measuring height/weight over time and/or hand–wrist films, is warranted, even though this may not be justified in the early pre-pubertal or post-pubertal phase due to the radiation.

In the present study, BALP peak salivary levels seem to correlate with the growth spurt, since higher values were observed in CS3, which were gradually reduced in the following stages. Likewise, OC salivary levels seem to follow a similar pattern with BALP, with higher values observed in CS2 and CS3 and lower values in CS4 and CS5.

To summarize, BALP has not been a widely acceptable method of pubertal estimation according to the literature, while OC has not been investigated in oral biofluids before. The results of this study are not positive about the use of these biomarkers for growth assessment in children and come in agreement with the updated evidence-based literature. It is affirmed that there is a high necessity for basic research in the field of biomarker discovery that may be used as a diagnostic tool in adolescent growth.

5. Conclusions

According to the results of the current investigation, the examined salivary biomarkers seem to be dubious for skeletal growth assessment:

- Bone alkaline phosphatase showed no statistically significant difference between pre-pubertal, pubertal, and post-pubertal patients.
- Osteocalcin levels showed no statistically significant differentiation among the pubertal growth phases.
- Slightly differentiated levels of those biomarkers through the different maturation stages, with higher concentrations at the pubertal phase, have been noticed.

More studies are needed to clarify the exact potential role of these biomarkers or novel ones as predictors of pubertal onset.

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