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SALIVARY CHANGES ASSOCIATED WITH HYPOMINERALIZED SECOND PRIMARY MOLARS (HSPM) AND MOLAR INCISOR HYPOMINERALIZATION (MIH)

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SALIVARY CHANGES ASSOCIATED WITH HYPOMINERALIZED SECOND PRIMARY MOLARS (HSPM) AND MOLAR INCISOR HYPOMINERALIZATION (MIH)

Dissertação apresentada à Universidade Estadual Paulista (UNESP), Faculdade de Odontologia de Araçatuba, para obtenção do título de Mestra.
Área de Concentração: Saúde Bucal da Criança

Orientador(a): Profa. Dra. Cristina Antoniali Silva

Coorientador(a): Profa. Dra. Cristiane Duque

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Eu dedico esse trabalho à minha família. Sem vocês, esse sonho não seria possível.

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“Science is more than a body of knowledge; it is a way of thinking. It is a bright light at the end of a dark tunnel – the darkness of fear, superstition, ignorance, dogma, and manipulation. The light of reason, criticism, and the pursuit of truth. The fact that we are here is a testament to our ability to escape that darkness.”

Carl Sagan

HORTA, H.F. **Alterações salivares associadas à Hipomineralização de Segundos Molares Decíduos (HSMD) e Hipomineralização Molar Incisivo (HMI).** 2025. Dissertação (Mestrado) – Faculdade de Odontologia, Universidade Estadual Paulista (UNESP), Araçatuba, 2025.

RESUMO

Este estudo teve como objetivo avaliar as alterações salivares e os fatores associados em crianças com ou sem Hipomineralização de Segundos Molares Decíduos (HSMD) ou Hipomineralização Molar-Incisivo (HMI). O estudo incluiu 45 crianças (com idades entre 2 e 11 anos), divididas em três grupos: controle (n = 15), HSMD (n = 15) e HMI (n = 15), classificadas de acordo com a Academia Europeia de Odontopediatria (EAPD), considerando opacidades demarcadas e a extensão do defeito. Um questionário foi utilizado para coletar informações sobre condições sistêmicas nos primeiros três anos de vida e durante a gestação materna. A saliva foi coletada utilizando Salivette®. As análises incluíram taxa de fluxo salivar, pH, capacidade tampão da saliva, concentrações de cálcio, fosfato, flúor, proteínas, atividade da amilase, capacidade oxidativa total, peroxidação lipídica (TBARS), capacidade antioxidante total e ácido úrico. Para avaliar os parâmetros salivares relacionados às opacidades demarcadas (Branco/Creme ou Amarelo/Marrom) e a extensão do defeito (menor que 1/3, menor que 1/3 e maior que 2/3 e maior que 2/3) nos parâmetros salivares, os grupos HSPM e MIH foram combinados para melhor compreensão. Os resultados obtidos foram analisados: Os dados qualitativos foram avaliados pelos testes de Kruskal-Wallis e Dwass-Steel-Critchlow-Fligner. Os dados quantitativos foram analisados pelos testes One-Way ANOVA e Tukey. Os dados não paramétricos (mediana e intervalo interquartile) foram analisados pelo teste de Kruskal-Wallis; os dados paramétricos (média ± DP) foram analisados pelos testes ANOVA e Tukey. As diferenças foram significativas quando $p < 0.05$. Trabalho de parto prolongado e febre no primeiro ano de vida foram os únicos fatores etiológicos associados aos grupos HMI e HSMD. As análises salivares mostraram um aumento nos níveis de cálcio e redução de fosfato no grupo HMI, enquanto o grupo HSMD apresentou menores níveis de proteínas e maior capacidade antioxidante total. Opacidades amarelas/marrons foram associadas ao aumento do dano oxidativo por peroxidação lipídica e à atividade antioxidante. Os resultados indicam que HSMD e HMI são influenciados por fatores individuais e ambientais. O trabalho de parto prolongado e a febre na infância foram

identificados como fatores associados importantes. Além disso, as alterações salivares relacionadas à composição mineral, aos níveis de proteínas e ao equilíbrio redox destacam sua relevância no manejo dessas condições.

Palavras-chave: Hipomineralização Molar Incisivo, Saliva, Estresse Oxidativo, Biomarcadores

HORTA, H.F. **Salivary changes associated with Hypomineralized Second Primary Molars (HSPM) and Molar Incisor Hypomineralization (MIH).** 2025. Dissertação (Mestrado) – Faculdade de Odontologia, Universidade Estadual Paulista (UNESP), Araçatuba, 2025.

ABSTRACT

This study aimed to evaluate salivary changes in children and the associated factors with, or without, Hypomineralized Second Primary Molars (HSPM) or Molar Incisor Hypomineralization (MIH) The study included 45 children (aged 2 -11 years), divided into control (n=15), HSPM (n = 15) and MIH (n = 15) classified by European Academy of Paediatric Dentistry (EAPD), considering demarcated opacities and extent of the defect. A questionnaire gathered information on systemic conditions during the first three years of life and maternal pregnancy. Saliva was collected using Salivette®. Analyses included salivary flow rate, pH, salivary buffering capacity, calcium, phosphate, fluoride, protein concentration, amylase activity, total oxidative capacity, lipid peroxidation (TBARS), total antioxidant capacity, and uric acid. To evaluate the salivary parameters related to demarcated opacities (White/Creamy or Yellow/Brown) and the extent of the defect (less than 1/3; greater than 1/3 and less than 2/3 and greater than 2/3) on salivary parameters, the HSPM and MIH groups were combined for a better understanding. The obtained results were analyzed: Qualitative data were evaluated by Kruskal-Wallis and Dwass-Steel-Critchlow-Fligner tests. The quantitative data were analyzed with One-way ANOVA and Tukey tests. Non-parametric data (median and interquartile range) were analyzed by Kruskal-Wallis' test; parametric data (mean ± SD) were analyzed with ANOVA and Tukey Tests. Differences were significant when $p < 0.05$. Prolonged labor and fever in the children's first year of life were the only etiological factors associated with MIH and HSPM groups. Salivary analyses revealed increased calcium and reduced phosphate in MIH, while HSPM exhibited lower protein levels and higher total antioxidant capacity. Yellow/Brown opacities were associated with increased lipid peroxidation and antioxidant activity. HSPM and MIH are influenced by individual and environmental factors. Prolonged labor and early-life fever were key associated factors. Salivary changes related to mineral composition, protein levels, and redox equilibrium, highlighting their importance in management of MIH and HSPM.

Keywords: Molar Hypomineralization, Saliva, Oxidative Stress, Biomarkers

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LISTA DE ABREVIATURAS E SIGLAS

AMELX	Amelogenin
AMNB	Ameloblastin
ANOVA	Analysis of Variance
ART	Atraumatic restorative treatment
CAAE	Ethical Appreciation Submission Certificate
CPP-ACP	Phosphopeptide-amorphous calcium phosphate
DNA	Deoxyribunucleic Acid
EAPD	European Academy of Paediatric Dentistry
ENAM	Enamelin
ESRRB	Estrogen-related receptor β
g/L	Grams per liter
HOPT	Hypomineralization of Other Permanent Teeth
HSPM	Hypomineralized Second Primary Molars
MDA	Malondialdehyde
mg/g	Miligrams per gram
MIH	Molar Incisor Hypomineralization
Min	Minutes
mL/min	Milliliters per minute
mM	Milimolar
mmol/L	Milimoles per gram
mol/L	Moles per liter
NADPH	Nicotiamide Adenine Dinucleotide Phosphate
nm	Nanometer
OR	Odds Ratio
OS	Oxidative stress
PEB	Post-eruptive breakdown
pH	Power of Hydrogen
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
SD	Standard Deviation
SOD	Superoxide dismutase
TAC	Total antioxidant capacity

TBARS	Thiobarbituric Acid Reactive Substances
TCLE	Informed Consent Form
TOC	Total Oxidant Capacity
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
UA	Uric Acid
WHO	World Health Organization
µL	Microliter
µmol Fe^{2+}/g	Micromoles of Fe^{2+} per gram
µmol/g	Micromoles per gram

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Salivary changes associated with Hypomineralized Second Primary Molars (HSPM) and Molar Incisor Hypomineralization (MIH)

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Abstract

Aim: This study aimed to evaluate the associated factors and salivary changes in children with, or without, Hypomineralized Second Primary Molars (HSPM) or Molar Incisor Hypomineralization (MIH)

Methods: The study included 45 children (aged 2 -11 years), divided into control (n=15), HSPM (n = 15) and MIH (n = 15) classified by European Academy of Paediatric Dentistry (EAPD). A questionnaire gathered information on systemic conditions during the first three years of life and maternal pregnancy. Saliva was collected using Salivette®. Analyses included salivary flow rate, pH, salivary buffering capacity, calcium, phosphate, fluoride, protein concentration, amylase activity, total oxidative capacity, lipid peroxidation (TBARS), total antioxidant capacity, and uric acid. To evaluate the salivary parameters related to demarcated opacities and the extent of the defect on salivary parameters, the HSPM and MIH groups were combined for a better understanding.

Results: Children in MIH group were older than those in the other groups, prolonged labor and fever in the children's first year of life were the only associated factors related to MIH and HSPM groups. Salivary analyses revealed increased calcium and reduced phosphate in MIH, while HSPM exhibited lower protein levels and higher total antioxidant capacity. Yellow/Brown opacities were associated with increased lipid peroxidation and antioxidant activity.

Conclusion: HSPM and MIH are influenced by individual and environmental factors. Prolonged labor and early-life fever were key associated factors. Salivary changes related to mineral composition, protein concentration, and redox equilibrium, highlighting their importance in management of MIH and HSPM.

Keywords: Molar Hypomineralization; Saliva; Oxidative Stress; Biomarkers

1. Introduction

Molar incisor hypomineralization (MIH) is a qualitative enamel defect that affects one to four permanent molars, with possible involvement of the incisors [1,2]. Nowadays, it is known that other permanent teeth, such as second premolars and canines [3,4] may also be affected, a condition described as Hypomineralization of Other Permanent Teeth (HOPT) [5]. Additionally, hypomineralization also affects primary teeth, such as second molars and canines (HSPM) and their presence are associated with 10.9 times likelihood of MIH occurrence, according to a recent systematic review and meta-analysis [6]. The clinical characteristics of MIH, HOPT, and HSPM are the same: well-defined opacities ranging from White/Creamy to Yellow/Brown. Since the characterization of MIH, studies on the condition have been aiming to understand its etiology, diagnosis, prevalence, and treatment [1].

The etiological hypothesis of hypomineralization is related to the pre-, peri-, and postnatal periods, as alterations in the functions of ameloblasts during the maturation phase can occur from the end of pregnancy up to four years old [7]. The associated factors related to HSPM include maternal tobacco use, prematurity, asthma, and childhood eczema [8,9]. Regarding MIH, the suggested associated factors include bleeding during pregnancy, illness and psychological stress during pregnancy, respiratory diseases, childhood illnesses, frequent fevers, and the use of medications during the first three years of life [10–14]. It is estimated that MIH and HSPM affect, respectively, about 2,8% to 40,2% [15,16] and 2,5 to 21,8% people worldwide [17,18]. In Brazil, prevalence ranges from 8,84% to 46,6% [19].

Hypomineralized enamel has reduced mineral content, higher porosity, and elevated levels of carbonate and protein, making it porous and susceptible to post-eruptive breakdown (PEB), primarily caused by the impact of masticatory forces [1,2,20]. In addition to this factor, the likelihood of finding a patient with MIH and dental caries is 2.1-4.6 times higher than in a patient with MIH without caries [21]. When associated, both conditions make dental

treatment even more difficult, since the low adhesion of the enamel teeth affected by hypomineralization to dental materials has been considered a clinical challenge [22]. This factor leads to frequent restoration failure, and patients with the condition are treated up to ten times more than children without the condition [23], negatively impacting the oral health-related quality of life of preschool-age children and their families [24]. Another clinical aspect that can impact on the integrity of enamel defects already compromised by hypomineralization is the composition of the patient's saliva [25]

Saliva is produced by three main pairs of glands: the parotid, submandibular, and sublingual glands [26]. It surrounds the hard and soft tissues of oral cavity [27] and is composed of a variety of electrolytes: sodium, potassium, calcium, bicarbonate, and phosphates. It also contains immunoglobulins, proteins, enzymes, mucins and nitrogenous products (urea and ammonia) [28]. Saliva plays an important role in oral health, acting in lubrification, maintaining the integrity of teeth, buffering capacity, antibacterial activity, and digestion [26]. Worrisomely, children with MIH have altered salivary calcium concentration [29]. Furthermore, a proteomic study using mass spectrometry showed 88 proteins uniquely identified in the saliva of MIH patients. Among the findings was glutathione reductase, an enzyme that acts as an essential antioxidant to maintain redox homeostasis balance [30]. The presence of glutathione reductase in the saliva of children with MIH suggests altered mechanisms that could possibly lead to the development of pathological conditions: anorexia nervosa [31], hypertension [32] and chronic kidney disease [33] caused by increased oxidative stress. Oxidative stress (OS) is a condition resulting from an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them through antioxidant systems. It can be assessed through salivary biomarkers, including the concentration/activity of enzymatic and non-enzymatic antioxidants or the concentration of products resulting from oxidative modifications of cellular components [34].

So far, it is unknown whether changes in composition and redox balance of saliva could be associated with HSPM or MIH. Salivary changes are often linked to oral diseases [35–37], and therefore, it is plausible to assume that the saliva of children with HSPM or MIH may differ from that of healthy children. While studies have evaluated changes in salivary patterns in children with MIH, to the best of our knowledge, there are no previous studies that investigated this alteration in children with HSPM. If confirmed, salivary analysis could be incorporated into routine diagnostic procedures for managing patients with hypomineralized lesions.

The aim of this study was to analyze associated factors related to HSPM and MIH and the biochemical components and biomarkers of oxidative stress in the saliva of children with MIH or HSPM, comparing them to those found in the saliva of children without this clinical diagnosis. He hypothesized that children with MIH or HSPM exhibit significant physiological alterations in the function and activity of the salivary glands, in addition to deficiencies in the formation of dental enamel.

2. Materials and Methods

2.1. Ethics statement

All the procedures described here were previously submitted for analysis and were approved by the local Human Research Ethics Committee, on July 28th, 2023 (CAAE: 71319123.2.0000.5420) (ANNEX A).

2.2. Study design and sample size

This research study was part of an extension project conducted by teams made up of professors, master and PhD students, undergraduate students at the School of Dentistry and dental surgeons from the municipal health department. The team assessed the oral health of children enrolled in multiple Municipal School of Basic Education in Araçatuba and Pediatric Dentistry clinic

of a School of Dentistry and provided guidance on the importance of dental hygiene/brushing.

Parents, teachers and caregivers of the children were called to explain the study protocol in detail and answer any questions raised by them. Informed consent forms were distributed to all guardians and only authorized children who submitted signed consent forms were included in the study (ANNEX B).

The children selected with MIH or HSPM were categorized according to European Academy of Paediatric Dentistry (EAPD) [7]. Oral health evaluations and the presence of MIH / HSPM were assessed by a single dentist (HFH) who was previously trained and calibrated (intra-examiner agreement $\kappa = 0.852$). The exclusion criteria included children's systemic diseases (other than respiratory diseases), those who were taking medication and those who had caries, fluorosis and hypoplasia. The inclusion criteria encompassed children with HSPM (n=15) or MIH (n=15), along with a control group of children without these conditions (n=15). According to the power calculation, including 13 children with HSPM or MIH would be sufficient to detect a statistically significant difference in phosphate concentration in saliva between control and MIH groups, with a power of test of 80% at a significance level of 5%. Since this number was close to the number of children assessed in previous studies, we decided to maintain 15 children per group [35,38]. For this research project, 45 children between 2 and 11 years old were selected among the children who participated in the extension project and Pediatric Dentistry clinic cited above.

2.3. Survey data collection

A questionnaire, including 11 questions (ANNEX C), was applied to parents and/or guardians of the 45 children selected for the study, gathered the child's general information and systemic conditions during the first three years of life (respiratory diseases, premature birth, birthweight, fever during the first three years of life, medication use, breastfeeding), as well as information about the mother's pregnancy. Responses to this section were recorded and

analyzed. The parents of the selected children were contacted by telephone or in person and informed about the purpose of the survey and the time required to complete the questionnaire. The names/contact details of the researchers were made available in case participants had any questions regarding the survey.

2.4. Clinical data collection

Dental examinations were conducted in the Pediatric Dentistry clinic or at the Municipal School of Basic Education. The teeth were kept moist, and excess saliva was removed with gauze pads. The dental examination was performed using a flat mouth mirror (Golgran®; Sao Caetano do Sul, SP, Brazil), an exploration probe recommended by the World Health Organization (WHO) (Golgran®; São Caetano do Sul, SP, Brazil) and an additional headlamp. The procedure started from the upper molars and proceeded in a clockwise direction.

Saliva collection took place in the morning, following a 2 hour-fast, and oral hygiene with toothbrush and water, without the use of fluoridated products (dentifrices or mouthwash). Unstimulated whole saliva was collected, using Salivette® with cotton swabs (Salivette®; Sarstedt, Numbrecht, Nordrhein-Westfalen, Germany), placed under the tongue to absorb saliva for 5 minutes. If the child cried or was uncooperative during the saliva collection, the procedure was halted, and no further attempts were made. The collection tubes were kept on ice during the examination to prevent proteolytic degradation of salivary proteins. To evaluate the salivary parameters related to demarcated opacities and the extent of the defect on salivary parameters, the HSPM and MIH groups were combined for a better understanding.

2.5. Quantification of unstimulated salivary flow rate

To assess the unstimulated salivary flow rate, saliva samples were collected over a 5-minute period using a Salivette® (Sarstedt, Numbrecht, Nordrhein-Westfalen, Germany) as described above. Following collection, the samples were maintained on ice. The salivary flow was calculated immediately after by the difference between the post-weight and pre-weight of Salivette®, then dividing the value by the collection time, 5 minutes. The volume was calculated by assuming a density of 1 mg/mL, and the flow rate was expressed in milliliters of saliva per minute (mL/min).

Saliva samples were centrifuged at 4000 RPM for 10 min using an Eppendorf R 5804 centrifuge (Eppendorf, Hamburg, Germany). The volume of supernatants was quantified, divided into six aliquots (100 – 500 µL), and stored at -80°C for later use in the biochemical analysis described below.

2.6. Salivary pH analysis and salivary buffering capacity

The pH value was determined in the salivary aliquots (200 µL) with a specific electrode (Analyzer®, São Paulo, SP, Brazil) connected to a pH meter (Thermo Fischer, Orion 720 A, MA, USA) previously calibrated with standard solution.

Buffering capacity was determined using the titulometric method, based on the volume of lactic acid (0,1 mol/L) required to reduce the salivary pH to 4,0. The obtained values were expressed in mL of lactic acid/mL saliva [39].

2.7. Salivary biochemical analysis

For the biochemical assays, 96-well plates filled with specific volumes of saliva and reagents were used. All reagents were sourced from Sigma-Aldrich (Germany/USA), Labtest Diagnostica S.A (Lagoa Santa, MG, Brazil) and Bioclin (Belo Horizonte, MG, Brazil). The absorbance was measured using a

UV-Vis-spectrophotometer (UV – 1203, Shimadzu, Japan) and a microplate reader (PowerWave 340, BioTek, USA). The measurements were conducted in duplicate. The values obtained in some biochemical analyses were standardized by total protein content. Normalization by total protein content allows for the assessment of differences in the proportions of biochemical analytes present in saliva.

2.7.1. Salivary calcium (Ca^{2+}) concentration

Ca^{2+} concentrations were determined by spectrophotometry with a plate reader (EON, BioTek, Winooski, VT, USA), as described previously [40]. Arsenazo III was used as the colorimetric reagent. Salivary aliquot (5 µL) or standard solution of calcium (5 – 80 mM Ca^{2+}, 5 µL) was mixed with 50 µL of Arsenazo III and 50 µL of deionized water in a 96-well microplate. The mixture was then shaken for 60 seconds before measuring the absorbance at a wavelength of 650 nm. Analyses were performed in duplicate, and values were expressed as µg Ca^{2+}/mL.

2.7.2. Salivary phosphate (PO_4^{3-}) concentration

PO_4^{3-} concentrations were determined using the standardized method [41]. Briefly, 100 µL of salivary samples, 50 µL of ammonium molybdate (Sigma-Aldrich; St Louis, MO, USA), was mixed with 20 µL of a reducing reagent (0.2 g of alpha-amino-naphthol-sulfonic acid, 1.2 g of sodium sulfite, and 1.2 g of sodium metabisulfite, to each 0.025 g of this mixture, 1 mL of deionized). The solutions absorbance was read at a wavelength of 660 nm using a microplate reader. Analyses were performed in duplicate, and values were expressed in µg PO_4^{3-}/mL.

2.7.3. Salivary fluoride (F^-) concentration

Saliva samples (25 µL) were buffered with TISAB III (Orion) and placed in the membrane of an ion-specific electrode (Orion 9409). A reference electrode (Orion Reference Electrode Double Junction 900100) was used to complete the circuit. The electrodes were calibrated with fluoride standard solutions ranging from 1 to 100 µM F^- (100 ppm F; Orion 940907) [42] and buffered with TISAB III. The values obtained from the samples, in millivolts (mV), were converted to µM F^- using Microsoft Excel software (Version 2010, Microsoft Corp., Redmond, Wash., USA), allowing the determination of parameters (linearity, slope, and coefficient of variation). Analyses were performed duplicate, and values were expressed in µM.

2.7.4. Salivary total protein concentration

Total protein concentration was determined in 15 µL aliquots of saliva using the Hartree-Lowry method [43] with standard curve of different concentrations of bovine serum albumin as the standard. If sample's concentrations above the highest point of the standard curve were detected, the samples were diluted, and the analysis was repeated. Absorbance values were determined at 660 nm. The results were expressed in g/L.

2.7.5. Salivary amylase activity

Amylase activity in salivary samples was determined by starch-iodine color reaction, as described previously [44], with a commercial kit (Labtest Diagnostica S.A, Lagoa Santa, MG, Brazil). The reaction mixture, containing the supernatant of a sample and 0,04% (w/v) soluble starch as the substrate in 0.2 mol/L phosphate buffer at pH 7.0, was incubated for 7.5 minutes at 37° C. The reaction was stopped by the addition of a solution containing

iodine/potassium iodide and HCl. Samples and blanks were measured at 660 nm. The results were expressed in U/g of total protein.

2.7.6. Salivary total oxidative capacity (TOC) analysis

In salivary samples, TOC was assessed using the Erel method, which is based on the oxidants present in the sample that oxidize the ferrous o-dianisidine complex to ferric ion. The ferric ion forms a colored complex with xylenol orange in an acidic medium. The color intensity was measured spectrophotometrically at 560/800 nm. The assay was calibrated with hydrogen peroxide (H_2O_2), and the results were expressed as micromoles of H_2O_2 per gram of protein [45].

2.7.7. Salivary oxidative damage analysis

Lipid peroxidation products, a biomarker of oxidative damage, were assessed by measuring the concentration of substances reactive to thiobarbituric acid (TBARS) method, in which malondialdehyde produced during the breakdown of peroxidized lipids reacts with thiobarbituric acid, forming a reddish-colored chromogen. This chromogen was then analyzed through spectrophotometric reading at 532 nm [46]. Trichloroacetic acid (15% w/v) was added to the saliva samples (80 µL) to precipitate the proteins and acidify the solution. Thiobarbituric acid (TBARS, 0.67% w/v) was added to the reaction medium. The sample was incubated in a water bath at 100 ºC for 60 minutes. The amount formed were calculated using the molar extinction coefficient ($\varepsilon 532 = 1{,}56 \times 10^5\ M^{-1}\ cm^{-1}$). The results were expressed as mmol/g of total protein.

2.7.8. Salivary total antioxidant capacity (TAC) analysis

Total antioxidant capacity was determined using the ferric reducing antioxidant power (FRAP) method, as described previously by Benzie and Strain [47]. The method is based on the reduction of the ferric tripyridyl triazine complex (Fe^{3+} TPTZ) to form Fe^{2+} in an acidic medium. An aliquot of saliva (10 µL) was used for the assay. The absorbance of the solution was measured at 593 nm, using a ferrous sulfate standard curve (0,1 to 3 mM $FeSO_4.7H_2O$). The results were expressed as µmol Fe^{2+}/g total protein.

2.7.9. Salivary uric acid (UA) concentration

The UA concentration in salivary sample (10 µL) was measured using a commercial kit (Bioclin, Belo Horizonte, MG, Brazil), in which uricase was used in the assay to convert UA into allantoin and hydrogen peroxide [48]. The test was performed following the manufacturer's instructions. Results were expressed in mg/g of total protein.

2.8. Statistical Analyses

The results from the questionnaire were analyzed statistically: Frequencies (%) were compared using the chi-square (χ^2) test. Means (SD) obtained from age, gestational time and maternal feeding time were compared using Kruskal-Wallis and Dwass-Steel-Critchlow-Fligner tests (for non-parametric data) or One-way ANOVA and Tukey tests (for parametric data) (Table 1). The statistical analyses were performed using Jamovi - Version 2.3 (Sydney, Australia).

The results of the biochemical analyses (Fig. 2 – 4) were tested for normality using the using the Shapiro-Wilk test. For non-parametric results, the Kruskal-Wallis test was conducted. If the data passed the normality test, an ANOVA test was performed. Non-parametric results were presented as median

and interquartile range in box-plot graphs. Parametric results were presented in bar graphs as mean ± SD. For all analyses, $p < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc.; La Jolla, CA, USA).

3. Results

3.1. Population's characteristics and variables related to etiology of MIH/HSPM

Forty-five children aged 2 – 11 years participated in this study. The mean age (± standard deviation) of the participants was 5.29 ± 1.77 years in Control Group, 5.07 ± 0.99 years in HSPM Group, and 7.43 ± 2.17 years in MIH Group. There was a statistically significant difference in age among the groups ($p = 0.005$). Responses to the questionnaire completed by parents were analyzed, allowing the determination of population characteristics and variables related to etiology of MIH and HSPM (Table 1). Comparing the groups, revealed notable differences in certain factors: sex, age, prolonged childbirth, and episodes of fever during the first year of life. The average age was higher ($p < 0.05$) in children in the MIH group when compared with the control group or HSPM groups. Prolonged labor was not reported by parents of children with MIH, but it was described by parents of children in the control group and the HSPM groups. Similarly, episodes of fever in the first year of life were not reported by parents of children with MIH.

Table 1. Population´s characteristics and variables related to etiology of MIH/HSPM

		Control	HSPM	MIH	p value
Gender	Female % (n)*	26.7% (12)	22.2% (10)	22.2% (10)	0.655
	Male % (n)	6.7% (3)	11.1% (5)	11.1% (5)	
Age	Mean (SD)**	5.29 (1.77)	5.07 (0.99)	7.43 (2.17)	**0.005***
Systemic alterations during last gestational trimester	% (n)	7% (3)	16.3% (7)	9.3% (4)	0.301
Medications during the last gestacional trimester	% (n)	9.3% (4)	11.6% (5)	9.3% (4)	0.925
Intercorrences during the childbirth	% (n)	7% (3)	2.3% (1)	0	0.182
Cesarian section birth	% (n)	31.8% (14)	31.8% (14)	31.8% (14)	0.620
Prolongued Childbirth	% (n)	14% (6)	2.3% (1)	0	**0.008****
Gestational time	Mean (SD)	37.8 (3.29)	38.2 (3.05)	39 (3.04)	0.123
Preterm birth (before 37 weeks)	% (n)	4.4% (2)	6.7% (3)	0	0.889
Low birthweight (< 2.5kg)	% (n)	2.2% (1)	6.7%(3)	2.2% (1)	0.898
Maternal feeding time	Mean (SD)	5.88 (5.2)	6.77 (5.13)	2.46 (7.33)	0.112
Prolonged maternal feeding (> 2 years)	% (n)	2.2% (1)	2.2% (1)	0	0.998
Childhood diseases	% (n)	6.8% (3)	0	6.8% (3)	0.172
Respiratory problems	% (n)	15.9% (7)	13.6% (6)	6.8% (3)	0.354
Fever in the first year of the child´s life	% (n)	13.6% (6)	11.4% (5)	0	**0.032***
Use of antibiotics	% (n)	27.3% (12)	31.8% (14)	22.7% (10)	0.312

Hypomineralised second primary molars (HSPM); Molar incisor hypomineralisation (MIH); Frequency (%): chi-square (χ^2) tests ($p < 0.05$); Means (SD) - One-way ANOVA and post hocTukey tests ($p < 0.05$).

3.2. Patterns of presentation for HSPM and MIH

The frequency of hypomineralization in first permanent molars, incisors, and second primary molars are shown in Fig. 1A. The results revealed distinct patterns in the distribution of teeth affected by MIH and HSPM. The highest proportion of affected teeth was observed in molars with HSPM, with 40% of cases exhibiting four or more affected teeth. Among molars with MIH, most children evaluated had two affected teeth, also accounting for 40%. Conversely, incisors with MIH displayed the highest proportion of unaffected teeth (46.67%), indicating a lower prevalence of alterations in this region compared to molars.

3.3. Clinical presentations

White/Creamy demarcated opacities were the most common presentation in both HSPM (27,5%) and MIH (31%). None of the participants with MIH exhibited structural loss due to hypomineralization, whereas this feature was observed in 6,8% of HSPM cases. Both conditions shared an equal prevalence of Yellow/Brown demarcated opacities (17,2%) (Fig. 1B).

The defect sizes were also documented (Fig. 1C). Hypomineralized defects were classified into three categories: (1) affecting less than one-third of the clinical surface, (2) affecting more than one-third but less than two-thirds of the clinical surface, and (3) affecting more than two-thirds of the clinical crown surface. Among children with MIH, lesions covering more than one-third but less than two-thirds of the clinical crown surface were the most common (31%), followed by lesions affecting more than two-thirds (10.3%) and less than one-third (6.9%) of the surface. In contrast, for HSPM, defects affecting less than one-third of the clinical surface were most frequent (20.7%), followed by lesions covering more than one-third but less than two-thirds (17.1%) and more than two-thirds (13.7%) of the surface.

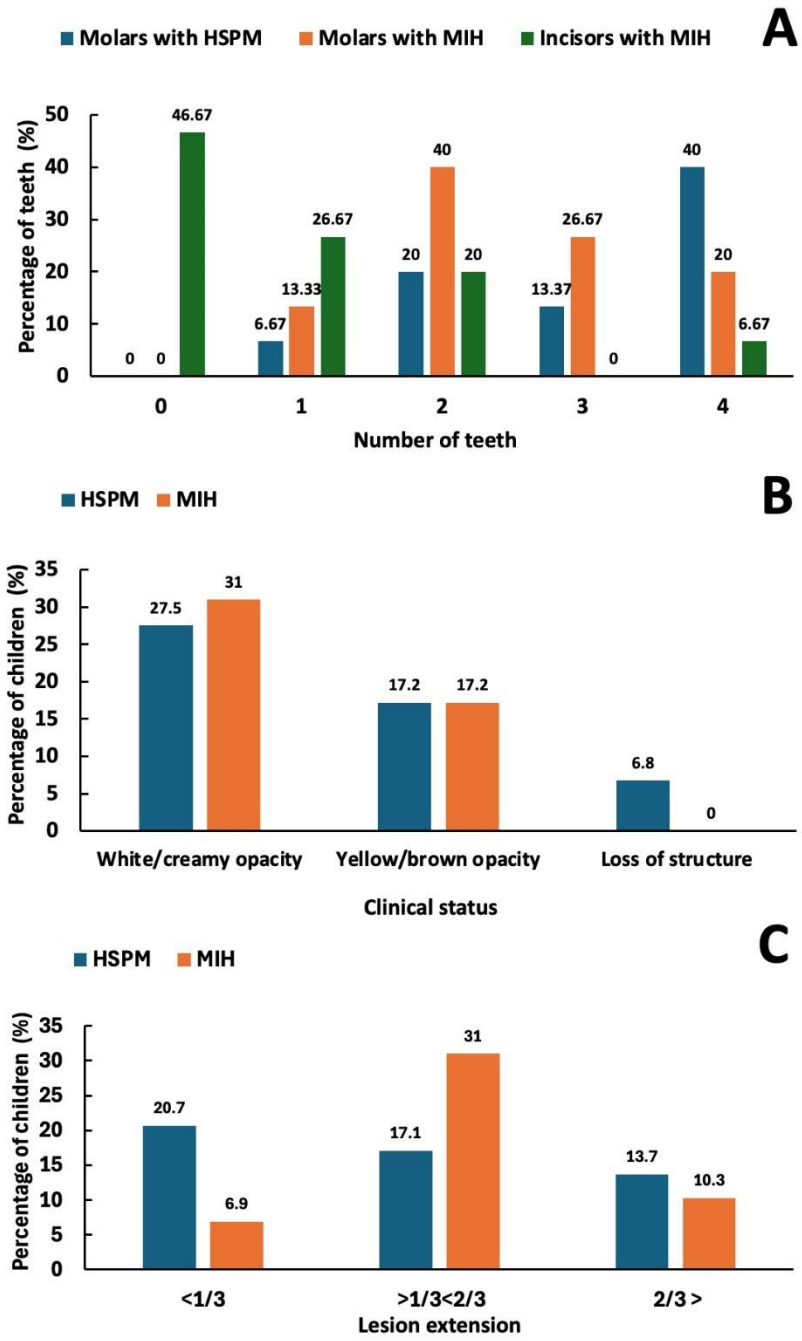

Fig. 1 - Percentage of molars with HSPM and molars/incisors with MIH (A) in the children. Percentage of children presenting HSPM or MIH, according to the color (B) and severity (C).
Only the tooth with the highest score for HSPM and MIH was considered for the analysis

3.4. Salivary parameters and oxidative stress biomarkers in children with MIH and HSPM

Children with MIH or HSPM exhibited similar salivary flow rates (Fig. 2A), pH (Fig. 2B), and buffering capacities (Fig. 2C) compared to the control group.

The secretion of electrolytes in saliva, with an increase in calcium (Fig. 1D) concentration was observed in the MIH group when compared with HSPM group ($p < 0.05$). A decrease in phosphate secretion (Fig. 2E) was noted in MIH compared to the control group ($p < 0.05$). Fluoride concentrations were similar between the groups (Fig. 2F).

Regarding salivary biochemical composition, the HSPM group showed a significant reduction in total salivary total protein concentration ($p < 0.01$) compared to the control group. No significant differences were observed between the control and MIH groups, or between HSPM and MIH groups (Fig. 2G). Amylase activity showed no differences between the groups (Fig. 2H).

Higher levels of salivary TOC were observed in the HSPM group compared to the control group ($p < 0.05$). No significant differences were identified between the control and the MIH groups or between the HSPM and MIH groups (Fig. 2I). However, lipid oxidative damage, as measured by TBARS, showed no significant differences between the study groups and the control group (Fig. 2J).

Salivary TAC levels showed no significant differences between the groups ($p > 0.05$) (Fig. 2K). Conversely, salivary AU levels were significantly higher ($p < 0.05$) in children with MIH or HSPM compared to the control group (Fig. 2L).

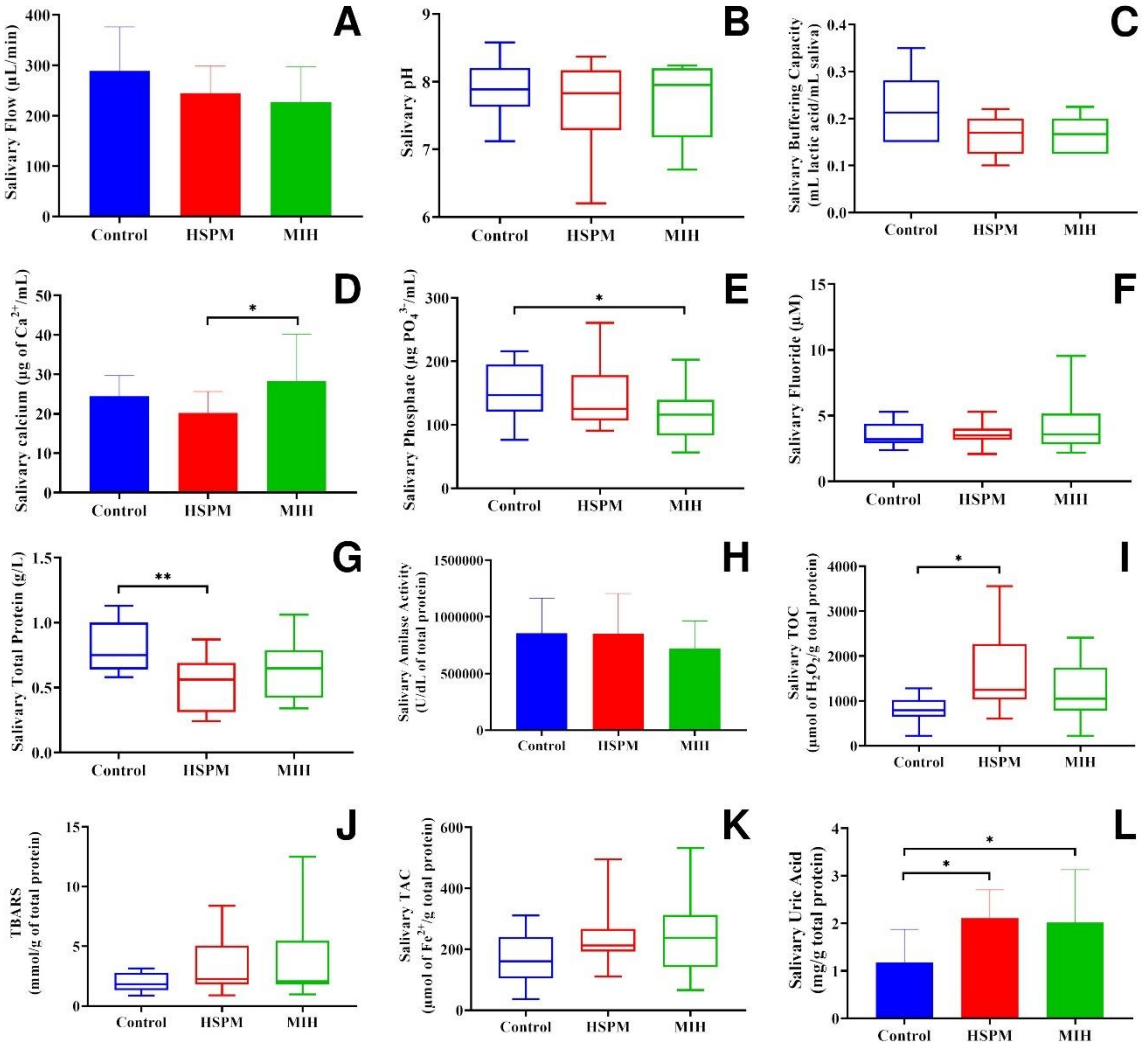

Fig. 2 - Comparison of salivary parameters among Control, HSPM (Hypomineralized Second Primary Molars), and MIH (Molar Incisor-Hypomineralization) groups. (A) Salivary flow (µL/min); (B) Salivary pH; (C) Buffering capacity (mL lactic acid/mL of saliva); (D) Salivary calcium (mg of Ca^{2+}/mL); (E) Salivary phosphate (µg of PO_4^{3-}/mL); (F) Salivary fluoride (µM); (G) Total salivary protein (g/L); (H) Salivary amylase activity (U/dL of total protein); (I) Salivary total oxidant capacity (TOC) (µmol of H_2O_2 g/total protein); (J) TBARS (mmol/g of total protein); (K) Salivary total antioxidant capacity (TAC) (µmol of Fe^{2+}/g of total protein); (L) Salivary uric acid (mg/g of total protein).
Data are presented as mean ± SD (bar graphs) or median and interquartile range (box plots).
*$p < 0.05$; **$p < 0.01$.

3.5. Salivary parameters and oxidative stress biomarkers in children with White/Creamy or Yellow/Brown opacities

Children with White/Cream or Yellow/Brown opacities exhibited no alterations in salivary flow (Fig. 3A), pH (Fig. 3B) or buffering capacity (Fig. 3C) compared to the control group ($p > 0.05$). Similarly, no significant differences were observed in calcium (Fig. 3D), phosphate (Fig. 3E), and fluoride concentrations (Fig. 3F), or salivary amylase activity ($p > 0.05$) (Fig. 3H).

On the other hand, Yellow/Brown opacities were associated with a significant reduction ($p < 0.05$) in total protein levels compared to the control group. In contrast, no significant differences were found between the control group and White/Creamy opacities or between the White/Creamy and Yellow/Brown opacity groups ($p > 0.05$) (Fig. 3G).

Yellow/Brown opacities were associated with a significant increase in salivary TOC (Fig. 3I) and TBARS (Fig. 3J) compared to the control group ($p < 0.01$). In both analyses, no significant differences were noted when comparing White/Creamy opacities to either Yellow/Brown opacities or the control group ($p > 0.05$).

Salivary TAC (Fig. 3K) did not show any differences between the groups ($p > 0.05$). However, higher salivary AU levels were observed in children with White/Creamy or Yellow/Brown opacities compared to the control group ($p < 0.05$). In contrast, no significant differences were found between the White/Creamy opacity and the Yellow/Brown opacity groups ($p > 0.05$) (Fig. 3L).

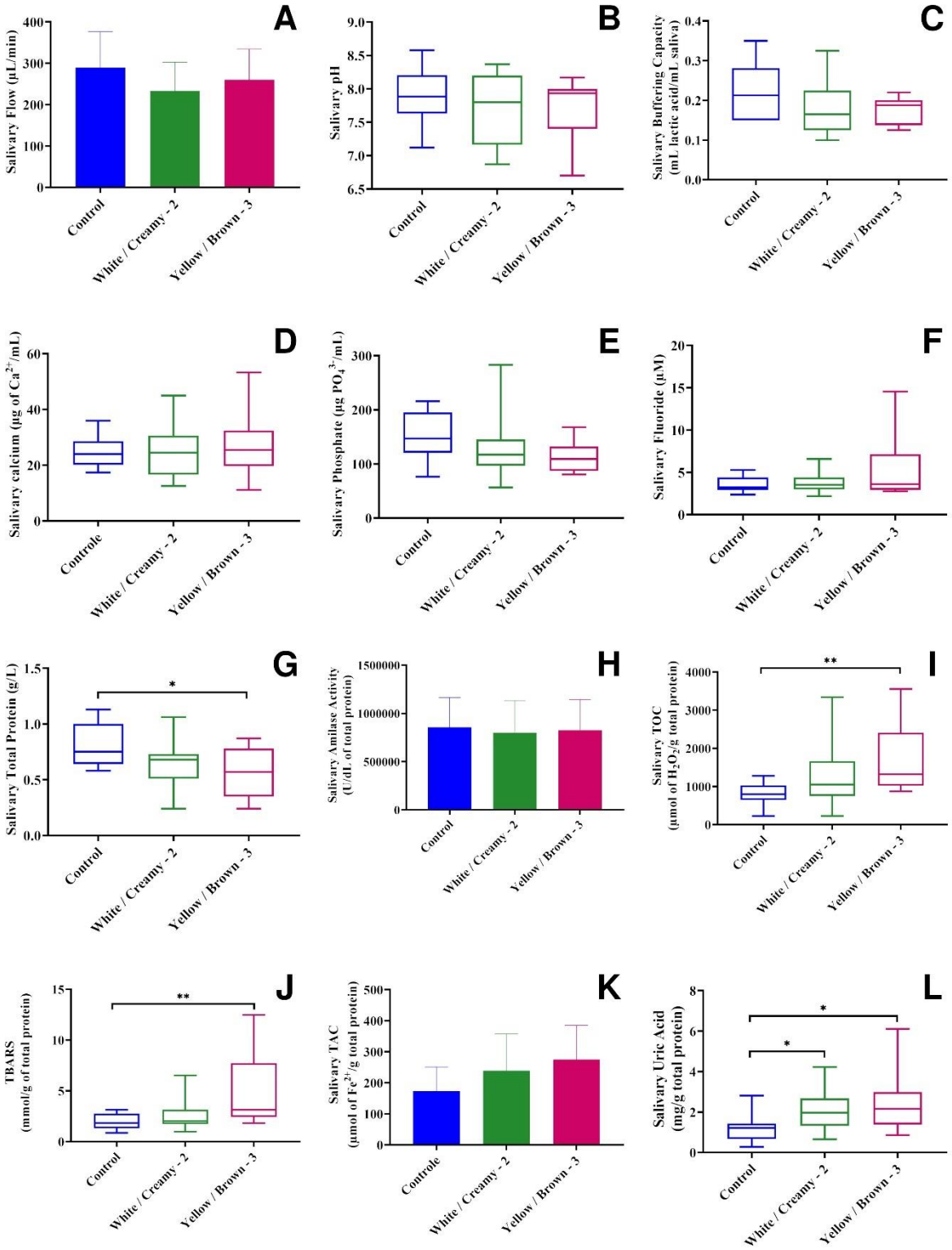

Fig. 3 - Comparison of salivary parameters among Control and demarcated opacities (White/Creamy and Yellow/Brown).
Data are presented as mean ± SD (bar graphs) or median and interquartile range (box plots). *$p < 0.05$; **$p < 0.01$.

3.6. Salivary parameters and oxidative stress biomarkers related to extension of the defect of the White/Creamy opacities

When evaluating the impact of the extension of HSPM or MIH with White/Creamy opacities on salivary biochemical parameters, no significant differences were observed compared to the control group across all analyzed parameters. These parameters included salivary flow (Fig. 4A), pH (Fig. 4B), buffering capacity (Fig. 4C), calcium (Fig. 4D), phosphate (Fig. 4E), fluoride (Fig. 4F), total protein (Fig. 4G) and amylase activity (Fig. 3H). Similarly, no differences were found in the salivary TOC (Fig. 4I) and TBARS (Fig. 4J) levels compared to the control group. Consistent with the other evaluated parameters, the extent of lesions in teeth with White/Creamy opacities showed no significant differences compared to the control group in salivary TAC (Fig. 4K) and UA (Fig. 4L).

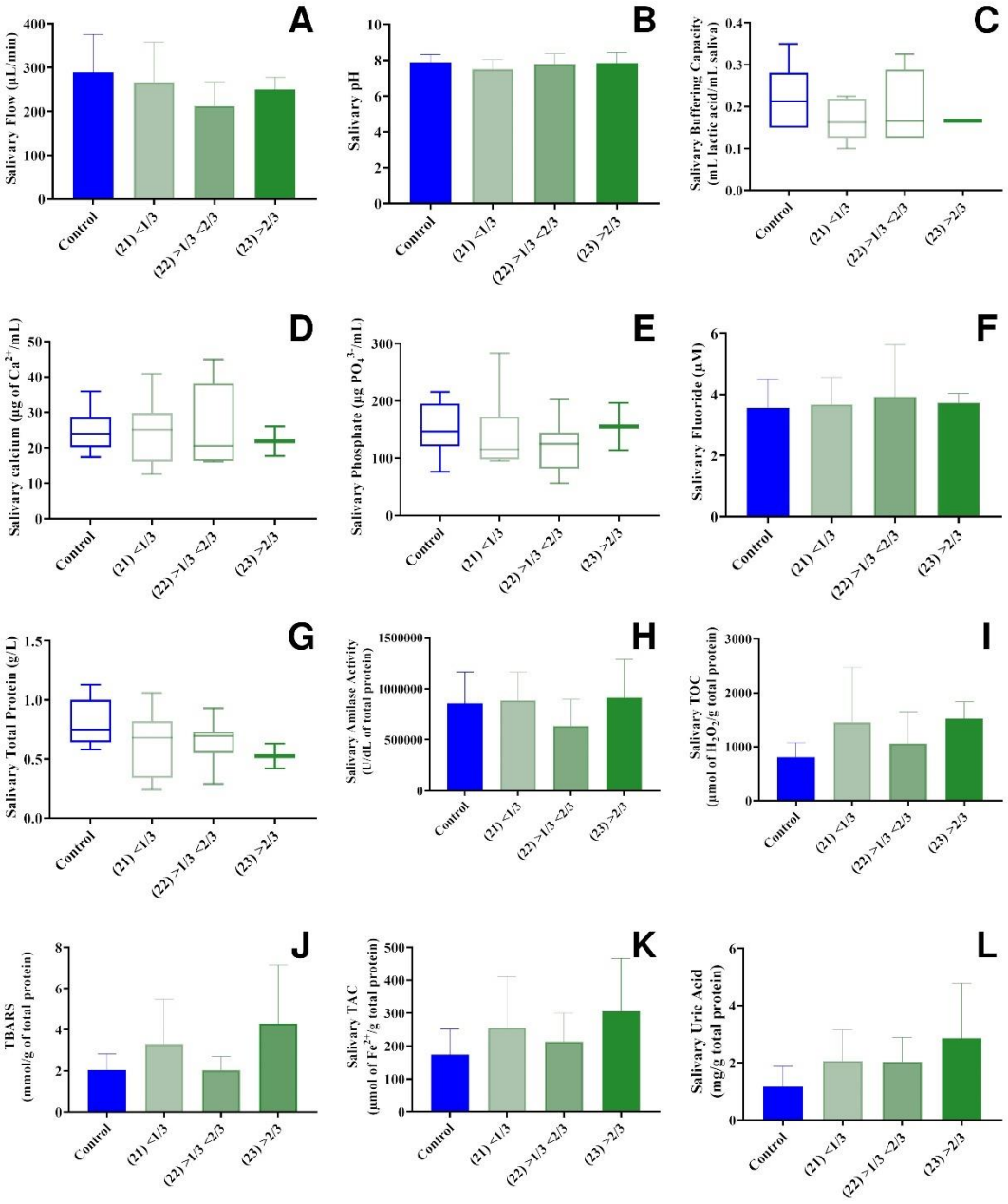

Fig. 4 - Comparison of salivary parameters among Control and extension of the defect of the White/Creamy opacities.
Data are presented as mean ± SD (bar graphs) or median and interquartile range (box plots). *p < 0.05; **p < 0.01.

3.7. Salivary parameters and oxidative stress biomarkers related to extension of the defect of the Yellow/Brown opacities

When assessing the impact of the extension of HSPM or MIH with Yellow/Brown opacities, no evidence was found that salivary flow (Fig. 5A), pH (Fig. 5B) and buffering capacity (Fig. 5C) influenced salivary biochemical parameters ($p > 0.05$). The results showed no significant differences when comparing the extent of the defect to the control group.

Nevertheless, regarding the electrolytes, the greater the extent, the lower the phosphate concentration ($p < 0.05$) (Fig. 5E). Salivary calcium (Fig. 5D) and fluoride (Fig. 5F) concentrations showed no significant differences between the groups ($p > 0.05$). In terms of salivary biochemical composition, amylase activity (Fig. 5H) did not differ significantly between the groups ($p > 0.05$). Notably, a reduction in total salivary protein concentration was observed in the group with Yellow/Brown opacity, with an extension greater than 2/3 [(33) > 2/3] (the group with the largest defect size), compared to the group with Yellow/Brown opacity, with an extension less than 1/3 [(31) < 1/3] and the control group ($p < 0.05$). No significant differences were observed between the control group and the group with Yellow/Brown opacity and with an extension less than 1/3 [(31) < 1/3], nor between the control and the group with Yellow/Brown opacity and with an extension greater than 1/3 and less than 2/3 [(32) > 1/3 <2/3] (Fig. 5G).

Turning now to the evidence on the effects of teeth with Yellow/Brown opacities, a significant increase in salivary TOC ($p < 0.0001$) (Fig. 5I) and TBARS ($p < 0.05$) (Fig. 5J), was observed compared to the control group when the extent of HSPM or MIH exceeded 2/3 (Yellow/Brown, with an extension greater than 2/3) [33) >2/3]. This significant increase in salivary TOC was also observed when comparing the groups (Yellow/Brown, with an extension greater than 2/3) [(33) > 2/3] and Yellow/Brown opacity and with an extension greater than 1/3 and less than 2/3 [(32) >1/3 <2/3] ($p < 0.0001$), as well as 2/3 (Yellow/Brown, with an extension greater than 2/3) [(33) > 2/3] and (Yellow/Brown, with an extension less than 1/3) [(31) <1/3] ($p < 0.0001$).

As highlighted in Fig. 5L, the salivary AU rate was significantly higher in the (Yellow/Brown, with an extension greater than 2/3) [(33) > 2/3] group compared to the control group ($p < 0.01$). However, no significant differences in salivary AU were found when comparing the control group with (Yellow/Brown, with an extension less than 1/3) [(31) <1/3], Yellow/Brown opacity and with an extension greater than 1/3 and less than 2/3 [(32) >1/3 <2/3] and Yellow/Brown opacity, with an extension greater than 2/3 [(33) > 2/3] groups ($p > 0.05$). Moving on to TAC analysis related to the extent of HSPM or MIH with Yellow/Brown opacities, the results indicate that salivary TAC increased as the extent of the defect increased. Significant differences were observed between the control group and the group with a larger extent of the defect Yellow/Brown opacity, with an extension greater than 2/3 [(33) > 2/3], with this group presenting the highest antioxidant capacity ($p < 0.001$). Additionally, a significant increase in salivary TAC was observed in the Yellow/Brown opacity, with an extension greater than 2/3 [(33) > 2/3] group, compared to Yellow/Brown opacity and with an extension greater than 1/3 and less than 2/3 [(32) > 1/3 < 2/3] ($p < 0.05$) and (Yellow/Brown, with an extension less than 1/3) [(31) < 1/3] ($p < 0.01$) (Fig. 5K).

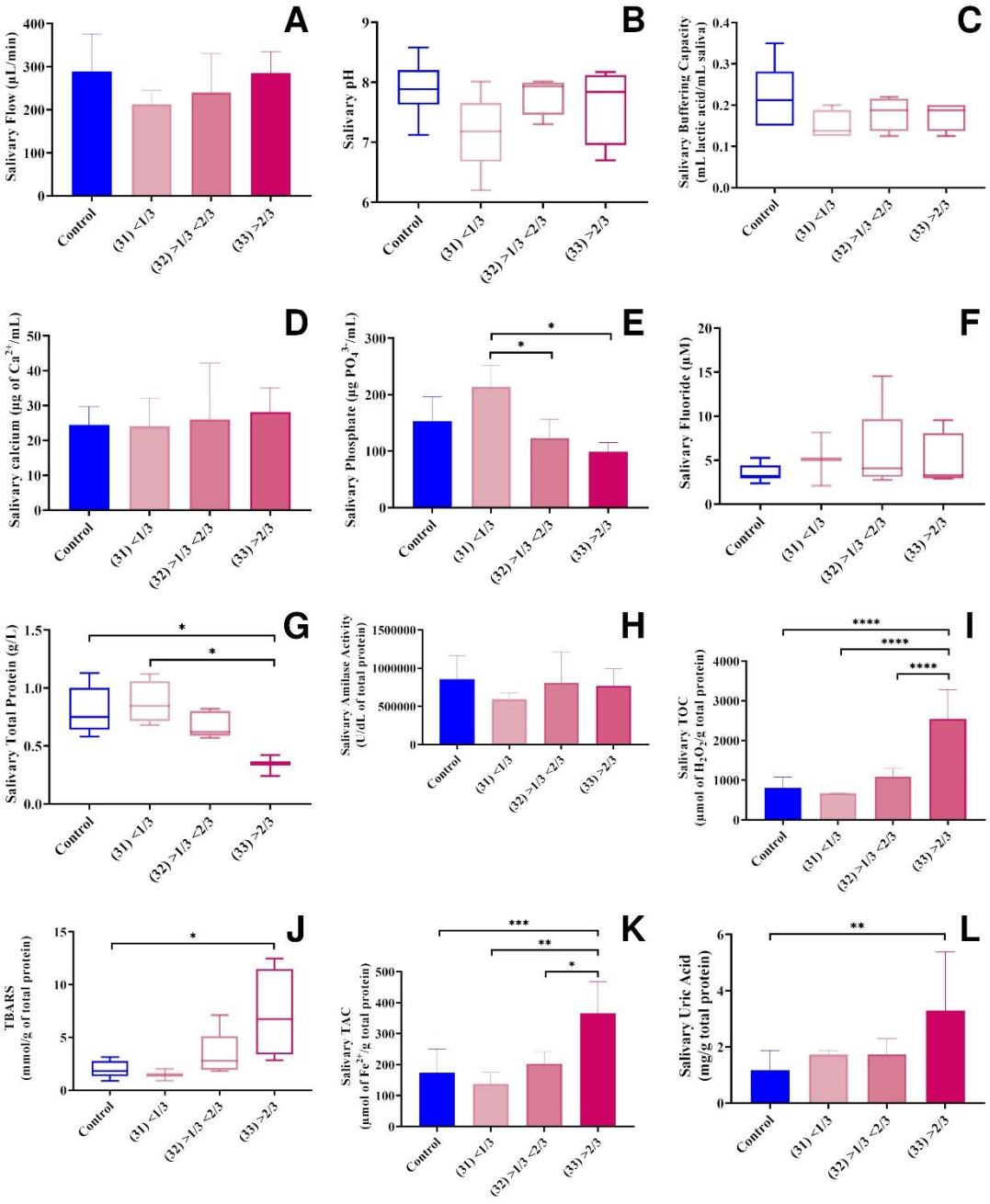

Fig. 5 - Comparison of salivary parameters across Control and extension of the defect of the Yellow/Brown opacities.
Data are presented as mean ± SD (bar graphs) or median and interquartile range (box plots). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

4. Discussion

In the present study, we evaluated variables in population characteristics related to associated factors of HSPM and MIH. Additionally, we assessed biochemical composition related to the functional characteristics and redox state of saliva in these children. The results indicated significant associated factors and salivary alterations associated with HSPM and MIH, confirming the alternative proposed hypothesis. Understanding the associated factors and salivary changes associated with these conditions is essential for management.

In the evaluated population, children with MIH were older than those with HSPM, a fact corroborated by other studies, as the prevalence of MIH tends to be higher in older children, especially after the eruption of the first permanent molars, which occur around the age of 6 [49]. On the other hand, HSPM is observed in younger children, usually after the eruption of the second primary molars [24].

Enamel developmental defects characterized by qualitative disturbances in enamel formation, leading to hypomineralized regions on affected teeth. These conditions often result in increased sensitivity and susceptibility to caries. In our study, we carefully selected children with MIH or HSPM but without caries, to specifically characterize the changes associated with these clinical conditions.

Among the etiological factors related to enamel development defects, systemic factors such as prenatal, perinatal, and postnatal conditions were evaluated. In our study, prolonged labor and fever during early childhood were the only perinatal and postnatal conditions, between the groups. Interestingly, the occurrence of intercurrences during childbirth, prolonged childbirth and preterm birth could not be associated with MIH, as no cases were reported by the parents. Furthermore, potential surgical differences, exposure to anesthetics [50,51], or peripartum events such as hypoxia during birth or delivery by cesarean section and suggested for the occurrence of MIH [50] were not relevant, as most children (14/15) in all three groups evaluated were

born via cesarean section. As mentioned previously, fever during early childhood has been associated with MIH [10] and HSPM [52]. However, it is challenging to distinguish between the causal factors of MIH among childhood illnesses, fever, and antibiotic use, considering that these factors may overlap in time. Additionally, information based only on the parents' recall, may introduce recall bias [10,20]. Contrary to expectations, none of the parents reported any childhood illnesses in HSPM group. Furthermore, differences were not observed in antibiotic use or respiratory problems between the HSPM or MIH and control group.

When comparing the distribution pattern of HSPM and MIH, first permanent molars and second primary molars were the most impacted teeth. These results don't match those observed in earlier studies, in which the first permanent molars were the most affected teeth, followed by incisors [53]. Analyzing studies involving only children with HSPM, the percentage of molars affected per child was lower for cases with one, two or four teeth affected by HSPM, and higher for cases with three affected teeth [54]. Regarding clinical presentations, White/Creamy demarcated opacities comprehended the most common presentation of HSPM and MIH, which are in line with previous studies, that describes this opacity as the main manifestation in MIH [49,53] and HSPM [9,53,54]. In the present study, both conditions exhibited the same percentage of children with the occurrence of Yellow/Brown opacities. This differs from the findings presented by Sidhu et al [53], which MIH showed a higher percentage of surfaces affected (22%) by Yellow/Brown opacities, compared to HSPM (9%). This study supports evidence from previous observations that PEB is more common in children with HSPM compared to MIH [53].

Concerning extent, most children in our HSPM sample presented small defects occupying less than 1/3 of the surface, consistent with findings from a study conducted in Teresina, Brazil [9]. Nonetheless, when assessing MIH, the present study found that most children had more than one-third but less than two-thirds of the dental surface affected. These results contrast with those from

a study in Lebanon, where most lesions were limited to less than one-third of the dental surface [55]. Sidhu et al [53] reported that lesions affecting less than one-third of the dental surface are significantly more common in HSPM compared to MIH.

The results of this work align with previous reports indicate no significant differences in buffering capacity between children affected by MIH or HSPM and the control group [25]. Surprisingly, this same study found that children with MIH or HSPM were more than twice as likely to exhibit a low resting salivary flow rate (OR: 2.64) and a moderately acidic saliva pH (OR: 2.77), a finding that contrasts with the results presented here. These differences could be attributed to variations in the methodologies employed in the studies (stimulated or unstimulated saliva, collection method, analysis method) highlighting the need for further research to better understand these salivary parameters in patients with MIH. Similarly to our study, Ismayilova et al [29], which investigated mineral composition of saliva in schoolchildren aged 6-15 years in Turkey, found no significant differences in buffering capacity, flow rate and pH in patients with MIH compared to the control group. Reinforcing the data, differences in these salivary parameters were not observed even when correlating the demarcated opacities based on the extent of the defect or their appearance as White/Creamy or Yellow/Brown opacities. Since no decrease in salivary pH and buffering capacity, or salivary flow, was observed between the groups, it is possible to suggest that the reduction in enamel protection or the impairment of the natural defense mechanisms of the oral cavity are not exacerbated in children with HSPM or MIH.

Concentrations of mineral elements in saliva may be a useful tool for assessing oral and systemic diseases (*apud* Ismayilova et al [29]). The levels of calcium and phosphate in saliva are regulated by several factors, including genetic factors. A study conducted by Küchler et al [56], evaluated the association between the genetic variations in genes expressed in enamel development with calcium level in stimulated saliva was associated with genetic variations in *AMELX* (amelogenin), *AMNB* (ameloblastin) and *ESRRB*

(*Estrogen-related receptor β*). *AMELX* and *AMNB* are involved in enamel maturation. A week association was observed between Enamelin (*ENAM)* allele distribution and phosphate levels in saliva. *ENAM* was associated with enamel hypoplasia.

Based on the available literature, there is limited data regarding the analysis of calcium, phosphate [29]. And there were no studies about fluoride levels in saliva of patients with MIH or HSPM. Our study found a higher concentration of calcium in patients with MIH, compared to those with HSPM. However, no significant differences were observed between the MIH and control group, suggesting that calcium homeostasis may be altered in children with hypomineralization and possibly linked to aforementioned genetic variations. Additionally, patients with MIH exhibited significantly lower phosphate levels in saliva compared to the control group, although no statistically significant differences were found between the MIH and HSPM groups.

In our study, we observed that the more extensive the Yellow/Brown lesion, the lower the salivary phosphate concentration in relation to the lesion [(31 < 1/3)]. These results partially align with those reported by Ismayilova et al [29], who observed higher calcium concentrations in the saliva of patients with MIH compared to the control group but found no differences in salivary phosphate levels between these groups. The discrepancies in findings may reflect methodological differences, variations in sample populations, or other influencing factors, excluding salivary flow and pH, which were not altered between the groups.

The reduction of [PO_4^{3-}] in the saliva of children could be associated with a possible demineralization [57] as well as dental sensitivity, given that dentifrice with casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), have shown a promising role in remineralization and dental desensitization [58]. Further studies are needed to clarify the role of salivary calcium and phosphate levels in the pathophysiology of HSPM and MIH and their potential implications for clinical management.

To our knowledge, this is the first study to evaluate salivary [F^-] in children with HSPM and MIH, showing no variations in fluoride concentrations among the groups, regardless of opacity or lesion extent. It is important to note that the children included in the study had not performed oral hygiene with fluoride-containing products before saliva collection, nor had they been treated with fluoride varnish, ensuring these factors did not influence quantified values.

Interestingly, although we did not observe changes in salivary [F^-] levels in children with HSPM or MIH, fluoride varnish has been used as a protocol to reduce sensitivity in these children. A study conducted by Biondi et al[59] showed that the use of sodium fluoride varnish (Duraphat®) may be more effective for moderate lesions, while tricalcium phosphate varnish (Clinpro®) may be more suitable for mild lesions.

Limited data on salivary composition in MIH pathology are available in the literature. These studies suggest that the protein composition of saliva is altered in MIH patients, reflecting a catabolic environment potentially associated with inflammation or systemic stress. We did not find any studies in the literature evaluating the total protein concentration in the saliva of children with hypomineralization, despite its relevance as a biomarker for various oral diseases, such as caries, periodontitis, and others. In our study, the total salivary protein concentration was reduced in children with HSPM compared to the control group, with no changes observed in children with MIH. Reduced protein concentration was also noted when analyzing White/Creamy and Yellow/Brown opacities, as well as their extents. We observed that the greater the severity of the lesion extent, the lower the protein concentration. These findings suggest that HSPM or MIH is associated with a reduction in salivary protein concentration. These results are noteworthy since salivary protein concentration typically increases with age and is positively correlated with the presence and severity of caries [35,38]. In our study, the mean age of children with HSPM did not differ from that of the control group, although it was higher in children with MIH, and none of the children had caries. Therefore, the

reduction in total salivary protein concentration observed in children with HSPM and MIH appears to be linked to this condition.

The influence of the salivary collection could be considered as a possible explanation for the lower protein concentration observed. This concern arises due to the potential selective absorption of proteins by cotton- or cellulose-based collection devices, as reported when using the Salivette® method [30,60]. However, this explanation can be ruled out, as the same collection method was consistently applied to all groups in this study.

Proteomic studies have evaluated differences in salivary protein expression in adolescents with MIH, of the same age, over 14 years old, with associated caries [61], and in children aged 6-4 years with MIH without caries but presenting sensitivity [30]. These studies showed that statistical comparisons identified differentially expressed proteins in the saliva of children with MIH, while pathway analysis indicated deregulation of inflammation, immune response mechanisms, and defense responses to bacteria [30,61].

When specifically evaluating salivary amylase activity, we did not find differences between children with HSPM or MIH and the control group, regardless of the type of opacity or the extent of the lesion. As we did not find studies in literature assessing amylase expression or activity in children with hypomineralization, we suggest that the lack of observed differences may be because the children in this study did not present caries, given that amylase contributes to biofilm development, which links increased amylase levels to a higher risk of caries [62].

Possible alterations in salivary gland development could be suggested for children with HSPM and MIH, as the lower concentration of total salivary proteins may indicate potential changes in the formation of submandibular or sublingual glands or in the function of salivary glands during protein synthesis/secretion. On the other hand, the absence of changes in amylase suggests no alterations in the parotid glands. However, these hypotheses should be further evaluated using more specific methodologies.

Some studies have investigated salivary biomarkers of oxidative stress in different populations, including children. One study highlighted the influence of dental caries severity on oxidative stress biomarkers in children [35]. Another study focused on whether atraumatic restorative treatment (ART) impacted salivary redox biomarkers in a population aged 4 to 6 years [36]. Additionally, a study assessed salivary glycoxidation products, oxidative damage to lipids, and nitrosative stress biomarkers in children with chronic kidney disease, both with normal and decreased saliva secretion [37]. These studies provide evidence of a connection between salivary oxidative stress and oral health.

A previous study showed that NAD(P)PX epimerase and gluthatione reductase were exclusively found in the saliva of children with MIH compared to the control group. NAD(P)HX epimerase (NAXE), an enzyme that repairs damaged forms of the antioxidant NADPH, acts as a potential cellular ROS scavenger (apud Sun et al [63]). Gluthatione reductase is involved in the regulation, modulation, and maintenance of cellular redox homeostasis [64]. The presence of both enzymes in the saliva of these patients suggests alterations in redox balance [30].

ROS are highly reactive and have extremely short lifetime. They can cause direct tissue damage, resulting in various metabolites from lipid peroxidation, DNA, and protein damage, which are commonly used to evaluate ROS-induced tissue destruction [65]. Although the TBARS values in the HSPM and MIH groups were not statistically different from the control group, a high variability in results was observed within these groups compared to the control, suggesting altered ROS production. When analyzing the type and extent of opacities, significantly higher TBARS values ($p < 0.01$) were observed in Yellow/Brown opacities and in lesions with an extent of [(33) > 2/3] compared to the control, indicating increased salivary ROS levels associated with these clinical conditions. This suggestion is further supported by the elevated TOC values found in the HSPM group compared to the control; in Yellow/Brown opacities compared to the control, and in lesions with an extent of [(33) > 2/3] compared to other yellow-brown extents and the control group.

Redox imbalance occurs due to the disproportion between excessive production of free radicals and the reduced activity of enzymatic antioxidant systems (SOD and glutathione) and non-enzymatic systems (Uric Acid).

Antioxidant systems are an essential defense of the body against damage caused by free radicals [66,67]. Disturbances in the balance between the lipid peroxidation system (caused by excess free radicals) and antioxidant protection may result from disruptions in the coordination of immunometabolism processes and could indicate a decrease in the body's adaptive reactions, which are associated with various diseases. Antioxidant systems in saliva appear to mirror those in plasma, where these systems are well-documented.

Total Antioxidant Capacity (TAC), defined as the moles of oxidants neutralized per liter of solution, is a biomarker used to measure the antioxidant potential of the body’s fluids [68]. Previous studies show that the increasing levels of antioxidants with age may be attributed to the improvement of a child's immune status as they grow older [69].

The values of TAC were not altered between HSPM or HMI and the control groups, but increased values were found when analyzing the Yellow/Brown opacities in relation to the extent of the lesion, suggesting that these changes are associated with the extent of the opacity.

In saliva, uric acid is the predominant salivary antioxidant, with albumin and ascorbate providing smaller contributions [70]. Uric acid concentrations were significantly higher in the saliva of children with HSPM and MIH compared to the control group, as well as in the White/Creamy and Yellow/Brown opacities when compared to controls. In the Yellow/Brown opacities, the extent > 2/3 showed a significantly higher value ($p < 0.01$) when compared to the control. Together, these results suggest that the non-enzymatic antioxidant action is increased in the saliva of children with HSPM and MIH, counteracting the increased concentrations of ROS.

Considering the importance of saliva in maintaining oral health, it is not surprising that disturbances in the redox balance of saliva dramatically increase

the risk of developing oxidative stress-related oral diseases, such as dental caries and oral inflammatory infections, among others.

This study has some limitations. Firstly, the sample only included children treated at the Faculty of Dentistry's Pediatric Dentistry clinic and those participating in the extension project. Children receiving care through private services were not represented in the sample. Therefore, these findings should only be extrapolated to children with characteristics similar to those analyzed here. Another limitation is the retrospective nature of the questionnaire given to parents, which may introduce observation and memory biases, making it difficult to establish temporal relationships. Additionally, the role of enzymatic antioxidant systems was not assessed. However, this study is the first to identify oxidative stress biomarkers in children with MIH/HSPM, thus expanding pediatric dentists about this condition.

5. Conclusion

HSPM and MIH are enamel formation defects influenced by individual characteristics and the oral environment. Prolonged labor and fever during the first year of life were the only factors, among the associated factors, related to the population of children evaluated. Among the salivary changes, differences in mineral composition (calcium and phosphate), total protein concentration, oxidant capacity, reactive species, antioxidant capacity, and non-enzymatic antioxidant activity were found. Understanding the etiological factors and salivary changes associated with HSPM and MIH are essential for their management.

6. References

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ANEXO A – COMITÊ DE ÉTICA

UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Alterações salivares associadas à hipomineralização dental em crianças
Pesquisador: Cristina Antoniali Silva
Área Temática:
Versão: 1
CAAE: 71319123.2.0000.5420
Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP
Patrocinador Principal: Universidade Estadual Paulista Júlio de Mesquita Filho

DADOS DO PARECER

Número do Parecer: 6.206.075

Apresentação do Projeto:
A hipomineralização é um defeito qualitativo, que se apresentada em molares e incisivos permanentes, é conhecida clinicamente como Hipomineralização Molar Incisivo (HMI). Quando ocorre na dentição decídua, pode ser observada nos segundos molares decíduos, e muitas vezes, pode englobar primeiros molares decíduos e caninos decíduos, sendo denominada Hipomineralização de Segundos Molares Decíduos (HSMD). A saliva contém biomarcadores que podem ser analisados e quantificados. A presença da glutationa redutase, uma enzima que participa da desintoxicação do peróxido de hidrogênio (H2O2) em pacientes com HMI, sugere uma alteração do estresse oxidativo salivar. Estudos prévios de nosso grupo mostraram que a cárie em crianças de 0-3 anos reduz o dano oxidativo salivar ao aumentar a atividade de sistema antioxidante enzimático e não enzimático. Além disto, mostramos também que há uma correlação positiva e forte entre a severidade das lesões de cárie e a capacidade antioxidante total (TAC), e entre severidade de lesão de cárie e atividade de sistema antioxidante não enzimático (ácido úrico, AU) e enzimático (superóxido dismutase, SOD), levando a redução progressiva do dano oxidativo salivar. Mais recentemente demonstramos que a severidade da lesão de cárie leva ao aumento progressivo da atividade de SOD sensível ao cianeto de potássio e consequentemente ao aumento, também progressivo, da biodisponibilidade de óxido nítrico (NO). Considerando que hipomeralização promove aumento da concentração de proteínas salivares, entre elas algumas

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relacionadas a ação antioxidante, levantamos a hipótese que esta alteração poderia alterar os diferentes biomarcadores salivares de estresse oxidativo. Não encontramos na literatura, até o momento, artigos que avaliaram alterações nos biomarcadores salivares de estresse oxidativo e sua possível correlação com a hipomineralização. O objetivo deste estudo será avaliar parâmetros bioquímicos da saliva de crianças com HSMD e HMI. Serão incluídos neste estudo 5 grupos de 15 pacientes de 0-6 anos de idade e de 6-12, matriculadas nas EMEBs da cidade de Araçatuba e atendidas na Clínica de Odontopediatria da Faculdade de Odontologia de Araçatuba, UNESP. O número amostral foi calculado com base em estudos prévios de nosso grupo utilizando como variável primária a concentração de ácido úrico salivar e um poder de teste de 80%, pelo programa Open Epi, versão 3. As amostras de saliva não estimuladas serão coletadas dos pacientes após 2 horas de jejum, no período da manhã. Experimentos bioquímicos, previamente padronizados para amostras de saliva, serão conduzidos para avaliação da concentração total de proteínas, concentração e/ou expressão de albumina, peroxidação de lipídios, proteínas carboniladas, capacidade antioxidante total, ácido úrico, atividade da superóxido dismutase (SOD) total, SOD sensível e insensível ao cianeto de postássio e concentração de nitrito (NO2-) salivar, de cálcio (Ca2+), fosfato (PO43-), e Flúor (F) salivar. Os resultados obtidos serão comparados entre os grupos por análise estatística aplicando teste de multivariância (ANOVA, com pós-teste de Student-Newman-Keuls para dados com distribuição normal ou pós-teste de Tukey, se não houver distribuição normal, ($p < 0,05$), além de correlação de Pearson e Spearman.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar a incidência de crianças com HSMD e HMI no município de Araçatuba e possível correlação com as alterações da composição bioquímica e biomarcadores de estresse oxidativo salivar.

Objetivo Secundário:

Quantificar a incidência de crianças de 0-6 anos com HSMD e de 6-12 anos com HMI no município de Araçatuba.Avaliar a associação entre a prevalência de HSMD e HMI e dados colhidos através de questionário sobre: gestação, nascimento, doenças e medicamentos administrados para a criança até os 3 anos. Será também perguntado o comportamento da criança frente às alterações e se há algum parente com esses quadros.Avaliar a hipersensibilidade dentinária causada por HMI/HSMD através de escala (0 a 2)Avaliar a presença de cárie dentária (lesão de dentina) associada à HSMD e HMIAvaliar possíveis alterações bioquímicas na saliva de crianças com HSMD e HMIAvaliar

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possíveis alterações de biomarcadores salivares de estresse oxidativo na saliva de crianças com HSMD e HMI

Avaliação dos Riscos e Benefícios:

Riscos:

Essa pesquisa oferece riscos mínimos. Os riscos serão os mesmos de uma consulta odontológica de rotina.

Benefícios:

Além dos benefícios para a ciência, a criança será encaminhada para tratamento clínico especializado na UBS e/ou Faculdade de Odontologia - Campus de Araçatuba UNESP

Comentários e Considerações sobre a Pesquisa:

PESQUISA APRESENTA-SE APTA PARA A SUA REALIZAÇÃO.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos foram adicionados de acordo com a resolução 466/12 DO CNS.

Recomendações:

NÃO HÁ.

Conclusões ou Pendências e Lista de Inadequações:

PESQUISA APRESENTA-SE APTA PARA A SUA REALIZAÇÃO.

Considerações Finais a critério do CEP:

Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 28/01/2014.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_2173910.pdf	14/07/2023 16:31:27		Aceito
Projeto Detalhado / Brochura Investigador	Projeto_de_pesquisa_PB.pdf	14/07/2023 13:21:53	HAYLLA DE FARIA HORTA	Aceito
TCLE / Termos de	Termo_de_consentimento_livre_e_es	14/07/2023	HAYLLA DE FARIA	Aceito

UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

Continuação do Parecer: 6.206.075

Assentimento / Justificativa de Ausência	clarecido.pdf	13:18:57	HORTA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Termo_de_assentimento_do_menor.pdf	14/07/2023 13:18:45	HAYLLA DE FARIA HORTA	Aceito
Folha de Rosto	Folha_de_rosto.pdf	14/07/2023 13:17:59	HAYLLA DE FARIA HORTA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

ARACATUBA, 28 de Julho de 2023

Assinado por:
André Pinheiro de Magalhães Bertoz
(Coordenador(a))

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ANEXO B – TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Título da Pesquisa: "**Alterações salivares associadas à hipomineralização dental em crianças**"
Nome da Pesquisadora: Haylla de Faria Horta
Nome da Orientadora: Cristina Antoniali Silva

1. **Natureza da pesquisa**: a Sra. (Sr.) está sendo convidada (o) a participar desta pesquisa que tem como finalidade avaliar a presença de anomalias dentárias e defeitos qualitativos específicos no desenvolvimento do esmalte que acometem incisivos e primeiros molares permanentes ou decíduos (hipomineralização molar-incisivo), além do acometimento pela cárie dentária e serão avaliados a concentração total de proteínas, concentração e/ou expressão de albumina, peroxidação de lipídeos, proteínas carboniladas, capacidade antioxidante total, ácido úrico, atividade da superóxido dismutase (SOD) total, SOD sensível e insensível ao cianeto de potássio e concentração de nitrito, cálcio, fosfato e flúor salivar.
2. **Participantes da pesquisa**: Serão selecionadas 75 crianças de 0-6 anos, entre as participantes do Projeto de Extensão Sorriso Feliz e de 6-12 anos, entre as atendidas na Clínica de Odontopediatria da Faculdade de Odontologia de Araçatuba – FOA/UNESP
3. **Envolvimento na pesquisa**: ao participar deste estudo a (o) Sra. (Sr.) permitirá que a pesquisadora realize o exame bucal em seu filho (a), aplique um questionário destinado aos pais e colete saliva da cavidade bucal do seu filho. A (O) Sra. (Sr.) tem liberdade de se recusar a participar e ainda se recusar a continuar participando em qualquer fase da pesquisa, sem qualquer prejuízo para o(a) seu filho(a). Sempre que quiser poderá pedir mais informações sobre a pesquisa através do telefone do (a) pesquisador (a) do projeto e, se necessário através do telefone do Comitê de Ética em Pesquisa.
4. **Sobre as entrevistas**: Não serão realizadas entrevistas
5. **Riscos e desconforto**: a participação nesta pesquisa não infringe as normas legais e éticas *(*a coleta de saliva será feita por um método que causa risco mínimo, não invasivo e indolor, utilizando um dispositivo com o nome comercial de SALIVETE®. Este dispositivo contém um pequeno rolo de algodão, que será posicionado na cavidade bucal). Os procedimentos adotados nesta pesquisa obedecem aos Critérios da Ética em Pesquisa com Seres Humanos conforme resolução n. 466/2012 do Conselho Nacional de Saúde – Brasília – DF. Não haverá riscos e desconfortos para os voluntários da pesquisa. Nenhum dos procedimentos usados oferece riscos à sua dignidade.
6. **Confidencialidade**: todas as informações coletadas neste estudo são estritamente confidenciais. Somente à pesquisadora Haylla de Faria Horta, a orientadora Prof. Assoc. Dra. Cristina Antoniali Silva e sua equipe de pesquisa composta pela Prof. Assoc. Dra. Cristiane Duque e Prof. Assoc. Dr. Antonio Hernandes Chaves-Neto, que terão conhecimento de sua identidade e nos comprometemos a mantê-la em sigilo ao publicar os resultados dessa pesquisa.

7. **Benefícios**: ao participar desta pesquisa a (o) Sra. (Sr.) autorizará que nós da equipe de pesquisa, realizaremos um exame clínico completo no menor, poderá ser realizado encaminhamentos. Além destes benefícios diretos, esperamos que este estudo traga informações importantes sobre diagnóstico e incidência sobre a hipomineralização molar incisivo (HMI), hipomineralização de segundos molares decíduos (HSMD) e cárie, de forma que o conhecimento que será construído a partir desta pesquisa possa auxiliar o Cirurgião-Dentista no tratamento desta patologia onde pesquisador se compromete a divulgar os resultados obtidos, respeitando-se o sigilo das informações coletadas, conforme previsto no item anterior.
8. **Pagamento**: a Sra. (Sr.) não terá nenhum tipo de despesa para participar desta pesquisa, bem como nada será pago por sua participação.

Após estes esclarecimentos, solicitamos o seu consentimento de forma livre para participar desta pesquisa. Portanto preencha, por favor, os itens que se seguem:
Obs.: Não assine esse termo se ainda tiver dúvida a respeito.

Confiro que recebi cópia deste termo de consentimento, e autorizo a execução do trabalho de pesquisa e a divulgação dos dados obtidos neste estudo.

Consentimento Livre e Esclarecido

Tendo em vista os itens acima apresentados, eu, de forma livre e esclarecida, manifesto meu consentimento em participar desta pesquisa:

_____

Nome do Participante da Pesquisa (Nome da Criança)

_____

Assinatura do Participante da Pesquisa (Responsável pela Criança)

_____ _____

Cirurgiã-Dentista Haylla de Faria Horta — Prof. Assoc. Dra Cristina Antoniali Silva

Pesquisadora: Haylla de Faria Horta (31) 99810-1579
Orientadora: Cristina Antoniali Silva
Coordenador do Comitê de Ética em Pesquisa: Prof. Dr. André Pinheiro de Magalhães Bertoz
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ANEXO C – QUESTIONÁRIO

Questionário Geral	
Paciente:	Sexo:
Nascimento: / /	Local:
Altura:	Peso:
Responsável pelas informações:	
Grau de parentesco com a criança: ( ) Mãe ( ) Pai ( ) Outro:	
Motivo da consulta:	
A mãe teve alguma doença ou alteração sistêmica durante o último trimestre de gestação?	
A mãe teve alguma intercorrência durante o parto? ( ) Não ( ) Sim Qual:	
A mãe da criança usou algum medicamento na gravidez? ( ) Sim ( ) Não Qual?	
O parto foi? ( ) Natural ( ) Cesariana ( ) Rápido ( ) Prolongado	
Seu filho nasceu com quantas semanas de gestação?	
Qual o peso do seu filho ao nascer?	
Quanto tempo teu filho foi amamentado exclusivamente com leite materno?	
Seu filho teve algum episódio de febre alta (>39°) durante os 3 primeiros anos de vida? ( ) Não ( ) Sim	
Seu filho teve alguma doença respiratória durante os 3 primeiros anos de vida? ( ) Não ( ) Sim Qual:	
Seu filho teve alguma doença de infância durante os 3 primeiros anos de vida? ( ) Não ( ) Sim Qual:	
Seu filho tomou antibióticos ou corticosteróides durante os 3 primeiros anos de vida? ( ) Não ( ) Sim Qual:	