Mediators of Inflammation

Inflammatory Biomarkers in Saliva

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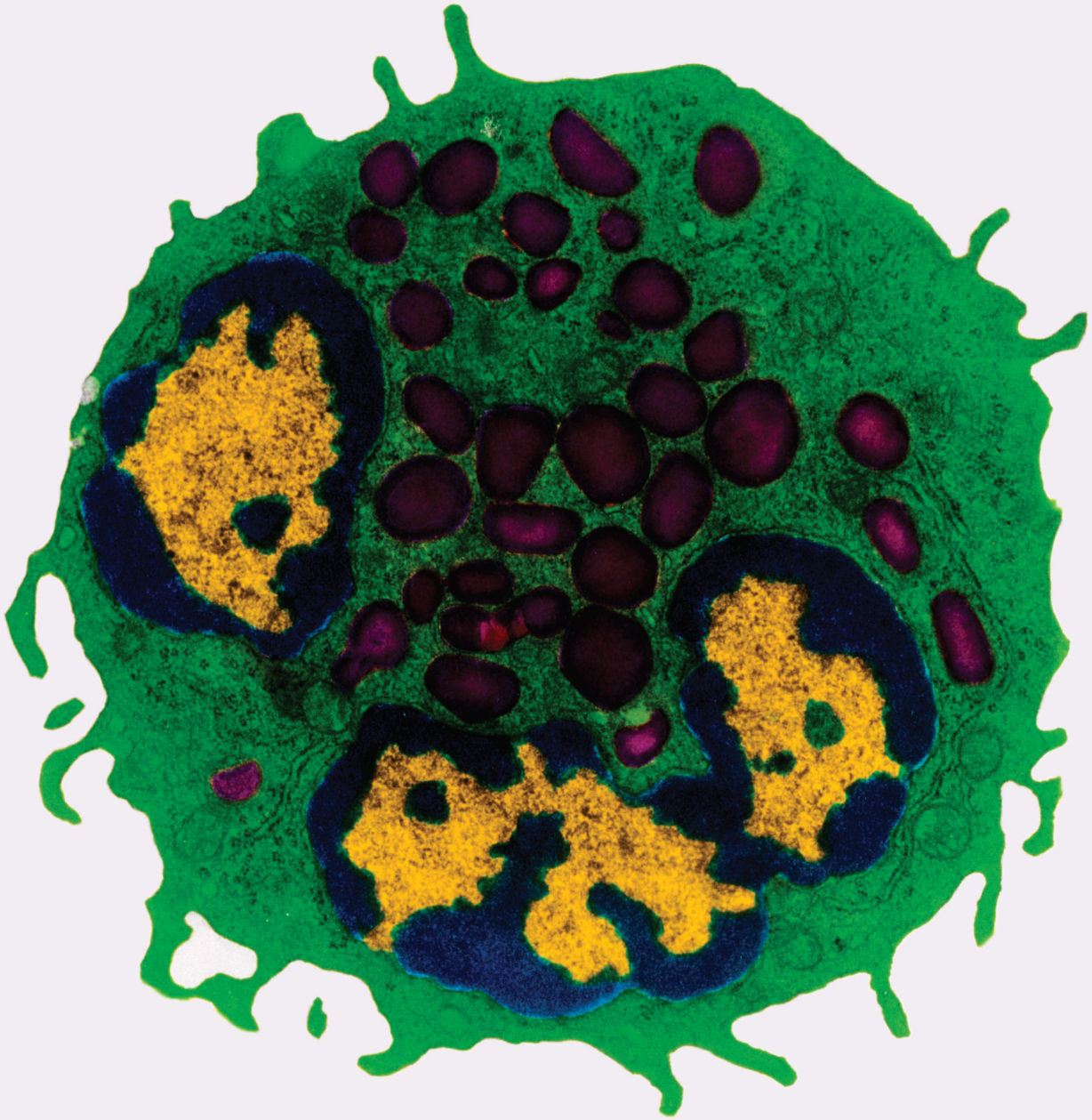

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Research Article

Metabolomic Evaluation of Chronic Periodontal Disease in Older Adults

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Periodontal disease is an infectious inflammatory disease related to the destruction of supporting tissues of the teeth, leading to a functional loss of the teeth. Inflammatory molecules present in the exudate are catalyzed and form different metabolites that can be identified and quantified. Thus, we evaluated the inflammatory exudate present in crevicular fluid to identify metabolic biological markers for diagnosing chronic periodontal disease in older adults. Research participants were selected from long-term institutions in Brazil. Participants were individuals aged 65 years or older, healthy, or with chronic periodontal disease. Gas chromatography/mass spectrometry was used to evaluate potential biomarkers in 120 crevicular fluid samples. We identified 969 metabolites in the individuals. Of these, 15 metabolites showed a variable importance with projection score > 1 and were associated with periodontal disease. Further analysis showed that among the 15 metabolites, two (5-aminovaleric acid and serine, 3TMS derivative) were found at higher concentrations in the crevicular fluid, indicating their potential diagnostic power for periodontal disease in older adults. Our findings indicated that some metabolites are present at high concentrations in the crevicular fluid in older adults with periodontal disease and can be used as biomarkers of periodontal disease.

1. Introduction

Molecules present in fluids in the oral cavity may indicate a relationship between processes linked to health and disease, as well as repair processes. Inflammatory proteins play different and important roles in oral cavity homeostasis including periodontal disease. Periodontitis is an infectious inflammatory disease that causes destruction of the tissues supporting the teeth. It significantly affects oral health and is the most common cause of tooth loss. Additionally,

preclinical and clinical studies have demonstrated clear associations between periodontitis and various other conditions as well as systemic diseases.[1–6].

The relationship between the microbiota and immune system is critical to the maintenance of periodontal health; therefore, certain groups, such as older adults, are more susceptible to the development of chronic periodontal disease [1]. Events related to biological senescence predispose older adults to infections and conditions that increase morbidity and mortality [7]. This increased susceptibility in older adults is associated with a decrease in the normal functioning of the immune system. The responsiveness of the T lymphocyte population decreases with advancing age, resulting in reduced efficiency of monocytes and macrophages in destroying invading pathogens [8, 9], thereby allowing for the development of periodontal diseases.

Recent advances in metabolic studies have enabled the identification and quantification of different metabolites in normal individuals and those with certain diseases using metabolomics [10–12]. Metabolites are chemically diverse and can be classified into ionic species, alcohols, hydrophilic carbohydrates, volatile ketones, lipids, and organic acids [13]. In disease diagnosis, several efforts have been made to identify metabolites in the saliva of individuals with diseases that would provide new biological markers to aid the diagnosis of periodontal disease [14]. However, the metabolic profile during the senescence period of individuals who have periodontal disease has not been defined.

Given the heterogeneous characteristics of the diverse immune responses to infection, efforts have been made to identify markers in the fluids in the oral cavity, such as saliva and crevicular fluid, to predict the presence of periodontal disease and its stage [11, 15]. Because of differences in the immune systems of different age groups, including during the senescence period, our study is aimed at identifying metabolic biological markers for diagnosing chronic periodontal disease in older adults.

2. Materials and Methods

2.1. Inclusion and Exclusion Criteria. Individuals aged ≥65 years, nonsmokers, and with or without chronic periodontal disease were included in the study. Individuals diagnosed with diabetes mellitus (controlled or not), arterial hypertension, without at least three absorbent cones collected, edentate individuals, those undergoing antibiotic therapy, or who had recently been treated by odontology intervention were excluded from the study.

2.2. Collection of Biological Materials. Initially, a minimum effect size of 50% was considered between the samples obtained from individuals with and without periodontal disease (independent-samples t-test) for the sampling calculation. With a power of 80% (a priori power analysis), it was possible to estimate a sample number of 102 participants. The percentage of possible losses was considered 18% due to nonadherence or dropout, leading to a total of 120 research participants ($\alpha = 5\%$). Therefore, individuals in the study were grouped into those with chronic periodontal disease (periodontitis group; $n = 60$) and those without (healthy group; $n = 60$). The mean age of the study subjects was 70 years (65–80 years). Diseased individuals were enrolled following clinical evaluations and crevicular fluid sampling using absorbent cones at a probing depth of ≥5 mm.

Selection of study subjects: all the teeth in the mouth were probed. The periodontal disease outcome measures included clinical attachment loss and periodontal pocket depth. The periodontal pocket was defined as the measurement starting at 5 mm from the gingival margin to the bottom of the pocket. The gingival margin was measured from the cementoenamel junction to the gingival margin. The examiners measured probing depth and gingival margin at six sites per tooth for each fully erupted tooth, except the third molars, in each patient. Two skilled examiners were calibrated for periodontal assessments, so that the measurements were comparable. The clinical attachment levels were calculated, a periodontal diagnosis was provided, and the patients were classified into two groups after periodontitis assessment: healthy and periodontitis [16].

After identifying the participants, three cones were obtained from the different sites of each patient and transferred into tubes containing 500 μL of protease inhibitor (complete; Roche, Basel, Switzerland). Immediately after collection, the tubes were submerged in liquid nitrogen and stored at -80°C. The volume used in each analytical run was determined after quantifying total proteins by the Bradford method [17].

2.3. Ethical Statements. All procedures were approved by the research ethics committee of the Federal University of Triangulo Mineiro (number: 017430/2014), registered in Plataforma Brazil, and followed National Health Council Resolution 466/2012. All participants provided a formal written consent to participate in the study.

2.4. Sample Processing for Metabolomics. The crevicular fluid samples were kept on ice until they were completely thawed, and a maximum of 100 μL of fluid (depending on the volume of proteins in each sample) was added to a tube containing 300 μL of metabolite extraction buffer (ice cold) containing acetonitrile, isopropanol, and ultrapure water (3 : 3 : 2). The mixture was then centrifuged at 15,800 × g at 0°C for 15 min to precipitating the proteins, and 350 μL of the supernatant was transferred to a new tube. As an internal standard, 5 μL of myristic acid (#366889; Sigma-Aldrich, St. Louis, MO, USA) was added at a concentration of 3 mg/mL. The metabolites were then dried in a SpeedVac (Thermo Fisher, Waltham, MA, USA) for 18 h and stored in a desiccator at 4°C until analysis.

The samples were subjected to shunt processes. First, 3 μL of fatty acid methyl ester was added to control the retention time alignment during sample processing. Subsequently, 30 μL of a solution of 40 mg/mL methoxyamine (#226904; Sigma-Aldrich) diluted in pyridine (#270407; Sigma-Aldrich) was added, and the pellet containing metabolites was homogenized and incubated for 16 h at 25°C under agitation at 650 rpm. After methoxyamination, 90 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (#69479;

Sigma-Aldrich) with 1% trimethylchlorosilane (#89595; Sigma-Aldrich) was added, and the mixture was incubated for 90 min at 25°C under agitation at 650 rpm. The metabolites were centrifuged at 15,800 × g for 5 min at 23°C, and 100 μL of the supernatant was transferred to a 2 mL amber vial. The samples were analyzed within 24 h of shunting.

2.5. Metabolomic Analyses. Samples were analyzed in triplicate using a gas chromatography/mass spectrometry (GC/MS) system (7890B GC/5977A MS; Agilent, Santa Clara, CA, USA). One microliter of the derivative was injected into the GC operating in splitless mode. The DB-5 ms column with a 10 m DuraGuard capillary (122-5532G; Agilent), which allowed for helium gas to flow at a pressure of 1.1 mL/min, was used to separate the metabolites. The injector temperature was set to 250°C, and column temperature was set to 60°C for 1 min and then increased to 310°C at a rate of 10°C/min. The effluent from the column was automatically inserted into the MS. The detector was operated in electron impact ionization mode (70 eV), and the mass spectrum was recorded after a solvent delay of 6.5 min. The temperature was set to 180°C and 280°C for the quadrupole MS and ion source, respectively.

2.6. Quality Control. An internal quality control was performed for all analyses using the following parameters: clear definition of objectives, procedures, norms, and criteria for tolerance limits; corrective actions and recording of activities; and the use of controls to evaluate analytical imprecision [18].

2.7. Statistical Analyses. The G*Power version 3.1.7 program was used for sampling the estimates and power of inferences. Initial data filtering was performed using the Microsoft Excel program (Redmond, WA, USA). Statistical normalization and analyses were performed using the MetaboAnalyst program [19]. A fold change calculation and t-test were also performed.

For multivariate evaluations, partial least squares-discriminant analysis (PLS-DA) components were determined to discriminate the healthy and periodontitis groups (PLS is a supervised method that uses multivariate regression techniques to extract information for predicting the class of a member (Y) using a linear combination of original variables (X)). To evaluate the significance of class discrimination, a permutation test was performed. In each permutation, a PLS-DA model was constructed between the data (X) and permuted classes (Y) using the ideal number of components determined by the model crossvalidation based on the original class.

3. Results

3.1. Whole Metabolite Profiles. In total, 969 metabolites were identified. Of these, 64 metabolites were detected in at least two of the three replicates and in at least 50% of either group (healthy or periodontitis group); these were considered for further statistical analyses. The metabolite intensities were normalized by self-scaling (Figure S1).

3.2. Fold Change Analyses of 64 Commonly Shared Metabolites. Fold change analysis was performed based on the ratio of the mean metabolite intensities in the periodontitis and healthy groups. Those metabolites with an intensity at least 2-fold larger in one group as in the other were considered. We identified nine metabolites with a hazard ratio of >2. Five metabolites were higher in the periodontitis than in the healthy group, with a fold change of >2 (2,3-dihydroxypropyl icosanoate, glycerol, serine, 5-aminovaleric acid, and putrescine), and four were higher in the healthy than in the periodontitis group, with a fold change of <0.5 (lactulose, oxalic acid, 1-benzoyl-2-t-butyl-5-ethyl-3-methyl-5-vinyl-imidazolidin-4-one, and maltose) (Figure 2S and Table 1).

3.3. Statistical Significance Assessment. A t-test was performed after excluding contaminants, which identified 10 metabolites among the healthy and periodontitis groups (Figure S3 and Table 2). Among these ten metabolites, three were among the five metabolites that showed a fold change > 2 in periodontitis (serine, 5-aminovaleric acid, and putrescine), and two were among the four metabolites that showed a fold change < 0.5 (1-benzoyl-2-t-butyl-5-ethyl-3-methyl-5-vinyl-imidazolidin-4-one and maltose) (Tables 1 and 2).

3.4. PLS-DA of Periodontitis and Healthy Groups. According to PLS-DA based on two main components, the periodontitis and healthy individuals were separated based on their metabolic profiles (Figure S4). Furthermore, crossvalidation analysis supported the results and showed that this separation was not random (Figure S5). The metabolites were then ordered based on the variable importance in projection (VIP) index according to their importance in the group separation in PLS-DA (Figure 1 and Table 3).

Overall, 5-aminovaleric acid and serine, which presented a fold change > 2 in the periodontitis group compared to in the healthy group, showed the highest VIPs, whereas the best VIP for a metabolite with a fold change < 0.5 was for 1-monopalmitin.

4. Discussion

Periodontal disease is a chronic inflammatory disease that affects the fixation and support structures of teeth. Over the past few decades, great efforts have resulted in advances in the diagnosis and treatment of periodontal disease [20–22]. However, epidemiological studies have shown that this disease remains among the main causes of tooth loss in adults [23], and its progression and development are age-related [24].

Because of improvements in the quality of life, the length of the senescence period, a stage of life in which various biological changes are initiated including in the immune system, has increased. In this study, we identified 969 metabolites correlated with aging in older adults with or without periodontal disease. Of the 969 metabolites, 15 were found to be associated with the presence of periodontal disease, as indicated by a VIP score of >1. Further evaluations showed that two metabolites (5-aminovaleric acid and

Table 1: Metabolites with a greater than twofold change in the periodontitis relative to the healthy group.

Metabolite	FC	$\log_2$ (FC)	HMDB ID
2,3-Dihydroxypropyl icosanoate	20.22	43.381	HMDB11572
Glycerol	6.79	27.643	HMDB00131
Serine	3.93	19.737	HMDB00187
5-Aminovaleric acid	2.54	13.442	HMDB03355
Putrescine	2.17	11.153	HMDB01414
Lactulose	0.48	-10.499	HMDB00740
Oxalic acid	0.47	-10.828	HMDB02329
1-Benzoyl-2-t-butyl-5-ethyl-3-methyl-5-vinyl-imidazolidin-4-one	0.42	-12.655	N/A
Maltose	0.13	-29.432	HMDB00163

FC: fold change; HMDB, Human Metabolome Database; N/A: not available in HMDB.

Table 2: Metabolites showing significant differences with a p value <0.05 according to t-test.

ID	Metabolite	p value	HMDB ID
4	5-Aminovaleric acid	0.0008	HMDB03355
60	Serine	0.0024	HMDB00187
30	1-Monopalmitin	0.0137	HMDB31074
14	Aspartic acid	0.0144	HMDB00191
15	D-mannitol	0.0178	HMDB00765
59	Putrescine	0.0179	HMDB01414
28	1-Benzoyl-2-t-butyl-5-ethyl-3-methyl-5-vinyl-imidazolidin-4-one	0.0209	N/A
17	Palmitoleate	0.0230	HMDB03229
16	Maltose	0.0263	HMDB00163
50	Lactic acid	0.0292	HMDB00190

HMDB: Human Metabolome Database; N/A: not available in HMDB.

serine) were found at higher concentrations in the crevicular fluid, which may be useful for predicting the diagnosis of chronic periodontal disease in older adults.

In a recent study, Moeller et al. evaluated longevity in a population and showed that periodontal disease was a mortality factor among the evaluated individuals [25]. Other studies also demonstrated that periodontal disease is associated with several pathological conditions, such as diabetes, cardiovascular diseases, and arthritis [26–28]. Therefore, improved diagnostic methods and new biological markers are needed for periodontal disease; the application and interaction of the -omic approaches will allow us to broaden our perspectives on the molecular mechanisms involved in periodontal disease [29] and enable the optimization of bold diagnostic and prognostic models.

MS with chromatography is useful for predicting periodontal diseases in different progression stages [30]. Considering the high sensitivity of identification and quantification of metabolites in our study, our results support the findings of the previous study.

This is the first study to identify molecules that may predict periodontal disease in older adults. A previous evaluation of younger adults individuals showed that different metabolites are linked with the development of chronic periodontal disease, including ornithine (VIP = 2.57), 5-oxoproline (1.99), valine (1.99), proline (1.35), spermidine (1.15), hydrocinnamate (1.07), histidine (1.04), and cadaverine (1.00) [11]. In contrast, we showed that in older adult patients with chronic periodontal disease, the following metabolites were more prominent: 5-aminovaleric acid (VIP = 2.37), serine (2.18), 1-monopalmitine, aspartic acid, D-mannitol, putrescine, 1-benzoyl-2-t-butyl-5-ethyl-3-methyl-5-vinyl-imidazolidin-4-one, palmitoleate, maltose, lactic acid, oxalic acid, edetic acid, contaminants, and D-glucose-6-phosphate, of which the first two were remarkably increased. The relationship between periodontitis and increased aminovaleric acid, in addition to lactic acid, certain sugars, and putrescine, a compound associated with tissue decay, has been reported previously [30]. The observed differences in metabolite profiles may be related to the different characteristics of the development of chronic periodontal disease, as well as to the host response to pathogens in the two populations.

In a survey of the literature over a 17-year period (Jan/2000 to Jan/2017), a study identified 90 different components in the crevicular fluid as diagnostic and prognostic markers for periodontal disease, including inflammatory mediators, oxidative stress markers, host-derived enzymes, tissue degradation products, and bone homeostasis mediators [31].

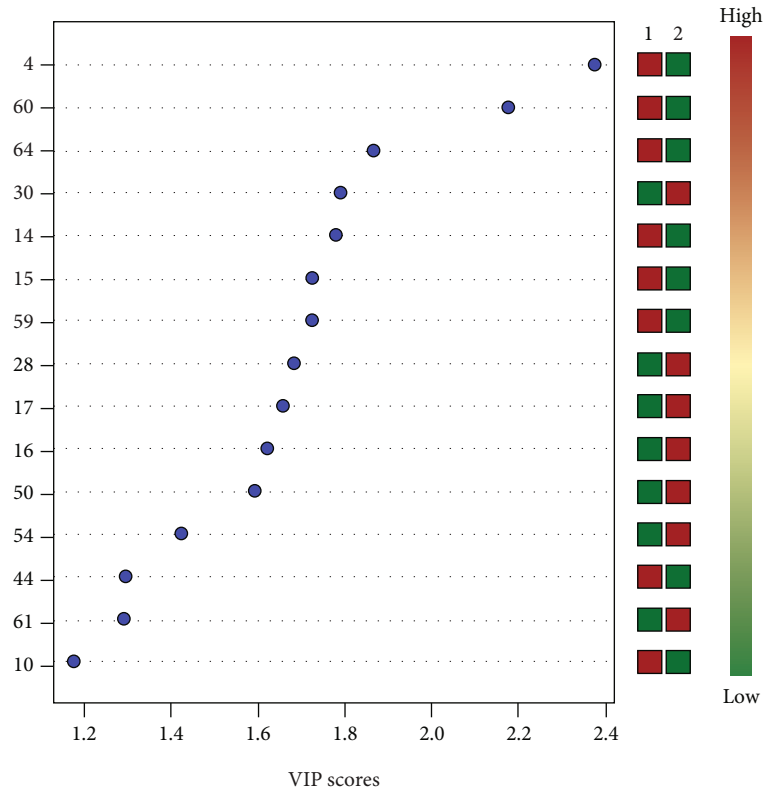

Figure 1: Effect of chronic periodontal disease on the distribution of VIP scores after the metabolome of inflammatory exudate present in the crevicular fluid of old adults patients with periodontal disease. Separation of periodontitis (1-red) and healthy (2-green) groups according to metabolite profile. Numbers 1 and 2 represent the healthy and periodontitis groups, respectively. The concentration of metabolites is represented by the color gradient between green (less concentrated) and red (more concentrated). To the left of the table are the metabolite IDs, identified in Table 3.

Table 3: Metabolites important for group separation according to partial least squares-discriminant analysis (PLS-DA) and their variable importance in projection (VIP) scores.

ID	Metabolite	VIP score
4	5-Aminovaleric acid	2.37
60	Serine, 3TMS derivative	2.18
64	*Contaminant	1.86
30	1-Monopalmitin	1.79
14	Aspartic acid	1.78
15	D-mannitol	1.72
59	Putrescine	1.72
28	1-Benzoyl-2-t-butyl-5-ethyl-3-methyl-5-vinyl-imidazolidin-4-one	1.68
17	Palmitoleate	1.66
16	Maltose	1.62
50	Lactic acid	1.59
54	Oxalic acid	1.42
44	Edetic acid	1.29
61	*Contaminant	1.29
10	D-glucose-6-phosphate	1.18

*Derivatives of chemical products (example: toothpaste).

We believe that metabolites from different metabolic pathways can guarantee diagnostic and prognostic specificity and sensitivity. In addition to metabolites from the inflamed site in the periodontium, inflammation-related metabolites from microorganisms are also present, as a marked dysbiosis is established in periodontitis; therefore, these molecules can be biomarkers, pointing at a potential strategy for the prediction, diagnosis, prognosis, and management of personalized periodontal therapy [32].

In our approach, we indicate that the metabolic arrangement found in crevicular fluid can be influenced by senescence, since there is an increased susceptibility to infections and inflammations in old age [33]. In a metabolomic evaluation of crevicular fluid from individuals with a mean age of 39 years (28 to 51 years old), the authors highlighted the association of two components, citramalic acid and N-carbamylglutamate, as markers of chronic periodontitis in an explanatory model with AUC = 87.6% [32]. In another study, metabolomic differences were identified between healthy (26.5 ± 1.7 years old) and periodontitis subjects (mean age = 29.4 ± 4.2 years old), changes in the concentrations of compounds associated with the biosynthesis of amino acids, galactose, and pyrimidine were observed, and correlation between the metabolic profile and microbial community was reported [34].

Multicenter studies or approaches in secondary studies that can guarantee the assessment of strata linked to the pathogen-host relationship, such as immunological status, age, and sociodemographic factors, will certainly provide consistent results in identifying the indicators for the diagnosis and prognosis of periodontal disease.

5. Conclusions

Our findings demonstrated that certain metabolites, such as 5-aminovaleric acid, serine, and 3TMS derivative, are likely present in the crevicular fluid of older adults with chronic periodontal disease. Thus, these metabolites can be used as biomarkers for diagnosing periodontal disease in these patients.

Data Availability

The supplemental data used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Wellington F. Rodrigues, Camila B. Miguel, Ferdinando Agostinho, and Gabriela V. da Silva contributed to the overall study concept and design, data management, chart adjudications, interpretation of data, and preparation of the manuscript. Javier E. Lazo-Chica, Sandra M. Naressi Scaping, and Marcelo H. Napimoga contributed to the overall study concept and design, data management and statistical analysis, interpretation of data, and preparation of manuscript. Carlos A. Trindade-da-Silvab, José E. Kriegere, and Alexandre da Costa Pereira contributed to the overall study concept and design, data management, chart adjudications, interpretation of data, and preparation of manuscript. Siomar de Castro Soares contributed to the subject identification and data acquisition and manuscript preparation. Carlo J. Freire Oliveira contributed to the statistical analysis and interpretation of results. Carlos Ueira-Vieira contributed to the overall study concept and design, result interpretation, and preparation of the manuscript. W.F.R., C.B.M., F.A., and G.V.S. contributed equally to this work.

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Supplementary Materials

Supplementary 1. Figure S1. Results of metabolite data normalization by self-scaling. Box and kernel density plots before (left) and after (right) normalization by self-scaling. Boxes indicate the top 50 metabolites, and densities are based on all samples.

Supplementary 2. Figure S2. Results of the fold change (FC) analysis. Metabolites with an intensity at least twice as large (dashed lines indicate the cut offs) in the periodontitis as in the healthy group or vice versa are indicated in pink. Data are presented in logarithmic scale.

Supplementary 3. Figure S3. Results of the metabolite *t*-test. Metabolites with statistical significance (p value <0.05) are shown in pink.

Supplementary 4. Figure S4. Separation of periodontitis (1-red) and healthy (2-green) groups according to metabolite profile.

Supplementary 5. Figure S5. Permutation test showing that the metabolic profile separation between the periodontitis and healthy groups was not random (p value <0.05).

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Research Article

Salivary Biomarkers (Opiorphin, Cortisol, Amylase, and IgA) Related to Age, Sex, and Stress Perception in a Prospective Cohort of Healthy Schoolchildren

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Background. The use of easily accessible biomarkers for assessing young patients' health is weighty. This cohort study is aimed at measuring stress/immune biomarkers in the saliva of healthy school-age children and comparing subgroups according to age, sex, and stress perception. *Material and Methods*. 503 children under 12 years old (8.7 ± 1.3) were included with anthropometric evaluation (height, waist, hip circumference, body weight, and body mass index (BMI)). Levels of opiorphin (OPI), free cortisol, alpha-amylase (sAA), and secreted immunoglobulin (sIgA) were determined by quantitative assays (ELISA) in unstimulated saliva. Unpaired *t*-test, Welch test, and Mann–Whitney *U* test were applied for appropriate group comparisons, and the correlation between variables was analyzed with Spearman's rank coefficient. Results were considered significant at $p < 0.05$. *Results*. sIgA and sAA exhibited significant differences depending on age and sex: IgA (ng/mL): 86 ± 68.6 vs. 104.9 ± 72.1 for (6-7 y.o.) and (8-11 y.o.), respectively, and 108.1 ± 80.1 vs. 94.6 ± 62.2 for male and females, respectively; sAA (U/mL): 78.9 ± 54.4 vs. 100.5 ± 81.2 for (6-7 y.o.) and (8-11 y.o.). No difference related to age or sex between groups was observed for cortisol and OPI. However, OPI levels were higher and correlated to prior stress exposure in children (0.31 ± 0.4 vs. 0.26 ± 0.5 ng/mL, $p = 0.031$). sAA was negatively correlated to low mood self-declaration in children in the last two weeks ($r = -0.10$, $p = 0.045$). *Conclusions*. sIgA and sAA can be used as sex- and age-related biomarkers in children 6-12 y.o., which is not the case for free cortisol and opiorphin. However, OPI reflected previous exposure to stress, suggesting its use for evaluating stress-related changes in children

1. Introduction

Psychological stress impairs homeostasis in many aspects, including immune dysregulation [1] with significant variability according to age and sex [2]. The use of easily accessible biomarkers is essential to assess these changes in fragile subjects, like children, especially in a context of exposure to stressful experiences during childhood, where invasive sampling procedures may increase psychological stress.

Saliva is a promising medium that has been used to evaluate both acute and chronic stresses [3]. Several salivary immune biomarkers are now considered to be sensitive

and reliable readouts of mental stress in adult patients [4], primarily cortisol, alpha-amylase (sAA), secretory immunoglobulin IgA (sIgA), and opiorphin (OPI).

Cortisol has been often used to assess the activity of the hypothalamic-pituitary-adrenal (HPA) stress axis system and received extensive attention in research on stress physiology [5]. Life situations described as unpredictable or dangerous cause increased cortisol release. Cortisol secretion, which physiologically peaks at awakening and gradually decreases throughout the day, increases in acute stress situations before returning to normal levels after cessation of stress [6]. Long-term high levels of cortisol may exert harmful, deleterious effects on mood [7], obesity [8], and blood glucose levels [9]. Low levels have also been linked to pain, fatigue, high stress sensitivity, and stress-related disorders such as posttraumatic stress syndrome [10].

Salivary amylase (sAA) was also recently suggested as an indicator of sympathetic nervous system (SNS) activation but significantly different from cortisol. Typical sAA concentration reaches a peak in the late afternoon or evening [11]. In acute stress situations, sAA secretion may have additional distinct peaks [12]. sAA showed positive correlations with heart rate, pain intensity, and cortisol, suggesting HPA and SNS coordination [13].

IgA is a secreted glycoprotein, part of the adaptive immune system, that acts synergistically with other inherited mucosal defense factors, such as alpha-amylase, lactoferrin, and lysozyme [14]. Similarly, to cortisol, sIgA peaks in the morning and then gradually declines till the evening. Acute stress increases sIgA release, whereas chronic stress has an inhibitory effect, emphasizing the ability of the immune system to protect the body against disease [15].

Opiorphin (OPI) is a polypeptide recently discovered in human saliva, increasing the bioavailability of enkephalins acting on μ- and δ-opioid receptors in the central nervous system (CNS), therefore displaying analgesic and antidepressant properties [16–25]. Salivary OPI secretion depends on ovarian cycles and hormonal status [18], which are known to affect the trophism of oral tissues [26–29]. OPI also plays a role in oral pain [26, 28, 30] and mental stress [27].

These four biomarkers may be easily measured in saliva, but in variable amounts depending on age and sex, as well as in response to psychological stress [31]. The main objective of this exploratory study was therefore to compare salivary levels of opiorphin, cortisol, sAA, and sIgA using enzyme-linked immunosorbent assay (ELISA), in two groups of healthy children, 6-7 and 8-11 years old, a developmental age critical for mental disorder development [32]. Secondary objectives were to compare salivary biomarkers' levels in subgroups of subjects stratified by age, sex, anthropometric parameters, stress perception of prior distressing events, and mood.

2. Materials and Methods

2.1. Ethics. The study was approved by the Ethics Committee, Poznan University of Medical Sciences (resolution no. 542/14 from 6 December 2014). Parents or legal guardians gave written informed consent for the participation of their children in the study. Prior to the examination, the purpose of the study was explained to the children. They were also asked to express their opinions on the study.

2.2. Subjects. Subjects were healthy schoolchildren attending an urban district school in the years 2019-2020 (the northern, southern, eastern, and western parts of the Poznan agglomeration). Ten primary public schools were randomly selected in the aforementioned area and invited to participate in the study. Six schools agreed. During meetings with the parents, professionals explained the details of the study and its noninvasive nature. Then, parents registered their children for final participation. After a general interview, children were screened for inclusion/exclusion criteria. The final sample was then divided into two subgroups according to age: 6-7 (group 1) and 8-11 (group 2) years old (y.o.). The rationale for age categories was based on Poland's educational system and hormone-related changes in children at those ages. As puberty might affect opiorphin production, we excluded children over 12 y.o. since sex steroid impregnation usually does not occur before this age [33]. The scholar system in Poland includes 6 years of early education (6-11) followed by 4 years of primary school. Each cycle is related to different intellectual and social requirements and skills. We divided the early childhood education period into groups of 6 to 7- and 8 to 11-year-old children, to obtain homogenous groups regarding socio-cultural parameters. The study flowchart is illustrated in Figure 1.

2.3. Exclusion/Inclusion Criteria. Inclusion criteria were as follows (Table 1): children >6 and <12 years old, consent signed by parents, and study participation orally approved by the child.

Exclusion criteria were divided into four categories: (1) school attendance: child absent from school more than 4 weeks in the recent period; (2) general health: permanent somatic diseases, mental or neurodevelopmental disorders, hereditary disorders (first-degree relatives), pharmacotherapy, endocrine therapy, and dietary supplements; (3) oral health: urgent or nonroutine dental treatment, ongoing orthodontic treatment/wear of an orthodontic appliance, and any general or oral treatment prone to alter salivary flow and composition; (4) communication: inability to answer questions and lack of cooperation during physical measurements or salivary sampling.

2.4. Children Assessment: Mental Evaluation. MINI-KID, a proprietary structured questionnaire completed by a specialist in child and adolescent psychiatry [34–36], was used to exclude children presenting mental and neurodevelopmental disorders from the study. The MINI-KID questionnaire is a widely used screening tool designed to quickly identify children at risk of psychiatric disorders [36] with well-supported reliability and validity in its Polish version [37]. Finally, child stress experience in the past 6 months and assignment to the SE1 or SE0 subgroups were based on the answers given in the following part of the

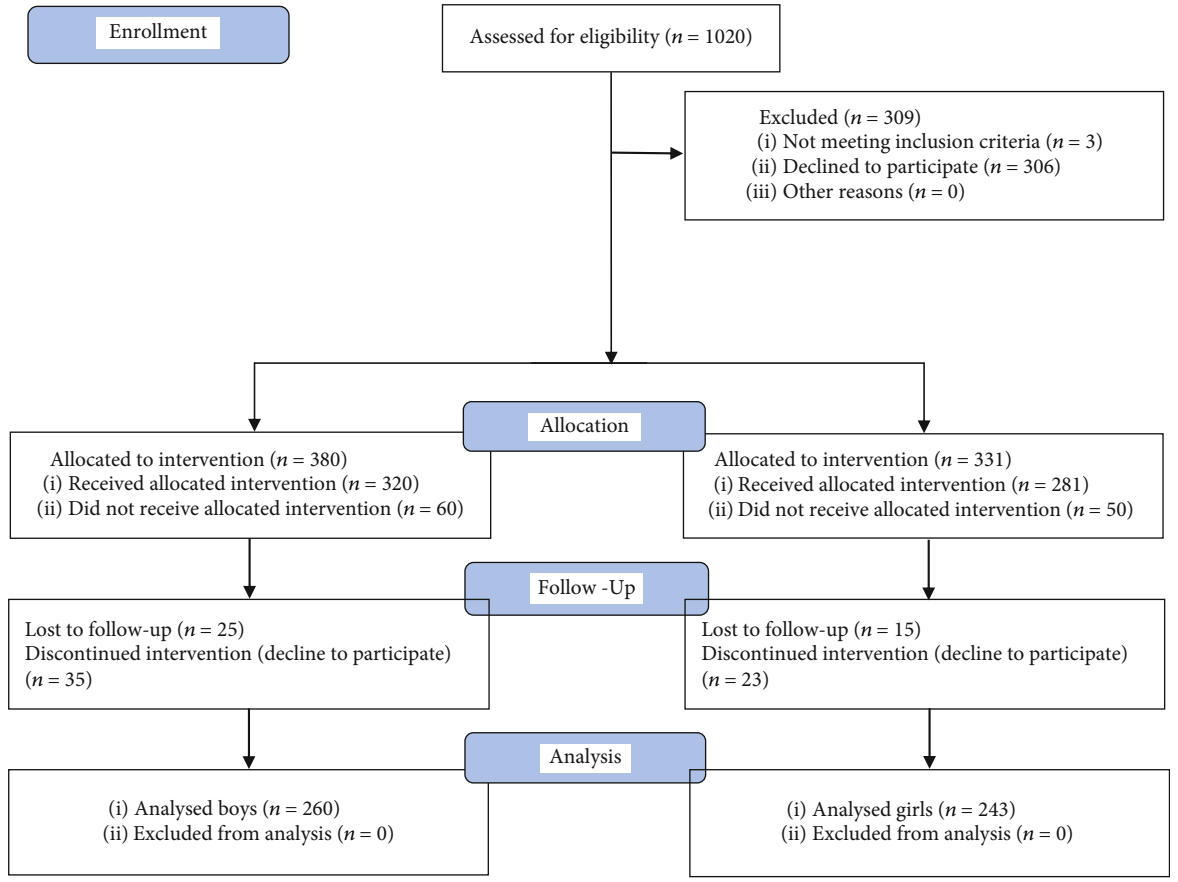


Figure 1: Study flowchart.

Table 1: Inclusion and exclusion criteria for both groups.

Criteria for inclusion into the study group 1	Criteria for inclusion into the study group 2	Criteria for exclusion from study groups
Children of both girls and boys aged 6–7	Children of both girls and boys aged 8–11	Children of age <6 and >12 years both girls and boys
Lack of mental disorders—assessment with the use of MINI-KID questionnaire [36]	Lack of mental disorders—assessment with the use of MINI-KID questionnaire [36]	Children with mental disorders—assessment with the use of MINI-KID questionnaire [36] (e.g., coexisting schizophrenia, bipolar affective disorder, and serious somatic disorders)
Parent or legal guardian approval	Parent or legal guardian approval	Lack of acceptance from parents or legal guardian
Children without hereditary mental disorders (first-degree relatives)	Children without hereditary mental disorders (first-degree relatives)	Children with disorders of the central nervous system (e.g., epilepsy, serious injuries, and CNS infections)
Healthy intellectual and physical ability to collaborate during the study	Intellectual and physical ability to collaborate during the study	Chronic pharmacotherapy, hormonotherapy, dietary supplements
Good oral health	Good oral health	Children attending urgent or nonroutine dental treatment prone to alter salivary flow and composition orthodontic treatment

MINI-KID: Mini International Neuropsychiatric Interview for Kids questionnaire; CNS: central nervous system.

questionnaire: Traumatic Event Screening Inventory (TESI) (see Supplementary 2). Confirmation by any declaration of the stress experience caused inclusion or exclusion to subgroup SE1 (yes) or SE0 (no). The list of analyzed experiences was as follows:

"…Tick the events that took place in the child's life in the last 6 months":

(1) Life or health of the child was seriously endangered (yes/no)

(2) Child witnessed an event in which the life or health of another person was at risk or someone died (yes/no)

(3) Child experienced physical violence (beating, jerking, pushing, burning, choking, forcing to sexual activity, etc.) or psychological violence (calling names, mocking, gossiping, someone shouted at them very strongly, threatened him, the child felt rejected by a loved him as a person) (yes/no)

(4) Child has witnessed physical or mental violence against another person (yes/no)

(5) Child has experienced the death of someone close to him (yes/no)

(6) There were severe problems in the family (quarrels, conflicts, breakups, alcohol problem, other addictions, emotional problems of family members, etc.) (yes/no)

(7) Child was separated from one of the parents for many days (yes/no)

(8) Serious problems with grades at school (yes/no)

(9) Another very stressful event (yes/no)

Evaluation of all the salivary biomarkers was performed according to subgroups SE1 and SE0.

2.5. Anthropometric Parameters. Body height, weight, waist, and hip circumferences were evaluated. Body height in a standing position was measured with the SECA 216 wall-mounted stadiometer with an accuracy of 0.1 cm. Body mass was recorded in lightweight clothing on a digital scale with an accuracy of 0.1 kg. Waist circumference was determined halfway between the lower edge of the rib arch and the upper iliac crest using metric tape [38]. Hip circumference was measured at a level parallel to the floor, at the largest circumference of the buttocks using metric tape [38]. All subjects were assessed for body mass index (BMI) according to the formula [39] $\text{BMI} = \text{weight}\,(\text{kg})/\text{height}^2\,(\text{m}^2)$ with cut-offs adopted by the International Obesity Task Force (IOTF) to estimate normal or abnormal weight according to age.

2.6. Salivary Collection. Special attention was paid to standardize collection. All participants were referred to the medical school offices between 9:00 and 10:00 a.m. for examination and saliva sampling, performed by the same qualified dentist (E.P.).

Unstimulated whole saliva was collected according to a previously described methodology [40, 41]. Recommendations neither to eat for one hour before the examination nor to undergo any medical or oral hygiene procedures had been given prior to the visit. For saliva collection, children were asked to spit unstimulated saliva into a sterile container for 2 minutes, which was discarded, followed by another collection in a separate flask for 10 minutes. Children were asked to focus on spitting and limiting other activities while leaning forward in a seated position during the examination. Immediately after collection, the samples were centrifuged; the separated supernatant was first frozen at -20°C and then at -80°C until further processing.

2.7. Cortisol. The concentration of free cortisol in the saliva was quantitatively determined by ELISA using the ELISA Kit DES6611 for the in vitro diagnostic (IVD) (Demeditec Diagnostics GmbH, Kiel, Germany). The standard curve ranged from 0 to 30 ng/ml, the intra- and interassay variability coefficients were assessed to be below 6%, and the standard curve was statistically significant ($r^2 = 0.998$, $p < 0.001$) [42, 43]. Cortisol levels are expressed in ng/mL.

2.8. Salivary Alpha-Amylase (sAA). The sAA concentrations were quantitatively determined by ELISA using the ELISA Kit DEEQ6231 for the in vitro diagnostic (IVD) (Demeditec Diagnostics GmbH, Kiel, Germany). The standard curve ranged from 0 to 500 U/ml, the intra- and interassay variability coefficients were assessed to be below 5%, and the standard curve was statistically significant ($r^2 = 0.982$, $p < 0.001$) [44, 45]. sAA levels are expressed in U/mL.

2.9. Salivary Secretory Immunoglobulin A (sIgA). Salivary IgA concentrations were quantitatively determined by the ELISA method using the ELISA Kit DEXK276 for the in vitro diagnostic (IVD) (Demeditec Diagnostics GmbH, Kiel, Germany). The standard curve ranged from 0 to 400 μg/ml, the intra- and interassay variability coefficients were assessed to be below 5%, and the standard curve was statistically significant ($r^2 = 0.995$, $p < 0.001$) [2, 46]. Levels of sIgA are expressed in μg/mL.

2.10. Opiorphin (OPI). The quantification of OPI in saliva was performed using a commercial enzyme immunoassay (ELISA test for measuring human opiorphin cat. no. EH1927, FineTest, Wuhan, Hubei, China) according to the manufacturer's instructions. The measuring range of the kit was 0.156–10 ng/ml and sensitivity 0.094 ng/ml. The intraplatelet coefficient of variation was <8% with an interplatelet variation coefficient of <10%, and the standard curve was statistically significant ($r^2 = 0.985$, $p < 0.001$). OPI levels are expressed in ng/mL.

All ELISA tests were performed according to the manufacturer's instructions, without any modification. All samples and standards were run in duplicates, and the mean value of the two assays was used for statistical evaluation. Optical density was read with a spectrophotometric plate reader (Asys UVM 340 Microplate Reader from Biochrom Ltd., Cambridge, UK) for a wavelength of 450 nm ± 10 nm. A four-parameter algorithm (4 parameters logistic) was used to assess concentration in the tested samples. All tests were performed by an investigator blinded to clinical data and the status of the samples (group affiliation).

Cortisol, sAA, and IgA were tested with validated *in vitro* diagnostic tests certified by CE IVD certificates. Measurements were selected only at the quantification level, and when two technical replicates were obtained, it was detected with an acceptable difference of <10%.

We used all the obtained nominal measurements for the calculations, treating them as individual natural variability,

as we did not observe extreme deviations in results. We have not adopted any arbitrary cut-off thresholds to distinguish between the correct and the abnormal levels, mainly due to the lack of diagnostic standards for the examined children age group.

2.11. Statistical Analysis. Statistical analyses were conducted in Statistica v13.3 (StatSoft, Poland). Normality of distribution was assessed with the Shapiro-Wilk test, and equality of variances was checked using Levene's test. Due to the lack of normality for most of the variables, more restrictive nonparametric tests were used. Comparison of two unpaired groups was performed using the Mann–Whitney U test. Spearman's rank correlation was used to detect the relationship between variables. In order to rule out interfering variables that may be biasing the correlation results, we used covariance analysis (a combination of analysis of variance and regression (ANCOVA)) [47] to check the effect of the interaction of qualitative variables (age, gender, being overweight, stress life event-interfering variables) on the concentration level of the studied proteins: cortisol, sIgA, sAA, and OPI. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Sample. The final sample consisted of 503 children (Figure 1), 6-11 years old, (mean age 8.7 ± 1.3), including 260 boys (51.7%) and 243 girls (48.3%), with no statistical difference between mean age (8.8 ± 1.4) vs. (8.6 ± 1.2) ($p > 0.05$, Mann–Whitney U test), respectively. Not surprisingly, the anthropological measurements of body weight, height, BMI, and waist size were significantly different according to age and gender ($p < 0.05$, Welch test). Children from both groups were Caucasians of Polish origin attending a public school for primary education. There were no differences between confessed religions (mainly catholic), domestic-owned animals (mainly dogs), or living places. The MINI-KID questionnaire excluded children at risk of mental disturbances, and the TESI questionnaire included children who experienced stressful events. More than half reported a stressful experience in the past and feeling low mood during the past two weeks (group SE1 $n = 411$, 81.7%). The main characteristics of the sample are summarized in Figure 2.

3.2. Salivary Biomarkers. Salivary measurements are shown in Figure 3. In unstimulated saliva, sIgA showed the most significant difference among children according to sex and age ($p < 0.038$ and $p < 0.026$, respectively). A significant difference ($p < 0.017$) was observed for sAA, found in higher concentration in group 2 aged 8-11 ($p < 0.017$), especially for boys ($p < 0.049$). No differences in the levels of cortisol and OPI were detected depending on sex ($p = 0.901$ and $p = 0.721$, respectively) and age ($p = 0.644$ and $p = 0.784$, respectively) or any anthropometric parameter. However, OPI was detected in statistically significant higher concentration in children having experienced distressing events (group SE1, $p < 0.031$) (see Figure 4). The rest of salivary biomarkers sIgA, sAA, or cortisol did not change ($p = 0.705$, $p = 0.711$, and $p = 0.491$, respectively) for this condition.

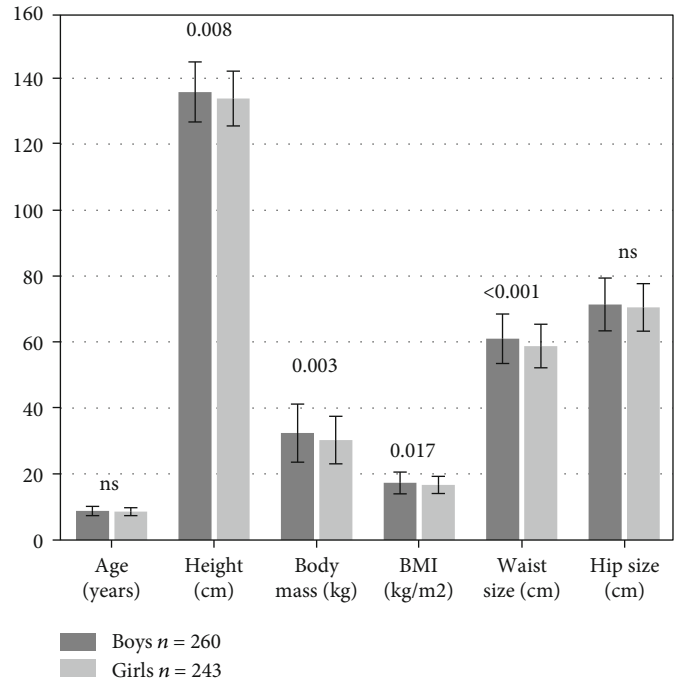
3.3. Correlations. Correlations are shown in Table 2. Spearman analysis revealed the correlation between sIgA and all measured anthropometric parameters, such as age, height, body mass, BMI, waist, and hip circumferences ($p < 0.05$) for all children (Table 2). A similar correlation between sAA and selective measured anthropometric parameters, such as age, height, and hip size in the total group and body mass with waist size in girls aged 6-11 ($p < 0.05$), was evidenced. OPI showed a significant correlation between age and height in group 1, together with sAA in the whole group of boys ($p < 0.05$). OPI was the sole marker positively correlated and sAA negatively correlated with children declaring prior distressing experiences and low mood ($p < 0.05$) (Figure 4). There was no significant correlation neither for cortisol according to age and sex differences nor for the other salivary biomarkers ($p > 0.05$) (Table 2).

3.4. Covariance Analysis. Four covariance models (ANCOVA) (shown in Table 3) were built ((1) sIgA, (2) sAA, (3) cortisol, and (4) opiorphin) for testing the effect of interaction between the following clinical factors: (1) age divided into younger and older children, (2) sex divided into boys and girls, (3) BMI divided into normal and abnormal indexes with cut-offs adopted by the International Obesity Task Force (IOTF), and (4) stressful experiences SE1 (yes) or SE0 (no). Statistical analysis showed that sAA and OPI were not dependent on variables such as age, gender, body weight (normal/obesity), and past stressful events, but sIgA was dependent on the age of children ($p < 0.001$) as cortisol on body mass index ($p < 0.046$).

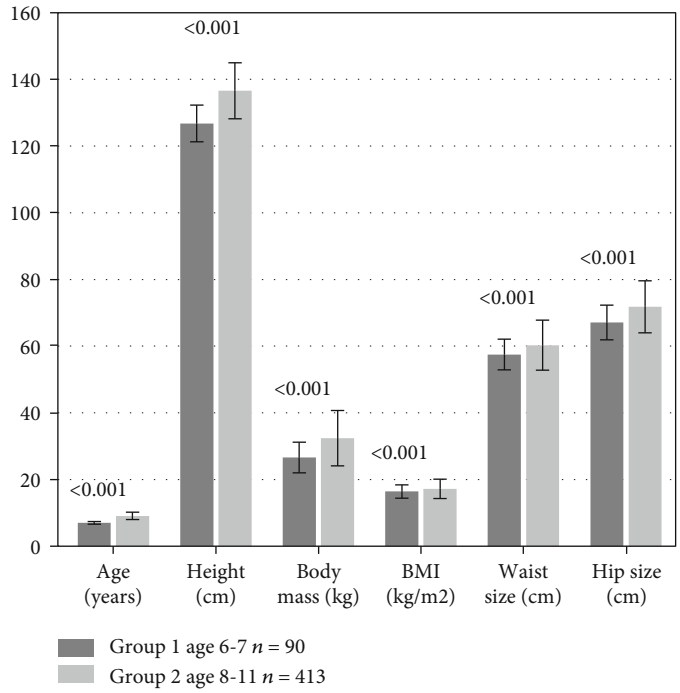
4. Discussion

The main results of this study are significant differences in sIgA and sAA among children depending on age and gender. Correlations between salivary immune biomarkers sIgA, sAA, age, sex, and parameters of developmental status were evidenced. Opiorphin was detected in statistically higher concentrations in children reporting previous experiences of stressful events.

In the present study, concentrations of sIgA and sAA were correlated to age and anthropometric parameters. The concentration of sAA showed significant developmental differences in particular for boys, especially for those of the older age group. Other studies focusing on human development have shown that salivary components and salivary gland function may alter with age [48]. The secretion of sAA changes with age, from low levels in neonates to levels similar to those of adults during adolescence; later, sAA remains stable throughout adulthood, even in old age. The second aspect of the present study concerning how mental stress may influence the secretion of sAA is also interesting. This salivary enzyme is mainly produced by the parotid glands but reflects the activation of the autonomic nervous system. Both physiological and psychological factors

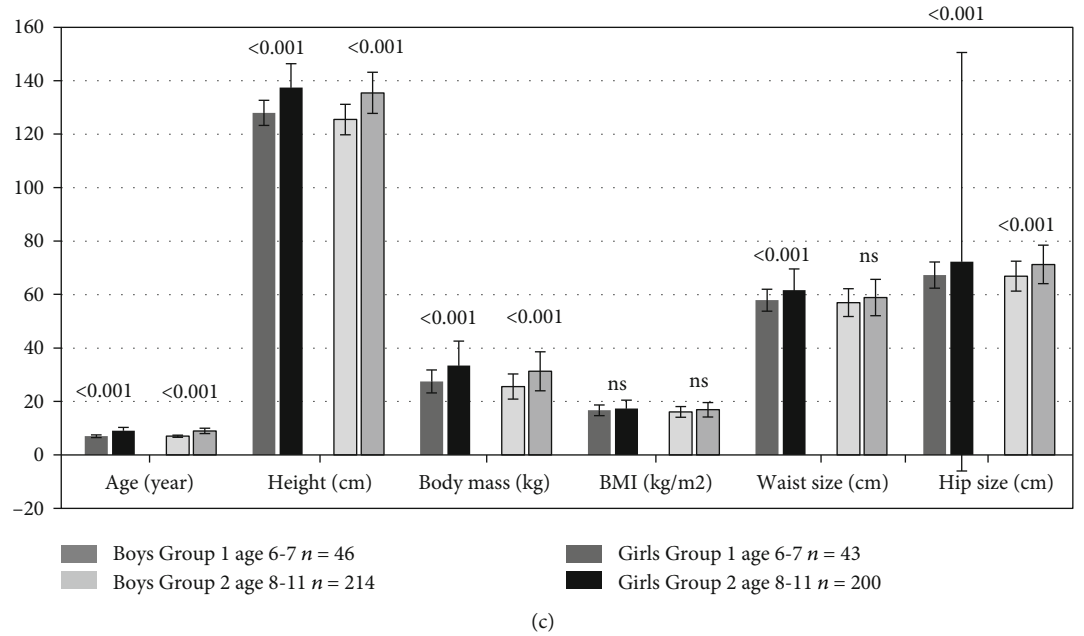


(a)



(b)

Figure 2: Continued.



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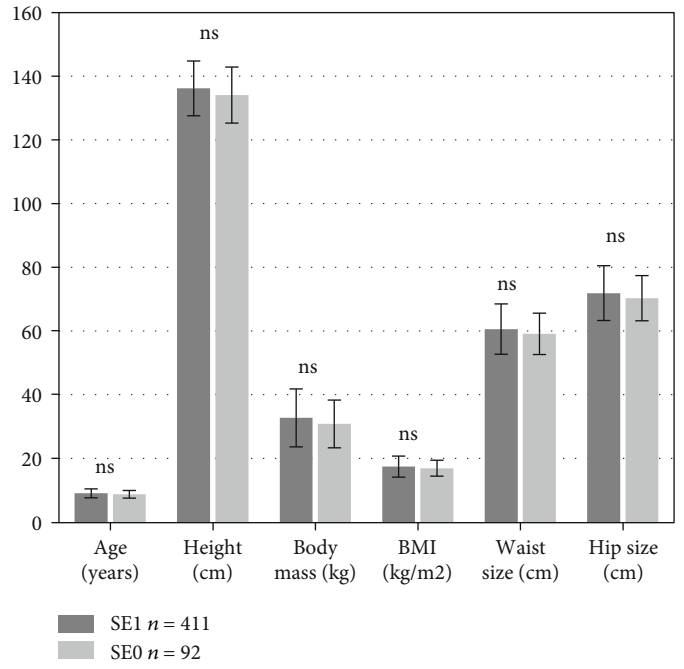


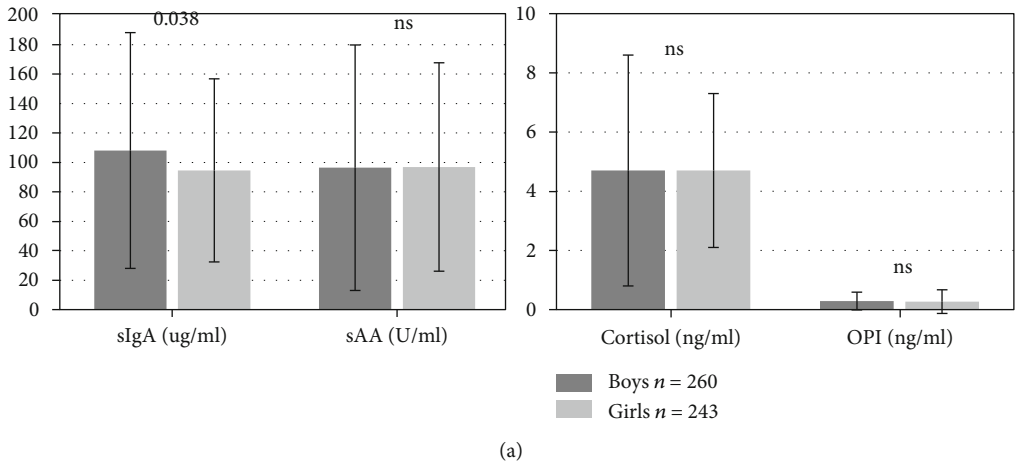
(d)

Figure 2: Anthropometric parameters according to (a) sex, (b) age group, (c) age group and sex, and (d) prior stress events (SE0 vs. SE1). One bar graph for each comparison (four graphs in total). Mean ± standard deviation and p value with statistical difference marked in values. n: number of examined children; ns: not significant value in statistical analysis; sIgA: salivary IgA; sAA: salivary alpha-amylase; OPI: opiorphin; SE1: group of children with stressful experience in the past; SE0: group of children without stressful experience in the past.

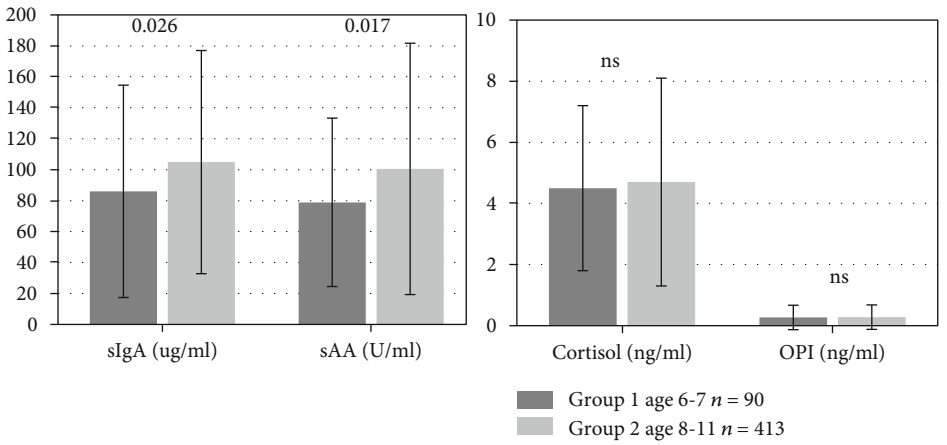
stimulate α-amylase release during stress [49, 50], and different modes of stimulation (taste stimulation for parotid gland activity, psychological stress, and physical effort) result in different patterns of sAA release [51–53].

In our study, the increase in sAA levels with age and physical developmental changes suggests a possible application of saliva biochemical analyses to monitor young children's health. Saliva has been increasingly suggested as a good, easily obtainable sample to assess the health status. In this study, the inverse correlation between mental stress among children in group SE1 and sAA was relatively weak. The covariance models (ANCOVA) testing the effects of interaction between clinical factors and salivary parameters did not indicate sAA as a significant one.

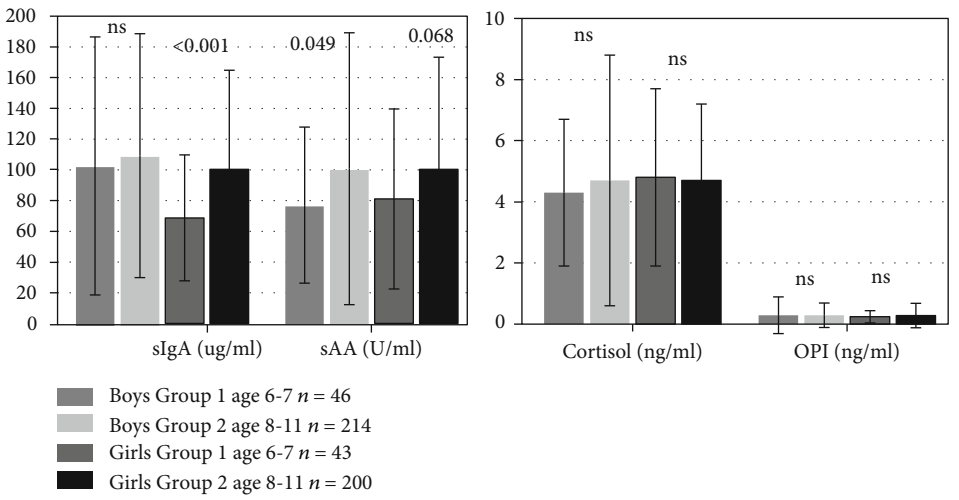
Similarly to sAA, sIgA levels in the present study were different depending on age. This finding is consistent with previous findings reported by other researchers among children and adult subjects [54–58]. However, none of these



(a)

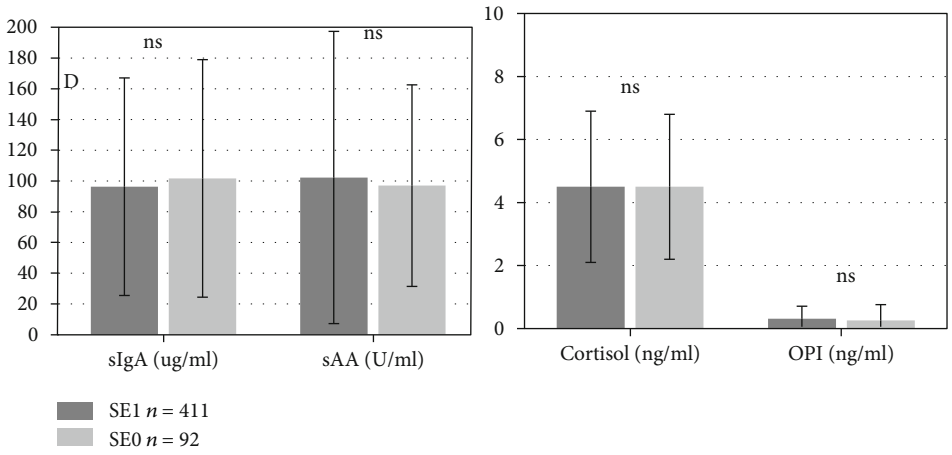


(b)



(c)

Figure 3: Continued.



(d)

Figure 3: Salivary parameters according to (a) sex, (b) age group, (c) age group and sex, and (d) prior stress events (SE0 vs. SE1). One bar graph for each comparison (four graphs in total). Mean ± standard deviation and p value with statistical difference marked in values. n: number of examined children; ns: not significant value in statistical analysis; sIgA: salivary IgA; sAA: salivary alpha-amylase; OPI: opiorphin; SE1: group of children with stressful experience in the past; SE0: group of children without stressful experience in the past.

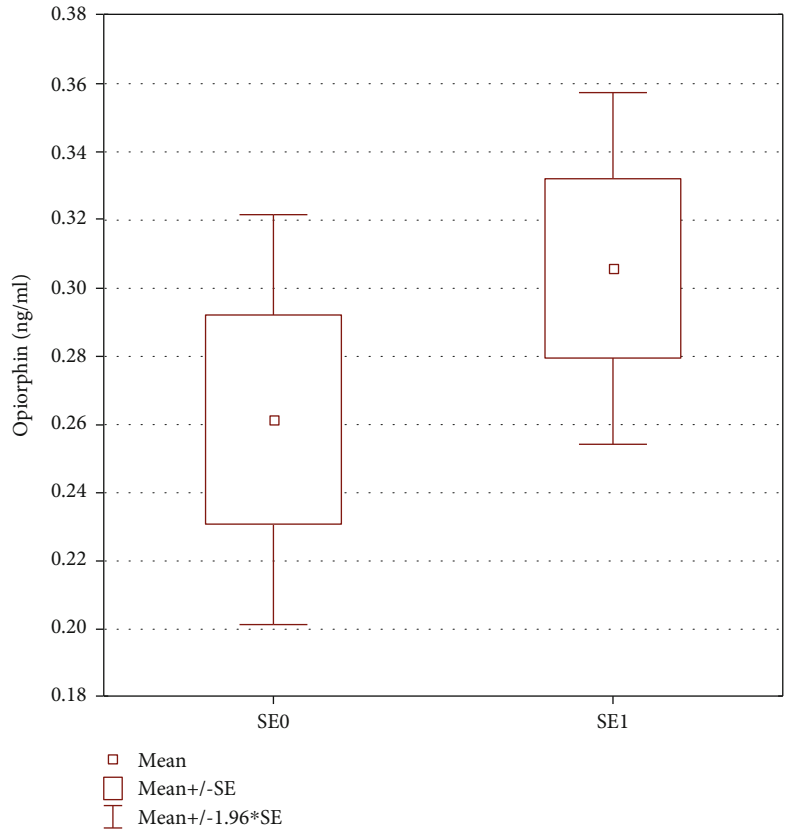


Figure 4: Comparison of salivary opiorphin (mean ± standard deviation). SE0: children free of stressful experiences in the past; SE1: children with stressful experiences in the past. Children from the SE1 group showed higher salivary concentration of this peptide. p value with statistical correlation, $p < 0.05$.

studies reported significant differences between adult men and women or boys and girls. Male subjects had higher sIgA levels than females. The reason for this sex difference may be found in salivary secretion level usually higher in males than females [59, 60]. Hormones might also play a role, although children under 11 years are usually not under hormonal influence, at least sex steroid impregnation. The present study included only healthy children in prehormonal

Table 2: Spearman's rank order correlations between total salivary parameters, divided by groups (young children group 1 and older children group 2), boys and girls.

Variables	Spearman's rank order correlations				
	Total (age 6-11) ($n = 503$)	Group 1 (age 6-7) ($n = 90$)	Group 2 (age 8-11) ($n = 413$)	Boys (age 6-11) ($n = 260$)	Girls (age 6-11) ($n = 243$)
	r_s	r_s	r_s	r_s	r_s
sIgA vs. age	0.19*	-0.21	0.17*	0.16*	0.22*
sIgA vs. height	0.24*	0.03	0.24*	0.22*	0.25*
sIgA vs. body mass	0.22*	-0.05	0.24*	0.21*	0.22*
sIgA vs. BMI	0.12*	-0.06	0.15*	0.11	0.13*
sIgA vs. waist size	0.15*	-0.08	0.17*	0.14*	0.15*
sIgA vs. hip size	0.21*	0.02	0.21*	0.19*	0.22*
sIgA vs. cortisol					0.14*
sAA vs. age	0.11*	0.12	0.03	0.10	0.12
sAA vs. height	0.08*	-0.09	0.05	0.05	0.12
sAA vs. body mass	0.10	-0.03	0.07	0.07	0.13*
sAA vs. BMI	0.09	-0.01	0.09	0.06	0.12
sAA vs. waist size	0.08	-0.03	0.08	0.04	0.13*
sAA vs. hip size	0.10*	0.04	0.08	0.08	0.12
sAA vs. SE1	-0.10*				
Cortisol vs. age	0.001	0.03	-0.05	0.00	0.01
Cortisol vs. height	-0.02	-0.03	-0.03	-0.02	-0.02
Cortisol vs. body mass	-0.01	0.01	-0.02	-0.02	0.00
Cortisol vs. BMI	-0.02	0.02	-0.03	-0.03	0.00
Cortisol vs. waist size	-0.01	-0.01	0.00	0.00	-0.01
Cortisol vs. hip size	-0.02	0.001	-0.03	-0.07	0.02
OPI vs. age	0.04	0.32*	0.04	0.02	0.06
OPI vs. height	0.10*	0.27*	0.10	0.08	0.11
OPI vs. body mass	0.09	0.04	0.09	0.06	0.12
OPI vs. BMI	0.05	-0.11	0.07	0.00	0.10
OPI vs. waist size	0.10*	0.11	0.09	0.08	0.12
OPI vs. hip size	0.05	-0.01	0.06	0.02	0.08
OPI vs. sAA				0.15*	
OPI vs. SE1	0.11*				

sIgA: salivary IgA; sAA: salivary alpha-amylase; OPI: opiorphin; SE1: children with distressing events in the past; BMI: body mass index; vs.: versus; n: number of patients; ns: statistically nonsignificant. *p value with statistical correlation, $p < 0.05$.

development, confirming the age-sex-related differences in IgA secretion. An interesting observation is that maximum secretion may be reached in elderly age, e.g., >80 years old [54, 56], suggesting that sIgA is not an aging-related event but rather a result of disease, including exposure to oral pathogens and subsequent pharmacotherapies [61, 62]. Indeed, the correlation evidenced here between sIgA and cortisol only among girls supports previous research highlighting the relationship between sex, HPA axis, and immunocompetency [27, 63]. Female patients might be more prone to the effects of stress in ages under 11 years old. Studies focusing on adult women, indicating that they are more sensitive to stress events and display higher anxiety levels when visiting the medical/dental facilities than men, may support this hypothesis [64–66]. The increase in sIgA among girls and correlation with age confirmed in the covariance model advocates that prophylactic mental health programs early in childhood might be directed towards girls because of their higher reactivity to mental stress in adult age.

OPI was the only salivary biomarker elevated in children who self-reported previous exposure to stress. Statistical analysis (ANCOVA test) showed that OPI levels were not dependent on age, sex, and body weight (normal/obesity), thus reinforcing the statistical link between OPI levels and past stressful events. Although declarative in nature, to our knowledge, no study to date has compared opiorphin to healthy children with and without self-declared past

Table 3: The covariance models (ANCOVA) testing effect of interaction between clinical factors and salivary parameters.

Variable	Model 1 (sIgA)		Model 2 (sAA)		Model 3 (cortisol)		Model 4 (OPI)	
	F	p value	F	p value	F	p value	F	p value
(1) Age: group 1 (age 6-7) and group 2 (age 8-11)	10.964	**0.001**	2.057	0.152	0.032	0.858	0.170	0.681
(2) Sex: boys and girls	1.770	0.184	0.218	0.641	0.430	0.512	0.337	0.562
(3) BMI: normal and abnormal	1.929	0.166	2.468	0.117	3.996	**0.046**	0.063	0.802
(4) Stressful experiences: SE1(yes) and SE0 (no)	2.246	0.135	0.856	0.356	0.660	0.417	0.004	0.952
1 ∗ 2	1.735	0.188	0.579	0.447	0.597	0.440	0.234	0.629
1 ∗ 3	1.191	0.276	0.413	0.521	0.046	0.831	0.232	0.631
2 ∗ 3	0.007	0.935	2.090	0.149	0.195	0.659	0.035	0.851
1 ∗ 4	0.001	0.977	0.000	1.000	0.943	0.332	3.122	0.078
2 ∗ 4	0.519	0.472	0.109	0.742	0.507	0.477	0.063	0.803
3 ∗ 4	0.837	0.361	1.699	0.193	0.478	0.490	0.924	0.337
1 ∗ 2 ∗ 3	0.723	0.396	3.679	0.056	1.252	0.264	0.020	0.889
1 ∗ 2 ∗ 4	2.958	0.086	0.861	0.354	0.169	0.681	0.187	0.666
1 ∗ 3 ∗ 4	0.428	0.513	0.131	0.718	1.246	0.265	0.055	0.814
2 ∗ 3 ∗ 4	1.196	0.275	0.000	0.987	2.501	0.115	0.789	0.375
1 ∗ 2 ∗ 3 ∗ 4	2.020	0.156	0.714	0.399	3.208	0.074	0.646	0.422

sIgA: salivary IgA; sAA: salivary alpha-amylase; OPI: opiorphin; SE1: children with distressing events in the past; SE0: children without distressing events in the past; BMI: body mass index. Significant p value effect was marked ($p < 0.05$); body mass index (BMI) cut-offs were adopted by the International Obesity Task Force (IOTF).

distressing events. OPI has been described as a potent endogenous antinociceptive [67] and stress-related peptide [28, 30, 67] in adults. Similarly, in female adolescents affected by eating disorders, salivary changes in OPI and sIgA were observed, suggesting a possible use of OPI as a neurobiological salivary biomarker of malnutrition [27]. The present results suggest that prepubertal children (<12 y.o.) might also have a measurable response to stress through OPI.

Therefore, further research specifically exploring the role of stress needs to be continued among such young individuals.

No evidence of specific reactive secretion differences was found between children for cortisol levels. Moreover, cortisol levels were unchanged among those who declared previous stressful events, suggesting a well-functioning HPA system. Cortisol levels, measured at the same time point as sIgA and sAA, did not show sex-related differences. However, the covariance models (ANCOVA) showed a dependence between BMI and salivary cortisol. This interrelation may suggest a dependent release from separate systems and individual diurnal settings [68, 69]. In the case of free cortisol, the daily fluctuation has to be taken into account, since in healthy adults, its concentration increases from 50% to 160% in the first 30 minutes after waking up [70]. In this study, no attempt to control the time that had elapsed after awakening in each individual was taken. We aimed at measuring differences in children subjected to a consistent routine in similar school conditions. In this study, the highest value for cortisol was 54 ng/ml, equal to 5.4 μg/dl and 148.98 nmol/l, which is within normal range [71].

4.1. Limitations of the Study. In the present study, much attention was paid to collect a well-controlled cohort matched according to age and sex, with similar demographic features, representative of the general population. The group aged 8-11 was the largest, while the group aged 6-7 was lower in all chosen schools. A possible explanation for such unequal distribution is the overlap between school education and kindergarten for the youngest children. The study excluded children attending kindergartens and focused on those attending schools. Schools share a similar education system regarding rhythms, activities, and programs, which is not the case for kindergartens.

We also used a standardized instrument for evaluating the psychological stress among such young children with professional analysis performed by the child and adolescent specialists. However, the exposure to stress was only declarative, as evaluated by the TESI questionnaire. Neither further investigations of the nature of the stress nor assessment of the severity of stress was undertaken. We may also assume that the level of perceived stress for the same stressor may differ from person to person [72, 73]. Further studies designed for this purpose could give insights into the variations of assessed biomarkers with type and perceived level of stress.

Another possible bias of the study was the acute psychological stress that may occur during physical measurement and salivary sampling in the school area, despite all precautions taken. We also limited our explorations to a quartet of salivary biomarkers when a larger set of biomarkers would have provided additional valuable information, especially inflammatory biomarkers as well as oral health indicators.

5. Conclusions

Results of the present study among healthy children indicate that sIgA and sAA can be used as possible sex- and age-related biomarkers in children 6-12 y.o., which is not the case for free cortisol and OPI. The importance of sIgA and sAA findings may support monitoring the age-sex differences, especially in the prehormonal period of life.

However, OPI levels reflected previous exposure to stress, suggesting its evaluation of stress-related changes in prepubertal children. Interactions between BMI and salivary cortisol release suggest the dependence of such secretion with abnormal body weight in obese children.

Data Availability

The datasets generated for this study are available on request to the corresponding author.

Conflicts of Interest

The authors declare that they have no conflicts of interests.

Authors' Contributions

Anna Krahel and Elzbieta Paszynska contributed equally to this work and shared the first authorship. Justyna Otulakowska-Skrzynska and Szymon Rzatowski contributed equally to this work and shared the second authorship. Maria Gawriolek and Monika Dmitrzak-Weglarz contributed equally to this work and shared the last authorship.

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Supplementary Materials

Supplementary 1. Supplementary Materials Tables S1, S2, and S3. Table S1: descriptive statistics of anthropometric and salivary parameters for the whole group according to female and male sex distribution. Mean ± standard deviation, median (Min–Max), and $^{*}p$ value with statistical difference. *n*: number of examined children; ns: not significant value in statistical analysis; vs.: versus; sIgA: salivary IgA; sAA: salivary alpha-amylase; OPI: opiorphin. Table S2: descriptive statistics of anthropometric and salivary parameters for the whole group in age groups (group 1 children between 6 and 7 years old and group 2 children between 8 and 11 years old). Mean ± standard deviation, median (Min–Max), and $^{*}p$ value with statistical difference. *n*: number of examined children; ns: not significant value in statistical analysis; vs.: versus; sIgA: salivary IgA; sAA: salivary alpha-amylase; OPI: opiorphin. Table S3: descriptive statistics of anthropometric and salivary parameters for the whole group in female and male subgroups (group 1 only girls or boys aged between 6 and 7 years old and group 2 only girls or boys aged between 8 and 11 years old). Mean ± standard deviation, median (Min–Max), and $^{*}p$ value with statistical difference. *n*: number of examined children; ns: not significant value in statistical analysis; vs.: versus; sIgA: salivary IgA; sAA: salivary alpha-amylase; OPI: opiorphin.

Supplementary 2. Supplementary Material: Traumatic Events Screening Inventory-Parent Report Revised (Polish language version).

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Review Article

Salivary Biomarkers in Lung Cancer

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A very low percentage of lung cancer (LC) cases are discovered at an early and treatable stage of the disease, leading to an abysmally low 5-year survival rate. This underscores the immediate necessity for improved diagnostic, prognostic, and predictive biomarkers for LC. Biopsied lung tissue, blood, and plasma are common sources used for LC diagnosis and monitoring of the disease. A growing number of studies have reported saliva to be a useful biological sample for early and noninvasive detection of oral and systemic diseases. Nevertheless, salivary biomarker discovery remains underresearched. Here, we have compiled the available literature to provide an overview of the current understanding of salivary markers for LC detection and provided perspectives for future clinical significance. Valuable markers with diagnostic and prognostic potentials in LC have been discovered in saliva, including metabolic (catalase activity, triene conjugates, and Schiff bases), inflammatory (interleukin 10, C-X-C motif chemokine ligand 10), proteomic (haptoglobin, zinc-α-2-glycoprotein, and calprotectin), genomic (epidermal growth factor receptor), and microbial candidates (*Veillonella* and *Streptococcus*). In combination, with each other and with other established screening methods, these salivary markers could be useful for improving early detection of the disease and ultimately improve the survival odds of LC patients. The existing literature suggests that saliva is a promising biological sample for identification and validation of biomarkers in LC, but how saliva can be utilized most effectively in a clinical setting for LC management is still under investigation.

1. Introduction

Lung cancer (LC) is the leading cause of cancer-related deaths globally [1]. The two main subtypes of LC are non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC), which account for 84% and 13% of LC, respectively [2–4]. Tobacco smoke is the single greatest risk factor of LC, though other less common risk factors include asbestos, radon, second-hand smoke, alcohol, arsenic, chromium, nickel, and polycyclic aromatic hydrocarbons [5, 6]. NSCLC can be further divided into adenocarcinoma (AC), squamous cell carcinoma (SCC), and large cell carcinoma (LCC). NSCLC has a poor five-year survival rate of 25%, often related to diagnosis of the disease at a late stage with frequent distant metastasis [4, 7]. There are two subtypes of SCLC, oat cell carcinoma, and combined-SCLC. The latter subtype is defined as SCLC with components of NSCLC [5]. SCLC has an exceptionally low five-year survival rate of less than 7% [4, 8] associated with its aggressive growth and high metastatic potential [9]. Early stages of SCLC may be treated by chemotherapy and radiotherapy, while NSCLC in its early stages may be treated successfully by surgical resection [10]. Indeed, if LC is diagnosed at an early and localised stage, the 5-year survival rate increases to 59%. Unfortunately, only 17% of all LC cases are diagnosed at this stage [4]. In order to improve treatment success in terms of reduced morbidity and mortality, early diagnosis of the disease is crucial.

Both low-dose computerized tomography (LDCT) and chest radiographs have been investigated as methods of LC-screening. In a randomized clinical trial comprising at least 53,000 heavy smokers, former and active, LDCT resulted in a 20% decrease in the LC mortality rate, as compared to the chest radiographs [11]. Consequently, several medical associations recommended LC-screening using LDCT for heavy active and former smokers [12–14]. However, LDCT-screening is not completely free from limitations, and it can result in false positive and negative results

and can cause a radiation hazard. The false positive results can lead to unnecessary further testing and invasive procedures, while the false negative results can delay necessary treatment [15, 16]. As a consequence of these limitations associated with LDCT, the development of complementary screening methods is highly coveted [17]. In this regard, molecular biomarkers are increasingly recognised as key knowledge not only to better understand LC biology but also to provide earlier and more precise diagnosis and to assign patients to the best targeted treatment available so that ineffective overtreatment is avoided.

Accordingly, several tumor markers such as mRNA [18, 19], microRNAs [20], cytokines [21], antioxidant enzymes [22], and fatty acids [23] spanning across several sample types such as blood/plasma [24, 25], sputum [26], and expired air [27, 28] have been investigated in LC. Although bodily fluids, such as blood, serum, urine, and sputum, have been extensively examined as liquid biopsy for diagnostic, prognostic, and predictive markers in LC, limited data exist on saliva as a potential liquid biopsy in LC [29].

Human saliva has been investigated as a biological fluid for diagnosis of diseases, including human malignancies. Saliva is a preferred biological sample as saliva collection is noninvasive and the procedure is quicker, cheaper, and more convenient for the patient as compared to invasive processes such as blood collection [30]. Importantly, saliva consists of a pool of biomolecules such as proteins, mRNA, miRNA, enzymes, and immunoglobulins coming from different sources, such as the salivary glands themselves [31, 32], secretions from nasal cavity and lower respiratory tract [33], gingival crevicular fluid [33, 34], and blood plasma as an ultrafiltrate [35] (Figure 1). Systemic diseases, including LC, may influence the salivary glands' function and subsequently the quantity and composition of saliva [36, 37]. In a lung cancer mouse model, a significant alteration of biomarkers in the saliva was observed. These observations suggest that tumors, even if not in close proximity, may release mediators affecting the salivary gland function and subsequently the composition of saliva [38]. In addition, saliva contains several types of bacteria, fungi, and virus species [39]. Change in the profile of these biomolecules and the microbiota in saliva in disease conditions forms the basis for the use of saliva in diagnosis and prognosis of human diseases.

The usefulness of salivary markers in both oral and systemic diseases has been investigated [40], though how markers of extraoral pathologies, like lung cancer, are found in saliva is not fully understood, and represents an important research area. This review is aimed at offering an overview of diagnostic and prognostic biomarkers in human saliva for LC (Table 1).

2. Methods

A literature search using the databases of PubMed and Google Scholar was performed. The search words involved the combination of the following terms from the Medical Subjects Headings (MeSH): "lung cancer," "biomarkers," and "saliva." The systematic search yielded 27 articles, in the timespan from 2011 to the 31st of December 2020. The inclusion criteria were as follows: (a) type of studies (human clinical studies) and (b) studies with full-text availability. The exclusion criteria were articles not related to LC and salivary biomarkers and/or articles for which full texts were not available in English. Additionally, individual articles retrieved manually from the reference list of the relevant papers were also included.

3. Metabolic and Inflammatory Biomarkers

Altered cellular metabolism has been identified as an emerging hallmark of cancer [41], opening an opportunity for biomarker discovery. Salivary metabolomics is a relatively new field, and accordingly, few studies have addressed the question of altered metabolic markers in saliva in cancer versus normal controls. It has, logically enough, primarily been applied to oral cancer but is increasingly expanding to more systemic diseases. The most frequently used techniques are ^{1}H+NMR and mass spectrometry [42]. Bel'skaya et al. performed a comprehensive biochemical analysis of unstimulated saliva from 425 LC patients (with no prior treatment) (consisting of AC, SCC, mixed ADC+SCC, neuroendocrine, and undifferentiated LC), 168 noncancerous lung disease patients, and 550 healthy controls [43]. A major shift in salivary metabolite composition, specifically those involved in lipid peroxidation and protein metabolism, as well as metabolic enzyme activity (increased alanine aminotransferase (ALT), decreased aspartate aminotransferase (AST), and decreased AST/ALT coefficient), was observed in LC as compared to healthy controls. The change in metabolic enzyme activity was also explored previously by Bel'skaya and Kosenok [44]. Although the histological subtypes were found to have similar metabolic enzyme activities, a significant difference was observed between LC (all subtypes) and healthy controls.

Bel'skaya et al. further investigated the value of additional markers for their diagnostic utility; however, none of the investigated biochemical salivary markers could be independently used in the early diagnosis of LC. The most informative biochemical parameters were catalase activity, level of triene conjugates and Schiff bases, pH, sialic acid, alkaline phosphatase, and chloride ion concentration in saliva. This panel of seven parameters could be used to diagnose LC with 69.5% and 87.5% sensitivity and specificity, respectively. Among these parameters, Bel'skaya suggested catalase activity to be the most important parameter for LC diagnosis [43]. In addition, Fourier transform infrared spectroscopy has been examined for its utility in detecting differences in biochemical salivary parameters between LC patients and healthy subjects. Most notably in the advanced stages of LC, a significant difference was evident at infrared spectra of 1070–1240 cm^{-1} [45].

The prognostic value of salivary biochemical markers was also investigated by Bel'skaya et al. [43]. An increased concentration of lactate dehydrogenase (LDH) activity and lower imidazole (IC) concentrations were found to be significantly associated with favourable prognosis of LC. A LDH concentration of more than 1133 U/L and less than

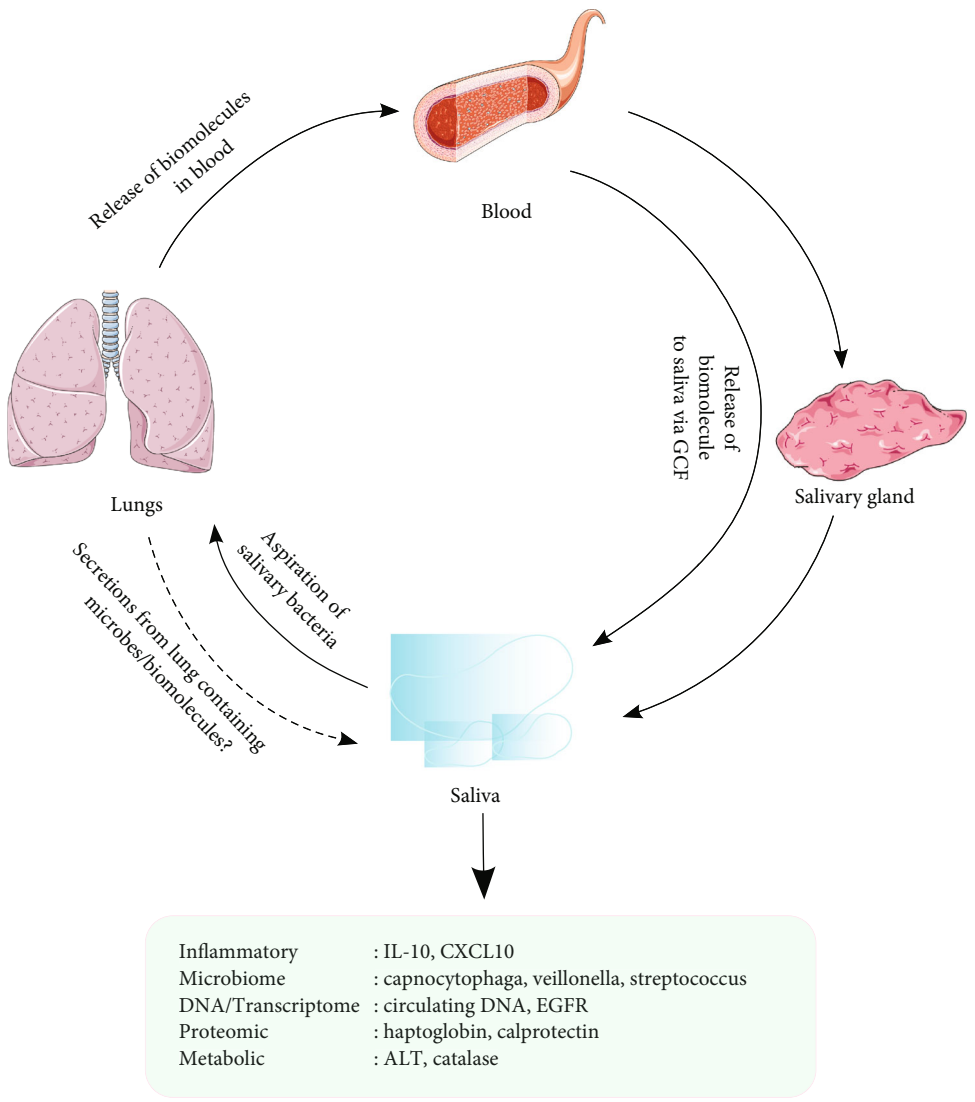

Figure 1: Schematic illustration showing possible pathways for enrichment of saliva for biomarkers in lung cancer [31–35].

0.311 mmol/L of IC, combined, could effectively predict a favourable outcome. Compared to patients with poor prognosis, the favourable outcomes were 1.4 (46.8% to 77.0%), 1.9 (27.1% to 47.5%), and 2.0 (18.0% to 43.3%) times more likely to survive at one, three, and five years. This was further studied in specific subtypes, and it was found that high LDH and low IC were favourable for SCC, but not for AC or neuroendocrine LC [46]. Instead, low IC levels combined with high seromucoids and uric acid were favourable for the prognosis of AC patients, and a combination of high NO, urea, and ALP was favourable for neuroendocrine tumors, as these values tend to decrease with the progression of the disease. As a predictive marker, C-reactive protein (CRP) may be of value as its concentration increases with tumor size and regional metastasis, especially in NSCLC [47].

One important caveat to using metabolic biomarkers for LC diagnosis was introduced in a systematic review of SCC of the aerodigestive tract [48]. Goh et al. found that the various classes of metabolites (branch chain amino acids, fatty acids, amino acids, carbohydrates, inorganic compounds, and lipids) showed considerable overlap in expression in LSCC, oesophageal SCC, and head and neck SCC, though predominantly between OSCC and HNSCC. In agreement with Bel'skaya et al., this further supports the need for a panel of metabolic markers, in conjunction with other proteomic and transcriptomic markers.

Inflammation is well known as both a cause and effect of tumor development. Chronic inflammation can lead to DNA damage and promote carcinogenesis. The inflammatory tumor microenvironment fosters invasion and metastatic potential of cancer cells [49, 50]. This makes inflammatory markers promising targets not only as diagnostic biomarkers but also valuable tools for determining prognosis. Several inflammation-related cytokines have been identified as significantly deregulated in NSCLC compared to healthy controls [47, 51]. Both proinflammatory and anti-inflammatory cytokines were overexpressed in the saliva of LC patients, including interleukin- (IL-) 1β, ILIRN,

Table 1: Summary of putative salivary markers in lung cancer.

	Author, year	Histological type	Sample size (LC/control)	Markers	Collection	Category	Sensitivity/ specificity
Metabolic	Bel'skaya, 2020 [43]	AC, SCC, AC +SCC, NEC	425/550*	Catalase activity, triene conjugates, Schiff bases, pH, sialic acid, alkaline phosphatase, chloride	Unstimulated WMS	Amino acids, biochemical	69.5%/87.5%
	Bel'skaya, 2017 [44]	AC, SCC, NEC	286/573	ALT, AST/ALT, ALP, GGT, ⍁-amylase	Unstimulated WMS	Biochemical	n/a
Inflammatory	Koizumi, 2018 [48]	NSCLC	35/35	IL-1β, ILIRN, IL7, IL10, CCL11, CXCL10, PDGF-BB, TNF	Unstimulated WMS	Protein	60.6%/80.8%**
Proteomic	Xiao, 2012 [53]	Not specified	26/26	Haptoglobin, zinc-⍁-2-glycoprotein, calprotectin	Unstimulated WMS	Protein	88.5%/92.3%
Transcriptomic	Zhang, 2012 [56]	NSCLC, SCLC	32/64	*CCNI*, *EGFR*, *FGF19*, *FRS2*, *GREB1*	Unstimulated WMS	mRNA	93.75%/82.81%
	Wei, 2014 [64]	NSCLC	40/n/a	*EGFR* 19-del *EGFR* 21-L858R	Unstimulated WMS	DNA	n/a
	Pu, 2016 [65]	NSCLC other, AC, SCC	17/n/a	*EGFR* 19-del *EGFR* 21-L858R	Not specified	DNA	n/a
	Ding, 2019 [66]	NSCLC other, AC, SCC	78/26***	*EGFR* 19-del *EGFR* 21-L858R	Unstimulated WMS	scfDNA†	n/a
Microbial	Zhang, 2019 [90]	AC, SCC	39/20	*Veillonella*, *Streptococcus*	Unstimulated WMS	16S rRNA	n/a
	Yan, 2015	AC, SCC	61/25	*Capnocytophaga*, *Veillonella*	Not specified	16S rDNA	84.6%/86.7%-78.6%/ 80.0%‡
	Yang, 2018 [92]	NSCLC	75/172	*Sphingomonas*, *Blastomonas*, *Acinetobacter*, *Streptococcus*	Unstimulated WMS	16S rRNA	n/a

*Also included 168 nonmalignant lung disease cases; **IL10 and CXCL10 only; ***also included 15 nonmalignant lung disease cases; †saliva circulating free DNA; ‡for SCC and AC, respectively. AC: adenocarcinoma; LC: lung cancer; n/a: not applicable; NEC: neuroendocrine cancer; NSCLC: non-small-cell lung cancer; SCC: squamous cell carcinoma; SCLC: small cell lung cancer; WMS: whole mouth saliva.

IL7, IL10, C-C motif chemokine 11 (CCL11), C-X-C motif chemokine ligand 10 (CXCL10), platelet-derived growth factor-BB (PDGF-BB), and tumor necrosis factor (TNF-α). Of these, the combination of IL10 and CXCL10 had the greatest diagnostic potential, with a sensitivity of 60.6% and specificity of 80.8%. The proinflammatory cytokines IL-6, IL-8, IL-18, and TNF-α have also been implicated in advanced LC [47].

4. Proteomic Biomarkers

Proteomic techniques have been predominantly used to analyse blood but have recently been adopted in salivary samples. Among such techniques are iTRAQ [52, 53] and two-dimensional gel electrophoresis (2-DE) [54, 55], which have been widely used to analyse the proteome of a number of LC subtypes. The salivary proteome has most often been profiled by two-dimensional gel electrophoresis with mass spectrometry (2DE-MS), though new techniques are being adapted to salivary proteomics as well. 2DE-MS was used in an investigation of 16 potential proteins as salivary biomarkers for early LC detection. Seventy-two subjects were enrolled in the study. The three proteins haptoglobin, zinc-2-glycoprotein, and calprotectin, combined, reached a sensitivity of 88.5% and specificity of 92.3% for diagnosis of LC [56]. Therefore, the combination of haptoglobin, zinc-α–2-glycoprotein and calprotectin represents a promising saliva-based diagnostic tool for LC.

Another mode of entry for biomolecules present in saliva is exosomal transport. These circulating exosomes contain lipids, proteins, and nucleic acids produced by tumor cells and can be transported in the blood as encapsulated membranes, the content of which resembles that of their parent tumor cells [57]. Sun and collaborators established a standardised method of exosome-isolation from saliva to compare their proteomic profiles. In saliva, 319 exosomal proteins were identified, along with 994 in serum, by liquid chromatography tandem mass spectrometry. Eleven exosomal proteins were discovered in saliva and plasma of LC patients that were not present in healthy subjects. This finding raises the possibility for the potential use of salivary exosomes as diagnostic biomarkers in LC [58].

5. Transcriptomic and Genomic Biomarkers

Several salivary transcriptomic and genomic biomarkers have received attention as molecules with diagnostic and prognostic potential. Among these are five mRNA candidates: *CCND1* (encoding for cyclin D1), *EGFR* (encoding for epidermal growth factor receptor), *FGF19* (encoding for fibroblast growth factor 19), *FRS2* (encoding for fibroblast growth factor receptor substrate 2), and *GREB1* (growth regulation by estrogen in breast cancer 1). The transcriptome signature of these genes was able to distinguish both NSCLC and SCLC from control subjects with a sensitivity of 93.75% and a specificity of 82.81% [59].

Currently, one of the most researched genetic markers for LC diagnostics is *EGFR*. *EGFR*-testing has traditionally been performed on surgically biopsied tissues. However, at the stage of biopsy taking, the LC has in most cases already progressed too far and frequent biopsies for monitoring *EGFR* mutations are impractical for these patients [60–62]. Therefore, the detection of *EGFR* by other means is highly sought after. *EGFR* is a membrane receptor frequently expressed in NSCLC that influences proliferation, angiogenesis, and chemoresistance, as well as inhibits apoptosis and promotes metastasis of NSCLC cells [63]. Identifying the presence and type of *EGFR* mutations is crucial in NSCLC patients as the common mutations, exon 19 deletion (19del) and exon point mutation 21-L858R (L858R) [64], are treatable by tyrosine kinase inhibitors such as erlotinib, gefitinib and osimertinib [65].

Electric Field-Induced Release and Measurement (EFIRM) has recently been introduced for the detection of mutations in *EGFR*. This method allows for cell-free DNA analysis using specific mutation-detecting probes, with improved sensitivity and specificity over PCR-based methods in NSCLC patients. Blood, urine, or saliva can be used as biological samples for EFIRM [66]. Two clinical studies, blinded, using EFIRM with saliva as a sample, identified the *EGFR* mutations exon 19del and L858R in NSCLC patients. The similarity between EFIRM-results and the gold standard of biopsy genotyping was high, 96-100% [67, 68]. Despite the promising results, the studies were of a small scale and need for large scale studies is evident, to explore the rate of false-positive and false-negative results [67, 68]. The method of *EGFR* detection by EFIRM fulfils many of the clinical requirements for successful and efficient detection and may become a clinical method in the future, on its own or with supplementary analysis of biopsies [67]. Another potential method of detecting *EGFR* mutations includes droplet digital PCR analysis of saliva-derived plasma cell-free DNA (plasma-cfDNA) and saliva cell-free DNA (saliva-cfDNA). No significant differences in the quantification or in concentrations of scfDNA were found between NSCLC, healthy or patients with benign lung lesions. However, the concordance rate of EGFR mutations between plasma-cfDNA and saliva-cfDNA was 83.78%[69]. Interestingly, a study by Li et al. [70] compared the concordance in detection of *EGFR* mutations of EFIRM and droplet digital PCR. The study involved thirteen patients with NSCLC, who donated plasma and saliva samples. Both *EGFR* mutations, exon 19del and L858R, were detected in both saliva and plasma samples with a sensitivity of 100%, while droplet digital PCR showed a sensitivity of 85.6% in plasma and 15.4% in saliva. The EFIRM-method was able to detect ultrashort (40-60 bp) circulating tumor-DNA fragments in saliva and plasma. This presents yet another promising and novel target for LC diagnosis in the earliest stages of the disease. In general, *EGFR* identification by EFIRM based on a simple saliva test provides high sensitivity. The method may be proven to be a great diagnostic supplement in the clinical setting.

6. Microbial Biomarkers

Bacterial homeostasis is important for normal bodily function, including in the oral cavity. The complex interaction

involved in homeostasis of normal oral flora is considered to minimize the growth of foreign microbial invaders and opportunistic microorganisms [71]. At least 700 unique bacterial species inhabit oral cavity, though more than half are currently impossible to culture [72]. When secreted, saliva is initially sterile [73] but is quickly contaminated by bacteria shed from the surfaces of tonsils, tongue, throat, and other oral surfaces [74, 75]. The normal oral microbiome, mainly comprised of the salivary microbiome and nonshedding bacteria on supra- and subgingival dental surfaces, has largely been characterised [76, 77]. The microbial profile of saliva mirrors the composition of microbiota on oral mucosa and on dental surfaces [76, 77]. The composition of oral or salivary microbiota has been suggested to reflect oral and general health status [78].

Bacterial dysbiosis is linked to the development of a number of diseases, not only at the site of bacterial imbalance but also at distant organs. Recently, there has been a growing interest in exploring the link between salivary bacteria and the incidence and severity of respiratory infections, including COVID-19 [79, 80]. An association between periodontal disease, an inflammatory condition in the gingiva and supporting structures of teeth induced by bacteria, and several respiratory infectious conditions has been reported previously [81]. It has been suggested that oral periodontopathic bacteria can be aspirated into the lung leading to pneumonia [82, 83]. Furthermore, microbial dysbiosis at different organs and in the body fluids including that of saliva has been linked to several types of cancer, such as oral, oesophageal, colorectal and lung cancer [77, 84–88]. As an example, in colorectal cancer specimens, significantly higher levels of *Prevotella*, *Escherichia coli*, *Bacteroides fragilis*, *Streptococcus gallolyticus*, *Enterococcus faecalis*, and *Streptococcus bovis* have been detected as compared to normal colon tissues [88–90]. Similarly, in another study, significantly higher levels of *Fusobacterium nucleatum* (*F. nucleatum*) and *Clostridium difficile* were observed in patients with colorectal cancer as compared to control subjects [91]. Interestingly, enrichment of some of these bacteria such as *Prevotella* and *F. nucleatum* has been shown in oral squamous cell carcinoma specimens [87].

Several studies, using 16S rRNA sequencing technology, have reported a differential profile of salivary microbiota in LC patient as compared to the control specimens. Zhang and collaborators, using 16S rRNA sequencing technology, reported a higher richness and lower diversity of salivary microbiota in NSCLC patients as compared to that of healthy subjects [92]. The authors also reported an increase in *Veillonella* and *Streptococcus* and a simultaneous decrease of *Fusobacterium*, *Prevotella*, *Bacteroides*, and *Faecalibacterium* genera in NSCLC patients compared to the control subjects [92]. Similar findings have been reported by Yu and coworkers in NSCLC specimens [93]. In parallel to the above observation in LC specimens, Yan et al. found increased abundance of *Veillonella* and *Capnocytophaga* in saliva from LC patients (SCC and AC) as compared to that of normal controls [94]. Of note, the enrichment profile of *Veillonella* and *Capnocytophaga* in saliva was able to distinguish control subjects from lung SCC with a specificity of 86.7% and sensitivity of 84.6%, and from AC with 80% and 78.6%, respectively [94]. Interestingly, saliva from non-smoking female LC patients was reported to be enriched with *Sphingomonas* and *Blastomonas* and diminished with *Acinetobacter* and *Streptococcus* as compared to normal controls [95]. These observations, although different from other studies using saliva from LC patients, could be related to that fact that LC in non-smokers is considered to be a different disease compared to smoking related LC [54, 96–98]. Overall, these observations indicate that LC might be associated with microbiome dysbiosis in saliva and profiling of salivary microbiome might have a diagnostic value in LC.

Despite the association between microbial profile in saliva and LC as described above, the possible contribution of salivary microbiota to LC carcinogenesis is not understood. Nevertheless, salivary microbiota has been shown to influence p53 and apoptosis signalling pathways in LC tumor cells [95]. In addition, salivary microbiota has been shown to influence systemic inflammatory status in LC patients [92]. A positive correlation between *Veillonella* in saliva from NSCLC and neutrophil-lymphocyte ratio and a negative correlation between *Streptococcus* and lymphocyte-monocyte ratio have been reported. The same authors reported a decrease in folate biosynthesis and an increase in xenobiotics and amino acid metabolism in salivary metabolites from NSCLC patients [92]. Given the association between inflammation and metabolic deregulation and LC [49, 50, 92, 99, 100], the above observations indicate a possible link between dysbiotic salivary microbiota and LC carcinogenesis. However, larger and longitudinal studies are needed to clarify these suggestions.

The potential use of salivary microbiota as a diagnostic biomarker has several limitations. The microbiota is dynamic, and it continuously changes to local and systemic conditions. Moreover, its composition depends on the host's age, ethnicity, diet, oral hygiene, dental status, antibiotic use, and smoking habit [101–105]. Most of the studies so far on this topic have been conducted in Chinese population. As a result, a standardised set of microbial diagnostic markers for LC is still in its infancy.

7. Conclusions

Saliva as a biological sample offers several advantages. Saliva collection is a noninvasive procedure, is quicker, cheaper and more convenient for the patient as compared to invasive procedures such as blood collection. Importantly, saliva consists of a pool of biomolecules coming from different sources, such as the salivary glands themselves, secretions from nasal cavity, and lower respiratory tract. The composition of saliva is suggested to reflect local and systemic health and disease conditions. In line with this, several studies have supported a link between LC and qualitative and quantitative changes in salivary composition. Accordingly, there is a growing interest in the identification of saliva-based biomarkers in LC. Recent studies have identified a number of saliva-based protein, genomic and transcriptomic, and microbial biomarkers with diagnostic and prognostic value in LC. Among them, mutation status in *EGFR* in saliva from

LC patients has emerged as potential diagnostic/prognostic marker in LC. Additionally, the salivary microbiome is a growing and fresh research area which may provide identification of microbiome-based markers in LC. However, the diagnostic and prognostic value of individual salivary markers for LC seems limited. This supports the need for identification of panel of markers, preferably combining inflammatory, genomic, transcriptomic, and microbial markers in saliva. At present, studies exploring the use of salivary diagnostic biomarkers for LC are limited to small-scale studies. Studies with larger patient groups are needed to assess salivary biomarkers' diagnostic reliability in larger and more diverse populations.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Childhood Allergy Disease, Early Diagnosis, and the Potential of Salivary Protein Biomarkers

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Allergic disease has risen to epidemic proportions since the last decade and is among the most common noncommunicable, chronic diseases in children and adolescents worldwide. Allergic disease usually occurs in early life; thus, early biomarkers of allergic susceptibility are required for preventive measures to high-risk infants which enable early interventions to decrease allergic severity. However, to date, there is no reliable general or specific allergy phenotype detection method that is easy and noninvasive for children. Most reported allergic phenotype detection methods are invasive, such as the skin prick test (SPT), oral food challenge (OFC), and blood test, and many involve not readily accessible biological samples, such as cord blood (CB), maternal blood, or newborn vernix. Saliva is a biological sample that has great potential as a biomarker measurement as it consists of an abundance of biomarkers, such as genetic material and proteins. It is easily accessible, noninvasive, collected via a painless procedure, and an easy bedside screening for real-time measurement of the ongoing human physiological system. All these advantages emphasise saliva as a very promising diagnostic candidate for the detection and monitoring of disease biomarkers, especially in children. Furthermore, protein biomarkers have the advantages as modifiable influencing factors rather than genetic and epigenetic factors that are mostly nonmodifiable factors for allergic disease susceptibility in childhood. Saliva has great potential to replace serum as a biological fluid biomarker in diagnosing clinical allergy. However, to date, saliva is not considered as an established medically acceptable biomarker. This review considers whether the saliva could be suitable biological samples for early detection of allergic risk. Such tools may be used as justification for targeted interventions in early childhood for disease prevention and assisting in reducing morbidity and mortality caused by childhood allergy.

1. Introduction

1.1. The Allergy Epidemic. Allergy is a hypersensitivity reaction triggered by immune system mechanisms. Atopy is defined as a personal or familial tendency to develop IgE antibodies in response to low-dose common environmental antigens (allergens), such as pollens, dust, and various food kinds, as confirmed by a positive skin prick test (SPT). These allergens are environmental factors in which the immune system generally does not develop an immunological response [1, 2]. Allergic sensitization normally starts in childhood, and the number of allergens to which a patient is sensitized might grow with time [3]. Allergic diseases include asthma, atopic dermatitis (AD), allergic rhinitis (AR), food allergies (FA), and anaphylaxis.

Allergic disease has risen to epidemic proportions since the last decade [4–7]. Recent data suggest that prenatal events, such as environmental influences on placental function and fetal programming, have a critical role in determining disease susceptibility. Furthermore, evidence suggests that allergic disease is associated with immune system deviations that occur *in utero* [8, 9]. Th1-dependent antimicrobial

immunity suppression in the neonatal period is a consequence of fetomaternal tolerance to prevent fetus rejection, leading to Th2 dominance and, in combination with other events and exposures, could predispose children to allergic disease [10, 11].

Most concerning is the increase in allergic and autoimmune diseases in the last 50 years indicating the susceptibility of immune pathways to modern environmental influences. Allergic diseases affect more than 20% of the population worldwide, especially in industrialised countries [5]. Furthermore, allergic diseases are among the most common chronic noncommunicable diseases in children and adolescents worldwide, with an estimated 50% of all schoolchildren suffering from allergic diseases [12]. Thus, allergy is a complex and heterogeneous disease that presents a significant burden to human health and preventative measures to reduce this burden are urgently required. Early detection of allergic susceptibility may be an approach by which prevention or interventions could be introduced to decrease allergic severity.

In children, asthma frequently coexists with allergies and other allergies which includes AD, AR, and FA [13, 14]. Most young children with severe AD have an increased risk of developing asthma and rhinoconjunctivitis. However, most cases of AD are mild to moderate in the general population. The "atopic march" is a pattern of progression through multiple allergy illnesses in early childhood in which individuals who first present with AD later develop AR and eventually atopic asthma [15]. Saunes et al. investigated the risk of the current asthma and the coexistence of allergy-related diseases in children aged six [16]. According to their findings, although most cases of AD in the general population were mild to moderate, early AD was linked to an increased risk of developing childhood asthma [16]. These findings support the hypothesis of an atopic march in the general population that includes the progression of atopic diseases, consisting of asthma, AD, AR, and FA. Moreover, evidence shows strong epidemiological and pathophysiological association between AR and asthma in adults and children. In both adult and paediatric populations, the ARIA classifications of symptom duration (intermittent and persistent) and severity (mild, moderate, and severe) have been validated [6]. Furthermore, clinical characteristics and comorbidities play a significant role in the atopic march and the progression from AR to asthma is commonly documented in chronological order.

1.2. Treatment of Allergy. Paediatric immune responses are more flexible and may respond better to treatment; thus, interventions made early in life have a greater likelihood of changing the natural history of respiratory allergies [17]. A study revealed that 48% of children with persistent wheezing (the hallmark symptom of childhood asthma) and positive SPT had symptoms of AR but none of AR symptoms in children with early-transient wheezing [18]. Another study reported that prescribing inhaled fluticasone propionate to preschool children for wheezing had no effect on the natural history of asthma or wheeze later in childhood and that it did not prevent lung function decline or lower airway responsiveness [19]. There is substantial evidence of a relationship between AR and asthma, as well as a link between childhood AR and adult asthma [17]. The hypothesis is that AR may itself be an asthma risk factor. Therefore, asthma burden in later life may be reduced by more focused treatment of AR in childhood because the mechanism of AR usually underlies the clinical syndrome of asthma [17].

Current therapies can control allergic symptoms but are not a cure for allergic diseases. Current drugs, such as $\beta 2$ agonist inhalers, antihistamines, and adrenaline, are used for allergy treatment and act on Th2-immune responses to inhibit the allergic disease [3]. Recent findings indicate that other immune responses are involved in allergic disease, including Th17-cells, Th1-type cytokines, and innate immune system, suggesting a promising therapeutic role of new agents that can block the action of these specific cytokines to improve the management of allergy and asthma. Specific immunotherapy to desensitise patients to allergens has been used for many years. However, risks of allergic reactions, including anaphylaxis, can occur as a consequence of this therapy and result in a life-threatening situation for the patient [3]. Other therapies being developed include targeting intrinsic structural defects, such as in the bronchial epithelium [5]. Nonetheless, the most effective way of reducing the overall burden of allergic disease is to implement early preventative strategies targeting allergic disease in children.

The administration of increasing amounts of specific allergens to which the patient has type I immediate hypersensitivity is known as allergen-specific immunotherapy (ASIT). AR, allergic asthma, and hymenoptera hypersensitivity are all treated with this disease-modifying therapy [20, 21]. Indications for ASIT include (1) insufficient symptom control despite pharmacotherapy and avoidance measures, (2) a goal to reduce AR and/or asthma morbidity, as well as the risk of anaphylaxis from future exposure, (3) when the patient is experiencing unfavourable pharmacotherapy side effects, and (4) when avoidance is not possible. Moreover, ASIT is cost effective compared with pharmacotherapy over a duration of time [20]. In AR and asthma therapy, the mode of ASIT administration is via the subcutaneous route by the physician or via the sublingual route and oral route by the patient [21]. Recent studies reported that immunotherapy appears to prevent the development of new allergy sensitizations and/or asthma in children with AR [17, 20]. Humoral, cellular, and tissue level changes occur with ASIT. These include large increases in anti-allergen IgG antibodies, a decrease in postseasonal rise of anti-allergen IgE antibodies, lower numbers of nasal mucosal mast cells and eosinophils, induction of Tregs, and inhibition of Th2 lymphocytes more than Th1 lymphocytes. A rise in IL-10 and TGF is shown as a result [20]. These recent studies suggest ASIT remains an important disease-modifying therapy in patients with allergic disease.

2. Early Detection of Allergy in Children

As allergic diseases usually occur in early life, early biomarkers of atopic susceptibility are required to target allergy and introduce preventive measures to high-risk infants. However, to

date, there is no reliable general or specific allergy phenotype detection method that is easy and noninvasive for children. Most reported allergic phenotype detection methods are invasive, such as skin prick test (SPT), oral food challenge (OFC), and blood test, and many involve not readily accessible biological samples, such as cord blood (CB), maternal blood, or newborn vernix [22–27].

Early markers of atopic predisposition, such as cord serum IgE (CS-IgE) levels and maternal blood concentrations of IgE, have been used to target allergy-preventive measures in high-risk infants [24]. A high level of CS-IgE is thought to be a risk factor for subsequent allergies in children, and it can be used to predict atopic symptoms up to the age of 20. In Finnish populations, the combination of increased CS-IgE and a positive family history of allergy is strongly associated with subsequent atopic manifestations [24]. Nabavi et al. studied 181 Iranian neonates and their mothers showing that IgE maternal blood concentration was correlated with IgE concentrations in CB [25]. Further results showed that the presence of any kind of allergic disorder in the mother and elevated maternal blood IgE level was associated with CB IgE in the child [25].

In AD, SPT is the gold standard method for allergy diagnosis. SPT is invasive but is the main tool in allergy diagnostics. However, there is a mixed opinion regarding the clinical usefulness of SPT [27]. SPT enables the identification of people who are at risk for FA as well as the specific allergen that is causing the eczematous flare-up. Positive SPTs to foods, when performed by a nonspecialist, might lead to prolonged elimination diets, which can result in nutritional deficiencies, loss of tolerance to avoided foods, and increment of healthcare costs [22]. Therefore, there is a tremendous need for early, noninvasive biomarkers to identify individuals who are at risk of AD. Protein abundances in newborn vernix, such as polyubiquitin-C and calmodulin-like protein 5, show a strong negative correlation to the AD group [26]. Polyubiquitin-C and calmodulin-like protein 5 have the potential to replace SPT as a noninvasive allergy diagnosis in children and are promising candidates as biomarkers for identifying newborns predisposed to develop AD.

FA can be diagnosed using diagnostic decision levels and component-specific IgE. OFC remains the gold standard diagnostic for FA, but it is time consuming, expensive, and risky in terms of the child developing a severe allergic reaction during the test [27]. Nevertheless, OFC may also be an alternative way to reduce parental anxiety and improve education [23]. An ideal *in vitro* test, such as the clinical performance of microarray for specific IgE detection in children with challenge-proven/excluded cow's milk protein allergy (CMPA) or hen's egg (HE) allergy, could be a safer alternative to OFC [28]. D'Urbano et al. showed that in children with suspected CMPA or HE allergy, the microarray has a good ability to predict OFC results [28]. Furthermore, this approach decreases the number of OFCs performed and decreases the risk of a severe reaction; however, it is not cost effective [28]. Therefore, owing to severe reaction risk that may be caused by OFC, another cost-effective and noninvasive pretest is needed.

Anaphylaxis is diagnosed mostly based on clinical criteria and not on aberrant results from laboratory testing such as serum total tryptase levels. Anaphylaxis diagnosis is not fully excluded regardless of normal results in laboratory tests [29]. Asymptomatic sensitisation is common in the general population; thus, positive SPT or increased serum-specific IgE levels that test for potential triggering allergens confirm sensitisation but do not diagnose anaphylaxis [29]. Thus, identifying a biological test that is noninvasive, safe, and cost effective is urgently required.

Saliva is a biological sample that is easy to collect via a painless procedure. Furthermore, saliva is the best approach for biomarker measurement as it is easily accessible, noninvasive, and an easy bedside screening for real-time measurement of the ongoing human physiological system [30–32]. All of these benefits highlight saliva as a very promising diagnostic candidate for detection and monitoring of disease biomarkers, especially in infants, toddlers, children, and anxious or uncooperative patients [33]. However, to date, saliva is not considered a medically acceptable biomarker. Interestingly, recent studies of saliva suggest that it can be used for the detection of head and neck carcinoma, breast and gastric cancers, salivary gland disease, Sjögren syndrome, systemic sclerosis, dental and gingival pathology, preeclampsia, and psychiatric and neurological diseases [30, 34–38]. Protein biomarkers have the advantages as modifiable influencing factors rather than genetic and epigenetic factors that are mostly nonmodifiable factors for allergic disease susceptibility in childhood. Saliva has great potential to replace serum as a biological fluid biomarker in diagnosing clinical allergy, especially in infants, toddlers, children, anxious, and uncooperative patients [33]. Moreover, saliva is an easily accessible, noninvasive, real-time measurement of the ongoing human physiological system and consists of an abundance of biomarkers, such as genetic material and proteins [30–32].

This review considers whether the saliva could be suitable biological samples for early detection of allergic risk. Such tools may be used as justification for targeted interventions in early childhood for disease prevention and assist in reducing morbidity and mortality caused by childhood allergy.

3. Biological Marker (Biomarker)

3.1. Criteria for a Biological Marker (Biomarker). A biological marker (biomarker) is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmaceutical responses to therapeutic intervention, according to the National Institutes of Health [39, 40]. Any biomolecule or a specific characteristic, feature, or indicator of a change in any biological structure and function that can objectively measure the state of a living organism is referred to as a biomarker [41].

The criteria for biomarkers include

(1) A significant oxidative modification product that may be directly linked to the onset of illness

(2) A stable product that is resistant to artefact induction is difficult to lose and does not change throughout storage

(3) Representative of the balance between oxidative damage generation and clearance

(4) Verified by an analytical assay that is specific, sensitive, reproducible, and robust

(5) Free of confounding and interference variables from dietary consumption

(6) Accessible in a target tissue or a valid surrogate tissue such as a leukocyte

(7) Detectable and quantifiable within the limits of detection of a reliable analytical procedure [40, 42]

3.2. Criteria for Potential Salivary Protein Biomarkers for Allergy. Therefore, to choose salivary protein targets for this current study, specific criteria for selection were defined. The criteria include

(1) Exclude proteins that are

  (a) Abundantly expressed in saliva

  (b) Associated with other pathologies

  (c) Altered in an inflammatory response in a nonspecific manner

  (d) That change with age and sex

(2) Include proteins that are

  (a) Acknowledged to be associated with the disease of interest

  (b) Not commonly observed in saliva

In addition, a confounding factor that should be considered includes proteins that are produced by the salivary glands versus proteins that enter saliva by diffusion from the circulation.

4. Saliva Samples

4.1. Types of Saliva Samples. There are two types of salivation, unstimulated and stimulated salivation. Unstimulated salivation is watery saliva produced by the salivary gland at rest, reflects the basal salivary flow rate, and is stimulated by parasympathetic innervation. Unstimulated salivation occurs for about 14 hours a day, and 90% of this saliva is produced by major salivary glands [43]. On the contrary, stimulated salivation represents the thicker secretion during food intake and is stimulated by sympathetic innervation. This saliva is present in our mouths for up to two hours and contains more salivary protein in the afternoon than in the morning; its concentration follows this diurnal pattern [43].

Stimulated salivation is preferable especially in children, in which the children can easily chew on the swab to stimulate saliva production.

4.2. Saliva Sample Collection Method. Four commercially available saliva collection devices are described in Figure 1 and include

(1) Unstimulated saliva: drool and SalivaBio Oral Swab (SOS) (Salimetrics, Carlsbad, California, the United States)

(2) Mechanically stimulated saliva: Salivette with a cotton swab (Sarstedt, Thermo Fisher Scientific, California, United States)

(3) Mechanically stimulated saliva: Salivette with a synthetic swab (Sarstedt, Thermo Fisher Scientific, California, United States)

(4) Acid stimulation saliva: GBO Saliva Collection System (Greiner Bio-One, Kremsmünster, Austria)

There are variations of salivary protein composition and salivary flow rate depending on the methods used in saliva sample collection [44]. The standard drool method (Figure 1(a)) had significantly higher salivary protein concentration as compared with the GBO Saliva Collection System method (Figure 1(d)). Furthermore, when compared to mechanically stimulated methods, salivary flow rates were significantly lower in unstimulated saliva, which includes drool (Figure 1(a)) and SalivaBio Oral Swab (SOS) (Figure 1(b)). These findings revealed significantly relevant differences in analyte levels and the salivary flow rates are determined by the saliva collection method [44].

However, based on the current study population having children between 6 months to 5 years old, mechanically stimulated saliva using Salivette with a cotton swab (Sarstedt, Thermo Fisher Scientific, California, the United States) (Figure 1(c)) was the most suitable saliva collection device to be used in children. This method was chosen as it was an easy, noninvasive, and painless procedure [32]. Unstimulated saliva using drool or SOS and acid stimulation saliva using the GBO Saliva Collection System is not feasible and can be extremely difficult to perform on these children.

4.3. Children Saliva Sample Collection Protocol Using Salivette. There are two methods to collect the children saliva, depending on the child's age, using Salivettes with a cotton swab (Sarstedt, Thermo Fisher Scientific, California, United States) or the standard drool method. These two methods were chosen and conducted according to the flexibility and cooperation of the child during the saliva sample collection procedure. The saliva sample collection procedure was conducted during a fixed time between 8.30 in the morning to 12 noon to enable the researcher to minimize the baseline variations, thus reducing the diurnal variations of salivary proteins.

If the child is less than 3 years old, the drooling saliva was collected from the child's mouth using the tube or swab. First, the Salivette was held at the rim of the

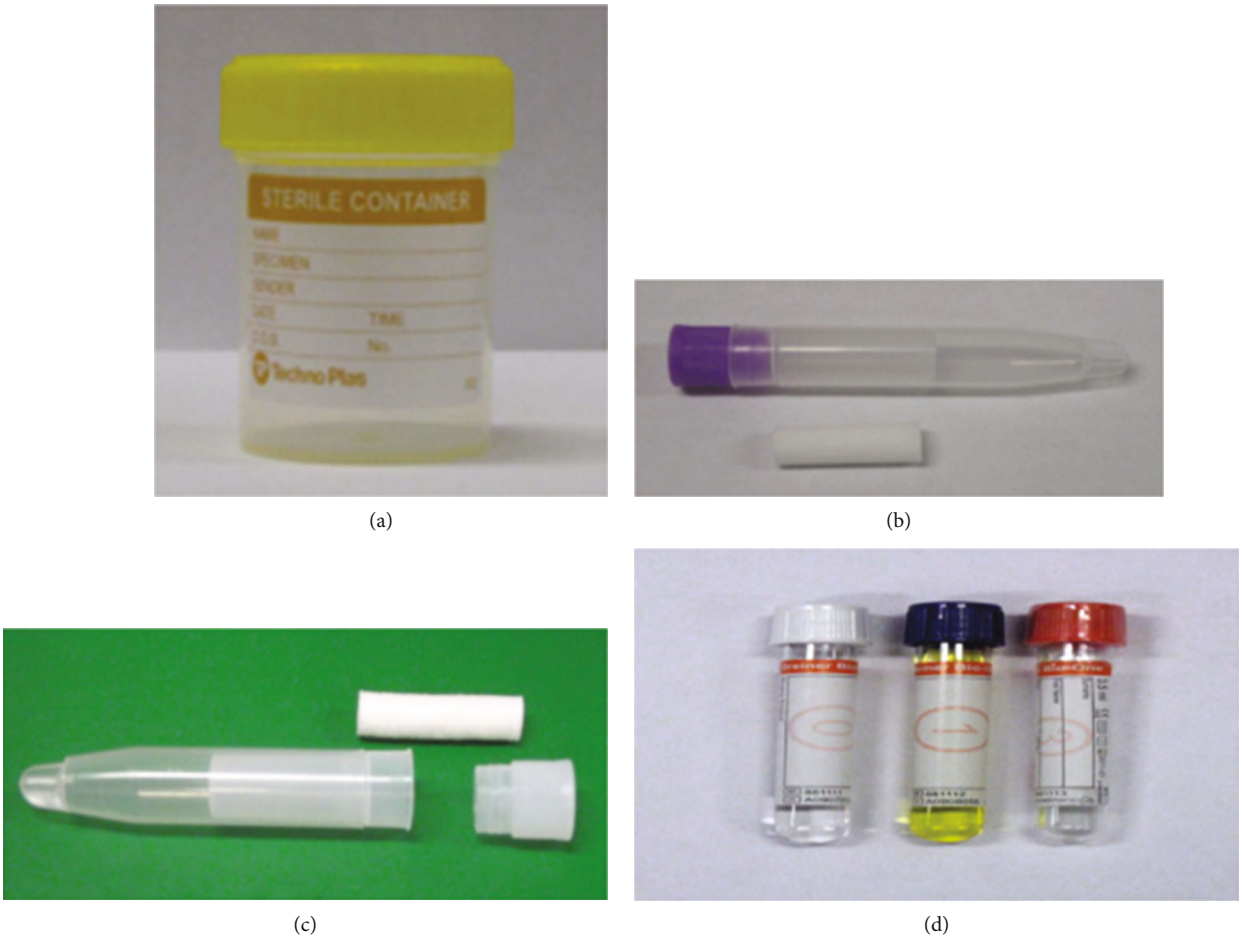

Figure 1: Commercially available saliva collection devices [44]. (a) Drool collected in a sterile specimen container. (b) SalivaBio Oral Swab (SOS) (Salimetrics, Carlsbad, California, the United States). (c) Salivette: cotton and synthetic swab (Sarstedt, Thermo Fisher Scientific, California, United States). (d) GBO Saliva Collection System (Greiner Bio-One, Kremsmünster, Austria).

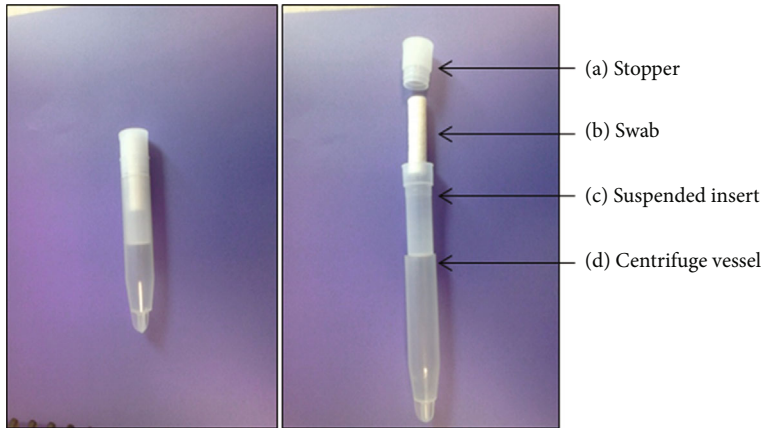

Figure 2: Salivette's parts. (a) Stopper. (b) Swab. (c) Suspended insert. (d) Centrifuge vessel.

suspended insert (Figure 2(c)) and the stopper is parted (Figure 2(a)) by slightly pushing it to the side. Then, the swab (Figure 2(b)) is removed from the Salivette and the tube is placed at the tip of the child's mouth to collect the drooling saliva in younger children (Figure 2).

Older children were asked to spit inside the tube, or saliva was collected from the child's mouth by inserting the cotton swab and allowing them to hold or chew it in their mouth for 1 minute (Figure 3). Approximately 1 ml of saliva volume was collected with both methods.

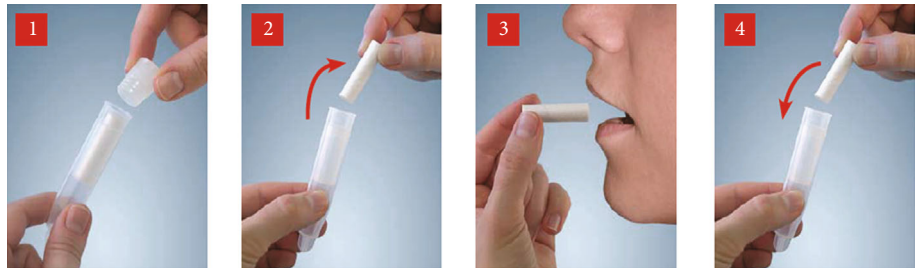

Figure 3: Steps of Salivette collection method. (1) The Salivette is held at the rim of the suspended insert, and the stopper is parted by slightly pushing it to the side. (2) The swab is removed from the Salivette. (3) Let the child chew the swab in his or her mouth for 1 minute, or the tube is placed at the tip of the child's mouth to collect the drooling saliva. (4) The swab is put back to the suspended insert, and the Salivette is closed firmly with the stopper.

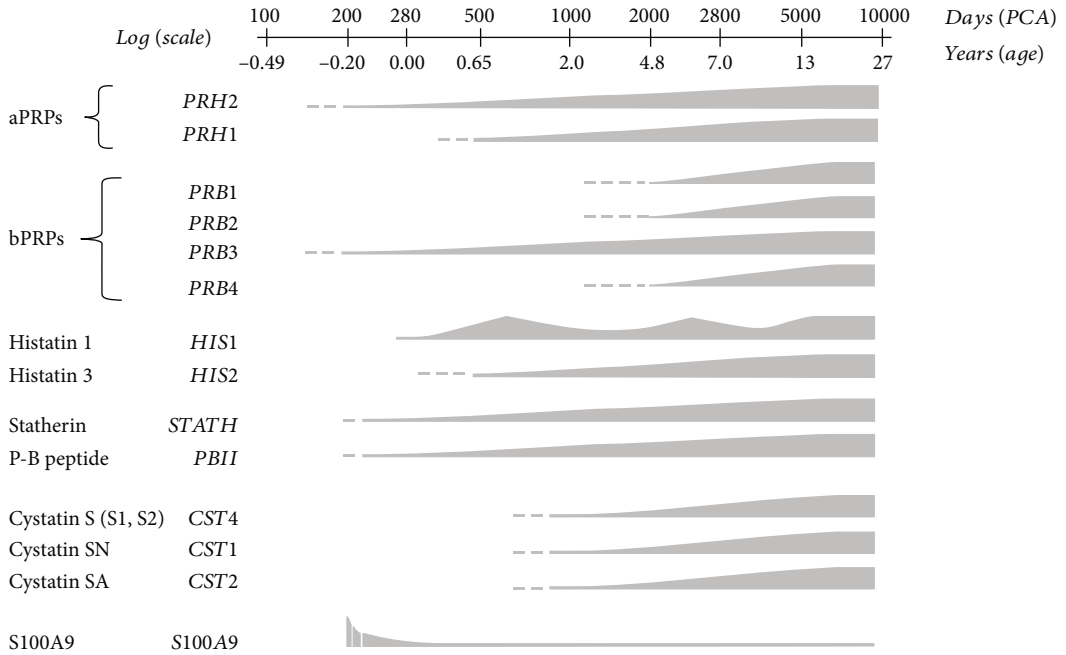

Figure 4: Scheme describing the approximate time course of different salivary proteins and peptides [48]. A function of age is the sum of extracted ion chromatogram (XIC) peak regions of all members belonging to the same family of proteins/peptides that are grouped based on the locus. A logarithmic scale was used in the abscissa axis to better highlight major changes in preterm newborns during the first months of life, which corresponds to the last months of fetal development, and those that occur immediately after the normal term of delivery and in the first years of life. Dashed lines indicate the age range in which the proteins were detected in at least one baby. The highest limit of this range corresponds to the age at which all saliva of the babies, even in small amounts, displayed the protein in the chromatographic profile.

After completion of the saliva collection, the Salivette was centrifuged at 3500 rpm for 30 minutes at 4°C. The centrifugation was performed within 4–6 hours after the saliva collection to avoid further protein degradation. A 10 μl aliquot of protease inhibitor cocktail (PIC) (v/v 1 : 100), Sigma Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, Missouri, the United States), was added to the Salivette before saliva collection to inhibit protein degradation. The Salivette and saliva were kept on ice (0 to −4°C) during the whole collection procedure as PIC will degrade if kept at a temperature higher than 4°C. After centrifugation, the solution of saliva and PIC was mixed and aliquoted to several cryotubes containing at least 200 μl of saliva and stored at −80°C. Approximately 200 μl of saliva was required for the proteomic analysis; therefore, only 1 tube was thawed at one time and this minimized the protein degradation effect on thawing and refreezing of the saliva samples.

4.4. Saliva Sample Handling. Sample handling imposes a real challenge to saliva analysis. Saliva samples need to be collected and stored under conditions of minimal proteolysis, deglycosylation, and dephosphorylation to minimize protein degradation [45]. Protein degradation may be caused by several factors: A variety of factors can cause protein degradation including bacterial proteases that may present in saliva, high temperature, pH conditions, and the freezing and thawing cycle. [46]. The recommended protocol to prevent any degradation is that sample processing should

be performed on ice (0 to 4°C), and PIC is added immediately, centrifuged to remove insoluble material, and stored in −20 or −80°C [47]. However, without PIC, short-term storage (less than 24 hours) of freshly collected samples on ice is effective in preventing protein degradation without compromising the chemistry of the proteome [46]. Furthermore, minimizing the time elapsing between sample collection and analysis will potentially reduce the risk of protein degradation. All these influencing factors must be carefully considered to prevent protein degradation as minimal as possible.

5. Salivary Protein Confounding Factors and Childhood Allergy

In the process of identifying potential salivary biomarkers for allergy, meticulous attention should be given to some salivary proteins that are dependent on age, sex, or state of disease, especially caries lesions or periodontal disease [48, 49]. Messana et al. investigated that the human salivary proteome was studied in a large sample of subjects of various ages, ranging from preterm newborns of 180 days of postconceptional age to 17 year olds. The study defined the appearance and level changes in proteins typically found in adult saliva from the last months of fetal development to adulthood [48]. Evidence suggested that individual salivary proteome diversity is particularly significant in paediatric age, especially in preterm newborns, such as S100 proteins (Figure 4) [48]. This was founded on the principle that proteome variability, which occurs as a result of physiological changes over time, has a significant impact. Exploration of proteomic temporal changes is termed "chrono-proteomics" [48]. In relation to sex specificity, a study evaluated interindividual biochemical variation in a population of 268 systemically healthy young students [49]. Findings revealed that female participants had lower salivary pH, buffering capacity, protein content, MUC5B, secretory IgA, and chitinase activity than male subjects, whereas male subjects had higher MUC7 and lysozyme activity [49]. The findings demonstrate that essential salivary biochemical variables have distinctive distributions and interrelationships in a systemically healthy young adult population, emphasising significant gender variations in salivary biochemistry [49].

6. Saliva as Potential Diagnostic Fluid in Childhood Allergy

Previously, there has been controversy related to the use of saliva as a diagnostic fluid due to the low concentration of analytes in saliva compared to blood. Nevertheless, technological advances, increased sensitivity of molecular methods, and nanotechnologies, such as mass spectrometry (MS); have reduced these limitations [50]. The advantages of using saliva as a diagnostic fluid are listed in Table 1 [33].

Approximately 2700 salivary proteins have been identified, and this number is expected to increase dramatically in the future due to current advances in detection methods for biomarkers in saliva [30, 51]. Saliva proteins identified to date have been categorised according to their functions (Table 2). The main function is immunity that contributes 21% of all known salivary proteins [34]. This suggests that this particular fluid is a suitable biological fluid for identifying a biomarker of allergic risk.

Most diseases, such as cancer and allergy, are likely to be treated efficiently if the disease can be diagnosed early. For example, ovarian cancer, the fifth most frequent malignancy and the leading cause of death in females, has a five-year survival rate of 25% when detected at stage 4, compared to 93% when diagnosed at stage 1 [52]. Saliva has been used widely to detect a variety of diseases using proteomic approaches, including head and neck carcinoma (oral cavity, oropharynx, larynx, and salivary glands), breast and gastric cancers, salivary gland function and disease, Sjögren syndrome, systemic sclerosis, dental and gingival pathology, preeclampsia, and psychiatric and neurological diseases [30, 34–38] (Tables 3 and 4). Childhood susceptibility to allergy is most likely possible to be detected in saliva using a proteomic approach to identify novel proteins that vary with allergy.

Table 1: Advantages of salivary testing for diagnosis [33].

Advantages of salivary testing for diagnosis
Noninvasive, simple to use, and low cost
Safer to conduct than serum sampling (needles are not required)
Diagnostic values in real time
No need for trained medical professionals
Obtaining many samples is simple
At-home collection and screening are possible
Cross contamination risks are minimal
More cost-effective sampling, shipping, and storage compared to serum
Requires less manipulation during diagnostic procedures compared to serum
Screening assays are commercially available

Table 2: Saliva proteins are identified according to their functions. The table is adapted from [34].

Salivary protein percentage (%)	Salivary functions
28.7	Uncertain function
21.0	Immunity
15.4	Unknown function
9.7	Signal transduction
7.1	Cytoskeleton and endomembrane
5.2	Metabolism
4.8	Cell mobility and secretion
4.2	Cell multiplication and cell cycle
2.3	Transcription and ribosomes
1.6	Protein replication and reparation

Table 3: Salivary proteins that are involved in oncological pathologies [30].

Author, year	Proteins involved	Proteins not involved	Site of tumor
Nakashima et al., 2006	(i) Maspin (ii) Stathmin	—	Salivary gland
Contucci et al., 2005	—	(i) Statherin	Salivary gland
An et al., 2004	(i) Transketolase (ii) Dim1p (iii) v-Ha-ras oncogene (iv) Type I collagen pro alpha (v) Tumor necrosis factor (ligand) superfamily member 4 (vi) Pirin	—	Salivary gland with metastasis
Oshiro et al., 2007	(i) Alpha-1-B-glycoprotein (ii) Complement factor B proteins	(i) Cystatin S (ii) Parotid secretory factor (iii) Poly-4-hydrolase beta-subunit proteins	Head and neck
Dowling et al., 2008	(i) Beta fibrin (ii) S100 calcium-binding protein (iii) Transferrin (iv) IG heavy chain constant region γ (v) Cofilin-1 (vi) Transthyretin	—	Head and neck
Mizukawa et al., 2001	(i) Alpha-defensins (ii) Beta-defensins	—	Oral cavity
Contucci et al., 2005	(i) Statherins	—	Oral cavity
Wong, 2006	(i) Interleukin-8 (IL-8) (ii) Thioredoxin	—	Oral cavity
Pickering et al., 2007	(i) Endothelins	—	Oral cavity
Streckfus et al., 2000	(i) c-erbB-2 protein (ii) CA15-3	—	Breast
Tabak, 2001	(i) 15-3 cancer antigen	—	Breast

7. The Role of Saliva in Early-Life Allergy Detection

Saliva is a human body fluid with complicated constituents and various biological functions [31, 34, 53]. The concentrations of analytes in saliva are 1000-fold lower than those in human serum [53]. Like the serum, saliva contains hormones, amino acids, electrolytes, immunoglobulin(Ig), antibodies, growth factors, enzymes, microbes, and their products. The majority of these constituents enter saliva via passive diffusion, active transport, or extracellular ultrafiltration through blood barriers of capillary walls [53]. Furthermore, expectorated saliva contains a significant amount of total gingival crevicular fluid (GCF) from periodontal pockets throughout the mouth [54]. GCF is an inflammatory serum exudate produced by periodontal tissue inflammation that originates from the blood vessels in the gingival connective tissue [55]. It contains biological molecular markers accumulated from the systemic and surrounding circulation [38]. The GCF is composed of a complex combination of molecules from the blood, the host tissues, and the subgingival biofilm, including leucocytes, proteins, enzymes, tissue breakdown products, inflammatory mediators, and cytokines produced locally in reaction to the bacterial biofilm [55]. GCF is a good source of biochemical disease markers because it can accurately reflect the ongoing response of periodontal cells and tissues. Hence, saliva, which also contains GCF, is an important bodily fluid that reflects the physiological and pathological function of the human body [31].

Saliva's essential functions in the oral cavity are lubrication and binding, followed by solubilization of dry food, oral hygiene, starch digestion initiation, and immunity [36]. Lubrication aids in lubricating the hard and soft oral surfaces and is vital for speaking, mastication, and swallowing by providing a lubricious layer that contains mucins, proline-rich proteins, and water [53]. Saliva aids in bolus formation by moistening food, which protects the oral mucosa from mechanical damage. In addition, saliva aids in the preliminary digestion of food by containing salivary

Table 4: Salivary proteins that are involved in dental pathologies [30].

Author, year	Proteins	Related pathology
Dowd, 1999; Van Nieuw et al., 2004	(i) Mucins (ii) Proline-rich glycoprotein (iii) Statherin	Dental caries
Vitorino et al., 2006	(i) Proline-rich proteins (ii) Lipocalin (iii) Cystatins (iv) Amylase (v) Immunoglobulin a (vi) Lactoferrin	Dental caries
Rudney et al., 2009	(i) Statherin (ii) Truncated cystatin S	Dental caries and other diseases
Nishida et al., 2006	(i) IL-1 beta (ii) Albumin (iii) Aspartate aminotransferase	Periodontitis
Kibayashi et al., 2007	(i) Prostaglandin E(2) (ii) Lactoferrin (iii) Albumin (iv) Aspartate aminotransferase (v) Lactate dehydrogenase (vi) Alkaline phosphatase	Periodontitis
Fábián et al., 2007; 2008	(i) Immunoglobulin (ii) Molecular chaperone hsp70 (iii) Cystatin S (iv) Salivary amylase (v) Calprotectin (vi) Histatins (vii) Lysozyme (viii) Lactoferrin (ix) Defensins (x) Peroxidases (xi) Proline-rich proteins (xii) Mucins	Periodontitis
Ito et al., 2008	(i) Cystatins (ii) Lysozyme	Periodontitis

alpha-amylase, which breaks down carbohydrates into sugars, while salivary lipase initiates fat digestion [53]. It also facilitates taste perception and digestion of carbohydrates by salivary alpha-amylase. This process allows soluble food-derived molecules to enter the gustative papillae and buffer the acidity of the food with the bicarbonates [50].

Saliva also contains lysozyme, an enzyme that lyses bacteria and inhibits oral microbial populations from overgrowing [53]. The antibacterial and antiviral properties, as well as its maintenance of tooth and mucosal integrity, are mostly due to salivary mucins, which bind to bacteria and prevent bacterial adhesion to tooth enamel. Saliva proteins are identified according to their functions. Interestingly, the main known function of salivary proteins is immunity, with 21% of salivary proteins being involved in immune function [34]. Therefore, saliva is a promising biological sample that may contain biomarkers of allergic disease risk.

Proteomic technologies, which combine modern instrumentation and enhanced analytical procedures, are widely used in clinical applications involving biomarkers. Due to its high sensitivity and precision for mass measurement, mass spectrometry- (MS-) based approaches for salivary biomarker identification have become one of the core technologies for proteomics in the last decade [56]. These include a variety of MS techniques, such as two-dimensional gel electrophoresis–mass spectrometry (2-DE/MS), liquid chromatography-tandem mass spectrometry (LC-MS/MS), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS), and surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF/MS), which have been used to identify biomarkers in saliva on a large scale, but not in relation to allergic diseases (Table 5) [56]. Although MS has been used extensively, most studies have moderate sample sizes (less than 45) as MS is an expensive method. Therefore,

Table 5: Summary of MS-based methods used for salivary biomarker identification. The table is adapted from [56].

Disease	Saliva	Stimulation	Proteomics approach	Biomarkers	Verification methods	References	Sample size
Breast cancer	Whole	Stimulated	SELDI-TOF/MS	(i) c-erbB-2	(i) ELISA (ii) Western blot	Streckfus et al., 2006	Control—3 Disease—3
Caries	Whole	Unstimulated	2-DE/MS	(i) Statherin 5 (ii) Cystatin	(i) Western blot	Rudney, et al., 2009	Control—18 Disease—23
Gastric cancer	Whole	Unstimulated	MALDI-TOF/MS	(i) 1472.78 Da (ii) 2936.49 Da (iii) 6556.81 Da (iv) 7081.17 Da	—	Wu, 2009	Control—18 Disease—23
Graft versus host disease	SM/SL	Stimulated	SELDI-TOF/MS MALDI-TOF/MS	(i) Lactoferrin (ii) SLPI (iii) IgA (iv) b2-microglobulin	(i) ELISA	Imanguli et al., 2007	Control—0 Disease—41
HNSC	Whole	Stimulated	LC–MS/MS	(i) Complement factor B	(i) Western blot	Ohshiro et al., 2007	Control—5 Disease—3
Oral lichen planus	Whole	Unstimulated	2-DE MALDI-TOF/MS	(i) Urinary prokallikrein (ii) PLUNC	—	Yang et al.,2006	Control—6 Disease—6
OSCC	Whole	Unstimulated	2-DE/MS LC–MS/MS	(i) M2BP (ii) Catalase (iii) Profiling1 (iv) CD59 (v) MRP14	(i) ELISA (ii) Western blot	Hu et al., 2008	Control—64 Disease—64
Pulmonary disease	Whole	Unstimulated	2-DE/MS	(i) Lipocalin (ii) Apolipoprotein A1	—	Nicholas et al., 2010	Control—20 Disease—25
Type 1 diabetes	Whole	Stimulated	2-DE MALDI-TOF/MS	(i) α-amylase (ii) Cystatin (iii) PIP	—	Hirtz et al., 2006	Control—8 Disease—8
Type 2 diabetes	Whole	Unstimulated	LC–MS/MS	(i) A1AT (ii) α-2-macroglobulin (iii) Cystatin C (iv) Transthyretin	(i) ELISA	Rao et al., 2009	Control—10 Disease—30

SM represents submandibular; SL represents sublingual; HNSC represents head and neck squamous carcinoma; OSCC represents oral squamous cell carcinoma; SS represents Sjögren's syndrome.

validation methods used, such as ELISA and Western blot, with larger sample sizes have been used to confirm MS findings (Table 5).

8. Conclusions

Saliva sampling is a noninvasive and stress-free alternative to blood collection; thus, there is no discomfort or pain associated with blood venepuncture. It is a readily accessible secretion that is generally recognised as a possible clinical diagnostic medium [56]. Moreover, as compared with blood, saliva contains fewer proteins which reduce the possibility of nonspecific interference and hydrostatic interactions [40]. Protein concentrations in the blood can range from a few seconds to several months or longer, with protein half-lives ranging from a few seconds to several months or longer. Interestingly, the composition of saliva is less complex and variable than serum; therefore, it should accurately reflect the current condition of the body at any given time [40]. Furthermore, as compared to blood, other advantages include easy and multiple sampling opportunities, less need for sample preprocessing and hence cost effectiveness, and minimal risk of contracting infectious organisms, and it is also an ideal biofluid for collecting specimens from patients in developing countries [56, 57]. Thus, saliva has the promising potential to

replace blood as the gold standard in diagnosing allergic diseases.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Cytokine/Chemokine/Growth Factor Profiles Contribute to Understanding the Pathogenesis of the Salivary Gland Dysfunction in Euthyroid Hashimoto's Thyroiditis Patients

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Hashimoto's thyroiditis (HT) is one of the most common autoimmune diseases. It is suggested that, in addition to thyroid gland dysfunction, HT is responsible for impaired secretion from the salivary glands. The aim of this study was to evaluate the extent of symptoms of salivary gland dysfunction. We also assessed the relationship between the levels of selected cytokines, chemokines, and growth factors in unstimulated whole saliva (UWS) and the rate of UWS secretion and symptoms of xerostomia in HT patients. The study group consisted of 25 female patients diagnosed with Hashimoto's disease in its spontaneous euthyroid state who had never received hormonal treatment. In more than half of the examined patients, we observed the level of UWS secretion below 0.2 mL/min, indicating impaired secretory function of the salivary glands. Moreover, we demonstrated that the clinical symptoms of salivary gland dysfunction worsen with disease duration. Nevertheless, the inflammatory changes occurring in these glands are independent of general inflammation in the course of HT. Our results clearly indicate an abnormal profile of cytokines, chemokines, and growth factors in the UWS of HT euthyroid women as well as the fact that concentrations of IL-6 and IL-1 as well as INF-γ, TNF-α, and IL-12 may be potential biomarkers for salivary gland dysfunction in the course of HT. Furthermore, salivary IL-12 (p40) may be helpful in assessing the progression of autoimmunity-related inflammation in the course of HT. In conclusion, secretory dysfunction of the salivary glands is closely related to autoimmunity-related inflammation in the course of HT, which leads to objective and subjective symptoms of dry mouth.

1. Introduction

Hashimoto's disease (HT) is classified as T cell mediated and concerns the thyroid gland. It is considered as one of the most frequent autoimmune diseases [1]. It affects approximately 2% of the population and is 5 to 10 times more common in women than men [2]. HT is characterized by the presence of thyroid autoantibodies, such as thyroid peroxidase antibodies (TPO-Ab) and thyroglobulin antibodies (TG-Ab), which leads to the destruction of THE thyroid tissue. Untreated HT may result in the development of papillary thyroid cancer and thyroid carcinoma [3]. HT is also one of the factors leading to the development of hypertension, cardiovascular diseases, dyslipidemia, obesity, insulin resistance, and depression [4–6].

Moreover, salivary gland involvement in patients with HT has been described in numerous studies. Considering the role of saliva in maintaining oral health, this observation is of utmost importance for both patients and dentists [3, 7, 8]. The quantitative and qualitative deficiency of saliva is a serious problem for the patient, even more so when accompanied by other symptoms of the underlying disease. Commonly observed symptoms associated with salivary gland dysfunction include a fissured, reddened, and sore tongue, atrophic and dry oral mucosa, and increased caries incidence, mainly in the cervical region of the teeth [9, 10]. Patients report difficulty swallowing food bites and eating foods with distinct flavors as well as problems with phonation [11]. The results by Agha-Hosseini et al. [4] as well as our own research [3] demonstrated that the secretory function of the submandibular glands in HT women is impaired. This submandibular gland disturbance of function manifested significantly reduced rate of unstimulated saliva secretion (UWS). By means of objective and quantitative salivary gland scintigraphy, the secretory function of the salivary glands was found to be significantly worsened in HT patients with xerostomia (subjective sensation of dryness) compared to HT patients without xerostomia and healthy subjects with or without xerostomia [7, 12]. Syed et al. [13] argue that clinical suspicion of thyroid disease should be considered in case of a chronic reduction in salivary secretion. Therefore, these authors suggest performing a thyroid function test (hormones, antibodies) if other causes of hyposalivation have been excluded. Our own study also confirmed that both the parotid and submandibular glands of women with euthyroid HT have an impaired ability to maintain redox balance, resulting in increased oxidative modification of salivary proteins, lipids, and genetic material [3]. We associated salivary gland antioxidant dysfunction with autoimmunity-related inflammation, not with levels of thyroid hormones and TSH. Oxidative stress was not the reason for reduced salivary secretion in the course of euthyroid HT [3]. The similarity in genetic and immunopathological background between Sjögren's syndrome (SS) and HT [14–16] suggests that, by analogy with SS, the secretory function of the salivary glands in HT may have an immunological basis. It could be a consequence of impaired secretion of cytokines, chemokines, and growth factors. A decrease in the salivary gland size and increase in the inflammatory infiltrate in the salivary glands were observed in hypothyroid HT tyrosylprotein sulfotransferase knockout mice [17]. Our previous papers showed that salivary gland immunological imbalance leads to secretory dysfunction of these glands not only in the course of SS but also other autoimmune diseases, such as psoriasis [18], rheumatoid arthritis [11], and systemic sclerosis [19].

Salivary levels of selected cytokines, chemokines, or growth factors in the course of HT have not yet been determined. In the light of the aforementioned facts, such determination seems necessary in order to understand the involvement of immunological mechanisms in the development of salivary gland dysfunction in this disease. One of the aims of the presented publication was to assess the degree of salivary gland dysfunction and the occurrence of subjective and objective symptoms of salivary gland dysfunction in spontaneously euthyroid HT patients never subjected to hormonal treatment.

The study described below was further designed to assess whether or not the obtained salivary concentrations of selected cytokines, chemokines, and growth factors may be connected with the rate of UWS and symptoms of xerostomia. Furthermore, given that salivary biomarkers are used in the diagnosis of numerous systemic diseases, the secondary objective of this study was to determine whether salivary levels of cytokines, chemokines, and growth factors may present any diagnostic value when comparing the saliva of euthyroid HT female patients to that of women in the control group.

2. Materials and Methods

The study was approved by the Bioethics Committee of the Medical University of Bialystok (permission number: R-I-002/386/2016). Each patient and control group participant had been informed about the aims and methodology of the presented experiment and gave written consent to participate in the study.

The study group consisted of 25 female patients diagnosed with Hashimoto's thyroiditis. The diagnosis of the disease was based on a positive result of an ultrasound examination (confirming the presence of parenchymal heterogeneity) and finding blood levels of TPO-Ab and TG-Ab antibodies above the laboratory norm. The selected patients were women with euthyroid HT (free thyroxine, fT4 and thyroid stimulating hormones, TSH within the laboratory norm levels) who, importantly, had never been treated with synthetic or natural thyroid hormones or had any other treatments applied. Patients were referred for periodic check-ups to the Department of Endocrinology, Diabetology, and Internal Medicine at the Medical University of Bialystok. The control group consisted of 25 generally healthy women, matched to the study group in terms of age and BMI, who attended dental check-ups at the Department of Restorative Dentistry at the Medical University of Bialystok. The participants from the control group had normal results of thyroid ultrasound as well as blood levels of TPO-Ab, TG-Ab, fT4, and TSH in normal range for healthy individuals.

Eligibility for the study was preceded by the collection of whole blood from an ulnar vein (for hormone and antibody as well as biochemical determinations), ultrasound examination, and dental examination. Participants of the study had 10 mL samples of venous blood collected into ethylenediaminetetraacetic acid (EDTA) tubes. Blood for biochemical determinations was centrifuged (1500 × g, 4°C, 10 minutes), and the obtained plasma was stored at -84°C for no longer than 4 months, until assayed. A total of 82 female HT patients and 82 control patients were studied. 10 patients had hypothyroid HT, 25 had euthyroid HT treated with hormones, and 15 had BMI > 25. Therefore, 34 patients with HT in the state not requiring hormonal treatment and 34 control subjects of corresponding age and BMI were qualified for the study.

Serum/plasma TSH, free T4 and T3, TPO-Ab, TG-Ab, SSA/Ro-Ab, SSB/La-Ab, glucose, insulin, HOMA-IR, vitamin 25 (OH)D3, TG, and CRP were quantified by using an Abbott analyzer (Abbott Diagnostics, Wiesbaden, Germany).

On the same day, a dental examination was performed, including the measurements of DMFT (Decay, Missing,

Filled Teeth) index, API (Approximal Plaque Index) index of oral hygiene, pocket probing depth (PPD), and gingival sulcus bleeding index (SBI) (Table 1). The examination was performed by one dentist (K. M.) who had obtained a suitable training beforehand, and an interrater examination was conducted in 15 randomly selected study participants with another dentist (A. Z.). The interrater reliability for DMFT was $r = 0.97$, for SBI $r = 0.93$, for API $r = 0.96$, and for PPD $r = 95$.

Based on the dental measurements, 9 HT patients were excluded from the study group due to the presence of periodontal disease and 8 from the control group because of poor oral hygiene and presence of multiple dental deposits.

HT patients had no other diseases, including autoimmune diseases, and—as noted above—their BMI was within the range indicating normal body weight (18.5–25). Both the patients and control group members were individuals with healthy periodontium and had no candidiasis and inflammation in the oral mucosa. HT patients and control subjects did not take medications on a permanent basis, including those affecting saliva secretion. The examination was performed in the second phase of the menstrual cycle, precisely between the 18th and 25th day. During 6 months preceding the research, they had not been on a weight-loss diet and had not significantly changed their lifestyle (in terms of diet, physical activity, and place of work or residence). The subjects were nonsmokers, consumed alcohol only occasionally (a glass of wine or a pint of beer at the weekend), and were not addicted to other stimulants. The clinical characteristics of patients and participants from the control group are presented in Table 2.

2.1. Collection of UWS. Saliva was collected one day after blood collection and dental examination, i.e., only from participants qualified for the study. UWS was collected between 7 a.m. and 9 a.m. The subjects had the collection performed on an empty stomach (last meal at least 10 hours before) and did not perform any oral hygiene procedures on this day other than rinsing the mouth with water. Saliva collection took place in a separate surgery. Upon arrival, each participant rested for 5 minutes and rinsed the mouth 5 times with water at room temperature. In a sitting position, the participant spat out saliva accumulated at the bottom of the mouth into a centrifuge tube placed in a container with ice. The saliva collection time was 15 minutes. Prior to centrifugation, the volume of obtained saliva was measured (with a calibrated pipette). This measurement allowed the rate of saliva secretion to be determined, by dividing the volume of collected saliva by the time taken to obtain it. The saliva was centrifuged for 20 minutes at 4°C, 10000 × g. Next, the supernatant was collected and frozen at -84°C for no longer than 4 months, until assayed.

To assess the prevalence of xerostomia, participants were asked to complete a questionnaire regarding the presence of dry mouth symptoms listed in the American-European classification criteria for Sjögren's syndrome [11, 20], and the Clinical Oral Dryness Score (CODS) was measured (K. M.) according to Osailan et al. [10] (Table 1). An interrater examination with another dentist (A. Z.) was conducted in 15 randomly selected study participants. The interrater reliability for CODS was $r = 0.96$.

2.2. Biochemical Methods. Salivary and plasma cytokines, chemokines, and growth factors were analyzed using the Bio-Plex® Multiplex System according to the manufacturer's instructions. Bio-Plex Pro Human Cytokine Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) is a multiplex assay based on magnetic beads whose performance can be compared to a typical ELISA. Captured antibodies directed against a specific biomarker are covalently bound to magnetic beads. The coupled beads then react with the sample containing the selected biomarker. A series of rinses is performed in order to remove the unbound protein, and then a biotinylated detection antibody is added to create a sandwich complex. The final complex is formed by adding streptavidin-phycoerythrin (SA-PE) conjugate. Data from the reactions are acquired using a dedicated plate reader (Bio-Plex 200) and high-speed digital signal processor.

The concentration of total nitric oxide (NO) in saliva and plasma was determined indirectly using sulfanilamide and NEDA·2HCl (N- (1-naphthyl) ethylenediamine dihydrochloride). In this assay, stable degradation products of NO_3^- were measured. Absorbance was analyzed at 490 nm [21, 22]. Total protein concentration was determined colorimetrically (Thermo Scientific Pierce BCA Protein Assay Kit, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard.

2.3. Statistical Analysis. GraphPad Prism 8.3.0 for MacOS (GraphPad Software, Inc. La Jolla, USA) was used for statistical analysis. The normality of the distribution was assessed using the Shapiro–Wilk test. For comparison of the quantitative variables, Mann–Whitney U test was used. The statistical significance level was established at $p < 0.05$. The relationship between the assessed parameters was evaluated using the Spearman rank correlation coefficient. In order to determine the diagnostic utility of salivary/plasma cytokines, receiver operating characteristic (ROC) curves were drawn, and the area under the curve (AUC) was calculated. Multivariate analysis of the simultaneous impacts of many independent variables on one quantitative dependent variable was made by the means of linear regression. HT duration, TPO-Ab, TPO-AB, UWS flow, and CODS were included as independent variables. The number of subjects in the groups was determined based on our previous experiment, assuming that the power of the test was 0.9 and $\alpha = 0.05$. The ClinCalc online calculator was used, and the minimum number of participants per group was 21.

3. Results

3.1. Stomatological Findings. The secretion of UWS by female patients with Hashimoto's disease was significantly (66%) lower than in the control group ($p = 0.009$). Moreover, 15 women from the study group had UWS level below 0.2 mL/min, which means that hyposalivation, i.e., secretory dysfunction of the salivary glands, could be confirmed in 60% of the study participants (Table 1).

Table 1: Stomatological characteristics of patients and control group participants (UWS: unstimulated saliva; TP: total protein; CODS: Clinical Oral Dryness Score; DMFT: Decayed, Missing, Filled Teeth; API: approximal plaque index; SBI: sulcus bleeding index, PPD: periodontal pocket depth; NS: statistically insignificant).

	Control, $n = 25$	HT, $n = 25$	p
UWS mL/min	0.67 (0.46–0.89)	0.27 (0.1–0.61)	<0.0001
No. of women (%) with hyposalivation (UWS < 0.2 mL/min)	0 (0)	15 (60)	<0.00001
TP NWS (mg/mL)	895 (658.23–1250.1)	1400 (1002.1-1600.56)	<0.0001
CODS (1–10)	0	6 (1–10)	<0.0001
No. of women with CODS ≥ 5	0	13	<0.0001
No. of women with CODS ≥ 1 ≤ 4	0	8	<0.001
Subjective oral dryness, *n* (%)	0 (0)	20 (80)	<0.0001
DMFT	18 (15–25)	16 (14–25)	NS
API	24.56 (0–30)	21.54 (0–32)	NS
SBI	0.45 (0–1)	0.33 (0–1)	NS
PPD (mm)	2.0 (0.5–2.5)	2.0 (0.5–2.5)	NS

Table 2: Clinical characteristics of the participants of the study (HT: Hashimoto patients; BMI: body mass index; TSH: thyroid-stimulating hormone; TPO-Ab: thyroid peroxidase antibody; TG-Ab: thyroid peroxidase antibody; SSA/Ro-Ab: anti-Sjögren's syndrome type A antibody; SSB/La-Ab: anti-Sjögren's syndrome type B antibody; HOMA-IR: homeostatic model assessment index; TG: triglyceride; NO: nitric oxide; UWS: unstimulated whole saliva; ND: not detectable; NS: statistically insignificant).

	Control, $n = 25$ M (min-max)	HT, $n = 25$ M (min-max)	p
Age (years)	34.3 (27.2–42)	34.5 (27.8–41.5)	NS
BMI (kg/m^2)	20.5 (19.35–23.96)	21.7 (19.2–24.61)	NS
TSH (μU/mL)	1.23 (0.48–2.3)	1.56 (0.7–2.99)	NS
Free T4 (ng/mL)	1.36 (1.1–1.39)	1.26 (0.76–1.62)	NS
Free T3 (pg/mL)	2.26 (1.75–3.96)	2.86 (2.63–4.51)	NS
TPO-Ab (IU/mL)	0.5 (0.36–2.51)	625.6 (132.4–768)	<0.0001
TG-Ab (IU/mL)	0.4 (0.23–1.91)	318.3 (145.8–437)	<0.0001
SSA/Ro-Ab	ND	ND	
SSB/La-Ab	ND	ND	
Glucose (mg/dL)	79.56 (72.35–85.01)	83.01 (73.56–88.05)	NS
Insulin (mU/mL)	4.25 (3–11.2)	3.89 (3–10.9)	NS
HOMA-IR	0.89 (0.53–2.41)	0.8 (0.51–2.39)	NS
Witamin 25-OH D3 (ng/mL)	47 (32–51.1)	42.98 (30–50)	NS
TG (mg/dL)	52 (39–79.56)	49 (36–85.21)	NS
CRP mg/L	0.4 (0.2–0.8)	0.63 (0.23–0.92)	NS
HT duration (years)	0	5.8 (3.8–8.3)	<0.0001
Thyroid gland's nodules	ND	ND	
NO UWS (μmol/mg protein)	0.15 (0.01-0.08)	0.26 (0.02–0.56)	0.0011
NO plasma (μmol/mg protein)	0.09 (0.014- 0.19)	0.08 (0.016–0.24)	0.9082

Protein concentration in UWS of HT patients was significantly (56%) higher than in the control group ($p = 0.008$) (Table 1).

CODS demonstrated a significantly higher intensity of objective dry mouth symptoms in HT patients compared to the control group ($p = 0.001$). Subjective sensation of dry mouth was reported only in the group of patients with Hashimoto's disease and affected 80% of the subjects ($p = 0.001$) (Table 1).

The analysis of the results obtained during the dental examination revealed no significant differences in the DMFT, API, SBI, and PPD indices (Table 1, Table S7).

3.2. Saliva

3.2.1. Cytokines. Significantly elevated levels of cytokines associated with Th1 lymphocyte activation (IL-3, IFN-γ) were found in UWS of HT patients compared to the control

group (↑35%, $p = 0.0074$, ↑85% $p \leq 0.0001$, respectively). The study group achieved considerably higher levels of interleukins connected with Th2 lymphocyte activation (IL-5, IL-6) compared to healthy controls (↑40%, $p = 0.015$, ↑54%, $p = 0.0187$, respectively). Similarly, significantly increased levels of TNF-α (which is a cytokine associated with Th17 lymphocyte stimulation) were observed in UWS of HT patients compared to the control group (↑93%, $p = 0.0003$). In addition, significantly higher levels of IL-12 (p40), HGF, IL-1α, IL-1β, and IL-1RA were found in UWS of study group patients compared to the control group (↑57%, $p \leq 0.0001$, ↑38%, $p = 0.0012$, ↑38%, $p = 0.0223$, ↑54%, $p = 0.0223$, ↑34%, $p = 0.0071$, respectively). Of all cytokines tested, only salivary IL-8 and IL-10 levels reached significantly lower values in UWS of HT patients compared to healthy controls (↓21%, $p = 0.0429$, ↓69%, $p = 0.0049$, respectively). Concentrations of the other cytokines measured in UWS: IL-2, IL-2RA, IL-4, IL-7, IL-9, IL-12 (p70), IL-13, IL-15, IL-16, IL-17, IL-18, IFN-α2, MIF, and TNF-β did not differ significantly between the two groups (Table 3).

The levels of IL-18, IFN-α2, MIF, and TNF-β were not significantly different between both groups (Table 3).

3.2.2. Chemokines. Concentrations of both CCL27/CTACK and CXCL1/Gro-α were significantly higher in UWS of HT patients compared to the controls (↑19%, $p = 0.0195$, ↑74%, $p = 0.0005$, respectively). Salivary levels of CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, β-NGF, CCL5/RANTES, SCF, SCGF-β, CCL7/MCP-3, CCL11/Eotaxin, CXCL9/MIG, CXCL10/IP-10, LIF, and CXCL12/SDF-1α reached similar values in both study groups (Table 4).

3.2.3. Growth Factors. Concentrations of G-CSF and M-CSF in plasma of HT patients were significantly higher compared to the controls (↑29%, $p \leq 0.0001$, ↑30%, $p = 0.0223$, respectively). The levels of the other growth factors assayed: FGF, GM-CSF, PDGF-BB, VEGF, and TRIAL reached similar values in both study groups (Table 5).

3.3. Plasma

3.3.1. Cytokines. Plasma concentrations of cytokines responsible for Th1 lymphocyte activation (IL-3, IFN-γ, TNF-β) in HT patients were significantly elevated compared to healthy controls (↑36%, $p \leq 0.0001$, ↑43%, $p = 0.0001$, ↑46%, $p = 0.0028$, respectively). Plasma levels of IL-5, IL-6, and IL-13 (responsible for activating Th2 lymphocytes) in the study group were considerably higher compared to the control group (↑36%, $p \leq 0.0001$, ↑51%, $p = 0.003$, ↑26%, $p = 0.0161$, respectively). Significantly higher plasma levels of TNF-α (which participated in the activation of Th17 lymphocytes) were observed in HT patients compared to the controls (↑79%, $p = 0.0001$). Furthermore, plasma levels of IL-1β, IL-1RA, IL-2RA, IL-12 (p40), IL-12 (p70), and HGF in study group patients were significantly higher compared to the control group (↑39%, $p = 0.017$, ↑43%, $p = 0.02$, ↑9%, $p = 0.0389$, ↑28%, $p = 0.0211$, ↑49%, $p = 0.0102$, ↑28%, $p \leq 0.0001$, respectively). Only IL-8, IL-10, and IL-17 concentrations were significantly lower in plasma of study group patients compared to the controls (↓60%, $p \leq 0.0001$, ↓31%, $p = 0.0004$, ↓18%, $p = 0.0466$, respectively). The cytokine levels of IL-1α, IL-2, IL-4, IL-7, IL-9 IL-16, IL-18, IFN- α2, and MFI reached similar values in the plasma of both HT and control subjects (Table 3).

3.3.2. Chemokines. Both CCL2/MCP-1 and SCF levels were significantly higher in the plasma of HT patients compared to the control group (↑75%, $p = 0.0189$, ↑74%, $p \leq 0.0001$, respectively). Similarly, plasma CCL27/CTACK and LIF concentrations in the study group were significantly increased compared to healthy controls (↑101%, $p \leq 0.0001$, ↑42%, $p = 0.0066$, respectively). Plasma levels of the chemokines CCL3/MIP-1α, CCL4/MIP-1β, β-NGF, CCL5/RANTES, SCGF-β, CCL7/MCP-3, CCL11/Eotaxin, CCL27/CTACK, CXCL1/Gro-α, CXCL9/MIG, and CXCL10/IP-10 did not differ significantly between the two groups (Table 4).

3.3.3. Growth Factors. Concentrations of G-CSF and M-CSF in plasma of HT patients were significantly higher compared to the controls (↑29%, $p \leq 0.0001$, ↑30%, $p = 0.0223$, respectively). The levels of the other growth factors: FGF, GM-CSF, PDGF-BB, VEGF, and TRIAL reached similar values in both study groups (Table 5).

3.4. NO. Concentration of NO in the UWS of HT women was significantly higher compared to the control ($p = 0.001$), whereas in plasma, it did not differ between groups (Table 2).

3.5. ROC Analysis. We used ROC analysis to examine the diagnostic utility of the assessed cytokines, chemokines, and growth factors. The data of ROC analysis are presented in the supplementary materials (Table S1-S6). Many biomarkers differentiate Hashimoto's patients from healthy controls with high sensitivity and specificity. However, salivary IFN-γ, IL-12 (p40), and TNF-α and plasma CTACK, SCF, G-CSF, HGF, IL-8, TNF-α, and IL-3 deserve special attention. Indeed, IFN-γ assessed in UWS with 84% sensitivity and 84% specificity distinguishes study group from healthy controls (AUC = 0.9104).

3.6. Multifactorial Regression Analysis. The results of multifactorial regression analysis of salivary inflammatory biomarkers are presented in Table S12. Interestingly, IL-6 and IL-1βlevels significantly depend on HT duration, UWS flow, and CODS, while IFN-γ on disease duration and UWS flow. Salivary TNF-α statistically depends on TG-Ab and UWS flow rate, while the IL-12 level only on salivary flow (Table S12).

3.7. Correlations. In the group of HT patients, a negative correlation was observed between TPO-Ab levels and the rate of UWS secretion, as well as a negative correlation between salivary concentration of IL-6 and the rate of UWS secretion. Similarly, in this group, the level of salivary IL-1α negatively correlated with the rate of UWS secretion.

Moreover, in the study group, we found a negative correlation between the CODS and the rate of UWS secretion and a positive correlation between the CODS and the disease duration. In patients with euthyroid HT, there was a positive

Table 3: Effect of Hashimoto's disease on cytokine levels in saliva and plasma. IL: 1α, 1β, 1RA, 2, 2RA, 3,4, 5,6,7,8,9, 10, 12 (p40), 12 (p70), 13, 15, 16, 17, 18-interleukin: 1α, 1β, 1RA, 2, 2RA, 3,4, 5,6,7,8,9, 10, 12 (p40), 12 (p70), 13, 15, 16, 17, 18; IFN-γ: interferon-γ; IFN-α2: interferon-α2; TNF-α, β: tumor necrosis factor α, β; HGF: hepatocyte growth factor; MIF: macrophage migration inhibitory facto;, ND: no detectable.

	Saliva					Plasma				
	Control group		Study group		p value	Control group		Study group		p value
	Mean ± SD	Median (min-max)	Mean ± SD	Median (min-max)		Mean ± SD	Median (min-max)	Mean ± SD	Median (min-max)	
IL-3	35.11 ± 11.41	38.88 (7-56)	47.33 ± 17.3	45 (13-80)	0.0074	26.72 ± 4.009	27.47 (19.79-35.24)	36.28 ± 9.588	36.01 (19.1-58.72)	<0.0001
IFN-γ	25.12 ± 8.914	23.57 (12.23-54.5)	46.5 ± 15.09	43.54 (19.11-81.46)	<0.0001	164.4 ± 50.86	169.5 (43.5-236)	235.9 ± 69.13	233.2 (123-427.9)	0.0001
IL-5	6.065 ± 1.85	6.05 (0.6-9.4)	8.49 ± 3.975	8.6 (1.2-15.65)	0.015	106.4 ± 75.49	94.43 (7.968-320.2)	160.3 ± 67.12	158.5 (41.24-301.3)	0.003
IL-6	72.01 ± 27.06	73.59 (19-136)	110.8 ± 69.45	103 (17-297)	0.0187	3.065 ± 0.6913	3.147 (1.568-4.517)	5.051 ± 2.172	5.202 (0.7955-9.66)	0.0002
TN-α	8.173 ± 5.213	6.975 (0.625-21.68)	15.79 ± 8.211	15.15 (1.28-37.39)	0.0003	12.5 ± 5.202	12.63 (6.019-27.13)	22.32 ± 8.808	21.27 (6.815-38.84)	<0.0001
IL-12 (p40)	12.45 ± 4.186	12 (6.614-21.75)	19.5 ± 5.242	19.5 (8.004-29.9)	<0.0001	13.61 ± 3.629	13.81 (8.131-21.28)	17.41 ± 6.56	17.59 (6.718-33.75)	0.0211
HGF	3.908 ± 0.9919	3.842 (1.599-6.273)	5.412 ± 1.614	5.373 (3.063-8.694)	0.0012	60.7 ± 6.446	60.69 (43.81-74.71)	77.82 ± 9.324	75.83 (66.35-103.7)	<0.0001
IL-1α	760.6 ± 360.9	766.1 (189.2-1375)	1054 ± 441.2	1148 (408.2-2182)	0.0223	328 ± 227.5	271 (15-970)	458.5 ± 346	417.5 (39.38-1619)	0.0741
IL-1β	99.04 ± 65.47	74.05 (22.53-245.4)	153.2 ± 86.18	153.3 (28.93-343.5)	0.0223	17.54 ± 9.707	17.28 (1.302-33.61)	24.46 ± 8.32	24.38 (8.663-48.13)	0.017
IL-1RA	5170 ± 1847	5071 (917-7936)	6951 ± 2239	6707 (3196-10575)	0.0071	13.49 ± 7.875	12.01 (0.7689-31.98)	19.32 ± 8.643	17.87 (4.071-40.51)	0.02
IL-8	1564 ± 913.3	1479 (87.75-3855)	1230 ± 1167	882 (21-5426)	0.0429	24.43 ± 8.201	25.36 (6.595-38.56)	9.871 ± 7.801	7.724 (-5.24-27.19)	<0.0001
IL-10	28.49 ± 10.39	26.5 (10.22-47)	19.69 ± 9.166	19 (2.235-40.22)	0.0049	34.42 ± 9.252	35.14 (13.04-49.94)	23.61 ± 11.09	21.76 (4.953-47.42)	0.0004
IL-2	27.58 ± 10.46	30 (4-46)	30.23 ± 12.54	29 (9.218-58)	0.6685	116.7 ± 48.75	129.7 (18.51-189.3)	140.9 ± 64.07	144.7 (25.42-298.6)	0.1213
IL-2RA	39.98 ± 20.56	37.13 (3.823-86.66)	40.09 ± 20.86	39 (2.598-87)	0.9578	28.35 ± 3.504	27.91 (22.57-34.75)	30.79 ± 5.045	32.49 (19.24-37.89)	0.0389
IL-4	16.22 ± 7.095	15 (2-33.5)	19.85 ± 7.338	18 (9-36)	0.0625	23.97 ± 4.79	24.39 (15.87-31.69)	26.25 ± 11.21	26.8 (1.017-47.67)	0.3067
IL-7	17.91 ± 4.275	18 (9.391-25)	17.8 ± 8.887	16.5 (4.807-42)	0.455	5.099 ± 2.223	4.495 (1.972-10.08)	7.364 ± 4.663	6.964 (0.6749-17.65)	0.1035
IL-9	45.98 ± 17.78	47 (16.48-82)	53.85 ± 21.96	55 (12.07-99)	0.1806	49.19 ± 20.28	48.04 (21.43-91.61)	55.94 ± 25.79	58.96 (8.061-92.59)	0.2799
IL-12 (p70)	22.59 ± 8.404	22.79 (3.101-36.6)	25.25 ± 7.734	24 (9.988-36.5)	0.2907	16.17 ± 6.58	16.96 (3.493-30.17)	24.02 ± 11.11	20.41 (2.664-44.17)	0.0102
IL-13	1.013 ± 0.4813	1.027 (0.3138-1.833)	1.287 ± 0.7352	1.137 (0.1965-2.41)	0.2022	9.829 ± 4.507	9 (3.549-21.14)	12.41 ± 3.768	12 (5.41-22)	0.0161
IL-15	10.72 ± 5.484	10.2 (0.473-23)	12.35 ± 9.511	9.6 (2.85-35.3)	0.7619	ND	ND	ND	ND	ND
IL-16	981.3 ± 515.6	941.6 (80.27-2125)	1095 ± 594.9	1012 (235.8-2163)	0.5253	161.6 ± 127.1	129 (11.15-587)	175.3 ± 165.5	141.5 (5.34-780)	>0.9999
IL-17	ND	ND	ND	ND	ND	33.74 ± 11.03	29.5 (21.41-58)	27.58 ± 12.11	26.6 (8.334-56.7)	0.0466
IL-18	369.3 ± 379.1	331.5 (10.73-1999)	399.7 ± 403.2	280 (4.705-1829)	0.9847	20.7 ± 9.127	22.17 (3.895-33.62)	23.2 ± 14.99	21.47 (2.131-57.12)	0.6581
IFN-α2	4.61 ± 0.9551	4.539 (2.918-7.328)	5.053 ± 1.844	5.054 (1.601-10.47)	0.3859	22.81 ± 6.25	22.29 (9.482-34.99)	22.71 ± 7.463	21.31 (2.789-39.93)	0.8777
MIF	759.7 ± 323.3	661 (258.2-1424)	750.5 ± 538.5	601 (119.5-2221)	0.4185	196.5 ± 78.99	208 (44.66-340.8)	206.2 ± 79.61	184.4 (81.59-359.2)	0.8929
TNF-β	5.702 ± 1.994	5.986 (0.7912-9.791)	7.135 ± 2.727	7.033 (1.758-11.87)	0.0925	6.692 ± 2.137	6.997 (1.33-10.08)	9.793 ± 4.96	10.01 (-2.785-17.23)	0.0028

Table 4: Effect of Hashimoto's disease on chemokine levels: CCL27/CTACK: chemokine ligand 3/monocyte chemoattractant protein-1; CCL3/MIP-1-α: chemokine ligands 3/macrophage inflammatory protein*1*-alpha; CCL4/MIP-1 β: chemokine ligands 4/macrophage inflammatory protein*1* β; β -NGF: β-nerve growth factor; CCL5/RANTES: chemokine ligand 5/regulated on activation, normal T cell expressed and secreted; SCF: stem cell factor; SCGF-β: stem cell growth factor-β; CCL7/MCP-3: chemokine ligand 7/monocyte-chemotactic protein *3*; CCL11/eotaxin: chemokine ligand 11/eatoksin; CCL27/CTACK: chemokine ligand 27/cutaneous T cell-attracting chemokine; CXCL1/GRO-α: chemokine (C-X-C motif) ligand 1/growth-regulated oncogene-alpha; CXCL9/MIG: chemokine (C-X-C motif) ligand 9/monokine induced by gamma interferon; CXCL10/IP-10: chemokine (C-X-C motif) ligand 10/interferon gamma-induced protein *10*; LIF: leukemia inhibitory factor; CXCL12/SDF-1 α: chemokine (C-X-C motif) ligand 12/stromal cell-derived factor 1; ND: no detectable.

	Saliva					Plasma				
	Control group		Study group		p value	Control group		Study group		p value
	Mean ± SD	Median (min-max)	Mean ± SD	Median (min-max)		Mean ± SD	Median (min-max)	Mean ± SD	Median (min-max)	
CCL27/CTACK	17.80 ± 5.689	18.08 (8.970-33.69)	21.23 ± 6.034	20.27 (11.53-36.62)	0.0195	70.22 ± 15.1	72.93 (39.18-111.6)	141.2 ± 71.73	128.3 (60-375.5)	<0.0001
CXCL1/Gro-α	8545 ± 3904	8235 (1768 -15622)	14898 ± 6761	15456 (3393-30180)	0.0005	17772 ± 7093	18731 (3763-33741)	19882 ± 7155	21785 (4134-29760)	0.2092
CCL2/MCP-1	703 ± 360.5	707.8 (198-1490)	727.7 ± 618.5	677 (24.52-2896)	0.7583	4.51 ± 2.51	4.557 (0.2395-9.026)	7.915 ± 4.844	5.865 (1.748-19.22)	0.0189
CCL3/MIP-1α	33.89 ± 22.89	28 (5.895-118.5)	44.79 ± 67.19	28 (7.238-354)	0.7914	123.6 ± 71.94	120.1 (11.45-268.1)	141.2 ± 92.17	141.4 (5.762-375.2)	0.5768
CCL4/MIP-1β	63.07 ± 26.34	54.5 (27.5-138)	61.73 ± 31.01	63 (2.401-129.5)	0.8362	124 ± 79.66	117 (16.15-276.7)	136.5 ± 87.9	140.4 (16.26-332.6)	0.6721
β-NGF	62.03 ± 23.58	66 (8.513-116.3)	70.33 ± 36.36	61 (8.457-143.3)	0.4973	84.27 ± 48.49	82.24 (6.667-179.1)	103.6 ± 52.8	99.81 (33.11-275.2)	0.263
CCL5/RANTES	2.94 ± 1.038	2.927 (0.394-4.531)	3.48 ± 1.46	3.201 (1.084-6.698)	0.2218	1550 ± 620.2	1470 (519.6-2704)	1978 ± 915.4	1740 (465.3-4047)	0.0916
SCF	77.67 ± 39.69	72 (5.96-150)	89.36 ± 79.58	76 (6.221-314)	0.7914	32.98 ± 9.754	33.06 (17.22-50.29)	57.39 ± 19.22	55.61 (27.62-89.57)	<0.0001
SCGF-β	63.07 ± 26.34	54.5 (27.5-138)	61.73 ± 31.01	63 (2.401-129.5)	0.8362	539.4 ± 316.7	560.9 (43.81-1213)	660.2 ± 270.4	658.4 (150.4-1260)	0.2467
CCL7/MCP-3	18.04 ± 5.184	18 (9.874-30)	20.1 ± 5.504	19.5 (10.93-30.9)	0.1931	639.5 ± 136.9	619.5 (275.5-879.5)	697.3 ± 195.3	731.6 (206.5-952.3)	0.1077
CCL11/Eotaxin	20.05 ± 6.526	19.99 (8.792-37.74)	21.89 ± 9.195	18.03 (9.54-43.23)	0.8475	72.15 ± 22.91	68.86 (15.32-120.1)	82.09 ± 32.24	75.98 (39.9-159.7)	0.441
CXCL9/MIG	608.7 ± 655.1	333.5 (17.5-3001)	1090 ± 1552	542.5 (74.97-7798)	0.3254	101.1 ± 92.73	68.06 (4.366-413.3)	133.7 ± 72.15	144 (18.91-307.8)	0.0568
CXCL10/IP-10	6686 ± 3147	6608 (1730-12452)	9053 ± 5665	7868 (833.7-23044)	0.1954	566.8 ± 346.5	542 (12.02-1490)	742.9 ± 648.9	574. (12.51-2896)	0.444
LIF	20.42 ± 10.76	19 (2.161-45.5)	25.68 ± 13.15	23 (8.214-53.5)	0.2329	24.86 ± 9.771	24.31 (9.054-42.78)	35.38 ± 16.87	35.87 (2.65-71.33)	0.0066
CXCL12/SDF-1α	53.34 ± 18.2	55 (17.55-91.5)	56.4 ± 22.92	53.48 (13.95-101)	0.6756	ND	ND	ND	ND	ND

Table 5: Effect of Hashimoto's disease on growth factor levels: FGF: fibroblast growth factor; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; M-CSF: macrophage colony-stimulating factor; PDGF-BB: platelet-derived growth factor–BB; VEGF: vascular endothelial growth factor.

	Saliva					Plasma				
	Control group		Study group		p value	Control group		Study group		p value
	Mean ± SD	Median (min-max)	Mean ± SD	Median (min-max)		Mean ± SD	Median (min-max)	Mean ± SD	Median (min-max)	
G-CSF	493.9 ± 72.24	497 (363.1-649.2)	574.1 ± 115.8	587.8 (344.1-894.8)	0.0066	603.7 ± 91.3	590.2 (454.4-764.7)	777.1 ± 164	726.8 (455.9-1162)	<0.0001
VEGF	1443 ± 850.1	1221 (53.24-3643)	2631 ± 1480	2433 (634.8-6122)	0.0025	191.1 ± 64.73	179.4 (91.19-331)	271.3 ± 158.2	252.3 (24.03-608.9)	0.0842
TRIAL	519.3 ± 307.7	441.3 (16.85-1154)	1009 ± 1011	727.1 (24.71-4653)	0.0472	80.32 ± 30.77	78.42 (26.96-143.5)	101.6 ± 47.97	101.7 (12.02-191.1)	0.0878
FGF	5.371 ± 1.205	5.412 (3.418-8.347)	5.737 ± 1.197	5.54 (3.717-8.09)	0.3571	34.45 ± 7.104	33.35 (19.34-46.92)	33.85 ± 9.314	31.99 (17.48-54.19)	0.6443
GM-CSF	24.12 ± 6.793	22.65 (11.77–37.98)	25.6 ± 10.25	25.17 (5.405 –50.55)	0.5637	98.28 ± 15.9	100.2 (70.8-140.6)	102.9 ± 26.21	103.9 (48.13-146.8)	0.4762
M-CSF	228.5 ± 137.5	206 (13.03-582.5)	309.7 ± 268.6	238 (18.8-1301)	0.2182	65.97 ± 30.89	69.24 (4.661-135.7)	85.52 ± 34.76	98.87 (5.619-131.4)	0.0223
PDGF-BB	480.9 ± 165.9	490 (103.6-740)	534 ± 262.3	548.2 (111-1060)	0.5096	1041 ± 337.1	1092 (351.6-1689)	976.5 ± 552	1095 (39.4-2135)	0.6305

Table 6: Statistically significant correlations in the study group (TG-Ab: thyroid peroxidase antibody; UWS: unstimulated saliva; IL-1α: interleukin 1α; IL-6: interleukin 6; IL-12: interleukin 12; INF-γ: interferon γ; CODS: Clinical Oral Dryness Score, NO: nitric oxide; IL-12 (p40): interleukin 12 (p40); TG-A: thyroid peroxidase antibody).

Pairs of variables	r	p
TPO-Ab and UWS flow	-0.865	<0.0001
IL-6 and UWS flow	-0.951	<0.0001
IL-1α and UWS flow	-0.864	<0.0001
CODS and UWS flow	-0.885	<0.0001
CODS and duration of Hashimoto's disease	0.751	<0.0001
IFN-γ and CODS	0.488	0.013
IL-12 and NO	0.723	<0.0001
INF-γ and NO	0.442	0.027
IL-12 (p40) UWS and plasma TG-A	0.557	0.004

correlation between salivary INF-γ concentration and the CODS.

We demonstrated that in UWS of HT patients, IL-12 levels were positively correlated with NO concentration. Similarly, a positive correlation was observed between INF- γ concentration and an increase in NO level in UWS.

In the study group, we observed a positive correlation between IL-12 (p40) concentration in UWS and plasma TG-Ab concentration (Table 6).

3.8. Immunoassay Method Validation. Representative assay working ranges, assay sensitivity, and precision are presented in Table S13.

4. Discussion

Hashimoto's disease (Hashimoto's thyroiditis, HT) is a chronic autoimmune disease with complex and heterogeneous course, primarily leading to the destruction and dysfunction of the thyroid gland. However, according to available studies on the subject, it also negatively affects the function of the salivary glands, which is manifested by reduced saliva properties as well as rate of its secretion. The reason for the disturbed function of the salivary glands in the course of HT has not yet been determined. Considering the role of saliva in maintaining the health of structures present in the oral cavity as well as general well-being, explaining these pathomechanisms is of the utmost importance for both patients and doctors.

In our experiment, we narrowed down the study group to female patients who had never been treated for HT and whom did not need to include hormonal supplementation in the course of the disease. Detection of HT in most instances was accidental, e.g., during breast ultrasound or so-called periodic examinations. In such cases, it would seem that patients only require observation and prophylactic visits to an endocrinologist. However, problems of HT patients with subjective sensation of dryness in the oral cavity that appeared during dental visits, most frequently manifested by the need to drink water during the night and problems with swallowing dry food, indicated a more serious problem. These observations encouraged us to perform the present study. One of the objectives of this publication was to assess the severity of salivary gland dysfunction and the presence of subjective and objective symptoms of salivary gland impairment in spontaneously euthyroid HT patients who had never undergone hormonal treatment. Considering the involvement of immunological disturbances in the development of salivary gland disorder in the course of numerous autoimmune diseases, the study is also aimed at assessing whether the severity of salivary gland dysfunction was correlated with the concentration of selected salivary cytokines, chemokines, and growth factors. We attempted to determine whether these salivary parameters could be helpful in determining the progression of HT disease.

Since SS may be one of the causes of salivary gland dysfunction in the course of autoimmune diseases, we performed a number of diagnostic tests (Schirmer's test–data not shown, antibody determination–Table 2, questionnaire on dryness in the eyeball–data not shown–and in the oral cavity), except the labial salivary gland biopsy for which we did not obtain the consent of the Bioethics Committee. The exclusion of SS as well as other general diseases and coexisting oral inflammation led us to the conclusion that all of the observed abnormalities are due to HT.

We confirmed that the median of UWS secretion of HT patients was significantly lower compared to the control group. More importantly, 60% of them had the rate of UWS secretion below 0.2 mL/min, which is clinical evidence of salivary gland secretory insufficiency. The values of UWS < 0.2 mL/min are commonly a cutoff for assessing salivary gland dysfunction [9, 11, 23]. We demonstrated a negative correlation between TPO-Ab levels and UWS flow in the group of HT patients. This result suggests increased salivary gland secretory dysfunction with the progression of this autoimmune thyroid disease, similarly to other autoimmune diseases. In the case of SS, it was observed that increased autoimmunity, expressed by increased levels of in SSA/Lo and SSB/Ra antibodies, led to lower sensitivity or density of muscarinic receptors responsible for saliva secretion [24]. In the course of SS and another autoimmune thyroid disease—Graves' disease, elevated concentration of antimuscarinic antibodies was confirmed parallel to the progression of the disease [25, 26]. These antibodies directly inhibit the carbachol-evoked increase of intracellular calcium ions, which suggests a possible direct role of these antibodies in reducing saliva secretion [27]. Women with low UWS flow rate (below 0.2 mL/min) demonstrated the highest CODS (≥5, data not shown). The CODS is a semiquantitative clinical dry mouth score that enables the assessment of the severity and progression of oral dryness. All the female patients with a secretion rate lower than 0.2 mL/min also responded positively to all 3 questions concerning subjective symptoms of dry mouth; only 5 patients with a secretion rate of more than 0.2 mL/min responded positively to just one of the questions regarding their subjective perception of dry mouth. However, CODS values within the range of 1–4 in HT patients with UWS flow > 0.2 mL/min suggest that oral dryness symptoms may also result from changes in the coating properties of saliva, e.g., from salivary content of glycoproteins, particularly mucins, as evidenced by a positive

correlation between salivary INF-γ concentration and the CODS in the group of HT patients. It was proven that high expression of INF-γ strongly correlates with reduced mucin production in acinar cells of SS patients [28]. It should undoubtedly be remembered that HT patients with UWS within the normal range and with a positive CODS test had experienced over a 50% reduction in their personal baseline UWS flow. Indeed, it is known that ≥50% reduction from personal baseline UWS flow can result in the perception of dry mouth [10]. Furthermore, we demonstrated a negative correlation between the CODS and the rate of UWS secretion in the entire group of HT patients. A negative correlation was also observed between the CODS and the disease duration, which is not surprising since objective symptoms of dry mouth result from its poor hydration or lack of cleaning effect provided by normal flow of saliva. The duration of HT in the subgroup of patients with hyposalvation was significantly longer than in the group of patients with HT with normal saliva flow (Table S8). The severity of dry mouth increases with time, as confirmed by the results of Zalewska et al. [23]. Participants from the control group reported no subjective symptoms of reduced saliva secretion, with zero values of objective dryness symptom scores (CODS = 0), which was also confirmed by the results of Osailan et al. [9].

We did not obtain similar relationships (as we described above) in the subgroup of patients with hyposalvation/normosalivation; although given the small size of this subgroup, these relationship deficiencies should be confirmed in a larger number of patients. As the ClinCalc online calculator test showed, the minimum size of the group should be 21 women.

Although it is commonly believed that HT is Th1 mediated [29], the obtained results do not indicate a dominance of either branch of the immune response. Indeed, both in plasma and UWS, a significant increase was observed in the concentrations of interleukins connected with the activation of Th1 lymphocytes (IFN-γ ↑88% in saliva and ↑43% in plasma; IL-2RA ↑9% and TNF-β ↑46% in plasma), Th2 (IL-5 and IL-6 ↑40% and ↑54%, respectively, in saliva and ↑51% and ↑65%, respectively, in plasma; IL-13 ↑26% in plasma), or Th17 (IL-6 and TNF-α ↑54% and ↑93%, respectively, in saliva and ↑65% and ↑79%, respectively, in plasma) with reduced secretion of Th2 cytokines (IL-10 ↓69% in saliva and ↓17% in plasma) [30]. We further observed increased salivary and plasma concentrations of IL-1α, IL-1β, IL-1RA, IL-3, IL-12 (p40), and plasma IL-12 (p70). Many of the tested cytokines did not differ between the study and control groups, both in saliva and plasma. Taking into account the subgroups of HT patients, we observed significantly higher levels of IFN-γ, IL-6, IL-12 (p40), IL1α, IL-1RA, IL-4, IL-7, and lower IL-10 concentration in the UWS of HT patients with hyposalivation compared to normal salivation HT patients (Table S9). However, we would like to emphasize that these results should be interpreted very carefully due to the insufficient number of cases in the subgroups.

We noted a significant increase in the concentration only of some salivary chemokines (CCL27/CTACK, CXCL1/Gro-α) and found no correlation between the concentrations of the assayed chemokines and interleukins. When analyzing the concentration of chemokines in the subgroups of HT patients, we did not find significant differences (Table S10). As leukocyte migration is the dominant biological process regulated by chemokines, the obtained results are highly likely to demonstrate enhanced migration of white blood cells from blood vessels into the salivary gland parenchyma [31]. It was shown that enlargement of the thyroid gland in the course of HT is accompanied by significantly increased blood flow, vascularization of this gland, and vascular permeability due to boosted expression of VEGF [32]. VEGF is a recognized angiogenic factor and enhancer of vascular permeability [33]. It is not known whether similar morphological changes occur in the salivary glands as in the thyroid gland. The significant increase of salivary VEGF levels may prove increased permeability of salivary gland blood vessels. Interesting but not surprising overall, higher VEGF concentrations were noted in the UWS of patients with hyposalivation compared to patients with HT and without objective salivary gland dysfunction (Table S11). As mentioned above, we observed increased salivary concentrations of IL-1α, IL-1β, IL-1RA, and IL-12, which can be derived from inflammatory cells infiltrating the salivary glands. It is well known that these cytokines are released by incoming stimulated monocytes, activated macrophages, and vascular endothelial cells. Previous studies demonstrated that elevated level of salivary IL-6 also correlates significantly with the degree of lymphocytic infiltration in the labial salivary glands of SS patients [34, 35], implicating the role of this cytokine in SS progression [35]. In the presented experiment, we did not perform labial gland biopsy. Spearman rank correlation coefficient showed the positive correlations between salivary IL-6 and UWS flow, IL-1α, and UWS flow as well as INF-γ and the CODS. Multifactorial regression analysis of salivary inflammatory biomarkers revealed that IL-6 and IL-1β level significantly depend on HT duration, UWS flow, and CODS, while IFN-γ on disease duration and UWS flow. Salivary TNF-α statistically depends on TG-Ab and UWS flow rate, while the IL-12 level only on salivary flow. Hypothesizing, these results suggest that increased levels of IL-6, IL-1α, and INF-γ in UWS may be related to the level of salivary gland degeneration, leading to reduced saliva secretion. It has been evidenced that IL-6 and IL-1 as well as INF-γ, TNF-α, and IL-12 play an important role in the destruction of salivary gland tissue [36–38]. INF-γ not only enhances remodeling of the extracellular space of salivary glands through upregulation the production of metalloproteinases [39] but, similar to TNF-α and IL-12, activates intrinsic apoptosis pathways [37, 38, 40], and IL-6 and IL-1 boost apoptotic damage by exposing cytosolic autoantigen present in HT [41]. IL-6 also significantly intensifies the local inflammatory process by inducing T cell proliferation and B-lymphocyte differentiation and decreasing the number of Treg cells [42, 43]. Moreover, it has been proven that increased secretion and concentration of IL-1, TNF-α, or INF-γ in the inflamed salivary gland microenvironment may inhibit acetylcholine release, resulting in an attenuated acinar cell response and reduced saliva secretion [44]. Furthermore, it is well established that NO controls the secretion of saliva. NO has been demonstrated to exert a stimulatory effect on salivation in normal male rats [45], whereas excess NO is detrimental to numerous cells,

including acinar cells and cells of ducts within the salivary glands, causing apoptosis of these cells and acute impairment of salivary gland function [37]. In the presented study, we observed that increased salivary IL-12 and INF-γ levels correlated positively with elevated salivary NO concentration, indicating that IL-12 and INF-γ may participate in the inhibition of saliva secretion by the NO-mediated pathway. The obtained results were not reported in previous studies, and their interpretation is a far-reaching assumption; therefore, further studies are necessary to assess the utility of IL-6 and IL-1 as well as INF-γ, TNF-α, and IL-12 as biomarkers for salivary gland dysfunction in the course of HT.

The limitation of the current publication is the small number of women with HT; however, as the ClinCalc online calculator shows, the number of 25 is sufficient for the analysis. However, these are selected patients: without a history of hormone therapy, with a healthy periodontium, no other inflammatory diseases of the oral cavity, and no other diseases, including autoimmune diseases. The composition of saliva depends on the local condition in the oral cavity and the general health of the patient. Therefore, in order to assess the function of the salivary glands due to HT, patients should be very critically selected, which could results in a small group size.

In the current publication, we evaluated the utility of the assayed parameters for the diagnosis of HT. ROC analysis showed that some of the studied parameters can be helpful in differential diagnosis of female patients with HT in an untreated euthyroid state from healthy women matched by age and BMI (Table S1, S2, S3). Special attention should be paid to salivary INF-γ and IL-12 (p40) levels that distinguish the study group from the controls (AUC = 0.91, $p < 0.0001$, AUC = 0.86, $p < 0.0001$, respectively). Moreover, salivary IL-12 (p40) concentration correlated positively with plasma TG-Ab, which may suggest that changes in salivary IL-12 (p40) concentrations reflect the progression of Hashimoto's disease. Changes in the levels of the studied parameters do not reflect the changes observed in plasma. Moreover, we observed no correlation between the concentrations of the examined parameters in UWS and plasma. The lack of such correlations proves that inflammatory changes and the related salivary gland dysfunction are independent of general inflammation in the course of HT. Similarly, ROC analysis revealed that plasma proteins, different from those present in UWS, could be helpful in the diagnosis of HT (CTACK: AUC = 0.9, $p < 0.0001$, IL-3: AUC = 0.82, $p < 0.0001$, G-CSF: AUC = 0.87, $p < 0.0001$, HGF: AUC = 0.97, $p < 0.0001$, IL-8: AUC = 0.89, $p < 0.0001$, TNF-α: AUC = 0.84, $p < 0.0001$). However, despite the promising results of ROC analysis and given the low number of female HT patients participating in the experiment, this analysis should be tested on a wider population of euthyroid HT patients.

5. Conclusions

(1) The reduction of UWS secretion in female patients with euthyroid HT compared to the controls is a manifestation of impaired function of the salivary glands. Moreover, UWS flow values below 0.2 mL/min, observed in 60% of the HT patients participating in the experiment, are clinical evidence of secretory dysfunction of the salivary glands

(2) The severity of salivary gland secretory dysfunction is closely associated with autoimmunity-related inflammation in the course of HT

(3) Clinical symptoms of salivary gland dysfunction worsen with disease duration

(4) We demonstrated impaired profiles of cytokines, chemokines, and growth factors in the UWS and plasma of euthyroid HT women

(5) The evaluation of the levels of assayed cytokines in the saliva and plasma of patients with untreated euthyroid HT does not indicate the dominance of any of the branches of the immune response

(6) IL-6 and IL-1 as well as INF-γ, TNF-α, and IL-12 may be potential biomarkers for salivary gland dysfunction in the course of HT

(7) Inflammatory changes and the associated dysfunction of the salivary glands are independent of general inflammation in the course of HT

(8) Salivary IL-12 (p40) may be helpful in assessing the progression of autoimmunity-related inflammation in the course of HT

Data Availability

The datasets generated for this study are available on request to the corresponding author.

Ethical Approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethics Committee of the Medical University of Bialystok (code of permission: R-I-002/386/2016).

Consent

Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

A. Z., M. M., and K. M. performed the conceptualization. A. Z., M. M., K. M., and S. Z. performed the data curation. A. Z., M. M., and S. Z. performed the formal analysis. A. Z. performed the funding acquisition. K. M., A. Z., and M. M. performed the investigation. A. Z., M. M., K. M. performed the methodology. K. M., Ł. P., and A. P-K. performed the material collection. A. Z., A. P-K., A. K. performed the supervision. A. Z. performed the validation. A. Z. and M. M. performed the

visualization. K. M. and A. Z. performed the writing—original draft. K. M., A. Z., and M. M performed the writing—review and editing.

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Supplementary Materials

Table S1: receiver operating characteristic (ROS) analysis of salivary cytokines in patients with Hashimoto's disease and healthy controls. Table S2: receiver operating characteristic (ROS) analysis of plasma cytokines in patients with Hashimoto's disease and healthy controls. Table S3: receiver operating characteristic (ROS) analysis of salivary chemokines in patients with Hashimoto's disease and healthy controls. Table S4: receiver operating characteristic (ROS) analysis of plasma chemokines in patients with Hashimoto's disease and healthy controls. Table S5: receiver operating characteristic (ROS) analysis of salivary growth factors in patients with Hashimoto's disease and healthy controls. Table S6: receiver operating characteristic (ROS) analysis of plasma growth factors in patients with Hashimoto's disease and healthy controls. Table S7: stomatological characteristics of the HT subgroups. Table S8: clinical characteristics of the participants of the HT subgroups. Table S9: differences in salivary inflammatory profile in HT subjects with hyposalivation compared to normal salivary secretion. Table S10: differences in salivary chemokine profile in HT subjects with hyposalivation compared to normal salivary secretion. Table S11: differences in salivary growth factor profile in HT subjects with hyposalivation compared to normal salivary secretion. Table S12: multifactorial regression of selected salivary biomarkers in all enrolled patients. Table S13: LLOQ, ULOQ, LOD, and intra- and inter-assay precision %CV. *(Supplementary Materials)*

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Research Article

Monitoring Salivary Levels of Interleukin 1 Beta (IL-1β) and Vascular Endothelial Growth Factor (VEGF) for Two Years of Orthodontic Treatment: A Prospective Pilot Study

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Background and Objective. Vascular endothelial growth factor (VEGF) and interleukin 1 beta (IL-1β) perform functions in orthodontic tooth movement and can be measured in the saliva. This novel approach is aimed at monitoring continuous changes in IL-1β and VEGF levels in saliva during two years of orthodontic treatment. *Material and Methods*. Nine healthy females (15-20 years of age) with crowding requiring four premolar extractions and fixed appliances in both jaws were included in this prospective pilot study. A total of 134 stimulated and 134 unstimulated saliva samples were collected during two years of treatment: before tooth extractions (baseline) and then every 6-8 weeks at follow-up appointments. All saliva samples were analysed by the enzyme-linked immunosorbent assay. The mean levels of IL-1β and VEGF were calculated according to the different orthodontic treatment stages: alignment, space closure, and finishing. Repeated analysis of variance (ANOVA) measurements were used to compare the means between different treatment stages. The percentage difference in IL-1β and VEGF between the different treatment stages was analysed by Bland-Altman plots. *Results*. A gradual increase in IL-1β and VEGF was observed at alignment, reaching significance at space closure ($p = 0.002$ and $p = 0.025$, respectively). At finishing, both IL-1β and VEGF declined, however, without reverting to baseline values ($p = 0.172$ and $p = 0.207$, respectively). Bland-Altman analysis showed the agreement between IL-1β and VEGF in terms of a systematic increase, with a higher percentage difference for VEGF. *Conclusions*. The salivary levels of both IL-1β and VEGF increased following orthodontic treatment and reached their peaks during the treatment stage of space closure. This novel approach provides a hint on how and when to sample saliva during orthodontic treatment to analyse bone remodelling.

1. Introduction

The saliva is a biological fluid that has numerous functions in the oral cavity [1]. Recent developments have increased the use of the saliva as a diagnostic fluid for monitoring the overall systemic health of an individual as the content of the saliva changes continuously based on the physiological status of the body [2]. Various cytokines and growth factors found in the saliva have been suggested to monitor inflammation and the course of disease [3, 4]. Vascular endothelial growth factor (VEGF) and interleukin 1 beta (IL-1β) are found in the saliva. VEGF plays an important role in angiogenesis, bone formation, wound healing, and renewal of the oral epithelium [5–7]. IL-1β is a proinflammatory cytokine, and elevated levels of IL-1β can occur in many different processes in the body as an inflammatory response, including bone remodelling and wound healing [8, 9].

Orthodontic force applied during fixed appliance treatment causes an inflammatory process in the periodontal ligament (PDL) that initiates chain reactions that ultimately lead to bone remodelling, resulting in tooth movement [10]. This force initiates different signal cascades through

different cellular responses, thus initiating the remodelling process [11, 12]. In these cellular responses, IL-1β and VEGF are strongly associated with inflammatory processes and their levels affect the biological processes during the orthodontic treatment [12, 13]. In addition, VEGF has the ability to participate in the regulation of bone metabolism and wound healing in orthodontic treatment [14]. IL-1β belongs to the proinflammatory group of cytokines with the role to stimulate bone resorption [15]. Several studies have shown a strong correlation between IL-1β activity and bone remodelling [11, 12, 15, 16].

Gingival crevicular fluid, present in the gingival sulcus, is continuously secreted by the underlying connective tissue into the oral cavity [17]. Previous studies have shown increased levels of IL-1β in the gingival crevicular fluid (GCF) during orthodontic treatment [18]. However, GCF has not been widely used in clinical research due to a number of limitations, namely, the sensitive method requiring good calibration by the clinician collecting the GCF, the amount of collected fluid absorbed by the filter paper being too small and difficult to analyse, the excessive time needed, and the difficulty of using it as a chair-side method in the clinics [9].

Orthodontic tooth movement is a result of continuous inflammation followed by bone resorption and new bone formation. Following those active changes would give a better understanding of the biological processes during ortodontic treatment [10]. However, there are no studies monitoring the levels of cytokines and growth factors during different stages of orthodontic treatment. Therefore, this prospective pilot study was designed as a novel approach to monitor the continuous changes in VEGF and IL-1β levels in the saliva during the different stages of orthodontic treatment: baseline, alignment, space closure, and finishing.

Saliva analysis is a simple and fast method that can be used to examine the ongoing inflammatory processes associated with bone turnover during orthodontic tooth movement. The current study is aimed at showing how VEGF and IL-1β levels are associated with the different stages of orthodontic treatment.

2. Materials and Methods

2.1. Study Setting and Eligibility Criteria. This prospective pilot study consists of consecutively recruited individuals ($N = 9$) who were treated at the Specialist Orthodontic Clinic in Gothenburg, Sweden. All the participants were females between 15 and 20 years of age and had Angle Class II:1 (overjet ≥ 5 mm) malocclusion with moderate to severe crowding ($\geq$6 mm) that required two maxillary and two mandibular premolar extractions and fixed appliance treatment. Only healthy patients without any medication were included. Low caries activity was registered by the general dentist using radiological and clinical examinations prior to the orthodontic treatment. Good oral hygiene was achieved before and also during the orthodontic treatment.

All patients received written and oral information about the study and written consent was signed before the start of the study. The Swedish Ethical Review Authority approved the study protocol (reg.no. 2020-03047).

2.2. Orthodontic Treatment. The orthodontic treatment consisted of four stages: (1) baseline: diagnosis, treatment planning, and tooth extractions; (2) alignment: start with fixed appliance treatment; (3) space closure: closure of extraction spaces and sagittal correction; and (4) finishing. The duration of the four stages was recorded for each patient.

All patients had conventional fixed appliances (MBT system) on all erupted permanent teeth in the maxillary and the mandible for approximately two years. The orthodontic wires (SMART, Beijing Smart Technology Co., Ltd.) used for alignment and finishing were $0.016''$ HANT (heat-activated nickel-titanium, NiTi thermal wires, SMART hant) and $0.019'' \times 0.025''$ HANT (NiTi thermal wires, Smart hant). For space closure and sagittal correction, $0.019'' \times 0.025''$ SS (stainless steel, Smart SS) with e-links and CL II elastics (3.5-4.5 oz, size $3/16''$) were used. Extraction spaces were measured *in vivo* to the nearest 0.5 mm at every appointment; i.e., every 6-8 weeks, by the same orthodontist using a caliper (Dentaurum, Ispringen, Germany).

2.3. Saliva Sampling and Handling. A total of 134 stimulated and 134 unstimulated saliva samples were collected from nine female patients undergoing orthodontic treatment by the same orthodontist for about two years. Saliva samples were collected before tooth extractions, i.e., at baseline and at recalls with 6-8 weeks in between for about two years. All subjects were told to refrain from eating, drinking, or carrying out any oral hygiene procedures for at least one hour before the collection of saliva. For unstimulated saliva, the subjects were asked to expectorate into a 50 ml tube until 5 ml of saliva had been collected. A timer was used to record the sampling time of approximately five minutes. For stimulated saliva, the patients were instructed to chew paraffin wax while continuously gathering all the new saliva into a 50 ml tube until 5 ml of saliva had been collected. The sampling time of approximately three minutes was recorded. After that, the volume of saliva was measured for each individual and calculated as the secretory rate (ml/min). Protease inhibitor cocktail tablets (Sigma-Aldrich, S8830; one tablet diluted in 4 ml distilled water and used 25 μl/ml) and EDTA (Sigma-Aldrich, 2 mM) were immediately added to minimise protein degradation. The samples were immediately stored at -80°C pending analysis. The samples did not undergo centrifugation, as this would have led to loss of proteins [19].

2.4. Sandwich ELISA. An enzyme-linked immunosorbent assay was used to determine the concentration of IL-1β in stimulated saliva and VEGF in unstimulated saliva according to the manufacturer's instructions (R&D systems, USA), except in the case of pretreatment of unstimulated saliva samples with the anionic detergent sodium dodecyl sulphate (SDS, Sigma-Aldrich, 0.02%) in the detection of VEGF. SDS was used to dissociate the mucin particles, as described in our previous study for quantitative detection of salivary cytokines [19]. Unstimulated saliva samples were then incubated for 20 min in SDS (50 μl for 200 μl saliva) and placed on a 96-microwell plate in a duplicate dilution series (from 1/2 to 1/4). The detection of IL-1β was made in stimulated saliva

samples that were incubated on a 96-microwell plate in a duplicate dilution series (from 1/2 to 1/4). Pretreatment of stimulated samples with SDS (0.02%) had no effect on the quantitative detection of IL-1β. For this reason, SDS is only used in unstimulated (whole saliva) samples, where it exerts its effect of dissociating mucin particles. The amounts of IL-1β and VEGF were determined in picograms/millimeter (pg/ml) by measurement of optical density (OD) on a plate reader using a wavelength of 450 nm. A standard curve generated from the OD values of standards provided by the manufacturer was used to determine the concentration of IL-1β and VEGF.

As the capacity to secrete saliva varies greatly between individuals, the total secretory output (pg/min) of IL-1β and VEGF was calculated by multiplying the flow rate (ml/min) for the respective individual by the concentration of IL-1β and VEGF (pg/ml) [19]. The mean output (pg/min) of IL-1β and VEGF was presented according to the different orthodontic treatment stages: alignment, space closure, and finishing.

2.5. Statistical Analysis. Arithmetic means and standard deviations were calculated for IL-1β and VEGF levels in the saliva for all treatment stages. Repeated measurement analysis of variance (ANOVA) was performed to compare the means of treatment stages (baseline, alignment, space closure, and finishing). The Tukey multiple comparison test was performed after the ANOVA, to determine the statistical significance between the treatment stages.

Bland-Altman plots were used for the interpretation of the percentage difference of IL-1β and VEGF between the baseline and alignment, baseline and space closure, and baseline and finishing, using 95% limits of agreement [20]. The statistical significance level was considered to be 5%, and statistics software (version 25; SPSS, Chicago, Ill) was used for all statistical computations.

3. Results

3.1. Demographic and Clinical Data. Table 1 shows the demographic data of the patients. The mean age of the patients at baseline was 18.4 ± 1.4. During the total treatment time (24.4 ± 5.4 months), between 24 and 38 saliva samples were analysed for each patient, depending on the treatment duration (Table 1). In total, 268 samples were analysed to determine the salivary output of IL-1β and VEGF.

3.2. Salivary Output of IL-1β in Stimulated Saliva. The levels of IL-1β in unstimulated saliva samples were close or below to the detection limit of ELISA (data not shown). Therefore, the IL-1β analyses were performed in stimulated saliva samples.

Figure 1 and Table 2 show a highly significant increase in the mean output of IL-1β at space closure (228.49 ± 112.54 pg/min), compared with the baseline (59.53 ± 48.23 pg/min), $p = 0.002$. Although a higher mean output was seen for the alignment (131.28 ± 70.97 pg/min) than for the baseline, the difference did not reach significance, $p = 0.052$. At finishing (168.10 ± 135.31 pg/min), the mean output of IL-1β decreased compared with space closure ($p = 1.00$; however, without reverting to the baseline values ($p = 0.172$).

3.3. Salivary Output of VEGF in Unstimulated Saliva. The levels of VEGF in stimulated saliva samples were close or below to the detection limit of ELISA (data not shown). Therefore, the VEGF analyses were performed in unstimulated saliva samples.

A significant increase in the mean output of VEGF was observed at space closure (575.27 ± 103.67 pg/min), compared with the baseline (193.33 ± 43.77 pg/min), $p = 0.025$ (Figure 1, Table 2). At alignment, a higher mean output was seen (550.21 ± 135.14 pg/min) than at the baseline, although without a significant difference, $p = 0.414$. The mean output of VEGF at finishing (449.23 ± 132.45 pg/min) decreased compared with space closure ($p = 1.00$); however, the values were still high and did not revert back to baseline values ($p = 0.207$).

3.4. Agreement between Treatment Stages for Salivary Output of IL-1β and VEGF. The mean percentage difference between the baseline and alignment IL-1β and VEGF was 6.24, with 95% limits of agreement of 1.06 to 11.41% (Figure 2(a)). Between the baseline and space closure, the mean percentage difference of IL-1β and VEGF was -12.66, with 95% limits of agreement of -31.93 to 6.61% (Figure 2(b)). For the baseline and finishing, the mean percentage difference of IL-1β and VEGF was -1.68 with 95% limits of agreement of -2.92 to -0.45% (Figure 2(c)).

4. Discussion

The present pilot study used the saliva as a research material to monitor the levels of VEGF and IL-1β change during 2 years of orthodontic tooth movement initiated by the orthodontic forces of a fixed appliance. To the far of our knowledge this is the first in vivo study in human settings using the saliva to monitor VEGF and IL-1β in tooth movement. The results of the study showed that a few weeks after the start of treatment, the levels of VEGF and IL-1β increased gradually and reached their highest levels at space closure, when horizontal forces were applied to yield bone remodelling. After the force was removed at the finishing stage, the levels of VEGF and IL-1β were almost halved; however, they were still higher than the baseline levels when analysed six to eight weeks after the space closure.

Continuous pressure to the periodontal ligament stimulates the production of VEGF and thereby angiogenesis [7]. In an experimental study on mice, VEGF has been shown to regulate bone metabolism and tooth movement through the activation of osteoclasts in the periodontal ligament [14]. During orthodontic treatment, compression forces initiate the formation of new blood vessels in the periodontium by activation of VEGF. This, in turn, stimulates vascularisation of new bone formation by angiogenesis, which is achieved by increased levels of VEGF [14]. Accordingly, the current study supports the idea that VEGF via osteoclast activation and angiogenesis perform functions in bone

Table 1: Treatment duration of the different treament stages and spacings in the upper and lower jaw (UJ, LJ) before start of alignment and space closure.

Baseline		Alignment			Space closure			Finishing	Total treatment time (months)
Crowding (mm), Mean ± SD	Overjet (mm), Mean ± SD	Spaces (mm), Mean ± SD UJ	Spaces (mm), Mean ± SD LJ	Treatment time (months), Mean ± SD	Spaces (mm), Mean ± SD UJ	Spaces (mm), Mean ± SD LJ	Treatment time (months), Mean ± SD	Treatment time (months), Mean ± SD	Mean ± SD
3.9 ± 2.4	6 ± 1.7	14.7 ± 1.2	14.7 ± 1.2	7.3 ± 1.7	7.3 ± 2.1	8.8 ± 2.9	13 ± 4.3	4.1 ± 2.1	24.4 ± 5.4

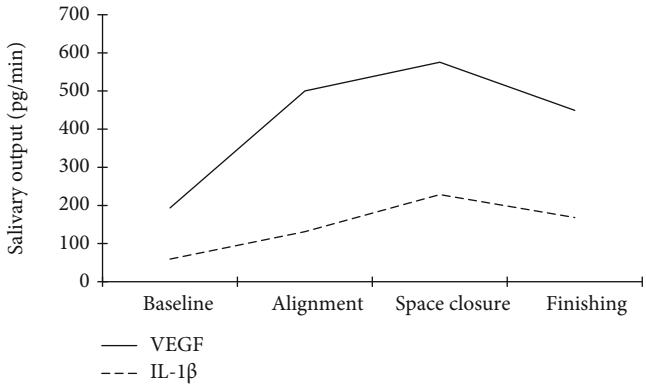

FIGURE 1: Mean salivary output (pg/min) of patients ($N = 9$) for IL-1β (dotted line) and VEGF (solid line) during the treatment stages: baseline, alignment, space closure, and finishing.

remodelling during orthodontic tooth movement, as the highest level of VEGF was found during space closure.

Physiological levels of VEGF are of great importance during bone repair in order to achieve a good result, where low levels have been shown to result in impaired healing [13]. However, very high levels of VEGF may also have harmful effects via inhibition of osteoblast maturation and bone mineralization. Moreover, sustained high levels of VEGF may activate osteoclast unnecessarily, which may result in resorption of the newly formed bone, whereas low plasma levels of VEGF in postmenopausal women have been associated with decreased oestrogen levels [21].

Another study in ovariectomised mice showed decreased levels of VEGF, further supporting the idea that hormonal factors may regulate the levels of VEGF as well as bone metabolism [22]. Furthermore, according to previous studies, there is a close relationship between osteoclast activity and oestrogen levels [23]. Low levels have been shown to accelerate tooth movement by increasing osteoclast activation and inducing bone resorption. In contrast, high levels have an inhibitory effect on tooth movement by increasing bone density and mineral content, which slows bone resorption [24]. In addition, the oestrogen level in plasma has been shown to correlate well with the level in the saliva [25]. In the present study, only young female patients who had their first menstrual period two years ago were included in order to exclude confounding factors such as gender and hormonal differences.

IL-1β is a proinflammatory cytokine and elevated levels are seen in different conditions as an inflammatory response [26]. Mechanical stress that occurs with force application leads to a sterile inflammation, which occurs during the process of bone remodelling in orthodontic treatment [11]. Bacteria-induced inflammation of the gingiva, so-called gingivitis, may also cause elevated levels of IL-1β in the saliva [27]. One of the inclusion criteria in this study was that patients should have optimal oral hygiene before starting treatment but also during the entire treatment period. All the patients in this study had good oral hygiene, except for three patients who showed mild gingivitis on three visits. Although the analysis of these samples showed no deviating values, they were still excluded from the study.

Bone remodelling during orthodontic tooth movement is suggested to be regulated, e.g., by cytokines that interact with bone cells directly or indirectly [15]. Orthodontic force application to the teeth leads to vascular changes in periodontal tissues, causing a sterile inflammation. As a result, proinflammatory molecules and leukocytes are released and migrate to the area of inflammation through the capillaries. IL-1β, which is a proinflammatory cytokine, is secreted by mononuclear cells in the early stages of induced mechanical stress [11]. Further, continued mechanical stress leads to increased levels of IL-1β in the areas of compression [28]. The IL-1β levels are closely correlated with osteoclast activation, which is crucial for the bone remodelling process and the speed of tooth movement [16]. This is in line with the results from the present study, where levels of IL-1β and VEGF started to increase already in the early phase of orthodontic treatment and then reached the maximum levels at space closure, where most of the continued compression forces are applied.

Another known signalling pathway for bone remodelling has been seen to be through RANK/RANKL interactions. In areas of mechanical stress, the levels of IL-1β and VEGF increase, which induces RANK/RANKL expression and activation of cells responsible for bone formation [13]. Studies show that in response to mechanical stress, osteoblasts increase bone resorption by upregulation of the VEGF and RANKL expressions [12]. In summary, both IL-1β and VEGF play important roles in bone remodelling through their effect on osteoblast and osteoclast activity [12, 13].

The lack of standardised laboratory protocols for analysis of saliva specimens [1] has made it difficult to perform quantitative measurements of small proteins such as EGF and IL-8 [19]. However, in the present study, we followed a methodology for detection of VEGF in unstimulated saliva, which we had previously shown would improve the accuracy and reproducibility of ELISA readings when sodium dodecyl sulphate (SDS), a detergent to dissolve mucin aggregates, was used [19]. However, pretreatment of stimulated saliva samples with SDS had no effect on the quantitative detection of IL-1β (data not shown). Therefore, SDS was only used in the pretreatment of unstimulated saliva to dissociate densely packed mucin aggregates, also known as micelles [29]. Low levels of VEGF were found in stimulated saliva, probably due to dilution of glandular proteins with water during high stimulation of paraffin chewing (data not shown). Therefore, the analysis of VEGF was performed in unstimulated saliva samples, where the main VEGF origin is from the salivary glands [6]. In contrast, analysis of IL-1β was performed in stimulated saliva samples, where the main supply of IL-1β present in the saliva is considered the gingival crevicular fluid [30] and can be gained in saliva by chewing [31].

Gingival crevicular fluid (GCF) could be used as a diagnostic and prognostic tool; however, there are several limitations, such as being too expensive and time consuming to be used in clinical settings [17]. On the other hand, saliva testing is simple, inexpensive, and easy to apply in clinics and enables monitoring of cytokines and growth factors originated from GCF and salivary glands. The present study

Table 2: Mean salivary output (pg/min) of patients ($N = 9$), 95% confidence interval, and significant values for IL-1β and VEGF during the treatment stages: baseline, alignment, space closure, and finishing. Comparisons by multivariate analysis of ANOVA: $^{*}p < 0.05$, $^{**}p < 0.01$.

	IL-1β				VEGF			
	Mean ± SD	95% confidence interval for mean		Analysis of variance	Mean ± SD	95% confidence interval for mean		Analysis of variance
		Lower bound	Upper bound			Lower bound	Upper bound	
Baseline (B)	59.5 ± 48.2	22.4	96.6	0.002** B/SC	193.3 ± 131.3	92.4	294.3	0.025* B/SC
Alignment (A)	131.3 ± 70.9	76.7	185.8	NS	500.2 ± 405.4	188.6	811.8	NS
Space closure (SC)	228.5 ± 112.5	141.9	315.0	0.002** SC/B	575.3 ± 311.0	336.2	814.3	0.025* SC/B
Finishing (F)	168.1 ± 135.3	64.0	272.1	NS	449.2 ± 397.4	143.8	754.7	NS

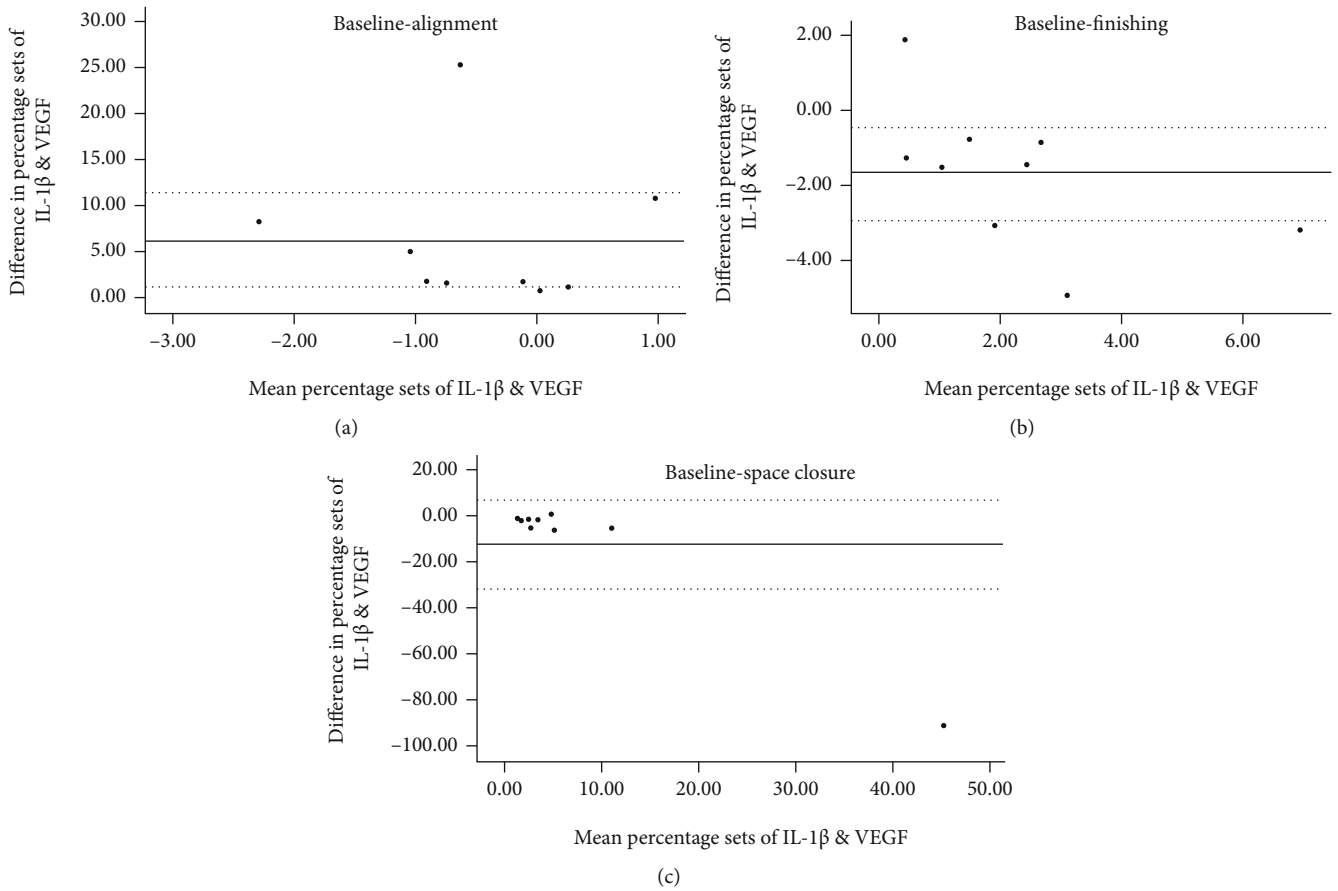

Figure 2: Bland-Altman plot comparing the percentage difference of IL-1β and VEGF between baseline and alignment (a), baseline and space closure (b), and baseline and finishing (c). The dotted line represents the upper and lower 95% limits of agreement, and the solid line represents the mean percentage difference between IL-1β and VEGF.

showed that IL-1β and VEGF can be monitored during orthodontic treatment and likely reflect the biological activity that occurs during orthodontic tooth movement and bone remodelling. The results from our study also show that the saliva as a biological fluid may be used to diagnose and monitor biological responses, as presented during bone remodelling in orthodontic treatment.

Since this is a prospective pilot study it has limitations, such as the small number of included subjects. The study was designed to collect saliva continuously during two years of orthodontic treament, which restricted the size of the study population. However, the results of this novel approach provide indications on how and when to sample saliva during orthodontic treatment. This is beneficial for future studies that may include larger study populations but with fewer saliva samples. It is important to get a better understanding of the biological processes that occur during orthodontic tooth movement.

5. Conclusions

This prospective pilot study is the first *in vivo* study in human settings using continuous sampling of the saliva to monitor VEGF and IL-1β during two years of orthodontic treatment. The salivary levels of both IL-1β and VEGF increased following orthodontic treatment and reached their peaks during space closure. This new knowledge shows how the levels of VEGF and IL-1β change during the different stages of an treatment, indicating their role in bone remodelling.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors report no conflicts of interest.

Authors' Contributions

HCA and JN did the conceptualization, methodology, software acquisition, data curation, validation, supervision, writing—original draft preparation, and reviewing and editing. HCA, FIA and HKT did the formal analysis.

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