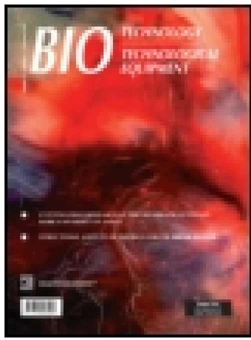

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Saliva diagnostics of sex hormones and subgingival microflora in children in puberty

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ABSTRACT

During puberty there are increased levels of sex hormones, which can affect the oral environment. At this period, there is a peak prevalence of periodontal pathology believed to be related to alteration in the subgingival microflora. This study investigated the interaction between sex hormones isolated in the saliva, and the subgingival microflora in children undergoing puberty. The study included 60 children aged 10–14 years who were monitored: 30 without gingivitis (up to 25% Papillary Bleeding Index—PBI) and good oral hygiene, and 30 children with plaque-induced gingivitis (over 50% PBI). All patients were registered with a periodontal status using a medical card developed for this purpose. For the study of sex hormones (oestradiol, progesterone and testosterone), samples of unstimulated saliva were taken under fasting condition in the morning and were tested by using labelled immunological analysis and liquid chromatography with mass spectrometry [LC-MS (MS(QQQ)]. Gingival sulcus from six teeth was taken with paper pins to test nine control strains (pooled sample) by real-time polymerase chain reaction (PCR). The results showed that, when comparing children with gingivitis with healthy children, only the oestradiol hormone had elevated values. In the children with gingivitis, the composition of the subgingival microflora was much more varied and complex, with a tendency to increase the species diversity of microorganisms from the red complex. The oestradiol levels in saliva correlated with the total number of subgingival microorganisms, as well as with some species of microorganisms, *Capnocytophaga gingivalis*, *Peptostreptococcus micros*, *Treponema denticola* and *Tannerella forsythia.*

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Introduction

During puberty there are increased levels of the sex hormones oestradiol, progesterone and testosterone, which can affect, in particular, the oral environment. There is a peak prevalence of periodontal pathology in children in puberty and is believed to be related to alteration in the subgingival microflora at this period. The bacterial counts increase in number and the subgingival microflora become more complex [1–3].

Among the risk factors for periodontal pathology in children in puberty, sex hormones are most predominant, as they are considered an important modifying factor which can influence the pathogenesis of plaque-associated gingivitis [1]. Sex hormones have a proven affinity toward the gingival tissue and the alveolar bone, and also have an influence on the formation of the subgingival microflora [4–6].

The influence of sex hormones on periodontal health is proven, but not completely studied due to the invasive nature of blood sample hormonal level studies. An alternative approach is 'oral based diagnostics'—the use of saliva as a non-invasive diagnostic environment. Oral based diagnostics makes it possible to study in detail the influence of various factors, among which the influence of sex hormones, on the periodontal health of children and adolescents [7–9].

Saliva diagnostics of hormones is an effective method for gathering biological material with the least amount of inconvenience on the part of the subject studied. The process is simple and can be performed multiple times. Furthermore, according to data in scientific literature, saliva offers a more biologically active free substance in regard to steroid sex hormones. This is confirmed by studies which show a high and statistically significant level of correlation between serum and salival concentration (total or free) of sex hormones [8–13].

It is considered that there is a direct correlation between the concentration of sex hormones in the

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saliva and in the blood serum. This gives basis to adopt saliva as an appropriate, noninvasive environment for the diagnosis of multiple biomarkers. This includes the study of the dynamics of sex hormones in the period of sexual maturation [8,9,12,13].

Microbiological identification of the bacteria from the subgingival microflora during periodontal diseases poses many problems. They are due to the inability of all periodontal pathogens, organized in microbial consortiums in a complex biofilm, to be cultivated by standard microbiological methods.

To date, polymerase chain reaction (PCR) is considered as the best method for studying microbial diversity without cultivation, basing the analysis on the isolation and amplification of specific microbial DNA. PCR is defined as a highly specific, highly sensitive and fast method for discovering periodontal pathogens in real time [14–16].

The aim of this study was to investigate the influence of sex hormones isolated in saliva, and the subgingival microflora in children undergoing puberty (10–14 year olds). Here, we performed quantitative analysis of the sex hormones in the saliva in children with and without plaque-induced gingivitis; isolated the subgingival microorganisms in children with and without plaque-induced gingivitis; and correlated the subgingival microorganisms with the sex hormone levels in the saliva of the studied children.

Subjects and methods

Clinical study

The study included 60 children between the ages of 10 and 14 with no systemic diseases and no antibiotic intake 3 months prior. The children were distributed into two groups:

- 30 children (mean age ± SD, boys 12 ± 1.464 years; girls = 12 ± 1.464 years) without gingivitis (Papilla Bleeding Index Saxer & Mulheman [17]—PBI $<$ 25%) and with good oral hygiene (Oral Hygiene Index-Green Vermilion—OHI-GV $<$ 1);
- 30 children (mean age ± SD, boys 12 ± 1.464 years; girls 12 ± 1.464 years) with plaque-induced gingivitis (over 50 PBI $>$ 50%).

We assumed that all children with a PBI spread $<$25% (percentage of bleeding papillae in all papillae studied) fall into the control group of children, not suffering from plaque-induced gingivitis. Children with PBI $>$ 50% fall into the group suffering from plaque-induced gingivitis, because they have more clear clinical manifestations and greater spread of gingival inflammation than 25–50% of PBI score patients. The children with a PBI score of 25–50% who we examined were very few and were excluded from the study.

The periodontal status of all children was registered through the use of a purpose made medical card. The following indices were assessed: OHI-GV and PBI (spread). The subjects of the study were tested for oestradiol, testosterone and progesterone levels in the saliva, and no plausible differences in the isolated quantities of the hormones were observed in regard to age and sex.

Sex hormone levels in the saliva

For the study of sex hormones in the saliva (oestradiol, progesterone and testosterone) samples of unstimulated saliva were taken in the morning, with the subjects being fasted prior to sampling. The saliva samples were collected in sterile, plastic containers and were frozen (-20°C).

Quantitative analysis of steroid hormones in the saliva

The sex hormones in the saliva of the subjects were studied through a immunological analysis and liquid chromatography-mass spectroscopy [LC-MS (MS(QQQ)]. The analyses were carried out with a Q Exactive (ThermoScientific, Germany) mass spectrometer equipped with a Transcent (ThermoScientific, Germany) autosampler, TurboFlow (ThermoScientific, Germany) ultra-effective chromatographic system and a source of chemical ionization—APCI (ThermoScientific, Germany). The data gathered from the analyses were registered, stored and processed with the Xcalibur 4.1 (ThermoScientific) software package.

Chromatographic conditions. Analysis of the steroid hormones was carried out through the TurboFlow method. Special polymer turbulent columns were used to carry out a preliminary purification and concentration of the samples, with the substances studied being adsorbed onto the column. After that, the substances were transferred to the analytical column. The process was carried out in the in-line (uninterrupted) mode of operation. To analyze the testosterone, progesterone and oestradiol levels in the saliva, the following chromatographic phases were used:

Phase A – 10 mm ammonia hydrogen carbonate in water;
Phase B – acetonitrile/phase A (9/1, (vol/vol)).

The samples were first concentrated in a TurboFlow C18 XL chromatography column, using 50 μL of sample (saliva diluted to 1/1 (vol/vol) in phase A). The analytical separation was carried out on an analytical column Syncronis C18 1.7 μm 50 × 2.1 mm in gradient elution mode.

Mass spectroscopy conditions. The analysis of testosterone, progesterone and oestradiol levels in the saliva was carried out using the parallel reaction monitoring (PRM) mode of operation of the mass analyzer. The analyzed substances were brought into a gaseous phase through chemical ionization, under atmospheric pressure and by using nitrogen as a carrier gas.

Quantitative analysis. The quantitative analysis was carried out through the external standard method using calibration curves. For testosterone and progesterone, the calibration curve range was 0.05–1000 ng/mL, for oestradiol the curve range was 1–1000 ng/mL. The quantity of the hormones in the samples was determined on the basis of these calibration curves. The analysis of each sample was repeated three times.

Microbiological study—real-time PCR

Real-time PCR was used for identifying the subgingival microorganisms in the studied samples and their relative quantity (microorganisms in samples). For the purposes of this study, samples were taken from the gingival sulcus of each child from six teeth (16, 13, 11, 26, 36, 43) with the use of paper pins. The samples were sent for analysis to a laboratory in Germany through MiPharma.

Nine control strains of subgingival microorganisms were studied: *Aggregatibacter actinomycetemcomitans* (purple complex); *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia* (red complex); *Prevotela intermedia, Peptostreptococcus (Micromonas) micros*, *Fusobacterium nuleatum*, *Eubacterium nodatum* (orange complex); *Capnocytophaga gingivalis* (green complex).

DNA isolation from gram-positive and gram-negative bacteria was done using a commercial kit (Roche Diagnostics) and a MagNA Pure 96 system (Roche Diagnostics). The reagents were packaged in cartridges, the system was closed. The PCR set-up was done with Hamilton's Microlab STARlet IVD. The final analysis of the samples was done using a LightCycler II 480 Roche.

The quantifying was carried out through the use of intercalating fluorescent dyes (e.g. SYBR-Green) or fluorescently marked probes (e.g. TaqMan probes), in which the increase of the fluorescent signal was recorded.

Data analysis

Data are presented as mean values with standard error of the means/standard deviation (±SD). Statistical analysis was performed using Independent Samples *t*-test (comparison between two groups). $p < 0.05$ indicated statistically significant differences. The results were analysed using SPSS–19 software.

Results and discussion

Quantitative description of the sex hormones in the saliva of children with and without gingivitis

The subjects of the study were tested for oestradiol, testosterone and progesterone levels in the saliva. Although gingival crevicular fluid (GCF) sampling provides more specific information about the periodontal inflammation than saliva, saliva offers a more biologically active free substance in regard to steroid sex hormones. There are a number of studies comparing serum and salival concentrations (total or free) of sex hormones that show a high and statistically significant level of correlation [8–13]. It is considered that there is a direct correlation between the concentration of sex hormones in the saliva and in the blood serum. This gave us a basis to adopt saliva as an appropriate, non-invasive environment to measure the sex hormones levels and to study their dynamics in the period of sexual maturation and their link to oral pathology.

The results (Table 1) showed that the average values of oestradiol in the healthy children (male and female) were 0.838 ± 0.53 ng/mL, of progesterone 0.105 ± 0.07 ng/mL, and of testosterone 0.102 ± 0.09 ng/mL. In the children with plaque-induced gingivitis, the oestradiol levels were higher when compared with the healthy children, 1.252 ± 0.37 ($p < 0.05$). The

Table 1. Quantities of sex hormones in children with and without plaque-induced gingivitis.

	Estradiol (ng/mL)		Testosterone (ng/mL)		Progesterone (ng/mL)	
	N	Mean ± SD	*N*	Mean ± SD	*N*	Mean ± SD
Healthy	30	0.838 ± 0.53	30	0.105 ± 0.07	30	0.102 ± 0.09
Gingivitis	30	1.252 ± 0.37	30	0.096 ± 0.06	30	0.125 ± 0.06
	$t = 2.988$ $p = 0.032$		$t = 0.598$ $p = 0.552$		$t = 0.738$ $p = 0.469$	

Table 2. Frequency of microorganisms according to gingival inflammation.

	Healthy		Gingivitis		Total		
Microorganisms	*N*	%	*N*	%	*N*	%	*Chi*-square test
A. actinomycetemcomitans	0	0	7	100	7	100	$\chi = 7.925$ $p = 0.005$
P. gingivalis	0	0	8	100	8	100	$\chi = 9.231$ $p = 0.002$
T. denticola	3	13.6	19	86.4	22	100	$\chi = 18.373$ $p = 0.000$
T. forsythia	1	8.3	11	91.7	12	100	$\chi = 10.417$ $p = 0.001$
P. intermedia	2	18.2	9	81.8	11	100	$\chi = 5.455$ $p = 0.020$
P. (Micromonas)micros	6	28.6	15	71.4	21	100	$\chi = 5.934$ $p = 0.015$
F. nucleatum	21	42.9	28	57.1	49	100	$\chi = 2.069$ $p = 0.150$
E. nodatum	0	0	2	100	2	100	$\chi = 5.455$ $p = 0.020$
C. gingivalis	30	50	30	50	60	100	

progesterone and testosterone levels were similar when comparing healthy children to children with gingivitis ($p > 0.05$).

These results are in agreement with previous reports that children at the age of sexual maturation exhibit heightened sex hormone levels and an increased frequency of gingival diseases [2–4,8,9]. The obtained data support the popular notion that gingival inflammation can be made worse by physiological conditions related to increased levels of sex hormones, such as puberty, pregnancy or when using oral contraceptives. Heightened levels of circulating sex hormones are considered to account for the severity of inflammation, as well as for the processes of hyperplasia of the gingiva [5,6].

Gingivitis at such an age is related to an increase in the quantity of dental plaque. Multiple studies point to the fact that changes in the hormonal levels further modify the gingival environment and have an influence over the type and the severity of the inflammation [4,7–9]. The results of this study point to a similar dependency in relation the quantities of oestradiol in the saliva of children with gingivitis. According to other authors, gingival diseases are not necessarily related to an increase in the quantity of dental plaque [2,3].

According to Goldie [5,6], the gingiva has progesterone and oestrogen receptors. Heightened plasma levels of oestrogen and progesterone cause an accumulation of these hormones in the gingival tissues. Sex hormones have an influence on the microcirculatory system and often lead to noticeable changes in the periodontium. As a whole, oestrogen is responsible for changes in the blood vessels, whereas progesterone stimulates the production of inflammatory mediators. Fluctuations in the concentrations of the hormones cause dilation of the gingival capillaries and an increase in their permeability, thus leading to an increase in the gingival exudate, oedema and accumulation of cells for defense and inflammation.

Identification of subgingival microorganisms in children with and without plaque-induced gingivitis

As a next step in our study, we identified the subgingival microorganisms (Table 2). The real-time PCR results showed that *C. gingivalis* (green complex) was found in all the subjects, whereas *F. nucleatum* (orange complex) was relatively evenly distributed between the ill and healthy children ($p > 0.05$). The other representatives of the orange complex identified in this study, *P. intermedia* and *P. micros*, were more frequently found in children with plaque-associated gingivitis ($p < 0.05$).

Some microorganisms from the red complex, such as *T. denticola* and *T. forsythia*, were identified in both groups of children, but the percentage of cases in the group with gingivitis was significantly higher ($p < 0.05$). Interestingly, three of the microorganisms tested were identified only in the children with gingivitis: *A. actinomycetemcomitans*, *P. gingivalis* and, only in two children, *E. nodatum*, which indicates their high pathogenicity.

Aggregatibacter actinomycetemcomitans (gam-negative microaerophilic) is considered as a primary microbial factor in aggressive periodontitis. In combination with various defects in the immune response, owing to various genetic polymorphisms, it leads to the loss of periodontal structures [18–20].

The red complex is comprised of the most aggressive periodontal pathogens: gram-negative, anaerobic microorganisms: *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Treponema denticola*, which colonize last and possess pathogenic potential for inflammation and periodontal destruction (in cases of chronic periodontitis) [21,22]. These microorganisms have specific pathogenic factors (proteinases, endopeptidases, leucotoxin A, cytolethal toxin and others). All of them participate in two ways in defeating the local immune response in the periodontium: (1) through stimulating immunopathogenic reactions—disrupting the balance between pro and anti-inflammatory interleukins, MMPs and their inhibitors, and (2) through a direct destructive action against the connective tissue and the alveolar bone in the area of the periodontium [22–24].

Table 3. Total microorganisms count in children with and without plaque-induced gingivitis.

	Healthy		Gingivitis		
	N	Mean ± SD	*N*	Mean ± SD	Ind *t*-test
Total	30	$3.7 \times 10^7 \pm 5.6 \times 10^7$	30	$9.1 \times 10^7 \pm 1.0 \times 10^8$	$t_{1,2} = -2.525$ $p = 0.014$

Table 4. Correlation between sex hormone levels and subgingival microorganisms.

		Oestradiol		Testosterone		Progesterone	
	N Children	Pearson Correl.	Sig.	Pearson Correl.	Sig.	Pearson Correl.	Sig.
Total microorganism count	60	0.233*	0.047	−0.041	0.364	−0.111	0.215
A. actinomycetemcomitans	60	−0.172	0.109	−0.021	0.442	−0.102	0.235
P. gingivalis	60	−0.069	0.311	−0.034	0.405	−0.096	0.246
T. denticola	60	−0.269*	0.026	−0.158	0.129	−0.161	0.125
T. forsythia	60	−0.254*	0.033	−0.127	0.182	−0.157	0.131
P. intermedia	60	−0.101	0.235	−0.119	0.198	−0.140	0.159
P. (Micromonas)micros	60	−0.256*	0.032	−0.188	0.089	−0.126	0.184
F. nucleatum	60	−0.167	0.116	−0.082	0.280	−0.158	0.130
E. nodatum	60	−0.096	0.245	−0.082	0.281	−0.111	0.214
C. gingivalis	60	−0.229*	0.049	−0.193	0.083	−0.135	0.167

*Level of significance $p < 0.05$.

According to Kumar [24], immediately after the onset of puberty, *P. intermedia* and *Prevotella melaninogenica* can be found more frequently in boys, whereas *Actinomyces odontolytius* can be found more frequently in girls. Despite this, these changes do not remain for the whole period, during which it is expected for sex hormone levels to rise. The proportions of these strains do not increase for the rest of a subject's life [25,26]. In children who have developed gingivitis, higher levels of spirochetes and *Eikenella corrodens* can be found. According to data found in scientific literature, an increase in *P. intermedia* can be observed as puberty progresses, especially in boys [25–27].

Through the use of ELISA and DNA hybridization, studies have shown that as children get older, the quantity of *P. gingivalis* increases; and whereas *P. gingivalis* colonizes only temporarily in children, in adults the microbial settlement and colonization is stable. Also about 50% of children between the ages of 13 and 18 are colonized by *T. forsythia* and *T. denticola*, which are the most frequently recognized periodontal pathogens [14,26]. Accumulating evidence shows that the real-time PCR method is becoming increasingly preferred to classical culture techniques for analysis of subgingival periodontal pathogenic microflora due to its ability for automated, fast and precise detection of the periodontal pathogens and assessment of their quantities [14,18,26,28–30].

Subgingival microbial load in children with and without plaque-induced gingivitis

The total microbial load was determined from the subgingival plaque samples taken from the subjects. The data are presented in Table 3. The average quantities of microorganisms in healthy children were 3.7×10^7 (microorganisms in samples). Higher quantities of microorganisms were isolated form the children with gingivitis, 9.1×10^7 ($t = -0.525$, $p < 0.05$). The total quantity of subgingival microorganisms increases with the development of the gingival inflammation during the period of sexual maturation.

Correlation between the subgingival microorganisms and the sex hormones in the saliva of the children tested

To assess the potential dependencies between the quantities of sex hormones in the saliva and the isolated subgingival microorganisms, the Pearson correlation index was used, with a level of probability $p < 0.05$ and $p < 0.01$. In this study, only oestradiol correlated with the growing number of subgingival microorganisms (Table 4). There was also a correlation between oestradiol and *C. gingivalis, P. micros* as well as with representatives of the red complex, *T. denticola* and *T. forsythia.*

Multiple studies have reported the influence of sex hormones on the composition of the periodontal microflora, which accounts for the observed peak in the spread and severity of gingivitis at that age [1,5,6,14]. Studies show an increase in the proportion of gram-negative anaerobes from puberty, up to the post-pubescent age, among which are black-pigmented anaerobic bacteria. The proportions of *P. intermedia*, *Capnocytophaga* and *E. corrodens* also increase [19,20,25,30].

It is known that the gingiva contains receptors for ovarian hormones, making this tissue a target for the

influence of oestrogen and progesterone. Gingival inflammations that develop during puberty can be, according to some authors, influenced by these hormones [5,6,14]. According to these authors, sex hormones influence the subgingival ecosystem as a whole, but the dependencies between specific hormones and specific subgingival microorganisms have not been explored in greater depth yet [5,6,14].

The effects of sex hormones on gingival inflammation have not yet been fully studied. While the primary role of the biofilm for the onset of the gingival inflammatory reaction is undisputed, the possibilities for the participation of sex hormones in the potentiation of this reaction have not been made fully clear [1,2,25]. The present study strongly suggest that the ages of childhood and adolescence are characterized by specific dynamically changing indicators for periodontal health and illnesses, related to tooth eruption, the stabilization of the periodontium and the oral microflora, especially that with periodontal pathogenic potential. The influence of primarily female hormones during puberty is related to the frequency of the gingival inflammation, which, in its less severe forms, need not necessarily be of the hyperplasic type.

Conclusions

Based on the obtained results, we speculate that oestradiol has an influence on gingival inflammation, whereas the effect of progesterone and testosterone is not well defined. In the studied subjects, during gingival inflammation, the subgingival microflora became more complex, with microorganisms from the red complex (*P. gingivalis*, *T. denticola*, *T. forsythia)* being isolated and an increase in the frequency of orange complex microorganisms. In comparing children with gingivitis to healthy children, the total microbial load of the microorganisms studied increased. A correlation was present between oestradiol and the total number of subgingival microorganisms, as well as with the following species of microorganisms—*C. gingivalis*, *P. micros*, *T. denticola*, *T. forsythia*. Future studies need to clarify how the levels of sex hormones in saliva affect the quantity, diversity and complexity of the subgingival biofilm in a larger group of subjects.

Disclosure statement

No potential conflict of interest was reported by the authors.

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