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Stress and salivary proteins as indicators of caries experience in children

SUMMARY OF Ph.D. THESIS

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Study hypothesis and objectives

Psychosocial factors induce a state of chronic stress to the individual, which has both psychological and physiological effects. Biological changes include responses of the neuroendocrine system, with multiple consequences, including at salivary level. Since saliva plays the role of a protective medium for the oral cavity, even minor changes in its composition might have an impact on the proper functionality of this ecosystem. Therefore, in the context of caries disease, loss of protective properties of saliva may lead to an increased incidence of dental caries.

Starting from these observations and from the fact that changes in the oral cavity reflect the general state of health, we developed the study hypothesis according to which the caries experience of children is influenced by stress and levels of salivary proteins. The general objective for the present study was to investigate whether stress and various salivary proteins levels have any influence on dental caries experience in children.

In the first study (**Chapter 5**) we evaluated the dental caries experience and the levels of salivary enzymatic activity – total protease activity and matrix metalloproteinase activity, in a group of schoolchildren, along with elucidation of the relationship between the above-mentioned. In addition, we aimed to investigate whether the levels of these salivary proteins reflect dental caries severity and differ between children of different age categories.

In the second study (**Chapter 6**) we evaluated the levels of salivary biomarkers for stress, salivary alpha amylase and salivary cortisol, in a group of schoolchildren, in relationship with dental caries experience and with salivary enzymatic activity.

In the third study (**Chapter 7**) we evaluated the level of dental anxiety in a group of schoolchildren presenting for dental treatment in a specialised paediatric dentistry clinic. Further, we aimed to determine the level of stress induced by two different dental treatments (a non-invasive intervention consisting of dental prophylaxis procedures versus an invasive intervention consisting of a cavity preparation and application of a dental restauration). The levels of stress were determined by combining psychometric evaluations (State-Trait Anxiety Inventory for Children scale) and observational evaluations (Frankl Behaviour Rating Scale) with measurements of salivary stress biomarkers levels (salivary alpha amylase and salivary cortisol).

General study methodology

Setting and ethical considerations

The studies were performed on a limited group of healthy schoolchildren, who presented at the Paediatric Dentistry Clinic of the Faculty of Dental Medicine, of Carol Davila University of Medicine and Pharmacy, Bucharest, for dental treatments between January 2019 and June 2022, taking into consideration the COVID-19 pandemic lock-down.

The studies were approved by the Research Ethics Committee of the Carol Davila University of Medicine and Pharmacy (no. 188/28 January 2019) and were carried out in accordance to the recommendations of the Declaration of Helsinki (2008) of the World Medical Association and to Good Clinical Practice guidelines. Prior being admitted to the study group, the parents or legal guardians of the participants were informed about the aims and protocols of the study and if they agreed to participating, they signed a written informed consent.

Study participants

Children presenting for the first time at the University Paediatric Dentistry Clinic were recruited for the study group if they met the following inclusion criteria:

- physically and mentally healthy;
- aged 5 to 14 years old;
- presence of at least one caries lesion on either primary or permanent teeth;
- absence of intraoral mucosal lesions or periodontal disease.

Participants were excluded based on the following criteria:

- a history of systemic diseases;
- the presence of mental disabilities;
- the administration of medical treatments that could have affected the activity of the salivary glands in the 3 months preceding admission in the study;
- refusal of participation from their parents or legal guardians accompanying them to the clinic;
- presence of a dental emergency that required immediate complex treatment (acute pain due to pulp disease, dental abscesses, traumatic injuries);
- children with previously traumatic experiences in the dental office.

Dental caries experience indices

Caries experience of the subjects included in the study was established through clinical assessments using standard examination instruments. Examinations were performed by a single trained examiner (R.-P.V.) in a dental office, respecting World Health Organization recommendations (Peterson et al., 2013). Afterwards, caries experience indices were calculated. The Decay-Missing-Filled indices (DMF/dmf) were calculated correspondent to teeth (DMFT/dmft) and surfaces (DMFS/dmfs). Considering that children with mixed dentition had their caries experience evaluated through four different indices that didn't reflect a global view of the actual situation, we also used Manifest Caries Lesion index (MCL) in order to gain a better understanding of the active caries experience and the overall severity of the disease. MCL scores were calculated by summing the number of teeth with untreated dental caries. Furthermore, we defined MCL% as the percentage of teeth with untreated dental caries out of the total number of teeth present in the oral cavity.

Saliva sampling

In order to standardize the saliva collection protocol, all saliva collections were performed between 8 and 11 a.m. This timing allowed the avoidance of major discrepancies between patients' recordings that could have been generated by diurnal variations of the investigated salivary parameters (Dawes, 1972). Prior to saliva sampling, specific written instructions were provided to the parents or legal guardians of the participants (Kelly et al., 2008). On the day of saliva collection, upon arrival, the children were invited in the dental office and were comfortably seated on the dental chair. Sterile and gradated polypropylene containers were provided to the subjects and the examiner indicated on the container the quantity of saliva that would be necessary to be deposited. The passive drooling method was carried on under the supervision of the examiner until approximately 2 mL of saliva was accumulated in the containers (Putnam et al., 2012). Saliva samples were centrifuged at 3500g for 15 minutes and aliquots of the saliva supernatants were transferred to 0.5 mL Eppendorf vials and deposited at -80°C, in order to avoid multiple freezing and thawing cycles of the same saliva sample.

Salivary enzymatic activity

Total protein content (TPC) was determined using the Pierce™BCA Protein Assay Kit (Thermo Scientific, Landsmeer, The Netherlands), as previously described (Prodan et al., 2015). The results were expressed in µg/mL.

Protease activity was detected using Fluorescence (or Förster) Resonance Energy Transfer (FRET)-coupled peptide substrates. FRET-peptide substrates used in this study were PEK-054 ([FITC]-NleKKKKVLPIQLNAATDK-[KDbc]) and PFU-089 ([FITC]-FR-[KDbc]), as previously described (Janus et al., 2015). Salivary total proteolytic activity was expressed as the increase in fluorescence per minute (ΔF/min).

Salivary levels of MMP-8 and MMP-9 were determined using Luminex xMAP (multianalyte profiling) technology for detection and quantification. A multiplex panel was obtained by combining two Simplex kits containing specific antibodies for each metalloproteinase considered (ProcartaPlex Multiplex Immunoassay, Affymetrix, eBioscience, Thermofischer, Vienna, Austria). The assay was carried out based on the protocol provided by the manufacturer (Vacaru et al., 2022a).

Salivary biomarkers for stress

Salivary alpha amylase activity was determined with a colorimetric-based enzymatic activity assay. The amylase specific substrate used was 2-chloro-4-nitrophenyl-α-D-maltotrioside (Sigma-Aldrich, Zwijndrecht, The Netherlands), which was dissolved in an Assay Buffer (100 mM MES, 350 mM NaCl, 6 mM Calcium Acetate, 900 mM Potassium Thiocyanate, 0.1% Sodium Azide) to obtain the substrate solution. The results were expressed in U/mL. (Vacaru et al., 2022a).

Salivary cortisol was assessed using an ELISA – Enzyme Linked ImunoSorbent Assay method with a commercially available kit (NovaTec Immundiagnostica GmbH, REF DSNOV20, Dietzenbach, Germany). The results were expressed in ng/mL (Vacaru et al., 2022b).

Observational behavioural rating

Situational fear was subjectively measured using the Frankl Behaviour Rating Scale (FBRS) (Frankl SN, 1962). The subject's reaction to dental treatment was assessed by the paediatric dentist performing the intervention (R.-P.V.) and it was classified into one of the

following categories of behaviour: definitely negative (--), negative (-), positive (+), definitely positive (++).

Psychometric assessments

For psychometric assessments, the validated Romanian version of the State–Trait Anxiety Inventory for Children scale (Mindgarden, Inc., Menlo Park, CA, USA, licence OL-00008675/ 2019-11.22 distributed by D&D Consultants/TestCentral) was administered (Spielberger CD, 1973). The test was composed of two forms, state and trait anxiety forms, each containing 20 items, which evaluated how the subject was feeling (Appendix 1). The questionnaires were administered at the end of the treatment sessions, in the same dental office, before post-treatment saliva sample was collected. The child was guided and supervised by the paediatric dentist, who also provided explanatory answers whenever the subject faced difficulties in understanding the terms used in the forms. The "state" anxiety form (STAIC S-Anxiety) evaluates how the subject is feeling in a given situation and the "trait" anxiety form (STAIC T-Anxiety) evaluates how the subject is feeling in general situations.

Data analysis

Statistical data analysis was performed using the program Stata/IC 16 (StataCorp.2019. Stata Statistical Software: Release 16. College Station, TX, USA: StataCorp LLC). Normal distribution was tested for quantitative variables using with the Shapiro–Wilk test. Data distributions were expressed as means, standard deviations (SD), medians, quartiles (Q1 and Q3), intervals and percentages. Correlations between variables were investigated using either Pearson (r) or Spearman (r_s) correlation coefficients. For categorical measures, Pearson Chi-squared tests were used. Fisher exact test was used when the expected frequency of any cell in the table was <5. Comparisons between pre-post levels were determined using a paired t-test or a sign test. Intergroup comparisons were performed using unpaired t-test or a one-way ANOVA test, with Scheffé correction test. Statistical significance was considered for p-values < 0.05.

Chapter 5. Evaluations of dental caries experience in children in relationship with salivary enzymatic activity

This was an observational clinical cross-sectional study performed on 22 healthy school children (mean age 8.2 ± 2.6 years old), who presented for the first time for dental treatments at the Paediatric Dentistry Clinic of the Faculty of Dental Medicine, of the Carol Davila University of Medicine and Pharmacy, Bucharest, between January 2019 and March 2020. During their first visit, objectives and protocol of the study were explained, written description of the study and specific instructions were provided, and if agreed on participating, clinical assessments were performed. Saliva collections were scheduled for a second visit for standardization purposes. On the second visit, clinical assessment and saliva sampling were conducted. Afterward dental caries indices were calculated and salivary analysis were performed to determine total protein content, total proteolytic activity and MMP-8 and -9 levels.

In **Chapter 5** we showed that there was a relationship between dental caries experience and salivary enzymatic activity. We observed that dental caries experience indices differed between different age groups. A plausible explanation is the fact that most of the children included in the study had a mixed dentition at the time of participation in the study. Therefore, the number of already untreated dental caries affecting primary teeth cumulated with the number of dental caries occurring in permanent teeth, most frequently on the first permanent molars. In the context of high caries activity, newly erupting first permanent molars often get affected by dental caries for several reasons: lack of a proper hygiene - the children are not able to brush the most posterior surfaces, exposure to cariogenic diets, and insufficient knowledge of parents - who might not be aware that the last molar is belonging to permanent dentition and for this reason they often ignore its necessity for immediate sealing or in some cases even treatment (Llena et al., 2020). Therefore, is of utmost importance to inform parents about appropriate dental hygiene and dietary habits, as well as to treat caries lesions in primary teeth as soon as possible, and to implement prophylactic therapies, such as local fluoridation (at home and in the dental office) and sealing of newly erupting permanent teeth.

Levels of salivary enzymatic activity did not differ significantly between different age categories. With respect to total proteolytic activity, the two substrates used, PFU-089 and PEK-054, showed different relationships with dental caries. This observation is justified by their difference in length and composition of amino acid sequence, and therefore by the

different types and number of salivary proteases that might degrade these substrates. Looking at our results, PEK-054 seems more probable to be an indicator of dental caries as opposed to PFU-089. The longer amino acid sequence of PEK-054, containing a variety of different amino acids, offers a much broad range of cleavage sites for different enzymes than PFU-089 with its unique Phenylalanine-Arginine combination. Considering the strong positive association between PEK-054 degradation and dental caries, further experiments to determine which part(s) of the sequence of PEK-054 substrate are cleaved in dental caries seem warranted, and could help to develop an even more specific substrate with an improved potential for dental caries diagnosis. Based on the cleavage preferences of MMP-8, it might be possible that PEK-054 is degraded by this matrix metalloproteinase (Rawlings et al., 2002). Unpublished results from preliminary experiments tend to confirm this hypothesis but additional research is needed.

Considering matrix metalloproteinase activity, high salivary levels of both MMP-8 and MMP-9 were associated to high caries experience. This observation could be justified by a bi-directional relationship between MMPs activity and dental caries. Firstly, during cavitation, due to local acidic pH, pro-MMPs are released from dentin and are activated, contributing to tooth degradation, but they also reach in the saliva, becoming detectable in the oral cavity. And secondly, MMPs originating from saliva or from gingival crevicular fluid, already present in the oral cavity, can penetrate through the enamel-dentin junction and become involved in the caries destructive process (Chaussain-Miller et al., 2006, Chaussain et al., 2013).

Considering that high salivary levels of MMP-8 and MMP-9 are indicators of existing destructive lesions in the oral cavity and that the subjects included in the present study did not exhibit any signs of periodontal disease or any other oral lesions, as well as the fact that the saliva samples were not contaminated with blood, we may conclude that, in this group of patients, high levels of MMP-8 and MMP-9 are indicators of caries activity.

Furthermore, in future studies, assessments of salivary levels of MMP-8 and MMP-9 in relationship with dental caries should be evaluated by comparison to a caries-free control group. Interestingly, the levels of salivary MMP-8 and MMP-9 registered in children affected by dental caries should also be compared to the levels registered in caries-free children that are affected by periodontal disease. Unfortunately, considering that the study was conducted in a university paediatric clinic, where usually are referred patients with various oral problems, it is rarely the case for a caries-free child to present, unless another oral pathology is associated, such as dental trauma or dental anomalies, for example. Therefore, in the present study no

caries-free control group was included. In order to create such a control group, future studies should also recruit children by other means, e.g., recruitment with the help of schools.

In addition, further research should be conducted using a longitudinal study design. One possible longitudinal design could be to recruit a large cohort of caries-free children and to determine their initial levels of salivary enzymatic activity. This would provide individual baseline levels for each child. Subsequently, changes in the enzymatic levels should be monitored during future re-calls. The objective would be to evaluate whether the salivary enzymatic activity changes in those children that develop dental caries during follow-up periods. Another possible longitudinal design might be to include children with various degrees of caries experience and to evaluate whether after treatment of dental caries the level of salivary enzymatic activity changes. An additional outcome would be to investigate whether after treatment of dental caries the enzymatic activity levels are comparable to the levels registered in caries-free children. The proposed longitudinal approaches should provide valuable information on the role of these salivary enzymes on dental caries progression over time. The salivary enzymatic activity might prove to be a practical indicator of caries experience levels, as well as an indicator of treatment's efficacy in reducing caries activity.

Optimistically, salivary enzymatic activity could be evaluated in the near future with chair-side diagnostic tests, similar to those used to determine salivary pH, buffer capacity or bacterial load. These tests might be used as practical instruments to monitor patients' caries activity over time as an integrated part of minimally invasive dentistry, in order to detect periods when caries activity levels increase. This provides the dentist with information to know when it is necessary to increase preventive methods or intervene therapeutically. Contrarily, in case of low caries activity as indicated by levels of salivary enzymatic activity, incipient or superficial caries lesions could simply be monitored and kept under control through routine prophylactic methods. Moreover, the availability of a chair-side test could help motivate patients to better comply to periodical dental check-ups and to implement healthy oral routines.

Chapter 6. Stress in children in association with dental caries experience and salivary enzymatic activity

This was an observational clinical cross-sectional study performed on the same group of 22 healthy school children (mean age 8.2 ± 2.6 years old) recruited at their first visit for dental treatments at the Paediatric Dentistry Clinic of the Faculty of Dental Medicine, of Carol Davila University of Medicine and Pharmacy, Bucharest, between January 2019 and March 2020, that was presented in *Chapter 5. Evaluations of dental caries experience in children in relationship with salivary enzymatic activity.* The research protocol included evaluations of dental caries experience and saliva collection. From the same saliva samples that were already collected, as previously described, besides salivary enzymatic activity analyses, additional biochemical analyses were performed in order to determine the salivary levels of the stress biomarkers alpha amylase and cortisol.

In **Chapter 6** we showed that the two stress response systems, HPA axis and ANS, appeared to have different effects on dental caries experience in children. The potential stressful event, as perceived by the subjects in this study, was the presentation to a dental clinic, and both stress biomarkers, salivary alpha amylase and salivary cortisol, responded accordingly, as was shown by the association between their levels normalized to total protein content. However, when their relationship to dental caries was further explored, these salivary biomarkers acted differently. In fact, salivary alpha amylase seemed to have a protective role, as higher levels were associated with lower dental caries experience, while levels of salivary cortisol showed no relationship whatsoever with dental caries experience. The negative relationship observed between salivary alpha amylase and dental caries could be justified by its enzymatic activity (Lahiri et al., 2021), although its levels are influenced by ANS system's responses to stressful stimuli (Ali and Nater, 2020). While amylase over cortisol represents a combined and coordinated response of HPA and ANS systems activity (Ali and Pruessner, 2012), its relationship with dental caries experience is most probably on account of salivary alpha amylase's relationship with dental caries.

Considering the long periods of time necessary for the development of a caries lesion, it is unlikely that situational stress has direct influence on occurrence of dental caries. In this study, we evaluated acute levels of salivary stress biomarkers, in association to a given situation and we did not evaluate chronic stress levels, which might be more plausible to have an influence on the development of dental caries. To obtain information about perceived chronic

stress, multiple saliva samples should be obtained from the participating children, at different time points throughout the day, as well as in different days over a longer period of time. This approach would enable a comparison between different given situations with different effects on stress levels. Such studies should also include evaluations of basal reference levels from saliva collected under low-stress situations, e.g., in the morning, at home, upon waking up, in several successive days.

Additionally, we investigated potential interactions between salivary stress biomarkers and salivary enzymatic activity. We showed that higher levels of salivary alpha amylase were associated with lower salivary total proteolytic activity and with lower salivary MMP-9 levels. Considering that we have shown previously that higher levels of salivary alpha amylase are associated with lower dental caries experience and that low levels of total proteolytic activity and MMP-9 are associated with low dental caries experience, the above-mentioned interaction seems plausible. To our knowledge, this relationship has not been explored previously in clinical studies, and the potential interactions between these salivary proteins and their influence on dental caries experience represent interesting and promising research topics that should be further explored.

Chapter 7. The effect of different types of dental treatments in caries management on stress and salivary protein levels

This was a pre-post clinical study performed on a group of 28 healthy school children, who presented at the Paediatric Dentistry Clinic of the Faculty of Dental Medicine, at Carol Davila University of Medicine and Pharmacy, Bucharest, for dental treatments between January 2019 and June 2022. Prior being admitted to the study group, the parents or legal guardians of the participants were informed about the aims and protocols of the study and if they agreed to taking part in the study, they signed a written informed consent. The children required dental treatments and according to their needs were allocated into two study groups: a prophylaxis group (PR) or a cavity preparation group (CP). During the following dental visit, children in PR group (n = 14) received a prophylactic treatment consisting of professional brushing with rotary instruments and topical fluoridation using fluoride gels. Children in the CP group (n = 14) received a restorative dental treatment. Before and 30 min after finalisation of the treatment session, the children were requested to provide a sample of unstimulated whole saliva for analysis of salivary stress biomarker levels. Additionally, anxiety levels were evaluated using observational and psychometric methods, as previously described.

In **Chapter 7**, we showed that different dental treatments have a different effect on salivary levels of stress biomarkers, alpha amylase and cortisol, and that the two stress systems, HPA axis and ANS, reacted differently to comparable stress levels. The more stressful the event was, the higher the increase in the levels of both salivary stress biomarkers was. However, these biomarkers still followed their diurnal pattern (McCarthy et al., 2009, Rohleder and Nater, 2009), which meant that regardless of being exposed to a stress-inducing event, such as the two dental treatments we performed, the post-treatment levels of salivary cortisol decreased, while the post-treatment levels of salivary alpha-amylase increased compared to pre-treatment levels. Considering that post-treatment levels registered a higher decrease for salivary cortisol and a lower increase for salivary alpha amylase in the case of dental prophylaxis compared to cavity preparation, it means that prophylaxis, a non-invasive intervention, was perceived as less stressful in comparison to cavity preparation, which was perceived as a more complex and invasive intervention. Age and caries experience did not influence the degree of stress or anxiety levels in children exposed to dental treatments.

In addition, children that exhibited a less cooperative behaviour during dental treatments registered higher levels of state anxiety. This observation shows that the more stressful a given

situation is perceived by a child, the less cooperative he or she will behave. However, State-Trait Anxiety Inventory for Children assessments failed to show any associations with salivary levels of stress biomarkers, alpha amylase and cortisol. Furthermore, in this study, psychometric evaluations were not sensitive enough to differentiate between children exposed to non-invasive and children exposed to invasive dental treatments. Nonetheless, on larger scale studies, these psychometric evaluations might show promising results and should be further explored in dental settings.

Moreover, an important strategy to prevent dental anxiety would be to include children in a dental education and prevention programme, and to initiate their first dental visits at a younger age, when no dental diseases had already occurred. Age and caries experience did not influence the degree of stress or anxiety levels in children, which means that more factors should be explored for understanding dental anxiety and stress levels induced by dental treatments. Children that exhibited less cooperative behaviour during dental treatments had higher levels of state anxiety, associated to a certain experience, but not of trait anxiety, that would define a chronic, general anxiety.

General discussions, conclusions and personal contributions

Taken altogether, these studies showed some interesting results despite the several limitations that are going to be discussed further. COVID-19 pandemic restrictions limited recruitment of participants, meaning that the sample size of this study was relatively small. The initial design of the study also had a longitudinal component, meaning that after the treatment of all caries lesions was achieved, re-evaluations of salivary levels of the investigated parameters were to be performed in order to assess whether the reduction of untreated dental caries might have affected their levels. However, COVID-19 pandemic restrictions made it impossible to follow-up all the included participants, or in some cases even to finish all dental treatments, limiting this study to a cross-sectional design. This was not the only reason for losing follow-up of patients, but also, after having all their caries lesions treated, some patients failed to come to periodical check-up in the absence of pain or discomfort.

An unexpected challenge that we faced was the collection of saliva samples. Saliva collection in general and the ability to provide the required volume of saliva seemed problematic for some children, especially younger ones who needed more time to complete this assignment. This is also one of the reasons why we excluded the possibility of having saliva samples collected at home by parents for baseline values. In that situation we would not have been able to control the collection process, as well as the afterward storage and transportation conditions of the samples.

Another methodological limitation is the fact that behavioural evaluations were performed by only one investigator, with the risk of introducing some subjectivity to these assessments. An option to reduce this risk would be to use video recordings of the treatment sessions, which could be used to have other investigators evaluating the behaviour of the subjects.

Lastly, we should also consider that the children were included in the study as they presented in the university paediatric dental clinic during the main investigators shift (R.-P.V.) and only if they met all the eligibility criteria. This meant that there were time gaps between different patients and therefore, the saliva samples collected from different subjects had different storage times in the freezer. In order to minimize these storage times, the biochemical analyses were performed in batches and executed as soon as a reasonable number of samples was collected. Although this procedure had reduced biochemical degradation of the salivary

parameters during storage, however, it had introduced a certain inter-assay variation associated with batch processing.

Within the limitation of these studies, our findings contribute to the existing knowledge of how various salivary proteins are associated with caries experience in children. In fact, the novelty of this study is represented by the fact that we conducted a comprehensive interdisciplinary study with multi-level assessments in a paediatric group. To our knowledge, the association of these evaluations taken together had not been previously explored.

This study provides valuable information on how different salivary proteins might interact and modulate each other's activity, and further influence the progression of dental caries. This makes them potential indicators for monitoring dental caries evolution. Out of the investigated parameters, salivary alpha amylase, MMP-8 and MMP-9 proved to be strong indicators of caries experience. Although determination of salivary total proteolytic activity with PEK-054 substrate lacks specificity, it shows the potential to be a promising indicator as well. Another valuable insight of the studies described in this thesis is the demonstration of the interactions of the two stress systems, HPA and ANS. From a practical approach, by integrating objective and subjective evaluations of children's behaviour and reactions during dental treatment sessions, paediatric dentists might be able to optimize their approach and to manage patients' levels of stress more efficiently, considering that a cooperative behaviour not always excludes the presence of dental anxiety.

Taken together, our results underline the need for better understanding the mechanisms through which stress and salivary proteins levels might influence caries experience and how various dental treatments affect children's behaviour and stress levels.

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LIST OF PUBLICATIONS

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