CROSS-SECTIONAL STUDY

Interaction Between Environmental Factors and Polymorphisms in a Hypoxia-Related Gene (HIF-1) Associated with Hypomineralized Second Primary Molars

Aluhê Lopes Fatturi, DDS, MSc[1] • Bruna Leticia Vessoni Menoncin, DDS, MSc[2] • Michelle Meger, DDS, MSc[3] • Rafaela Scariot, DDS, MSc, PhD[4] • João Armando Brancher, DDS, MSc, PhD[5] • Erika Calvano Küchler, DDS, MSc, PhD[6] • Juliana Feltrin-Souza, DDS, MSc, PhD[7]

Abstract: ***Purpose:*** *This study's purpose was to investigate whether polymorphisms in the HIF-1 encoding gene and hypoxia-related environmental factors were associated with hypomineralized second primary molars (HSPMs).* ***Methods:*** *From a total of 731 children from Curitiba, Paraná, Brazil, were selected, the prevalence of HSPMs in this population was 9.4 percent, representing 69 cases (HSPMs) and 662 controls. The environmental factors were collected via questionnaire. HSPMs were evaluated by calibrated examiners. Two genetic polymorphisms (rs2301113 and rs2057482) in the HIF-1 gene were genotyped by polymerase chain reaction in real time. Associations were tested by Poisson regression analysis (Prevalence Ratio$_{adjusted}$; P<0.05).* ***Results:*** *In the multiple variable model, including the environmental factors and genetic polymorphisms, maternal use of an illicit drug (Prevalence Ratio$_{adjusted}$; equals 4.52; P<0.001; 95 percent confidence interval [95% CI] equals 2.38-8.53), maternal diseases during pregnancy (Prevalence Ratio$_{adjusted}$; equals 1.97; P=0.034; 95% CI equals 1.05 to 3.71), and respiratory diseases during childhood (Prevalence Ratio$_{adjusted}$; equals 2.66; P=0.003; 95% CI equals 1.41 to 5.03) increased significantly the prevalence of HSPMs. In the presence of environmental factors, individuals carrying at least one C allele in rs2057482 had a lower prevalence of HSPMs (Prevalence Ratio$_{adjusted}$; equals 0.51; P=0.048; 95% CI equals 0.27 to 0.99).* ***Conclusions:*** *Children who had hypoxia-related factors presented with a higher prevalence of hypomineralized second primary molars. A C allele in rs2057482 served as protection against HSPMs in hypoxia conditions.* *(Pediatr Dent 2021; 43(3):185-90) Received August 17, 2020 / Last Revision January 26, 2021 / Accepted January 28, 2021*

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Hypomineralized second primary molars (**HSPMs**) are clinically represented by demarcated opacities in enamel involving one to four primary second molars.[1] The presence of HSPMs is associated with a higher prevalence of molar-incisor hypomineralization (**MIH**).[2] It is suggested that this association occurs due to the chronological coincidence between dental development of permanent first molars and primary second molars. Thus, MIH and HSPMs could share the same etiological factors, especially in prenatal life until the age of one year.[3] Recent studies show that these demarcated opacities have a multifactorial and complex origin in which systemic or environmental factors act in synergism with the individual genetic aspects.[4,5]

Amelogenesis is a complex process of ectomesenchyme interactions. The ameloblasts perform the synthesis function, enamel protein matrix secretion, mineralization, and maturation of the enamel matrix.[6] Although this process is genetically controlled, environmental factors could affect the amelogenesis, such as pH alteration, blood support, fever, intake of high fluoride concentration, antimicrobial medications, and dioxins.[7-11]

Drs. [1]Fatturi and [2]Menoncin are PhD students, and Drs. [4]Scariot and [7]Feltrin-Souza are professors, all in the Department of Stomatology, Federal University of Parana, Curitiba, Paraná, Brazil. [3]Dr. Meger is a researcher, and [5]Dr. Brancher is professor, Postgraduate Program of Dentistry, Positivo University Curitiba; and [6]Dr. Küchler is a professor, Department of Pediatric Dentistry, Ribeirão Preto Dental School, Ribeirão Preto, São Paulo, Brazil. Correspond with Dr. de Souza at julianafeltrin@hotmail.com

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An in situ and in vitro studies observed that hypoxia altered the amelogenesis, which could clinically result in an enamel defect.[7,12] Epidemiological studies also observed that environmental factors related to hypoxia, such as prolonged birth delivery, maternal use of nicotine and illicit drugs during pregnancy, and respiratory problems in early childhood, were associated with a higher prevalence of demarcated opacities in both the permanent and primary dentition.[7,13-15] Sidaly et al.,[15] in a case-control study, analyzed if a lower Apgar score could be associated with a higher frequency of MIH. The authors did not find a significant association between children who had an Apgar score greater than five at five minutes and MIH. They suggested that ameloblasts could be affected by hypoxia; however, in mild hypoxia, compensatory mechanisms were observed, including increased expression of HIF-1α, which is positively regulated in ameloblasts after hypoxia.[16]

Hypoxia-inducible factor (**HIF-1**) is a transcriptional factor regulating oxygen homeostasis. It consists of two subunits (HIF-1α and HIF-1β) that coordinate the response to hypoxia in normal tissues and tumors, allowing adaptation and cell survival in a hostile environment.[17] The HIF-1α gene encodes the hypoxia-inducible factor one alpha subunit, which plays a key role in oxygen homeostasis by activating the transcription of approximately 100 genes involved in energetic metabolism, angiogenesis, and apoptosis, whose protein products increase oxygen release or facilitate metabolic adaptation to hypoxia.[18]

During oxidative stress, cells respond through HIF-1, mediating the repair and adaptation mechanisms.[7,19] In an in vitro study, conditions of hypoxia in ameloblasts promoted an increase of HIF expression.[7] In addition, alterations in the expression of cytokines, such as IL -1α, IL-1β, IL-6, IL-10, IFN-γ, and MCP-1, that could affect the expression of enamel proteins are also observed.[16]

Evidence from twin studies,[20,21] epidemiological studies,[22,23] and genetic studies (phenotype-genotype design)[24-26] suggests that HSPM/MIH has a multifactorial etiology in which, environmental factors and genetic factors play a role in the etiology of HSPMs. In fact, some phenotype-genotype studies have already demonstrated that genetic polymorphisms in many genes with a small effect are involved in HSPMs/MIH phenotype.[24,25,27]

The actual evidence supports the hypothesis that genetic polymorphisms in a hypoxia-related gene are candidates for HSPM.[7,15,16]. HIF-1 is the main component that detects hypoxia in organisms; it is possible that HIF-1 polymorphisms influence adaptation to hypoxic events[28] during enamel development. The present study's authors hypothesize that interactions between hypoxia-related environmental factors and genetic polymorphisms in the HIF-1α are involved in the etiology of HSPM.

Therefore, the purpose of this cross-sectional study was to analyze whether genetic polymorphisms in the HIF-1α and hypoxia-related environmental factors were associated with the prevalence of HSPMs.

Methods

Ethical approval. This cross-sectional study was approved by the Health Sciences Research Ethics Committee of the Federal University of Parana (**UFPR**), Curitiba, Paraná, Brazil, and the Municipal Education Secretary of Curitiba, Paraná, Brazil. The protocol was approved by the human research committee of UFPR before the research had begun. Previous to the study, children and their caregivers agreed to participate in the study and signed the informed consent form. This article was reported according to Strengthening the Reporting of Genetic Association Studies (STREGA).[29]

Recruitment and eligibility criteria. Eight-year-old school children were included in the study. Children who had orthodontic appliances that would impair clinical examination, with the absence of one to four primary second molars, and with syndromes or imperfect amelogenesis were excluded.

Sample. For a representative sample of the city, the participants were selected from public schools in the city of Curitiba, with a population of 1,908,359 inhabitants and a Human Development Index of 0.823. The determination of the sample size and the sampling procedures were described by Lopes-Fatturi et al.[3]

Clinical data collection. Clinical data collection was performed in a school environment by four calibrated examiners using artificial light, a dental mirror, dental probe, and sterile gauze. Four examiners were trained and calibrated to diagnose HSPMs using a modified developmental defects of enamel (DDE) index. The examiner's calibration was described by Lopes-Fatturi et al.[3] Data collection was performed from November 2016 to September 2017. The dependent variable was HSPMs, the case was defined as children with at least one primary second molar affected by HSPMs, and the control was defined as children without HSPMs on the clinical exam.

Evaluation of environmental factors. Environmental factors were collected through a structured questionnaire completed by the children's caregivers.[3] The questionnaire was prepared using previous studies in literature and contained questions regarding medical history during pregnancy, the perinatal period, and the postnatal period, up to the age of three years.[30]

For this study, the hypoxia-related environmental factors were selected according to reports in the literature as producing hypoxia situations.[31] Hypoxia-related factors were considered as follows: drug use was considered the maternal use of alcohol, tobacco, and illicit drugs during the pregnancy; maternal problems

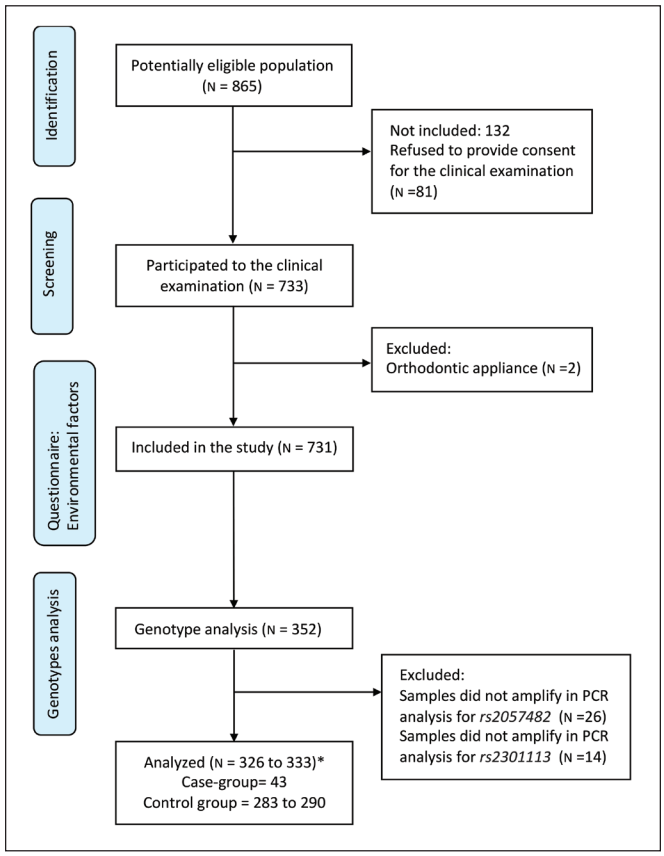

Figure. Flow diagram of study. Case-group included children with at least one second primary molar affected by HSPM. Control group included children without HSPM.

* The ranges is because some of the exclusions were not complete, some samples only did not amplify for rs2057482 and others only for rs2301113.

Table 1. CANDIDATE GENE DESCRIPTION AND ITS POLYMORPHISMS: HARDY-WEINBERG EQUILIBRIUM AND THE DISTRIBUTION OF OBSERVED FREQUENCY OF THE GENOTYPE ON THE STUDY POPULATION

Gene	Polymorphism	Locus	Change of base*	Minimum allele frequency	Genotype	N (%)	Hardy-Weinberg equilibrium chi-square
HIF-1	rs2301113	61739830	[A/C]	0.47	AA	157 (46.4)	1.712
					AC	130 (38.5)	
					CC	51 (15.1)	
	rs2057482	61747130	[C/T]	0.24	CC	226 (70.0)	0.256
					CT	86 (26.6)	
					TT	11 (3.4)	

* Nucleotide base change, adenine (A) to cytosine (C) in the rs2301113; cytosine (C) to thymine (T) in the rs2057482.

were considered disease or health conditions previously related to hypoxia, such as diabetes, hypertension, and prolonged labor during the delivery; and respiratory diseases were considered the presence of bronchitis, asthma, and pneumonia until the first three years of life.

Genetic polymorphisms in HIF-1α. For the genetic polymorphism's analysis, 352 children from the included sample were randomly selected in a case-control design as previously described in Fatturi et al.[24] In the previous study, all children were selected for the case group; for the control group, children were randomly selected, matching gender, age, and ethnicity at a ratio of three-to-one (control-to-case; Figure).[24]

Therefore, the schoolchildren's DNA was obtained from oral mucosal epithelial cells using a five ml mouthwash of three percent glucose solution, according to a previously published protocol.[32,33] Functional genetic polymorphisms in the HIF-1α were screened according to Cariaso and Lennon[34] based on their lowest allele frequency, which must have been greater than 30 percent, and linkage disequilibrium. Therefore, two genetic polymorphisms (rs2301113 and rs2057482) that were previously associated with different conditions,[35-37] indicating their possible clinical relevance, were selected. The description of the selected genetic polymorphisms is provided in Table 1.

Allelic discrimination analysis using real-time polymerase chain reaction and the Taqman test (StepOnePlus Real-Time PCR System, Thermo Fisher Scientific, Waltham, Mass., USA) were performed for genotyping.

Statistical analysis of data. The HSPM dependent variable was categorized as "case group" and "control group." The presence of HSPMs was calculated according to Ghanim et al.,[1] when at least one of the primary second molars is affected by demarcated hypomineralization. Genotypes were categorized as additive, dominant allele, and recessive allele. The Hardy-Weinberg equilibrium was evaluated by the chi-square test. The association among HSPMs, HIF-1α genotypes, and hypoxia-related environmental factors were analyzed by univariable and multiple Poisson regression analysis, with a robust variance used with its respective prevalence ratio (**PR** at a significance level of five percent. To perform a Poisson regression analysis for the multiple variables, independent variables with $P<0.20$ in the association with HSPMs were added to the multiple model. Statistical anaysis was performed using the SPSS 16.0 software for Windows (SPSS Inc., Chicago, Ill., USA) and STATA 14.0 software (StataCorp, Texas City, Texas, USA).

Table 2. DEMOGRAPHIC CHARACTERISTICS OF STUDY POPULATION (CURITIBA, PARANÁ, BRAZIL, 2017, N=731)*

Variables	Categories	N (%)
Gender	Male	374 (51.6)
	Female	357 (48.8)
Age	7	267 (36.5)
	8	464 (63.5)
Ethnicity	Caucasian	617 (84.4)
	African-descendant	89 (12.2)
	Asiatic	11 (1.5)
	Indian	14 (1.9)

* N=absolute frequency.

Results

Among the potentially eligible population of 865 schoolchildren, a total of 784 children and their caregivers agreed to participate in the study (response rate equals 90.6 percent); 733 children were clinically examined for eligibility and two children were excluded from the study due to the use of an orthodontic appliance), resulting in a final sample of 731 children.[3] The prevalence of HSPMs in this population was 9.4 percent, representing 69 cases (HSPMs) and 662 controls.[3] The present study is a sequential analysis of the same cohort.[3,24] For genotyping analysis, 43 cases and 279 to 287 controls were included (Figure). The genotype distribution was within the Hardy-Weinberg equilibrium (chi-square equals 0.256 and 1.712 for HIF rs2057482 and HIF rs2301113, respectively; Table 1).

Of this sample, 374 children were males (51.16 percent). Caucasian was the predominant ethnicity (617 children; 84.40 percent; Table 2).

In a univariable analysis, a significant increase in the occurrence of HSPMs was observed in children whose mothers used drugs (tobacco, alcohol, and illicit drugs) during pregnancy (Prevalence $Ratio_{crude}$ equals 2.14; $P=0.004$) or had some health problem during pregnancy (Prevalence $Ratio_{crude}$ equals 1.99; $P=0.006$) and children who had respiratory diseases (Prevalence $Ratio_{crude}$ equals 1.78; $P=0.016$). In this unadjusted analysis, the polymorphisms in rs2057482 and rs2301113 were not significantly associated with HSPMs (Table 3).

Table 3. CRUDE PREVALENCE RATIO OF THE ENVIRONMENTAL FACTORS AND GENOTYPE POLYMORPHISMS FOR HYPOMINERALIZED PRIMARY SECOND MOLARS (HSPMs)

		HSPMs		Total	PR_c* (95% confidence interval)	*P*-value**
		Case N (%)	Control N (%)			
Drug use during pregnancy†	Yes	17 (17.0)	83 (83.0)	100	2.14 (1.28-3.57)	**0.004**
	No	47 (7.9)	545 (92.1)	592	1.00	
Maternal problems‡	Yes	30 (14.5)	177 (85.5)	207	1.99 (1.21-3.25)	0.006
	No	27 (7.3)	344 (92.7)	371	1.00	
Respiratory diseases in childhood§	Yes	24 (14.5)	142 (85.5)	166	1.78 (1.11-2.85)	**0.016**
	No	42 (8.1)	476 (91.9)	518	1.00	
HIF-1α rs2057482 Genotypes	CT/CC	33 (11.0)	282 (89.0)	315	0.38 (0.13-1.06)	0.066
	TT	3 (27.3)	8 (72.7)	11	1.00	
HIF-1α rs2301113 Genotypes	AC/CC	24 (13.4)	155 (86.6)	179	0.87 (0.46-1.64)	0.671
	AA	16 (10.3)	138 (89.6)	154	1.00	

* Crude Prevalence rate (PRc) calculated by univariable Poisson regression analysis.

** Level of significance for *P*-value=0.05. Bold text indicates statistical significance difference for *P* value.

† Drug use was considered the mother's use of tobacco, alcohol, and illicit drugs during the pregnancy.

‡ Maternal problems were considered the presence of diabetes, hypertension during pregnancy, and prolonged labor delivery.

§ Respiratory diseases were considered the presence of bronchitis, asthma, and pneumonia until the first three years of life.

Table 4. ADJUSTED PREVALENCE RATIO OF THE ENVIRONMENTAL FACTORS AND GENOTYPE POLYMORPHISMS FOR HYPOMINERALIZED PRIMARY SECOND MOLARS (HSPMs; N=323)

		PR_c* (95% confidence interval)	*P*-value**
Drug use during pregnancy	Yes	4.52 (2.38-8.53)	<0.001
	No	1.00	
Maternal problems	Yes	1.97(1.05-3.71)	0.034
	No	1.00	
Respiratory disease during childhood	Yes	2.66 (1.41-5.03)	0.003
	No	1.00	
HIF-1α rs2057482 genotype	CT/CC	0.51 (0.27-0.99)	0.048
	TT	1.00	

* Adjusted prevalence rate (PRa) calculated by multiple variables Poisson regression analysis.

** Level of significance for *P*-value=0.05. To perform the multiple Poisson regression analysis, independent variables (with *P*<0.20 in the association with HSPMs) were added to the multiple model. The PRa was adjusted by the independent variables presented on the table: drug use during pregnancy, maternal problems, respiratory disease during childhood, and HIF-1α rs2057482 genotype.

Analyzing the factors in a multiple variables model (Table 4), children whose mothers used drugs during pregnancy were 3.52 times more likely to have HSPM than children whose mothers did not use drugs (Prevalence $Ratio_{adjusted}$ equals 4.52; *P*<0.01). In the same way, children whose mothers had suffered from health problems (such as diabetes and/or hypertension during pregnancy or prolonged labor delivery; Prevalence $Ratio_{adjusted}$ equals 1.97; *P*=0.03) had a 97 percent higher prevalence of HSPMs than mothers without these health problems. During childhood, children who suffered from respiratory diseases were 1.66 times more likely to have HSPMs (Prevalence $Ratio_{adjusted}$ equals 2.66; *P's* =0.03) than children without respiratory diseases. In the presence of hypoxia-related environmental factors, individuals who carried at least one C allele (with allele CC/CT genotype) in the rs2057482 polymorphism had a 50 percent lower prevalence of HSPMs (Prevalence $Ratio_{adjusted}$ equals 0.50; *P*=0.04) than allele TT children (Table 4).

Discussion

HSPM is an enamel development defect that is clinically similar to MIH, since the formation period of the permanent first molars and the primary second molars have a chronological coincidence. In this study, the authors assumed that, from the fourth month of intrauterine life to the first year, environmental factors could result in hypomineralization in the primary second molars as well as the permanent molars.[38] The present study analyzed the association between environmental factors related to hypoxia, as well as polymorphisms in the HIF-1α gene, which could be associated with a prevalence of HSPMs.

Although the MIH and HSPM etiologies have not been completely elucidated, previous studies have already noted some environmental and genetic factors associated with these defects.[3,30,39] Besides these environmental factors, the association of the mother's use of tobacco and alcohol during pregnancy,[3,39,40] maternal diseases,[30,41,42] complications in childbirth,[30,42] low birth weight, prematurity,[43-46] and respiratory problems in children up to three years of age[3,4,30,47] were observed. The present study's results corroborate previous studies, with a higher prevalence of HSPMs in children whose mothers had a health problem during pregnancy or mothers who were on drugs during pregnancy as well as a higher prevalence of children who had respiratory problems up to the age of three years. The presence of fever, dioxin factors, antibiotic use, nicotine, and alcohol may lead to changes in cell differentiation and enamel mineralization, and, consequently, be associated with enamel hypomineralization.[8,10,48,49]

Hypoxia may be caused by systemic problems during birth, prematurity, Caesarean section, respiratory problems, and prolonged labor.[31] An increased expression of HIF-1α has also been observed in pregnant women with preeclampsia,[50] which reinforces the hypothesis that maternal problems during pregnancy can lead to hypoxia-generating factors.

In the present study, hypoxia-related environmental factors significantly increased the prevalence of HSPMs. Some studies have already proposed that hypoxia at birth is a risk factor for enamel defects development.[42,51,52] It is possible to assume that some environmental factors associated with hypomineralization are hypoxia-generating situations.

The authors observed through multiple analyses that, when environmental factors analyze HIF-1α polymorphism (rs2057482), the strength of association increased significantly between the environmental factors and the HIF-1α rs2057482 polymorphism with HSPMs. This suggests a positive interaction between these events. In the presence of hypoxia-related environmental factors, individuals with C allele (CC/CT) at the HIF-1α rs2057482 polymorphism presented with a 50 percent lower prevalence of HSPMs, indicating that the C allele in the rs2057482 polymorphism acts as a protective factor for HSPMs in hypoxia conditions.

Adaptation to hypoxia in cells and tissues leads to the transcriptional induction of several genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival. The main factor mediating this response is the transcription factor HIF-1α, an oxygen-sensitive transcriptional activator.[53] In a rat study, Sidaly et al.[7] found that 24- and 48-hour hypoxia situations increased the expression of the structural genes: amelogenin (Amelx); ameloblastine (Ambn); enamelin (enamelina; Enam); and the matrix metalloproteinase-20 (Mmp20). This suggests that hypoxia situations may disrupt amelogenesis-related protein expression and, thus, cause an enamel disturbance.[7] Another factor that corroborates with this hypothesis is the fact that previous studies have shown that hypomineralized enamel has a substantial protein content higher than normal and a decreased mineral content,[54,55] suggesting alteration on these enamel proteins that were not removed in the mineralization stage.

Although retrospective design studies have memory bias limitations, this bias is likely not common in important health events such as pregnancy disorders, complications during childbirth, and illnesses in the child's early years.[56] It is believed that this bias was not a limitation in this study, and biological factor related to hypoxia was also analyzed. Thus, future studies should analyze both environmental factors and genetic variants for the etiology of HSPMs. Moreover, future investigations should consider the prevalence of HSPMs in a subset of children with medically documented hypoxia.

Conclusions

Based on this study's results, the following conclusions can be made:

1. Children who were exposed to hypoxia-related environmental factors presented with a higher prevalence of hypomineralized second primary molars.
2. Children carrying the C allele in the rs2057482 presented with a lower prevalence of HSPMs in the presence of hypoxia-related environmental factors.
3. These findings suggest a possible interaction between environmental and genetic factors on the etiology of HSPMs.

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