CLINICAL RESEARCH

Can Salivary Biomarkers Be Used as Predictors of Dental Caries in Young Adolescents?

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Data Collection B
Statistical Analysis C
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Background: Identifying caries predictors in the subpopulation at risk is one of the preconditions for developing effective caries prevention measures. The present exploratory study aimed to examine the significance of socio-demographic characteristics, dietary-hygiene habits, salivary pH, and salivary antimicrobial HNP-1, hBD-2, and LL-37 peptides as potential caries risk predictors in children ages 11–13 years.

Material/Methods: This prospective 1-year study enrolled 213 children ages 11–13 years. The subjects underwent a dental examination and their mothers were interviewed. Unstimulated saliva was collected from the subjects to determine its pH value, as well as the salivary levels of HNP-1, hBD-2, and LL-37 peptides in 85 of the subjects. After 12 months, the 1-year caries incidence rate was recorded. Logistic regression analysis was used to estimate the ability of selected variables to predict caries risk.

Results: The univariable logistic regression analysis determined that the most significant independent caries risk predictors were: sex (female) (OR=2.132, p=0.007), mothers' education (OR=1.986, p=0.020), salivary pH (OR=0.270, p=0.043), oral hygiene index (OR=1.886, p=0.015), and daily tooth brushing frequency (OR=0.565, p=0.042). The multivariable model showed that sex and oral hygiene-related variables were the most important caries predictors.

Conclusions: Salivary HNP-1, hBD-2, and LL-37 peptides were not found to have a significant predictive value. Therefore, socio-demographic and oral hygiene variables remain important caries predictors in early adolescents, suggesting the importance of the mechanical control of biofilm as the key measure for preventing caries. However, there is still a need for effective caries risk biomarkers, and additional research is needed in this area of caries risk prediction.

MeSH Keywords: **Adolescent • Dental Caries • Oral Hygiene**

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Background

Dental caries is the most common progressive disease of hard dental tissues, and it has a complex and multifactorial etiology. The epidemiolSogical data indicate that dental caries is still a major socio-economic and public health problem, and its prevention remains imperative for dental professionals [1,2]. Unfortunately, a simple method for predicting caries risk does not exist [3]. Therefore, along with the identification of a subpopulation at risk, the identification and knowledge of reliable caries predictors still remain one of the basic preconditions for the development of effective measures of primary prevention and control of this disease [4].

Early adolescence (10–14 years of age) is an important stage in the development of children and is the first step in the transition from childhood into adulthood [5]. In addition to biological, cognitive, and emotional changes, it is characterized by changes in children's habits and behavior that can negatively affect their general and oral health. Data show high caries prevalence in this period (60–90%), and that dental caries and its complications have negative effects on the development and quality of life of children in this age group [1,6,7]. Therefore, early adolescents should be considered a high-risk subpopulation of children, and they deserve special attention in the field of primary dental health protection of children. Due to multifactorial caries etiology, cross-sectional studies have identified diverse socio-demographic, biological, clinical, behavioral and other variables that may be relevant as caries risk indicators in early adolescents [8–12]. However, there is contrasting evidence for their caries-predictive importance [10,13–15]. Previous caries experience may represent the highest caries-predictive value [10,16,17], but its predictive importance is to some extent limited due to the need to identify people with high risk of caries prior to the onset of disease. Thus, further studies are needed to identify a highly reliable caries risk predictor to identify people at risk before the disease occurs.

In the field of caries risk prediction, particular attention has been focused on saliva, which, because of its composition, can be used as a medium for monitoring all 3 primary factors in the etiology of caries [18]. The importance of many salivary components as caries risk biomarkers has been analyzed, but due to the infectious nature of caries, particular attention has focused on salivary antimicrobial proteins, which are essential components of innate oral cavity immunity [19]. Recently, it has been suggested that salivary human α and β defensins and cathelicidin could be used for early identification of individuals at risk [20,21]. These are small cationic antimicrobial peptides that, through their complex mechanism of antimicrobial action, contribute significantly to the biological control of caries [22,23]. Saliva contains 3 α defensins (HNP 1–3) of almost identical antimicrobial activity, 4 β defensins (hBD 1–4),

and the only biologically active human cathelicidin, LL-37 peptide [24]. Human β defensin 2 has shown the strongest bactericidal effect on cariogenic microorganisms, primarily *S. mutans* species, which are most responsible for the development of caries [25]. In addition, human cathelicidin LL-37 peptide and α defensins also have this antibacterial effect [26]. However, it is unclear whether these antimicrobial peptides in saliva can be used as caries risk predictors.

The present study aimed to examine the ability of selected variables to predict caries risk and allow the identification of early adolescents at risk before the onset of caries. Therefore, the aim of this study was to investigate the significance of salivary antimicrobial HNP-1, hBD-2, and LL-37 antimicrobial peptides, salivary pH, socio-demographic characteristics, and dietary habits as potential caries risk predictors in the early adolescent period.

Material and Methods

Study design and subjects

This study was designed as a prospective 1-year study based on data collected at baseline (October–November, 2017) and 12 months later. The study included a population of young adolescents, ages 11–13 years, who were patients at the Department for Preventive and Pediatric Dentistry of the Dental Clinic of the Faculty of Medicine, University of Niš, Serbia, as well as students of 2 primary schools from the city center of Niš. The directors of the institutions were informed in writing about the objectives of the study, after which they gave their written consent for participation. Minor subjects and their parents received (orally and in writing) all necessary information about the study. The study included only children who voluntarily consented to participate, and who had a signed parental consent. The study was conducted following the principles of the Declaration of Helsinki, and was approved by the Ethics Committee of the Faculty of Medicine, University of Niš (approval number 12-14532-2/3).

The basic inclusion criteria were: (1) healthy individuals without history of medication use; (2) children with complete permanent dentition without structural dental defects and initiation of orthodontic therapy; (3) children with permanent residence in the city of Niš since birth (the average fluoride concentration in drinking water is <0.05 mg/mL [27]). The selection of the subjects was done first on the basis of data from school registers/medical documentations (place of birth and residence), and then on the basis of data obtained from mothers and clinical dental examination.

Sample size calculation

The sample size was calculated based on the prevalence of caries in the young adolescent population (86%) in Serbia, the desired level of absolute precision of 5%, study power of 80%, and a population of 6000 adolescents aged 11–13 years in the Nišava District, according to the census estimates of 2018. The sample size was calculated by using methods for proportion in the STATCALC program of EPI INFO version 7.2.2.6. The minimal sample size calculation was 180.

Data collection

This 1-year prospective study was based on baseline data and data obtained 12 months later. The study data were collected in dental offices of the Dental Clinic and primary schools included in the study (the schools have an organized professional dental service), by a well-trained examiner who was a specialist in preventive and pediatric dentistry.

Baseline data collection

After obtaining children's consent to participate, mothers filled in the data on subjects' basic socio-demographic characteristics (age, sex, date, and place of birth, permanent residence, and parents' education), as well as data on their systemic health, use of medications, oral hygiene, and dietary habits.

The subjects selected according to the inclusion criteria were scheduled for further clinical examination and saliva sampling in the morning 1 hour after the usual morning oral routine (children and their mothers were instructed to come to the check-up before breakfast (i.e., with an "empty stomach").

Clinical dental examination of subjects

The clinical dental examination was performed using a probe and a dental mirror. Subjects with structural defects and initiation of orthodontic therapy were immediately excluded from further study. The oral hygiene status of the subjects was assessed using a simplified OHI-S index [28]. Decayed, missing, and filled (DMF) teeth and surfaces were recorded according to the criteria of the World Health Organization (WHO) for epidemiological studies to assess DMFT (decayed, missing, and filled teeth) and DMFS (decayed, missing, and filled surfaces) indexes [29].

Saliva sampling and pH measurement

The samples of unstimulated saliva were collected immediately after clinical examination, by spitting saliva into sterile test tubes. The salivary pH value was determined using a digital pH meter (Hanna Instruments, USA).

Salivary antimicrobial peptides level measurement

The sampling of unstimulated saliva for determining salivary levels of HNP-1, hBD-2, and LL-37 antimicrobial peptides was performed immediately after determining the pH value of saliva. To identify these peptides as reliable caries risk predictors, additional criteria were set for the selection of subjects: (1) no antibiotic treatment at least 1 month before the examination; (2) no soft-tissue disease of the oral cavity; and (3) caries-free children and children with active, untreated caries (children with extracted and filled teeth were excluded).

According to the set criteria, 85 subjects were selected. The sampling of unstimulated saliva for antimicrobial peptide detection was performed immediately after the salivary pH measurement by spitting saliva for 5–10 minutes into sterile tubes to collect 2 ml of sample. The samples were stored at 2°C, and transported within 1 hour after sampling to the Scientific Research Center for Biomedicine of the Faculty of Medicine, University of Niš, for further analysis, where they were centrifuged at 10 000 rpm, at 4°C for 10 minutes. The supernatant was separated and the samples were divided and frozen at –82°C until the next stage.

The concentration of tested antimicrobial peptides was determined using the enzyme-linked immunosorbent assay (ELISA) with commercial ELISA kits (Human alpha-Defensin 1- DuoSet ELISA, DuoSet® Ancillary Reagent Kit 2, R&D System, Minneapolis, USA; Human β-Defensins 2 ELISA Kit, Cusabio Biotech, Houston, USA; Human LL-37 ELISA kit, HyCult Biotech, Uden, The Netherlands). According to the manufacturer's instructions, the detection range of ELISA kit for HNP-1 is from 0.50 to 32 ng/mL, for hBD-2 from 62.5 pg/mL to 4000 pg/ml, and for cathelicidin LL-37 the detection range is from 0.1 to 100 ng/mL. The concentration of all analyzed peptides was expressed in ng/mL.

Dental status follow-up

The dental examination was repeated after 12 months to re-evaluate decayed, missing, and filled (DMF) teeth and surfaces in the subjects, following the same method described above.

Statistical analysis

Data analysis was performed using the program package R [30]. Descriptive statistics included a mean value±standard deviation, as well as absolute and relative numbers.

The 1-year caries incidence rate was determined by directly monitoring changes in the DMFT index after 12 months. A comparison of continuous variables between the 2 independent or paired groups was made using the Mann-Whitney

test or Wilcoxon test, whereas a comparison of categorical variables was made using the chi-square and McNemar tests. The testing of potential caries risk predictors was done using univariable and multivariable logistic regression analyses. The calibration of the multivariable model was tested using the Hosmer-Lemeshow test. The hypothesis was tested with a significance level of $p < 0.05$.

Results

Out of 621 early adolescents, the basic inclusion criteria were met by 247 subjects and they were included in the study at baseline. However, 34 subjects stopped participating in the study (due to relocation, diagnosed systemic disease, started orthodontic therapy, and refusal to take part in the final examination), so the response rate for the final examination was 86.23%. Finally, this 1-year prospective study included 213 early adolescents, and 85 of them were selected for salivary antimicrobial peptides level measurement at the baseline. The basic demographic and clinical characteristics of the subjects are shown in Table 1 and Table 2. The post hoc analysis showed that the strength of the study was 99%.

At baseline, 70.9% of the subjects had DMF teeth. The mean DMFT and DMFS indexes were 4.06 and 5.88, respectively (Table 2). After 12 months, a statistically significant increase in caries was recorded ($p < 0.001$). An average 1-year caries incidence rate was 1.15 DMF teeth (Table 2).

The 1-year caries incidence rate was 56.3% (21.7% of subjects who at baseline were caries-free and 78.3% of subjects with DMF teeth at baseline) (Table 3). Caries incidence was significantly higher in: children with caries at baseline ($p < 0.001$), females ($p = 0.007$), children whose mothers had lower educational level ($p = 0.028$), children with higher OHI-S index values ($p = 0.014$), and children with tooth brushing frequency ≤1 per day ($p = 0.046$). The other examined variables, including salivary pH and salivary level of HNP-1, hBD-2, and LL-37 peptides, were uniform regarding the 1-year caries incidence. The correlation of examined variables with 1-year caries incidence is shown in Table 3.

The univariable logistic regression analysis determined that the most significant caries risk predictors in the study group were: sex (female) (OR 2.123, $p = 0.007$), low mothers' education (OR 1.986, $p = 0.020$), high OHI-S index (OR 1.886, $p = 0.015$), tooth brushing frequency ≤1 per day (OR 0.565, $p = 0.042$), and low salivary pH (OR 0.270. $p = 0.043$) (Table 4).

The multivariable model showed that statistically significant caries risk predictors in the early adolescent period were sex, high OHI-S index, and tooth brushing frequency ≤1 a day, adjusted for all other parameters in the model. The increase of the value of the OHI-S index for one unit in this population increased the risk for caries by 76% (OR 1.764). In females, the risk of caries was almost 3 times higher (OR 2.908). The tooth brushing frequency was a protective factor (OR 0.519), and it decreased the risk of caries by almost 2 times. The model was well-calibrated (Hosmer-Lemeshow test $p > 0.05$) (Table 5).

The interaction of sex, OHI-S index, and tooth brushing frequency ≤1 a day showed no statistically significant association with caries risk (OR 1.043, $p = 0.742$).

Discussion

As previously mentioned, early adolescence is a specific developmental period characterized by numerous changes, including ones related to children's habits and behavior that can adversely affect their oral health [5]. Caries is one of the most common oral diseases in this period, the prevalence of which increases with the age of children [1]. This was confirmed by this study, which registered a high annual caries incidence rate of 1.15 DMFT. Because the study included young adolescents from a middle-income country, the result confirmed

Table 1. Basic demographic characteristics of the study group.

Characteristic	Mean±SD
Age (years)	12.54±0.56
Sex	
Male (N, %)	99
Female (N, %)	114

Table 2. Prevalence of dental caries in study group of young adolescents.

Caries prevalence indexes	At baseline	Follow-up	p-value*/annual caries incidence
Subjects with DMF teeth (%)	70.9%	80.1%	<0.001**
DMFT index	4.06±3.54	5.21±4.44	<0.001/1.15 DMFT
DMFS index	5.88±6.35	8.28±8.61	<0.001

* Wilcoxon test; ** χ^2 test.

Table 3. Clinical, socio-demographic, dietary, oral hygiene, and salivary characteristics of subjects with regard to 1-year caries incidence.

Characteristics	One-year caries incidence				P*
	Yes		No		
	N	%	N	%	
	120	56.3	93	43.7	
DMFT index at baseline					
DMFT=0	26	21.7	35	37.6	<0.001**
DMFT ≥1	94	78.3	58	62.4	
Sex					
Male	46	38.3	53	57.0	0.007
Female	74	61.7	40	43.0	
Mothers' education					
≤12 years	88	73.3	54	58.1	0.028
>12 years	32	26.7	39	41.9	
Fathers' education					
≤12 years	73	61.3	50	53.8	0.332
>12 years	46	38.7	43	46.2	
The average number of daily meals (mean±SD)		4.72±1.01		4.56±1.00	0.250
Frequency of refined carbohydrates intake					
<1 a day	42	35.0	38	40.9	0.482
1 a day	23	19.2	20	21.5	
≥2 a day	55	45.8	35	37.6	
OHI-S index (mean±SD)	120	1.21±0.55	93	1.02±0.55	0.014
Tooth brushing frequency					
<1 a day	11	9.2	9	9.7	
1 a day	53	44.2	26	28.0	0.046
≥2 a day	58	46.7	58	62.4	
Tooth brushing duration					
<1 min	26	21.7	16	17.2	0.166
1–2 min	70	58.3	48	51.6	
>2 min	24	20.0	29	31.2	
Salivary pH (mean±SD)	120	6.94±0.34	93	7.07±0.30	0.702
HNP-1 ng/mL (mean±SD)	49	12.69±5.61	36	13.02±3.78	0.376
hBD-2 ng/mL (mean±SD)	49	2.84±1.30	36	2.84±0.91	0.554
LL-37 ng/mL (mean±SD)	49	1.74±2.03	36	1.35±1.30	0.569

* Mann-Whitney test; ** McNemar test.

Table 4. Potential caries risk predictors in the examined group of young adolescents (univariable logistic regression analysis).

Potential predictors	OR	95% CI	p-Value
Sex (Female)	2.132	1.228–3.699	0.007
Mothers' education ≤12 years	1.986	1.115–3.538	0.020
Fathers' education ≤12 years	0.733	0.423–1.270	0.268
Number of daily meals	1.168	0.892–1.528	0.259
Frequency of refined carbohydrates intake			
≤1 a day	1.040	0.495–2.187	0.917
>1 a day	1.422	0.772–2.617	0.258
OHI-S index	1.886	1.130–3.147	**0.015**
Tooth brushing frequency ≤1 a day	0.565	0.325–0.980	**0.042**
Tooth brushing duration			
<1 min		Reference category	
1–2 min	0.897	0.436–1.849	0.769
>2	0.509	0.223–1.162	0.109
Salivary pH	0.270	0.076–0.962	**0.043**
HNP-1	0.986	0.903–1.077	0.752
hBD-2	1.002	0.686–1.464	0.991
LL-37	1.145	0.875–1.498	0.322

OR – odds ratio; 95% CI – 95% confidence interval.

Table 5. Potential caries risk predictors in the examined group of young adolescents (multivariable model).

Potential predictors	B	OR	95% CI		p-Value
			Lower	Upper	
Sex (Female)	1.067	2.908	1.575	5.369	**0.001**
Mothers' education ≤12 years	−0.599	0.549	0.265	1.140	0.108
Fathers' education ≤12 years	−0.006	0.994	0.494	2.001	0.987
Number of daily meals	0.284	1.328	0.984	1.792	0.063
Tooth brushing frequency ≤1 a day	−0.656	0.519	0.274	0.984	**0.044**
OHI-S index	0.567	1.764	1.015	3.065	**0.044**
Salivary pH	−1.281	0.278	0.041	1.882	0.189
HNP-1	−20.437	0.000	0.000	30.264	0.688
hBD-2	85.109	9169167	0.000	47.231	0.688
LL-37	0.127	1.135	0.830	1.552	0.428
Constant	−2.222	0.108			0.035

B – regression coefficient; OR – odds ratio; 95% CI – 95% confidence interval; Hosmer-Lemeshow test: p=0.55.

that early adolescence is a high caries risk period, stressing the need for the identification of reliable caries predictors in this subpopulation of children.

The present study examined the caries-predictive significance of salivary HNP-1, hBD-2, and LL-37 antimicrobial peptides, salivary pH, and socio-demographic and hygiene-dietary variables that can identify a young adolescent at risk before the disease appears, which is why previous caries was not considered. The predictive significance of the analyzed variables was assessed in a 1-year prospective study, and the findings showed that the most important independent caries predictors were female sex, low mothers' education, OHI-S index (OR 1.886, p=0.015), tooth brushing frequency ≤1 per day, and low salivary pH. The study determined that sex and oral hygiene variables were the strongest predictors of caries risk.

The study findings confirm that in early adolescence, effective predictors of caries still need to be sought in socio-demographic characteristics and oral hygiene habits of children. This is mostly in line with the findings of other authors, who have often examined the caries-predictive significance of variables by both cross-sectional studies and retrospective longitudinal studies [10,13,14,31].

Sex and oral hygiene variables showed the highest predictive significance. Girls are 3 times more likely to have caries, which probably results from an earlier eruption of permanent teeth, and their longer exposure to cariogenic noxae during the post-maturation period when they are most susceptible to caries. The study shows that the tooth brushing frequency is a preventive factor and that children who brush their teeth twice or more times a day have a 2-fold lower risk of developing caries. The OHI-S index also was a good predictor of caries risk, which suggests that in addition to regularity and frequency, tooth brushing techniques are also important in predicting caries. It is interesting that despite the importance of nutrition in caries etiology, children's dietary habits did not show predictive significance, probably because their caries-predictive importance is often masked by fluoride use and oral hygiene habits [32].

An important goal of this study was to examined the caries-predictive significance of HNP-1, hBD-2, and L-37 cationic peptides. Cross-sectional studies have suggested their role as indicators of caries risk [19–21], indicating the need to determine their predictive significance. Therefore, in the present exploratory study, we examined the correlation of salivary levels of these peptides with the incidence of caries, and their possible caries-predictive significance, to confirm them as effective caries risk biomarkers at this age. Unfortunately, the study did not confirm that. As this is the first study to explore the predictive importance of these peptides in young adolescents, the result

is difficult to compare, but it is in a line with the finding of Simon-Soro et al. [33], who examined the importance of these peptides as predictors of early childhood caries.

Our results should not discourage further research in this area. First, the study examined the caries-predictive importance of these peptides in a 1-year prospective study, which could be considered an important limitation of the study. However, the small sample size tested for salivary biomarkers can be considered the major limitation of the study, and the main cause for the lack of statistical significance regarding the caries-predictive importance of these biomarkers. In addition, the mechanism of their antimicrobial activity is complex. Their antibacterial effect is achieved by direct action and by extracellular and intracellular mechanisms, as well as the ability to influence the acquired immune response and enhance its effect [22,26]. Therefore, further well-designed, longer-term studies are needed to clarify the importance of these peptides as caries predictors. In addition, further studies should consider the analysis of the predictive importance of other non-immunoglobulin antimicrobial proteins that saliva is rich in [34]. This could make a huge change in the prediction of caries risk and is therefore important in primary caries prevention.

This study has confirmed that young adolescents are a subpopulation of children at high risk and who need special attention in the field of primary caries prevention. Based on the present study, along with sex and mothers' level of education, the most important caries predictors in were oral hygiene-related variables, suggesting that the key to caries prevention still lies in the responsible behavior of children towards oral health and that mechanical plaque control remains a central caries preventive measure. The obtained results suggest that health education with children should start as early as possible, from young school age, and, in addition to children, should also include their mothers. However, a healthcare education program should not be based solely on providing information, as it usually offers only short-term results. Creating healthy habits is a complex and time-consuming process, the effectiveness of which requires strong motivation and remotivation of children to adopt them, and later apply them through life. This is the basis of oral health promotion, and it is especially important in this developmental period.

Conclusions

In conclusion, salivary HNP-1, hBD-2, and LL-37 peptides were not found to have a significant predictive value. Therefore, socio-demographic and oral-hygienic variables remain important caries predictors in young adolescents, suggesting the importance of the mechanical control of biofilm as the key caries-preventive measure. However, there is still a need for

effective caries risk biomarkers. Such biomarkers are likely to be found among the many salivary antimicrobial proteins that saliva is rich in, and additional research is necessary in this area of caries risk prediction.

Conflicts of interest

None.

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