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Hipomineralização molar-incisivo e doença cárie: Estudos de base genética e fatores associados

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Tese apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para obtenção do título de Doutor em Odontologia. Área de Concentração em Multidisciplinaridades em Saúde (Ênfase em Saúde Coletiva).

Orientadora: Prof ª. Dra. Renata Iani Werneck
Coorientadores: Prof ª. Dra. Simone Tetu Moysés
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TERMO DE APROVAÇÃO

TAYLA CAVALLARI

HIPOMINERALIZAÇÃO MOLAR-INCISIVO E DOENÇA CÁRIE: ESTUDOS DE BASE GENÉTICA E FATORES ASSOCIADOS

Tese apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos parciais para a obtenção do Título de **Doutor em Odontologia**, Área de Concentração em **Saúde Coletiva**.

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SUMÁRIO

RESUMO

Avanços no conhecimento da epidemiologia genética forneceram evidências de que doenças com herança multifatorial se desenvolvem por meio de interações entre os genes e o ambiente. Dessa forma, essa tese teve como objetivo explorar dois fenótipos de afecções bucais com envolvimento genético: a hipomineralização molar-incisivo e a doença cárie.

Artigo 1:

Objetivo: Identificar a associação de polimorfismos no gene *KLK4* na suscetibilidade à hipomineralização molar-incisivo (HMI). **Métodos:** Um estudo caso-controle foi conduzido com estudantes de Escolas Municipais de Curitiba entre 6 e 10 anos. Um total de 200 crianças foi recrutado, sendo 100 participantes do grupo caso (com HMI) e 100 do grupo controle (sem HMI). Células da mucosa bucal foram obtidas por meio de bochechos com solução de glicose 3%. Efetuou-se a extração do DNA com acetato de amônio e EDTA. A amplificação do material genético foi conduzida por meio da técnica de PCR em tempo real com uso de sondas TaqMan®. O teste qui-quadrado foi aplicado para comparar as frequências alélicas e genotípicas entre os grupos. **Resultados:** Na análise multivariada foi encontrada associação entre HMI e o marcador *rs*235091 para o modelo dominante do alelo A (p=0.002), além de um resultado *borderline* para a variável presença de infeções respiratórias (p=0.099). **Conclusão:** Polimorfismos genéticos no gene *KLK4* podem contribuir com a HMI.

Artigo 2:

Objetivo: Apresentar uma análise de interação genética e proteica associada à doença cárie. **Métodos:** Inicialmente uma revisão sistemática da literatura (RSL) foi conduzida por meio das bases de dados PubMed e BVS, para identificar estudos que investigaram genes associados com a doença cárie entre os anos de 1982 e 2017. A RSL foi realizada de acordo com os critérios estabelecidos no PRISMA e os estudos incluídos foram avaliados através da escala New Castle-Ottawa Scale (NOS). Utilizou-se a base *String Protein Interaction* para analisar a interação de proteínas e o *Ingenuity Pathway Analysis* (IPA) para verificar as vias biológicas associadas com a doença cárie. **Resultados**: Um total de 51 artigos foi incluído

nesta revisão e 27 genes foram associados ao desenvolvimento da doença cárie, sendo que destes, 23 interagem com pelo menos um outro gene. Os genes *TUFT1, VDR, TFIP11, LTF, HLA-DRB1, MMP2, MMP3* e *MUC5B* foram conectados por redes interativas com 10 outros genes. **Conclusão:** É essencial compreender o padrão multifatorial em algumas doenças humanas. Este estudo apresenta vias que podem estar diretamente correlacionadas com a susceptibilidade à doença cárie.

Palavras-chave: Genética; Hipomineralização; Doença cárie.

INTRODUÇÃO

Os frequentes progressos no campo das ciências da vida, sobretudo na biologia molecular e engenharia genética, mostram evidências promissoras de que muitas doenças são influenciadas por alterações em estruturas genéticas. Nesse contexto, estudos epidemiológicos associando variáveis ambientais com genéticas têm sido conduzidos para avaliar a interação e o risco no desenvolvimento de doenças com etiologia multifatorial (1, 2).

Por meio da genética molecular, é possível compreender que doenças complexas também podem ser causadas por alterações na sequência do DNA. Dessa forma, estudos genéticos de associação têm sido amplamente utilizados para melhor abranger os fatores causais destas doenças. Esses estudos avaliam genes candidatos e testam se um determinado alelo ou genótipo encontra-se em uma frequência significativamente maior entre os grupos de indivíduos afetados e não afetados (3–5).

Outro aspecto relevante trata-se da expressão de um fenótipo, esta pode ser modulada por vários genes, sendo assim, o mapeamento de redes de interação gene-proteína possibilita a melhor compreensão do efeito de cada gene no funcionamento celular (6).

Diante do exposto, essa tese teve como objetivo explorar dois fenótipos de doenças bucais com envolvimento genético: a hipomineralização molar-incisivo e a doença cárie.

2. HIPOMINERALIZAÇÃO MOLAR-INCISIVO

O esmalte dentário é o tecido mais mineralizado do corpo humano. Sua formação ocorre por meio de 3 estágios subsequentes: o secretório, de transição e de maturação, até que parte da matriz orgânica seja degradada e substituída por componente mineral (7).

Estudos têm demonstrado que as células responsáveis pela formação do esmalte, os ameloblastos, são extremamente sensíveis a estímulos térmicos ou mecânicos. Uma vez que a natureza do esmalte dentário não é remodeladora, qualquer distúrbio que ocorra durante a sua formação, este, poderá ficar registrado como defeito na estrutura dentária (8).

Defeitos no desenvolvimento do esmalte consistem em um importante marcador biológico, visto que estas alterações podem ser avaliadas por meio de um padrão de calcificação, inferindo o período em que provavelmente ocorreram. Dessa forma, defeitos como as hipoplasias dentárias, podem surgir quando há uma alteração durante a fase secretória da formação do esmalte (9). Contudo, se células forem afetadas na fase tardia da mineralização ou maturação, um defeito na translucidez deste tecido pode ser induzido, caracterizando uma hipomineralização (10).

A Hipomineralização molar-incisivo (HMI) consiste em um defeito qualitativo na mineralização do esmalte dentário, quando em dentes permanentes, pode atingir de um a quatro primeiros molares e frequentemente também está associada aos incisivos (10). Além disso, as hipomineralizações podem ser observadas em dentes decíduos, acometendo em caninos e segundos molares (11), uma vez que parte do desenvolvimento desses dentes ocorre de maneira simultânea com os primeiros molares permanentes (12).

A condição de hipomineralização foi descrita pela primeira vez na Suécia, na década de 1970, referida como “primeiros molares permanentes hipomineralizados” (9). Somente em 2001 surgiu o termo *Molar Incisor Hypomineralization* ou Hipomineralização Molar-Incisivo (MIH/HMI) em que ressalta o envolvimento dos incisivos permanentes (10).

Os dentes com HMI apresentam áreas com porosidade, tendo um maior teor de magnésio e carbonato, e menores concentrações de cálcio e fosfato em comparação com o esmalte, além de apresentarem valores de dureza significativamente mais baixos (13). Outro aspecto importante é que dentes com HMI possuem um conteúdo proteico de 3 a 15 vezes maior que o esmalte normal (14).

Um estudo de revisão sistemática e metanálise apontou uma prevalência global da HMI de 13,1% (11,8–14,5%) e uma incidência de 17,5 milhões de novos casos a cada ano (15). Há uma variação no que se refere a prevalência da HMI. No Brasil, um estudo realizado na cidade de Araraquara apontou uma prevalência de 12,3% (16). Já em outros países foram reportadas as seguintes prevalências: na Índia 13,12% (17), na Polônia 6,43% (18), no Japão 18,8% (19), nos Estados Unidos 9,6% (20), entre outras.

2.1. Características clínicas

Clinicamente, a HMI apresenta opacidades demarcadas, com alteração na translucidez variável em grau, tamanho e cor (figura 1). A tonalidade do defeito varia da cor branca ao amarelo/acastanhado. O esmalte dentário tende a romper facilmente devido aos esforços mastigatórios, favorecendo o desenvolvimento da lesão cariosa (21).

Os dentes acometidos por HMI podem apresentar sensibilidade, uma vez que estes dentes apresentam inflamação pulpar crônica. Essa inflamação é responsável pelo aumento da sensibilidade e está diretamente relacionada à maior inervação da região sob a área hipomineralizada (22).

A HMI pode se apresentar de maneira distinta (assimétrica) em um mesmo paciente, sendo possível observar uma variação de leve opacidade em um molar e grandes perdas estruturais em outro (10).

A classificação da HMI é determinada pela severidade do defeito, sendo leve quando há uma opacidade demarcada, sem fratura de esmalte, ou severa, quando associadas a fraturas pós-eruptivas e a hipersensibilidade dentária (figuras 1, 2, 3 e 4 – imagens do autor). De acordo com formulário simplificado de Ghanin *et al*., considera-se HMI os seguintes critérios (23):

1. Presença de opacidades demarcadas: alterações na translucidez do esmalte, podendo apresentar-se de cor branca, amarela ou castanha;

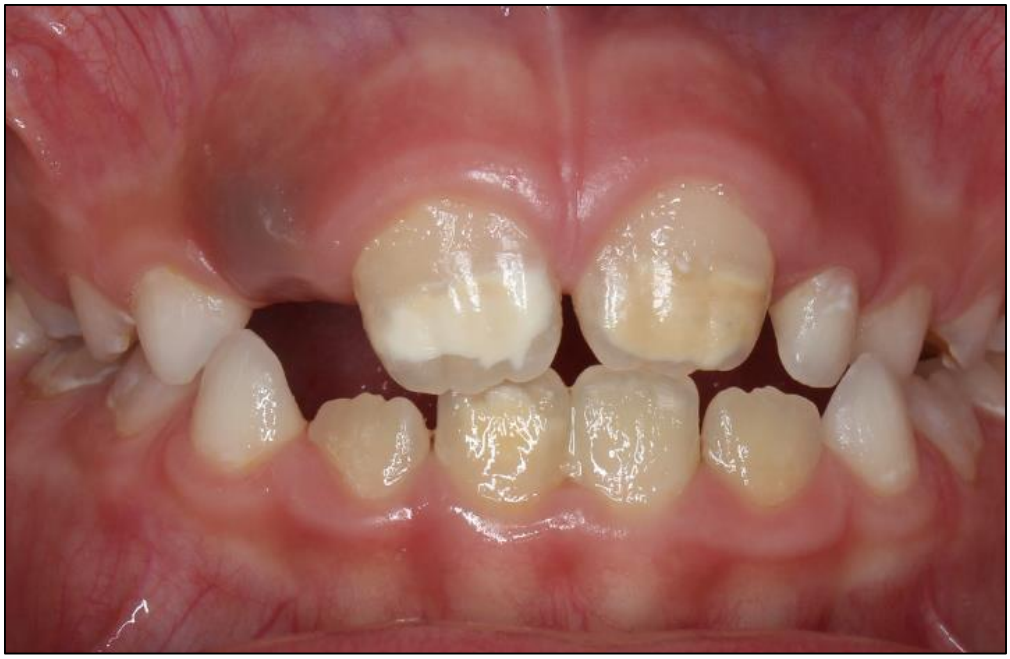

Figura 1: Imagem apresentando dentes com opacidades demarcadas nos elementos 11,21,41 e 42.

2. Fratura pós-eruptiva do esmalte: perda de superfície do esmalte após a erupção dos dentes;
3. Lesão cariosa atípica: a lesão cariosa existente encontra-se associada a opacidades demarcadas. A localização da lesão cariosa se estende a áreas menos acometidas

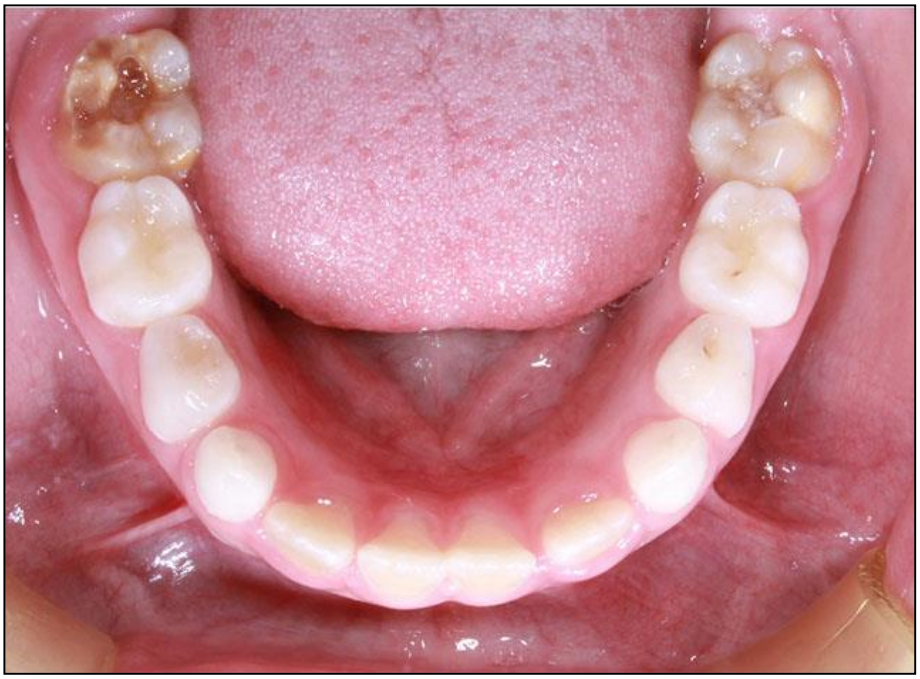

Figura 2: Imagem apresentando fratura pós eruptiva e lesão cariosa nos elementos 36 e 46.

4. Restaurações atípicas: frequentemente estendem-se para superfícies que não são normalmente afetadas por cárie. Além disso, na margem das restaurações observa-se uma opacidade (figura 4).

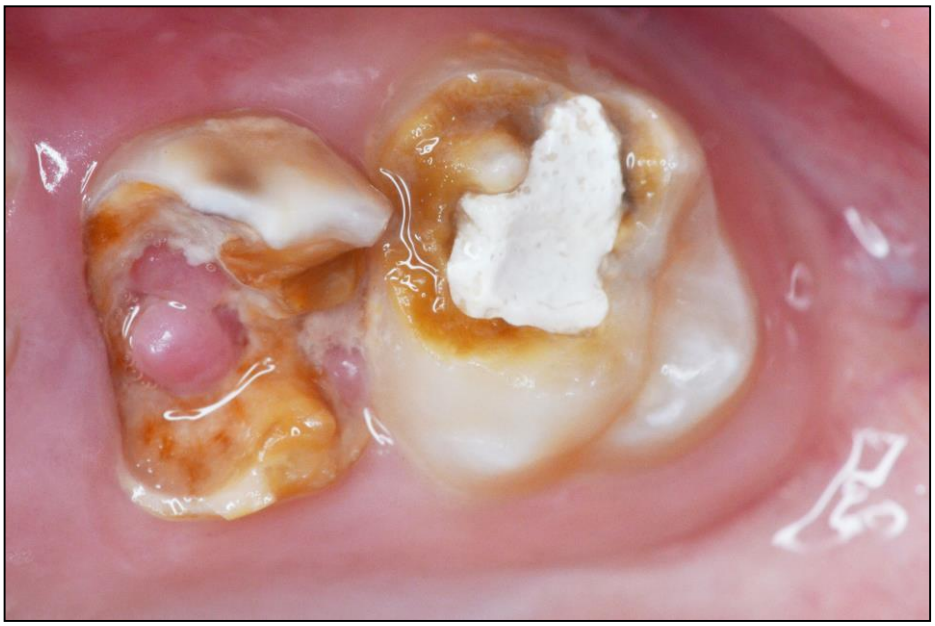

Figura 3: Imagem apresentando lesões cariosas atípicas com perda de estrutura severa nos elementos 55 e 26.

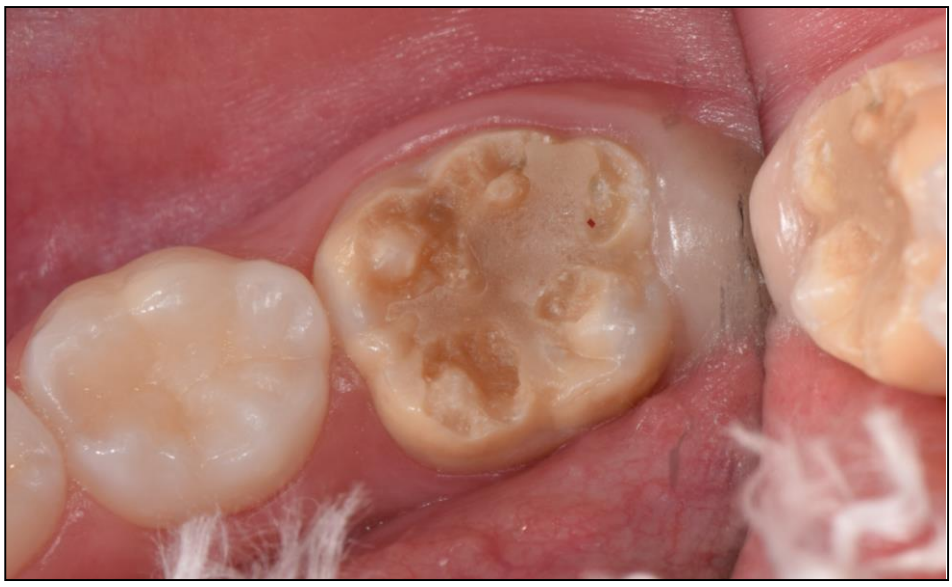

Figura 4: Imagem apresentando restauração atípica no elemento 46.

2.2 Diagnóstico diferencial

O aspecto clínico da HMI remete às hipoplasias do esmalte. Entretanto, nas hipoplasias, as bordas do esmalte adjacente são lisas e arredondadas. Jájá na HMI, as bordas apresentam-se ásperas e irregulares. Enquanto a HMI consiste em um defeito qualitativo do esmalte, as hipoplasias são caracterizadas como um defeito quantitativo, ou seja, a formação da matriz orgânica é deficiente, afetando a espessura do esmalte (9).

A HMI pode ser diferenciada da fluorose dental, uma vez que esta apresenta-se com opacidades difusas que afetam dentes homólogos de forma simétrica. Clinicamente, a fluorose é caracterizada por estrias esbranquiçadas, podendo variar para tons mais escuros em casos severos. Além disso, na fluorose há o relato de exposição a altos níveis de flúor (24,25).

O diagnóstico diferencial com a amelogênese imperfeita é fundamentado na distribuição das opacidades. Enquanto na HMI raramente os molares são afetados na mesma intensidade, na amelogênese imperfeita toda a dentição é afetada, tendo um padrão familiar genético correlacionado (8).

2.3 Etiologia

A etiologia da HMI ainda não está completamente elucidada. Durante o desenvolvimento dentário uma série de fatores pode interagir afetando os ameloblastos e matriz de formação do esmalte dentário. Embora ainda não existam dados conclusivos quanto aos fatores predisponentes, estudos apontam que os distúrbios causadores podem ocorrer no período pré-natal, neonatal ou durante a primeira infância (26, 27).

Em relação aos fatores etiológicos no período pré-natal há uma evidência correlacionando HMI com alterações na gestação (28). De acordo com uma revisão sistemática e metanálise, crianças cujas mães tiveram problemas de saúde durante a gestação, apresentaram uma maior probabilidade de ter HMI (29). A provável causa está relacionada à febre, interferindo dessa forma, na atividade ameloblástica (29). Outros aspectos já citados referem-se ao estresse psicológico materno e a exposição frequente a exames ultrassônicos durante o último trimestre gestacional (30). Sobre o aspecto psicológico, o autor cita que distúrbios

psicológicos podem ser acompanhados de anemia seguida de hipotensão. Ainda não está claro se as ondas de ultrassom, em termos de intensidade e duração, têm um efeito negativo sobre a saúde e desenvolvimento do feto. Uma possibilidade é que, devido a outras doenças sistêmicas, há uma necessidade de exposição frequente ao ultrassom (30).

No que se refere ao período neonatal, a literatura aponta uma relação entre à falta de oxigênio durante o parto e o parto por cesária (31). Sabe-se que a falta de oxigênio pode gerar hipóxia para os ameloblastos, ocasionando, dessa forma, defeitos no desenvolvimento do esmalte. Com relação ao parto por cesariana, ainda há um fator de confusão nos achados, uma vez que a eleição para o parto cesáreo, muitas vezes é feita pela condição de pré-eclâmpsia, prematuridade, pressão alta ou diabetes (32).

Problemas respiratórios, febre e doenças da infância associaram-se significativamente com HMI. Durante a infância, os fatores predisponentes consistem em problemas de saúde nos três primeiros anos de vida, período crítico para formação da coroa dos primeiros molares e incisivos permanentes. Estudos apontam que é difícil determinar ou dissociar se foi a enfermidade, as alterações sistêmicas, o medicamento utilizado ou febre que ocasionou a HMI, uma vez os ameloblastos são sensíveis a altas temperaturas (9, 27, 29).

Tendo em vista a característica multifatorial da HMI, autores sugerem que defeitos no desenvolvimento do esmalte podem ser resultados de variações genéticas em proteínas expressas durante a amelogênese (33). Segundo estudo realizado por Jeremias et al, os marcadores rs3796704 (ENAM), rs4694075 (AMBN); rs5997096/rs134136 (TFIP11), foram associados com a HMI ($p<0.05$) (34). Um estudo avaliou a relação da HMI entre pares gêmeos monozigóticos e dizigóticos e encontrou uma concordância maior entre gêmeos monozigóticos, indicando uma influência genética. (35).

2.4 Considerações

Tendo em vista os fatos apresentados, a HMI vem ganhando uma maior atenção no cenário clínico em decorrência dos desafios da prática odontológica e do impacto na saúde bucal. Dependendo da severidade e extensão do defeito do esmalte, o indivíduo com HMI pode desenvolver sinais e sintomas que interferem

na sua qualidade de vida, uma vez que estes dentes apresentam sensibilidade, dor, desconforto à mastigação, além da maior suscetibilidade à doença cárie. O conhecimento epidemiológico da doença é um importante indicador para definição de ações de promoção de saúde bucal. Uma vez que os fatores etiológicos ambientais e sistêmicos envolvidos ainda não estão totalmente esclarecidos e diante da complexidade de variáveis que envolvem a referida condição, explorar um possível polimorfismo genético torna-se fundamental.

3. DOENÇA CÁRIE

A cárie dentária consiste em uma doença crônica, de etiologia multifatorial, pertencente ao grupo das doenças complexas (36,37). No Brasil, de acordo com os últimos levantamentos epidemiológicos, houve uma redução no índice CPOD (dentes cariados, perdidos por cárie e obturados), na população em idade escolar (aos 12 anos). Na década de 1980, o CPOD era de 7,3 e em 2010, esse índice diminuiu para 2,1 (38). Apesar do evidente progresso no controle da doença, a cárie ainda é um importante problema de saúde bucal (39). Além disso, o declínio da cárie dentária foi acompanhado pelo fenômeno da polarização, que consiste na concentração da doença em parcela da população (40).

De acordo com Ferjeskov e Manji em 1990, o desenvolvimento da doença era influenciado por variáveis socioeconômicas, comportamentais e biológicas (41). Atualmente, sabe-se que os determinantes sociais de saúde estão interligados com desenvolvimento da cárie dentária, uma vez que estes envolvem aspectos sociais, culturais, econômicos e comportamentais de uma determinada população (42,43). De acordo com Dahlgren e Whitehead, determinantes sociais de saúde dispõem-se em diferentes camadas de acordo com seu nível de abrangência, sendo uma camada proximal, associada a determinantes individuais, e uma camada distal, na qual se encontram os macrodeterminantes (44). Dentre os fatores proximais no desenvolvimento da doença cárie podem ser citados: o hospedeiro, os microrganismos, a composição da saliva e a dieta. Os determinantes distais incluem, dentre outros, classe social, renda, escolaridade, comportamento e acesso aos serviços de saúde (36,37).

Com a consolidação da epidemiologia genética, estudos moleculares que utilizam técnicas de análise do DNA e mapeamento de genes envolvidos no desenvolvimento da doença foram iniciados (45,46). Desde a década de 1980, estudos tentam associar genes candidatos com a doença cárie, sendo que alguns já apresentaram associação. Grande parte das pesquisas foi desenvolvida analisando os genes relacionados com a formação, desenvolvimento e mineralização do esmalte. Estes genes são: Amelogenina (*AMELX*) (47, 48), Tuftelina (*TUFT1*) (49), Ameloblastina (*AMBN*) (50), Enamelina (*ENAN*) (51), Aquaporina (*AQP5)*, Calcicreína (*KLK4*) (52), Metaloproteinases *(MMP13* e *MMP20*) (53).

Alguns genes que possuíam relação com a resposta imune do hospedeiro também foram pesquisados: *Mannose-binding lectin* (*MBL*) (54), *HLA-DRB1*, *HLA-DQB1* (55), *DEFB1* (56).

Por fim, encontraram associação de genes relacionados com a composição da saliva: *saliva carbonic anhydraseVI* (*CA6*), *proline-rich protein gene (PRPs*) (57) e Lactotransferrina *(LTF*) (58).

A investigação de fatores sociodemográficos, comportamentais e genéticos é essencial para o entendimento da ocorrência da doença em determinados grupos de indivíduos. Sabendo que a doença cárie tem uma herança multifatorial, é importante compreender que a expressão do fenótipo pode ser condicionada pela ação conjunta de dois ou mais pares de genes com segregação independente, uma vez que redes de proteínas possuem vários tipos de interação e regulação.

4. OBJETIVO

O objetivo desse trabalho foi conduzir um estudo de associação (caso-controle) para investigar em uma população sul-brasileira a participação de polimorfismos na suscetibilidade a HMI. Além de realizar análises de redes de interação entre genes e proteínas com os genes previamente associados com a doença cárie.

4.1 Objetivos específicos:

1. Analisar as variáveis que predispõe a HMI;
2. Testar polimorfismos no gene *KLK4* e HMI;
3. Conduzir uma revisão sistemática de genes relacionados com a doença cárie;
4. Realizar uma análise de interação entre genes e proteínas com a doença cárie.

ARTIGO 1

The impact of *KLK4* gene on MIH

Abstract

Objective: to investigate polymorphism in *KLK4* gene in the susceptibility to molar incisor hypomineralization (MIH). Methods: a case control study was performed with 200 students recruited in municipal schools of Curitiba city, between 6 and 10 years old. A medical history questionnaire was applied to consider possible etiological factors associated to MIH. Buccal cells were also collected and DNA samples from 100 cases (with MIH) and 100 unaffected controls were studied. The diagnosis of MIH was performed according to Ghanin criteria. Five markers were genotyped with the polarized fluorescence by Real-Time Polymerase Chain Reaction. Chi-square test was used to compare allele and genotype frequencies between the groups. Results: the average age from the case group was 7.8 years old and for the control group was 7.6 years old. An association was found between MIH and the marker *rs*235091 in dominant allele A model (p=0.002). A borderline result for respiratory infection was found (p=0.099). Conclusion: *KLK4* gene appears to contribute to MIH in the south Brazilian population.

Keywords: Enamel; Hypomineralisation; Epidemiology; Genetic.

Introduction

Dental enamel is the most mineralized tissue in the human body and its formation is divided into secretory, transition and maturation stages. During the secretory stage, the ameloblasts, cells responsible for dental enamel formation, secrete mostly amelogenin, enamelin and ameloblastin. This process occurs until the organic matrix has been degraded and replaced by the mineral component (Bartlett 2013). There are different enzymes that contribute to this degradation (Simmer and Hu 2002), among them, there is the kallikrein-4, which is a protein encoded by *KLK4* gene expressed during the transition from the secretory to the maturation stage (Lu et al. 2008).

Studies have shown that ameloblasts are extremely sensitive to injuries and different kinds of enamel defects may occur depending on the stage of development. When ameloblasts are affected in the late amelogenesis stage of mineralization or maturation, a hypomineralization can occur (Weerheijm KL 2003; Lacruz et al 2017; Simmer and Hu 2001).

The Molar incisor hypomineralization (MIH) is a qualitative defect of dental enamel that affects the permanent first molars, often in association with the permanent incisor (Weerheijm KL 2003). Furthermore, second primary molars (HSPM) (Garot et al. 2018) and canines (Figueiredo Sé et al. 2017) can also be involved. Clinically, the MIH is presented as demarcated opacities that vary in color shades from white to yellow/brownish, with or without post-eruptive breakdowns (PEB). There is no conclusive data in relation to the MIH etiology. The various etiological factors depend on the prenatal, perinatal, and postnatal conditions of a child (Souza et al. 2013; Lygidakis at al. 2009).

It is known that the entire enamel formation process is under genetic control and individual genetic may be influenced by modulating the expression of genes, thus, it is plausible to hypothesize that genetic variations could be associated with MIH (Jeremias et al. 2013 /A). Genetic mutations have already been associated with different kinds of amelogenesis imperfecta (Smith et al. 2017). One study presented that there is more association to the occurrence of MIH among monozygotic twins brothers (genetically identical) than among dizygotic ones (Teixeira et al. 2018).

The identification of genes involved in human diseases is important to better understand the conditional. Testing candidate genes is an effective strategy for evaluating the relationship between genotype-phenotype. Thus, the aim of this research was to conduct a case control study in a South Brazilian school population to identify if polymorphism in *KLK4* gene influence the susceptibility to molar incisor hypomineralization.

Material and Methods

Ethical approval

This study was approved by the ethics committee of Pontifícia Universidade Católica do Paraná (number 1.971.986) and informed consent forms were signed by the children's legal guardian prior to beginning the data collection. The manuscript was written in accordance with the guidelines: Strengthening the Reporting of Observational Studies in Epidemiology (STROBE).

Sample recruitment

The study population was composed of children recruited in the municipal schools of Curitiba, Brazil. The sample recruitment was based on the MIH prevalence for a specific region in Brazil (Jeremias et al. 2013/B). A sample power calculation was performed considering the total of 85.000 children enrolled in municipal schools of Curitiba and the MIH prevalence of 12,3% (Jeremias et al. 2013). To obtain a consistent sample with an error margin of 6.35% and 95% confidence level, two hundred individuals would be sufficient to give power to the genetic analysis. The sample was divided into two groups: case (with MIH) and control (without MIH), each with a hundred individuals.

The recruitment occurred from August 2017 until August 2018 and a questionnaire was distributed to the parents to investigate the possible etiological factors associated to MIH. This questionnaire contained environmental, systemic and local variables. The risk factors explored were: birth, prematurity, ingestion of alcohol during pregnancy, exposure to cigarette smoke, family income, history of respiratory or urinary infections, otitis, and high fever. Children were not included when: in use of fixed appliance; the incisors and permanent molars were not present at the clinical exam or the evidence of another enamel defect linked to a condition was present such as: amelogenesis imperfect, fluorosis and hypoplasia.

Diagnosis

The diagnosis of MIH was carried out according to the criteria established by the simplified form of Ghanim et al. (Ghanim et al. 2017). Children who had one or more first permanent molar classified with codes 2 to 5 participated in the case group. These codes included: demarcated opacities, post-eruptive enamel

breakdown, atypical restorations and atypical caries lesions. The clinical assessment was performed by one investigator (T.C.). The calibration to determine the phenotype of MIH was done by an expert in pediatric dentistry who received an extensive training on clinical pictures of MIH by an experienced examiner.

The control group was composed of individuals without MIH or dental caries, since studies have already demonstrated that some genes are involved with dental caries development. To diagnose the control group, the researcher was calibrated according to the ICDAS criteria (International Cavities Detection and Assessment System (ICDAS) (Shivakumar et al. 2009), by means of a bank of extracted teeth. Patients who had all the dental elements with the scores between 0 and 2 were considered control group. The intra-examiner reproducibility was taken on 10% of the sample one month after the first analyses and the Kappa test was used to measure the reliability. The obtained values for Kappa test was 1. Clinical examinations were performed in an illuminated local and when it was necessary, a gauze was used to dry and clean the teeth prior to examination, besides the use of a flashlight and a mouth mirror. If there were doubts in the diagnosis of MIH, the patient was excluded from the research.

DNA collection

Buccal epithelial cells from the subjects were obtained using 5 ml of 3% glucose mouthwash for 1 min (Aidar and Line 2007), and then decanted by centrifugation at 2,000 rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 650 µl extraction buffer (10 mM Tris-HCl [pH 7.8], 5 mM EDTA, and 0.5% SDS). The samples were kept frozen at -20°C until the DNA was extracted. After defrosting, the samples were incubated overnight with 10 µl of proteinase K at 37°C. The following day, the DNA was extracted using 10 M ammonium acetate and 1 mM EDTA, in accordance with the established protocol by Trevilatto (Trevilatto and Line 2000). The concentration and purity of the DNA were determined by optical density in a spectrophotometer (NanoDrop™®) using1 µl of the extracted material.

Selection and genotyping markers

Target polymorphisms (Tag SNPs) markers for *KLK4* gene were selected based on the International HapMap Project, release 27. The linkage disequilibrium

(LD) was calculated by r^2<0.80 for multimarker with a minimum allele frequency of 0.05 on African population (YRI; Yoruba in Ibadan, Nigeria). Five markers were selected: *rs*2242670, *rs*2235091, *rs*2978642, *rs*2978643 and *rs*1654553.

The patient's genotypes were obtained using genetic discrimination based on the TaqMan® fluorescence system. Amplification reactions were performed using the real time polymerase chain reaction (PCR), as implemented in the QuantStudio™ 7 Flex Real-Time PCR System.

Statistical analysis

To investigate the association between variables and MIH, the Chi-square test and Fisher's exact test were conducted. A logistic regression model backward was used in the multivariate analysis. The variables that in the bivariate analysis had a p≤ 0.2, were included in the multivariate analysis. For greater plausibility of the data, each tag SNP was assessed for genotypic modes of transmission (additive, dominant and recessive models). The dominant model was assembled using the homozygous genotype associated allele compared to the grouping of the other genotypes (homozygous and heterozygous).

The analyses were performed using SPSS version 22.0 statistical software. Haploview 4.2 software was used to analyze the LD between the Tag SNPs of *KLK4* gene.

Results

Two hundred children were recruited, ninety-nine girls and a hundred one boys. The average age from the case group was 7.8 years old and for the control group was 7.6 years old. There were no statistically significant results related to the gender (p=0.322) or age (p=0.638). In relation to patients' ethnicity, 139 were caucasians, 49 dark skinned and 3 blacks (p=0.176).

The five *KLK4* markers analyzed were Tag SNPs, in accordance with the Hapmap. The analysis performed on the Haploview confirmed the existence of linkage disequilibrium among the markers (Figure 1).

Considering the cutting level of p≤ 0.2, the following variables were included in the multivariate analysis: urinary infection (p=0.174), respiratory infection (p=0.184), race (p=0.176), otitis (p=0.094) (Table 1), the additive model for the marker rs2235091 (p=0.028), the marker rs2235091 for A dominance (p=0.008) and the marker rs2978642 for T dominance (p= 0.172) (Table 2).

In multivariate analysis, for the marker rs2235091, the A allele in the dominance model remained a significant association with MIH (p=0.002). The frequency of the allele A in the control group was significantly higher showing a protection from the development of MIH. Individuals with the A allele are 0.196 more resistant in the development of MIH. Even in the multivariate analysis a borderline result for respiratory infection was found (p=0.099) (Table 2).

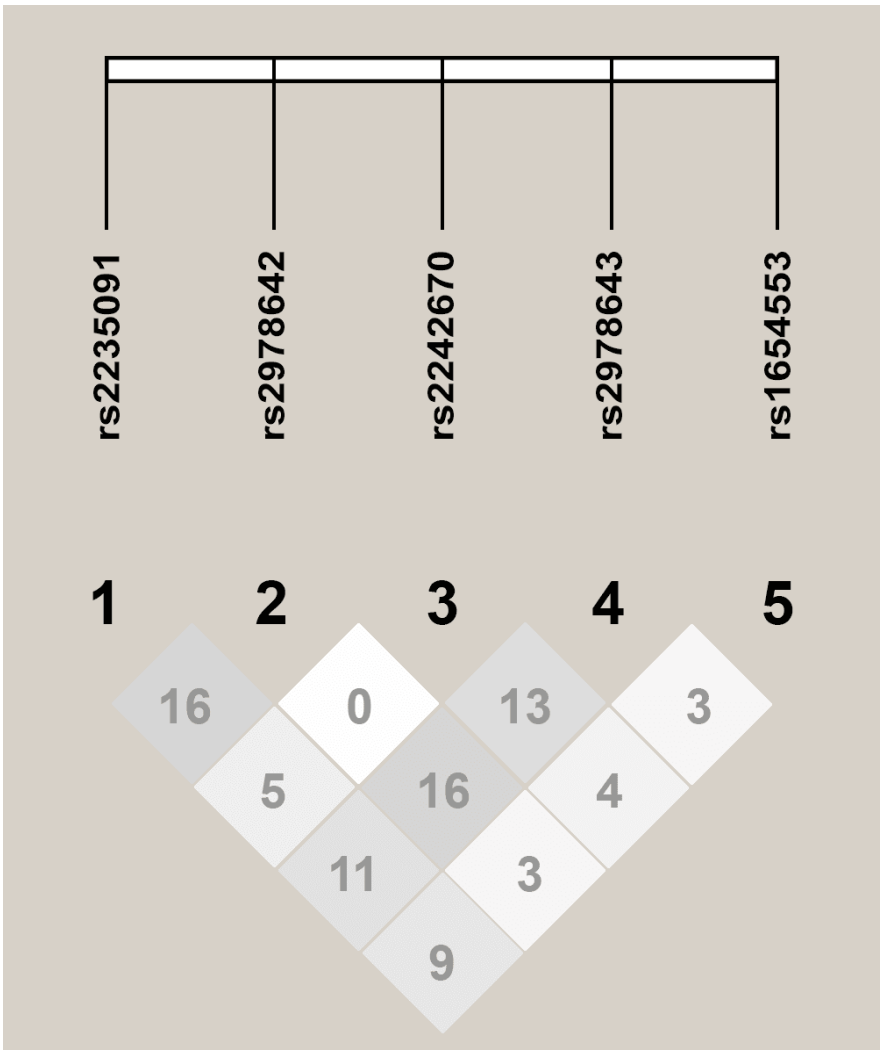

Fig. 01. Linkage disequilibrium (LD) among the SNPs.

Schematic representation of multimarker linkage disequilibrium (LD) (r2) between the five *KLK4* tag SNPs analyzed. The number inside the squares indicates the amount of LD. The color intensity of the squares reflects the strength of binding between the loci.

1 Table 01: Risk factors for MIH in the studies subject

Variable	Case (n)	Control (n)	P-value	Multivariate P-value	OR(CI)
Birth					
Natural delivery	47 (48.5%)	42 (44.7%)	0.601		
Caesarean delivery	50 (51.5%)	52 (55.3%)			
Prematurity					
Yes	89 (92.7%)	83 (92.2%)	0.900		
No	7 (7.3%)	7 (7.8%)			
Ingestion of alcohol during pregnancy					
Never	54 (70.1%)	62 (74.7%)	0.463		
Once a month	23 (29.9%)	21 (25.3%)			
Exposure to cigarette smoke					
Yes	27 (27.3%)	18 (18.8%)	0.225		
No	72 (72.7%)	73 (80.2%)			
Monthly income					
Up to US$400.00	69 (69.1%)	64 (66.7%)	0.741		
More than US$400.00	30 (30.9)	32 (33.3%)			
***Respiratory infection**					
Yes	27 (27.3%)	34 (36.2%)	0.184	0.099	0.555 (0.276-1.117)
No	72 (72.7%)	60 (63.8%)			
***Ottites**					
Yes	7 (7.4%)	14 (15.1%)	0.094	0.221	0.458 (0.131-1.602)
No	88 (92.6%)	79 (84.9)			
***Urinary infection**					
Yes	8 (8%)	13 (14.1%)	0.174	0.145	0.489 (0.187-1.278)
No	92 (92%)	79 (85.9%)			
***Asthma**					
Yes	13 (13%)	13 (14.1%)	0.819		
No	87 (87%)	78 (85.9%)			
***High fever**					
Yes	52 (52.5%)	56 (60.9%)	0.245		
No	47 (47.5%)	36 (39.1)			

* Until 3 years old

Statistically significant P-value < 0,05

OR - Odss Ratio

CI- Confidence interval 95%

2

1
2 Table 02: Case-control analyses of *KLK4* Tag SNPs

Variable		Model	Case (n)	Control (n)	Bivariate P-value	Multivariate P-value	OR (CI)
***KLK4* (rs2242670)**							
	AA - GG - AG	Additive	28 - 20 - 50	28 - 24 - 45	0.725		
	AA + AG vs GG	A Dominance	78 vs 20	73 vs 24	0.493		
	GG + AG vs AA	G Dominance	70 vs 28	69 vs 28	0.875		
***KLK4* (rs1654533)**							
	TT - CC - CT	Additive	29- 17 - 51	30 - 17 – 51	0.994		
	TT + CT vs CC	T Dominance	78 vs 20	73 vs 24	0.974		
	CC + CT vs TT	C Dominance	70 vs 28	69 vs 28	0.913		
***KKL4* (*rs*2235091)**							
	AA - GG - AG	Additive	29 - 21 - 48	37 - 8 -54	0.028	0.625	1.194 (0.587-2.427)
	AA + AG vs GG	A Dominance	77 vs 21	91 vs 8	0.008	0.002	0.196 (0.068-0.560)
	GG + AG vs AA	G Dominance	69 vs 29	62 vs 37	0.247		
***KLK4* (rs2978642)**							
	AA - TT - AT	Additive	44 - 6 - 48	35 -7- 57	0.393		
	AA + AT vs TT	A Dominance	92 vs 6	92 vs 7	0.789		
	TT + AT vs AA	T Dominance	54 vs 44	64 vs 35	0.172	0.182	1.559 (0.812-2.994)
***KLK4* (rs2978643)**							
	CC - GG - CG	Additive	44 - 6 - 49	52 - 6 - 41	0.502		
	CC + CG vs GG	C Dominance	93 vs 6	93 vs 6	0.616		
	GG + CG vs GG	G Dominance	55 vs 44	47 vs 52	0.160		

Statistically significant P-value < 0.05

Discussion

This study presented an association between the SNP rs2235091 of *KLK4* gene and MIH, supporting the thesis that MIH is a multifactorial disorder with genetic influence. It is known that an isolated event is not enough to develop a phenotype of a complex disease; however, a series of consecutive events may lead to some alteration of a previous functional allele.

Some studies have been conducted to better understand the nature of genetic component and MIH. In a study performed by Jeremias et al., a genetic evaluation of five genes related to Amelogenesis (*AMBN, Amelx, Enam, TUFT1, TFIP11*) were conducted, and it was found an association between the rs3796704 of *ENAM* gene and MIH (Jeremias et al. 2013). According to Bussaneli *et al*., there is an association with the rs10733708 of *TGFBR1* gene in severe cases of MIH, suggesting that polymorphism in genes related to the immune response may contribute to the development of MIH (Bussaneli et al.2019). Künish *et al.,* carried out a GWAS and identified a possible locus linked to MIH for the rs13058467 of S*CUBE1* (Kühnisch et al. 2014).

The *KLK4* gene is located at chromosome 19 (19q13.3–19q13.4), its main function, in dental enamel formation, is to facilitate the orderly replacement of organic matrix with mineral, producing an harder and less porous enamel layer (Lu et al. 2008). A study has shown that kallikrein mutation is associated with autosomal recessive hypomaturation, reinforcing that *KLK*4 function is critical for enamel mineralization. The loss of *KLK*4 function might affects the maturation stage of enamel development, inhibiting the growth of enamel crystallites, affecting the final deposition of mineral (Hart et al. 2004).

Environmental pollutants, such as Bisphenol A (BPA), have been associated with MIH. In an experimental study, rats were daily exposed to a low dose of BPA, to simulate the human exposure. The authors presented that this exposure might cause hypomineralizations through the modulation of *ENAN* and *KLK4* gene, since *KLK4* mRNA level were significantly decreased (Jedeon et al. 2013).

In addition, the *KLK4* gene has also been associated with dental caries development. A study revealed a significantly difference in the distribution of alleles in the same marker found in this study (Gerreth et al. 2018). Similarly, Wang et al., also associated the rs2235091 with the occurrence of dental caries (Wang et al.

2012). According to Lu et al, polymorphisms of *KLK4* gene can intensify the enamel porosity and aids the decrease of mineral (Lu et al. 2008).

In this study, the relationship between MIH and respiratory infection presented a result with borderline significance. Even without the correct statistical significance ($p<0.05$), this finding has a biological relevance, being an indicative to be observed. Perhaps with a larger population, this statistical issue could increase the significance level of the findings. According to the literature, abnormal oxygen levels might inhibit the action of enzymes and the development of the crystal hydroxyapatite resulting in hypomineralization (Sui et al. 2003). In addition, respiratory diseases during the childhood, produces prolonged episodes of high fever and it is clear that the ameloblasts are sensitive to high temperatures (Ghanim et al. 2013). The use of medications was also investigated as a possible etiologic agent factor. Nevertheless, it is difficult to establish whether the condition was caused by the medical treatment or by the disease (Souza et al. 2013).

A study estimated the correlation between the use of aerosol therapy in early childhood and the presence of MIH. It has been shown that the frequent use of aerosol therapy might be a risk factor for the development of MIH (Loli et al. 2015). It is important to consider that corticosteroids can disrupt amelogenesis. In experimental study, rats were chronically treated with low doses of corticosteroids and the disorders of the mineral metabolism in teeth of corticosteroid-treated were also affected by abnormal calcium and phosphorus in enamel (Pawlicki et al. 1992).

In this study, 31% of children that have MIH, also have HSPM. According to a systematic review, the presence of HSPM is predictive for MIH (OR=4.66, 95% CI 2.11–10.26) (Garot et al. 2018). The second primary molar and first permanent molar have a shared period of development and mineralization. If a risk factor occurred during the same period, a hypomineralization defect might occur concomitantly in the primary and permanent dentition. Thus, it is important to know that HSPM can increase the chances of a child to present MIH in order to prevent and/or manage.

Due to the fact that this research has a retrospective design, the potential limitations of the present study are bias of maternal recall, and time elapsed between tooth development and diagnosis of enamel defects. Moreover, further investigation is still necessary regarding the metabolic pathway of this gene.

Besides, future researches should be carried out involving patients from different populations.

In summary, MIH has a complex inheritance and is due to the interaction of genes and the environment. Whereas the MIH is a serious challenge in the clinical practice, since dental hypersensitivity can be present, coupled with poor restorative and inadequate effect of local anesthetics, understanding the genetic variants that cause MIH offer us new insights. The identification of MIH genes and other etiological factors will help clinical to improve an accurate prognosis and clearer risk information to patients.

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Review

Dental caries: Genetic and protein interactions

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ABSTRACT

Objective: To present a genetic and protein interaction analysis associated with dental caries.
Material and Methods: The first step was to conduct a systematic literature review (SLR) through an electronic database search. Case-controls that reported associations between genes and dental caries were the main type of study design used as inclusion criteria, retrieved from the PubMed and the Virtual Health Library databases, comprising the chronological range from 1982 to 2017. The SLR was guided by PRISMA protocol and the methodological quality of the studies was established through Newcastle-Ottawa Scale (NOS). In the second step, the String Protein Interaction (SPI) approach was used to analyze protein interaction (by esyN software) and also the Ingenuity Pathway Analysis (IPA) to check biological pathways associated with dental caries genes.
Results: A total of 51 articles were included to perform this SLR, describing a number of 27 genes associated with dental caries development. At the genetic level, 23 genes have at least one other gene with which they interact. The genes TUFT1, VDR, TFIP11, LTF, HLA-DRB1, MMP2, MMP3 and MUC5B were shown to be connected in interactive networks by at least 10 other genes.
Conclusion: It is essential to apprehend the multifactorial pattern of inheritance in human disease. This study presents pathways which may be directly correlated with several dental caries phenotype and this contributes to a better understanding of this disease, opening up a wider range of biotechnology options for its effective control in the future.

1. Introduction

Dental caries is a chronic disease with a multifactorial etiology. There is considerable evidence about the importance of environmental and behavioral factors in dental decay development (Fejerskov, 2014). However, the polarization of this disease is still occurring and important variances among different population groups are evident (Tanner et al., 2013).

Despite the available knowledge on several risk factors related to the predisposition of dental caries, there may be some individual variations that might help to explain why some people exposed to the same risk factors develop the disease and others do not. Therefore, studies have been conducted to better understand the nature of the genetic influence on dental caries (Vieira, 2012; Werneck, Mira, & Trevilatto, 2010).

The genetic case-control study design has been frequently used by researchers worldwide to investigate different types of diseases and also dental caries. These studies aim to compare affected and unaffected individuals as well as to test whether a particular allele occurs at a significantly different frequency between paired groups (Izakovicova Holla et al., 2017; Li, Hu, Zhou, Xie, & Zhang, 2015; Olszowski et al., 2017; Wendell et al., 2010; Valarini, Maciel, Moura, & Poli-Frederico, 2012; Choi et al., 2016; Cogulu et al., 2017; Ergöz et al., 2014).

Several studies have already reported findings associating different genes with dental caries (Banderas-Tarabay, Zacarías-D'Oleire, Garduño-Estrada, Aceves-Luna, & González-Begné, 2002; Filho et al., 2016; Haznedaroğlu et al., 2015; Kulkarni et al., 2013; Robino et al., 2015; Olszowski, Adler, Janiszewska-Olszowska, Safranow, & Chlubek,

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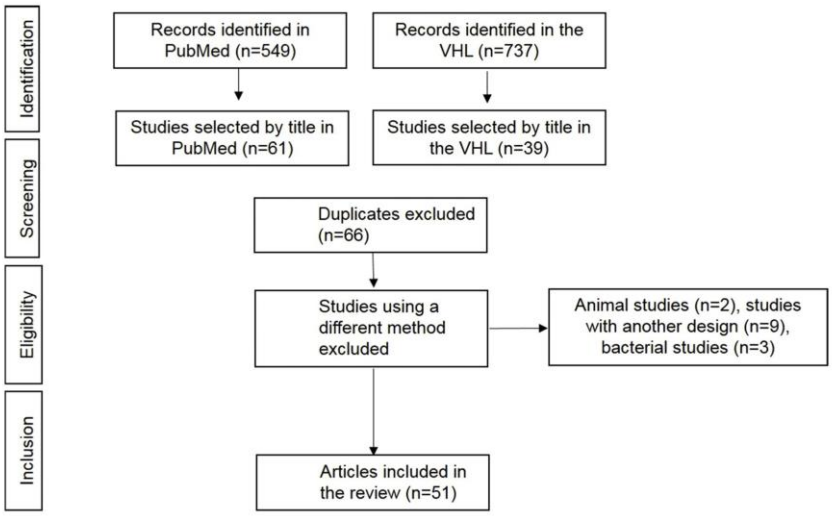

Fig. 1. Flowchart of the systematic review for the screening of genetic variants associated with dental caries.

2015; Yu, Bixler, Goodman, Azen, & Karn, 1986; Hu et al., 2015), yet there is a shortage of knowledge regarding the metabolic pathways involved and whether these pathways are directly correlated with dental caries phenotype. It is important to understand the set of different pathways and associated genes driving similar dental phenotypes, although different genes are affected. Since protein networks consist of various types of interaction and regulation, networks reflecting this complex scenario will provide a better understanding of the disease. Thus, the aim of this study was to present, for the first time, a genetic and protein interaction network analysis with the related dental caries genes assembled by means of a SLR.

2. Materials and methods

A SLR was performed to generate a catalog of genes already described in published works related to dental caries. The SLR followed the recommendations proposed in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, and sought to answer the following question: "Which genes are associated to the development of dental caries?" The approach used to structure the research question was based on the Population, Intervention, Comparison and Outcome framework (PICO), following the parameters:

P = genes
I = not applicable
C = individuals affected or unaffected by dental caries
O = association with dental caries

The search strategy included the following keywords: *dental caries* OR *dental decay* OR *dental caries susceptibility* OR *caries experience* AND *genetic* OR *genetic epidemiology* OR *genes* OR *polymorphism*. The following search strategy was used in the PubMed database: (((dental caries[Title/Abstract] OR dental decay[Title/Abstract]) OR dental caries susceptibility[Title/Abstract]) OR caries experience[Title/Abstract]) AND ((((genetic therapy[Title/Abstract] OR genes[Title/Abstract]) OR molecular epidemiology[Title/Abstract]) OR genetic [Title/Abstract]) OR polymorphism[Title/Abstract]) AND ("1982/01/01"[PDAT] : "2017/12/31"[PDAT]). For the VHL, the following search strategy was used: tw:((tw:(dental caries)) AND (tw:(genetic))) AND (instance:"regional") AND (la:("en" OR "es" OR "pt" OR "fr") AND year_cluster:("2015" OR "2014" OR "2017" OR "2013" OR "2012" OR "2016" OR "2011" OR "2008" OR "2010" OR "2002" OR "2005" OR "2004" OR "2007" OR "2009" OR "2006" OR "2000" OR "2001" OR "1993" OR "1995" OR "1998" OR "1994" OR "1999" OR "2003" OR "1990" OR "1996" OR "1991" OR "1997" OR "2018" OR "1986" OR "1987" OR "1989" OR "1992" OR "1983" OR "1985" OR "1982" OR "1988" OR "1984")).

This research included genetic studies on humans, with no restrictions regarding age or ethnicity. Case-control genetic studies were primarily selected because this design has methodological strengths that often allow the most relevant candidate genes to be revealed. Additionally, we also included well-conducted cross-sectional or cohort studies, when affected and non-affected individuals for dental caries were separated and analyzed to compare the allelic and genotypic frequencies between the groups. This SLR included articles published in Portuguese, English, French, or Spanish between January 1982 and December 2017. The electronic publication databases were PubMed and the Virtual Health Library (VHL), which included LILACS and Medline.

Two reviewers (T.C and L.Y.A) performed independent searches for articles in the databases and selected the eligible articles by reading their titles and abstracts. Following this first step selection, duplicate articles were excluded. The Newcastle-Ottawa Scale (NOS) was used to evaluate the methodological quality of the articles selected. The two reviewers read all articles in their entirety to determine whether they met the criteria for inclusion in the review. In the event of disagreement, a third reviewer was consulted to help disentangle the issue and to reach consensus (R.I.W). Animal studies, narrative reviews, studies that used a methodological design that did not adhere to the inclusion criteria and studies with bacteria were excluded (Fig. 1). After combining the findings of the SLR, the genes that showed association with dental caries were then evaluated in relation to their metabolic pathway. To perform the protein interaction analysis, it was used the String Protein Interaction (SPI) approach (von Mering et al., 2003). This was used to detect interactions reported from curated databases, experimentally determined and text-mining based interaction. Only medium confidence interactions were reported (M = 0.4). For genetic interaction analysis, it was used the esyN software (Bean et al., 2014) to

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Table 1
Studies included in the SRL relating genetic and dental caries.

Study	Country	Genes studied	Case/ control	Reported sample calculation	Average age	Caries diagnosis criterion	controlled risk factors	Statistical analysis	Summarized result	NCO
Anderson & Mandel, 1982	USA	*DB, PRP1,* and *PR*	26/28	No	Adults aged at least 24 years old (Not mention the average)	Control group with no caries history and case group with DMFT > 15	No	Chi-square	No difference in the distribution of phenotypes	6
Anderson et al., 1982	USA	*DB, PRP1, PR, PS, PMF,* and *PB*	47/46	No	Not described	Case group with at least 5 caries lesions	No	Chi-square	No difference in the distribution of phenotypes	6
*Yu et al., 1986	USA	*PR, PA PS, PMF, PIF,* and *DB*	Total of 306	No	9,7	DMFT	No	Regression coefficient homogeneity test, covariance analysis	The *PR* and *PA* genes may be associated with susceptibility to dental caries	6
*Banderas-Tarabay et al., 2002	Mexico	*MG1, MG2,* and *PRP1*	Total of 120	No	19	Control group: DMFT ≤4 and case group DMFT: ≥10	No	ANOVA, t test, Pearson's correlation	DMFT ≥ 10 showed a significant reduction of *MG1, MG2* and *PRP1* protein	6
Pehlivan et al., 2005	Turkey	*MBL*	42/40	No	9.78 case group and 10.7 control group	Not reported	No	Chi-square	No difference between groups	6
Slayton et al., 2005	USA	*AMELX, ENAM, AMBN, 4TUFT1, TFIP11,* and *KLK4*	92/92	No	Children from 3 to 5 years old. (Not mention the average)	Control group: DMFT ≤4 and control group without evidence or history of caries (including white spot lesions)	No	Chi-square	There was no association	7
*Deeley et al., 2008	Guatemala	*AMELX, ENAM, AMBN, TUFT1,* and *TFIP11*	66/44	Yes	32.6 case group and 23.5 control group	Control group: DMFT ≤2 and case group DMFT: ≥3.	No	Chi-square and Student's t	*AMELX* may be related to the susceptibility with dental caries.	8
*Patir et al., 2008	Turkey	*AMELX, ENAM, AMBN, TUFT1,* and *TFIP11*	91/82	No	4.8 case group and 5.9 control group	Case group: DMFT : ≥4 and control group without evidence or history of caries (including white spot lesions)	No	Chi-square and Student's t	*AMELX, AMBN,* and *TUFT1* may be associated with dental caries	8
*Azevedo et al., 2010	Brazil	*LTF*	48/62	No	12	Case group DMFT: ≥1 (white spots were considered), and control group with no dental caries experience	No	Chi-square	Protective effect on dental caries experience	8
*Ozturk et al., 2010	USA	*DEFB1*	201/95	No	40.23	Younger than 30 years old (control group with DMFT: < 14 and case group: DMFT ≥14). Older than 30 years old (control group DMFT: < 9 and case group DMFT: ≥ 9).	No	Chi-square, Fisher's exact test, multiple logistic regression, Z-test	*DEFB1* polymorphisms are potential markers for dental caries	7
Brancher et al., 2011	Brazil	*LTF*	25/25	Yes	12	Control group: DMFT = 0 and case group DMF: ≥4	No	Chi-square	There was no association	7
* Kang et al., 2011	South Korea	*AMELX*	87/33	No	21.3 case group and 23.2 control group	Control group with DMFT ≤2 and case group with DMFT ≥3	Yes OH	Fisher's exact test, multiple logistic regression	SNPs of *AMELX* might be associated with dental caries	7
Buczkowska-Radliska et al., 2012	Poland	*MUC7*	31/22	No	20-21 years old (Not mention the average)	Control group: DMFT≤7 and case group DMFT: ≥15	Yes OH, B	Mann-Whitney U test, chi-square	There was no association	7
*Olszowski et al., 2012	Poland	*AMELX, ENAM, MBL2,* and *MASP2*	380/376	No	Children aged 5 years (deciduous dentition) and 13 years old (permanent dentition)	Control group: DMFT < 3 and case group DMFT: ≥3	No	Chi-square and Fisher's exact test	*MBL2* was associated with dental caries experience	7
*Shimizu et al., 2012	Philippines, Turkey, Argentina, Brazil	*AMELX, ENAM, AMBN, TUFT1,* and *TFIP11*	742/ 1077	No	25,8	Children: control group DMFT = 0 to 2 and case group: DMFT ≥3. Adolescents: control group DMFT = 0 to 5 and case group: DMFT ≥ 6. Adults: control group: DMFT = 0 to 8 and case group: DMFT ≥9	No	Chi-square, Fisher's exact test	*AMELX, AMBN, TUFT1,* and *ENAM* may be associated with dental caries experience	8
*Tannure, Küchler et al., 2012	Brazil	*MMP20*	227/ 161	No	8.7 case group and 9.4 control group	Control group: DMFT = 0 and case group divided into low caries experience (DMFT =1, moderate (DMFT ≥ 2 and ≤ 3) and high (DMFT ≥ 4)	Yes OH, DH	Chi-square, t-test, binary logistic regression	*MMP20* may be associated with dental caries	8
*Tannure, Küchler et al., 2012; Tannure, Kuchler et al., 2012	Brazil	*MMP2, MMP9, MMP13,* and *TIMP2*	293/ 212	No	8.8 case group and 9.4 control group	Case group: DMFT ≥ 1 and control group: DMFT = 0	Yes OH, DH	Student's t, chi-square, binary regression	*MMP13* may be associated with dental caries	8

(*continued on next page*)

Table 1 (*continued*)

Valarini et al., 2012	Brazil	*HLA-DR4, HLA-DQ4, HLA-DQ5, HLA-DQ6* and *HLA-DQ2*	99/65	No	15.9 case group and 15.7 control group	Case group: DMFT ≥1 and control group: DMFT = 0	Yes S	logistic regression, chi-square	There was no association	8
*Wang et al., 2012	USA	*ENAM, TUFT1, DSPP, KLK4, MMP20, AQP5*, and *SPP1*	82/251	No	5,2	Case group: DMFT ≥1 and control group: DMFT = 0	Yes OH, S	Linear regression, logistic regression	*DSPP, KLK4, and AQP5* may be associated with dental caries protection	8
*Kulkarni et al., 2013	Canada	*GLUT2* and *TAS1R2*	Total of 80	No	26,4	WHO, ICDAS, and interproximal radiographic analyses	Yes OH, B, G, ST	T test, chi-square, ANOVA	*GLUT2* and *TAS1R2* were associated with dental caries susceptibility.	6
Gasse et al., 2013	France	*AMELX*	212/146	Yes	7.6 case group and 22 control group	WHO, ICDAS, and interproximal radiographic	Yes OH, DH, S	multivariate regression, Fisher's exact test, odds ratio	There was no association in this population	8
Yang et al., 2013	China	*MBL*	62/68	No	3,5	Case group with ECC and control group with no history of caries	No	Student's t test, chi-square	There was no association of *MBL* with early childhood caries	8
*Chaussain et al., 2014	France	*ENAM*	212/146	No	7.6 case group and 22 control group	According to the WHO	Yes OH, DH, S	Logistic regression, odds ratio, multivariate analyses	*ENAN* is a candidate gene for susceptibility to dental caries	8
Volckova et al., 2014	Czech Republic	*LTF*	482/155	No	Children aged 11-13. (Not mention the average)	Case group DMFT: ≥1 and control group: DMFT = 0	Yes G, B	Fisher's exact test, Pearson's test, odds ratio	There was no association in this population	7
Doetzer et al., 2015	Brazil	*LTF*	346/331	Yes	12	Control group: DMFT = 0 and case group: DMFT ≥1, (low caries experience) or DMFT ≤2 (high caries experience)	Yes G, B, ST	Chi-square, Fisher's exact test, Mantel Haenszel's exact test	*LTF* were associated with dental caries protection	8
*Izakovicova Holla et al., 2015	Czech Republic	*GLUT2* and *TAS1R2*	482/155	No	Children aged 11-13. (Not mention the average)	Case group: DMFT ≥ 1 and control group: DMFT = 0	Yes G, B, OH, B	Fisher's exact test, odds ratio, logistic regression	*GLUT2* and *TAS1R2* may influence the risk for dental caries	7
*Li et al., 2015	China	*CA-VI*	164/191	No	51.16 case group and 47.44 control group.	Case group: DMFT ≥ 3 and control group: DMFT ≤ 2	Yes S	Chi-square, odds ratio, logistic regression	The ACA haplotype of the *CA-VI* gene may be associated with susceptibility to dental caries	7
*Robino et al., 2015	Italy	*TAS1R2* and *GLUT2*	647	No	44.9	DMFT and panoramic radiography analyses	Yes OH	Association analysis, Regression Analysis	It was identified a direct association between variants in TAS1R2 and GLUT2 genes and caries prevalence	8
*Abbasolu et al., 2015	Turkey	*AMBN, AMELX, ENAM, KLK4, MMP20 TUFT1, DEFB1, LTF*, and *ALOX15*	136/123	No	4.6	Case group: DMFT≥1 and control group: DMFT = 0	Yes OH, DH	Student's t, chi-square, Fisher's exact test, multivariate analysis, logistic regression	The TT genotype (*ALOX1*) was associated as a risk factor for the development of early childhood caries. The GG (*ENAM*), AG and GG (*KLK4*), CT (*LTF*), and GG (*TUFT1*) genotypes showed protection	8
*Antunes et al., 2015	Brazil	*MMP2, MMP3, MMP9, MMP20, TIMP1,* and *TIMP2*	786	No	3.9	The presence of WSL (White spot lesions and/or ECC) and without disease (the absence of WSL or ECC	Yes OH, DH	Chi-square, Fisher's exact test	*MMP9* and *MMP20* are involved in WSL and ECC development	8
*Olszowski et al., 2015	Poland	*ACE I/D*	120/41	No	Children aged 15 years old. (Not mention the average)	Case group DMF: ≥1 and control group DMFT = 0	No	Chi-square, Fisher's exact test	The DD genotype of *ACE I/D* polymorphism might be protective against dental caries	8
*Hu et al., 2015	China	*VDR TaqI*	264/219	No	50	Case group: DMFT ≥ 1 and control group: DMFT = 0	No	Odds ratio and chi-square	The T allele may be a genetic determinant factor for the development of caries disease	7
*Haznedaroglu et al. 2015	Turkey	*TAS1R2* and *TAS1R3*	Total of 184	No	Children aged between 7 and 12 years (Not mention the average)	According to the WHO	Yes OH, DH, S	Fisher's exact test, chi-square, Student's t, logistic regression test	Moderate caries disease experience for the *TAS1R3* rs307355 gene and high for the *TAS1R2* rs35874116 gene	6

(*continued on next page*)

Table 1 (*continued*)

*Gerreth et al., 2016	Poland	*ENAM*	48/48	No	Children aged 20-42 months. (Not mention the average)	Diagnoses included cavitated lesions and non-cavitated incipient initial lesions or white spots	No	Chi-square, Fisher's exact test	Polymorphisms in the *ENAM* gene may be a predictor of caries disease in children	8
*Saha et al., 2016	India	*AMELX*	30/30	Yes	Children aged 5-12 years. (Not mention the average)	Case group: DMFT ≥ 4, control group had no history of dental caries	No	Chi-square, t-test	High correlation with dental caries	7
*Filho et al., 2016	Brazil	*MMP20*	184	No	Not described the avarege. Children aged 4-7 years.	Low caries experience: DMFT = 1. Moderate caries experience DMF = 2–3, and high caries experience: DMFT = 4 or higher	No	Chi-square, Fisher's exact test	*MMP20* rs1784418 C > T appears to protect against dental caries	7
*Karayasheva et al., 2016	Bulgaria	*MMP2* and *MMP3*	41/41	No	Children aged 20-32 years. (Not mention the average)	Control group: DMFT ≤ 5 and case group: DMFT ≥ 14	Yes OH, DH, G, ST, B	Chi-square, odds ratio	The genes are probably associated with caries disease	7
*Linhartova Borilova et al., 2016	Czech Republic	*ACE I/D*	561/182	No	Children aged 13-15 years. (Not mention the average)	Case group: DMFT ≥ 1 and control group: DMFT = 0.	Yes G, B	Chi-square, Fisher's exact test	ACE I/D polymorphism may be associated with caries in permanent dentition	8
*Yildiz et al., 2016	Turkey	*AMELX, CA-VI, DEFB1*, and *TAS2R38*	77/77	Yes	28.0	Control group: DMFT ≤ 5 and case group: DMFT ≥ 14,	Yes OD, DH, B, ST	Chi-square, multiple regression, Mann-Whitney U test	Polymorphisms in the *CA6, DEFB1*, and *TAS2R38* genes associated with biofilm and lactobacilli can increase the risk of developing caries	8
*Cogulu et al., 2017	Turkey	*APAL, FOKI, CDX2* and *TAQI.*	112/38	No	10.19	High caries risk group: DMFT > 4, moderate caries risk group: DMFT= 1–4) and caries-free group.	Yes OH, DH, B	Chi-square, Fisher's exact test	No statistically significant differences were detected	8
*Gerreth et al., 2017	Poland	*AMELX, AMBN, TUFT1, TFIP11, MMP20* and *KLK4*	48/48	No	30.26 mounths	Dental evaluation concerned the occurrence of teeth with carious cavities as well as teeth with initial (incipient) caries lesions (non cavitated,white spot)	Yes D,OH	Chi-square, Fisher's exact test	Polymorphisms in AMELX, AMBN, TUFT1, KLK4 genes may be genetic markers that contribute to dental caries	8
*Alyousef et al., 2017	Saudi Arabia	*MMP9, MBL2, MMP2*, and *TIMP2*	102/100	No	Children aged 5-13 years. (Not mention the average)	According to the WHO	Yes OH,DH, S	Chi-square, odds ratio, T-test	*MBL2* gene was shown to be associated with a high prevalence of caries	8
*Cavallari, Salomão et al., 2017	Brazil	*MUC5B*	100/100	Yes	18.43 case group and 18.52 control group	According to ICDAS. Control group: zero score for all dental elements	Yes OH, DH, B, S	Chi-square, Fisher's exact test	Genetic polymorphisms of the *MUC5B* gene can influence dental caries development	8
*Cavallari, Salomão et al., 2017	Brazil	*KLK4*	100/100	Yes	18.43 case group and 18.52 control group	According to ICDAS. Control group: zero score for all dental elements	Yes OH, DH, B, S	Chi-square, Fisher's exact test	Genetic variations in the *KLK4* gene may contribute to dental decay	7
Lips et al., 2017	Brazil	*DEFB1*	162/342	No	3.2 control group and 4.7 case group	According to DMFT	Yes OH, DH, B	Shapiro-Wilk test, Chi-square, Fisher's exact test	There was no association	8
Olszowski et al., 2017	Poland	*FCN2*	82/175	No	15	According to DMFT	No	Chi-square, Fisher's exact test	There was no association	8
Izakovicova Holla et al., 2017	Czech Republic	*VDR Taq I*	235/153	No	Children aged 13-15 years. (Not mention the average)	Case group: DMFT ≥ 1 and control group: DMFT = 0	Yes B, G	Chi-square, Fisher's exact test	There was no association	8
Kastovsky et al., 2017	Czech Republic	*BMP2* and *DLX3*	Primary dentition 113/83 Permanent dentition542/176	No	Children aged 2-6 years old (primary dentition) and 13-15 (permanent dentition) (Not mention the average).	According to DMFT	No	Chi-square, Fisher's exact test	Variability in *BMP2* and *DLX3* was not associated with caries	7

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Table 1 (*continued*)

*Yu, Jiang, Sun, Kong, & Chen, 2017	China	*VDR-FOK*	200/200	No	12.24 case group and 12.20 control group	According to DMFT	Yes OH, G	Chi-square	*VDR-Fok I* gene polymorphisms may be associated with susceptibility to permanent tooth caries in Chinese adolescent	8
Linhartova Borilova et al., 2017	Czech Republic	*ENAM*	Primary dentition 109/78 Permanent dentition541/177	No	Primary dentition 3.8 Permanent dentition 13-15 (Not mention the average)	Case group:DMFT ≥ 1 and control group DMFT = 0	No	Chi-square, Fisher's exact test	*ENAM* variant (rs12640848) cannot be used as a risk factor of dental caries in the Czech population	8

*Articles with association for dental caries.

Legends:Oral Hygiene Habits: OH; Dietary Habits: DH; Gingivitis: G; Biofilm: B; Socioeconomic/ demographic characteristic; Salivary Tests: ST.

show complex interaction, enhancing genetic interaction, suppressing genetic interaction and physical interaction. It is based on several biological databases available, such as KEGG (Kanehisa & Goto, 2000) and Reactome (Fabregat et al., 2016). To check the biological pathways associated with a list of genes (regarding dental caries), it was used Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com) with default parameters.

3. Results

After applying the SLR protocol, it was found 549 articles in the PubMed database and 737 in the VHL database. Next, by reading the titles and abstracts, 61 articles were selected from PubMed and 39 from the VHL. Duplicated articles and those that did not meet the inclusion criteria were excluded. A final number of 51 articles were included in this SLR (Fig. 1). These articles where then scrutinized by full-text analysis to collect the following information: authors and year of publication, country where the research was performed, studied genes, number of participants in each group (case-control), average age of individuals, dental caries diagnostic criteria used, controlled risk factors, statistical analyses performed, and the assigned score according to the NOS, which are presented in Table 1.

The SLR counted in 54 different genes (ACE I/D, ALOX15, AMBN, AMELX, APAL, AQP5, BMP2, CA-VI, CDX2, DB, DEFB1, DLX3, DSPP, ENAM, FOKL, FCN2, GLUT2, HLA-DR4, HLA-DQ2, HLA-DQ3, HLA-DQ4, HLA-DQ5, HLA-DQ6, KLK4, LTF, MASP2, MBL MBL2, MG1, MG2, MMP2, MMP3, MMP9, MMP13, MMP20, MUC5B, MUC7, PA, PB, PIF, PMF, PR, PRP1, OS, SPP1, TAS1R2, TAS1R3, TAS2R38, TIMP1, TIMP2, TUFT1, TFIP11, VDR-TAQI, VDR-FOK). Furthermore, 27 of those genes were found to be associated either with protection or risk of dental caries (PRP1, PR, PA, MG1, MG2, AMELX, ENAM, TUFT1, KLK4, HLA-DR4, TAS1R3, TAS2R38, MBL2, MMP20, MMP2, MMP9, MMP13, GLUT2, TAS1R2, CA-VI, DEFB1, ALOX15, VDR-TAQI, MMP3, CA6, MUC5B,VDR-FOK) (Table 1).

Most studies analyzed genes related to enamel formation, development and mineralization, including amelogenin (*AMELX*) (Kang, Yoon, Lee, & Cho, 2011; Slayton, Cooper, & Marazita, 2005; Shimizu et al., 2012; Gasse et al., 2013), tuftelin (*TUFT1*) (Partir et al., 2008), ameloblastin (*AMBN*) (Deeley et al., 2008; Shimizu et al., 2012), enamelin (*ENAN*) (Gerreth, Zaorska, Zabel, Borysewicz-Lewicka, & Nowicki, 2016; Linhartova Borilova et al., 2017), aquaporin (*AQP5*) (Wang et al., 2012), kallikrein (*KLK4*) (Wang et al., 2012; Cavallari et al., 2017) and metalloproteinase genes (Karayasheva, Glushkova, Boteva, Mitev, & Kadiyska, 2016; Tannure, Kuchler et al., 2012; Tannure, Küchler et al., 2012). Other studies analyzed genes related to the host immune response, including mannose-binding lectin (*MBL*) (Alyousef et al., 2017; Pehlivan et al., 2005) and defensin beta 1 (*DEFB1*) (Lips et al., 2017; Ozturk, Famili, & Vieira, 2010). Genes related to the composition of saliva were also examined, including salivary carbonic anhydrase VI (*CA6*) (Li et al., 2015), lactotransferrin *(LTF*) (Azevedo et al., 2010; Brancher et al., 2011; Doetzer et al., 2015) and mucins (*MUC7*) (Buczkowska-Radliska, Pol, Szmidt, & Bińczak-Kuleta, 2012) and (*MUC5B*) (Cavallari, Moysés, Moysés, & Werneck, 2017; Cavallari, Salomão, Moysés, Moysés, & Werneck, 2017).

The major part of the included studies was conducted with adults. However, studies with preschool children related to early childhood caries were also explored (Bagherian, Nematollahi, Afshari, & Moheghi, 2008; Yang, Wang, & Qin, 2013; Abbasoğlu et al., 2015; Antunes et al., 2016;Linhartova Borilova et al., 2016; Kastovsky et al., 2017; Gerreth, Zaorska, Zabel, Borysewicz-Lewicka, & Nowicki, 2017).

The genes associated with dental caries also showed a correlation at genetic and protein levels (Table 2). At the genetic level, it was found that 23 genes have at least another interacting gene (Fig. 2). The genes *TUFT1, VDR, TFIP11, LTF, HLA-DRB1, MMP2, MMP3* and *MUC5B* are all central elements (known as HUB), but they are connected to interacting networks formed by at least 10 other genes. The genes *TAS1R2,*

Table 2
Associated metabolic pathways of genes associated with dental caries.

Canonical Pathways	Molecules
Inhibition of Matrix Metalloproteases	MMP20,MMP3,MMP13,MMP2
HIF1α Signaling	MMP20,MMP3,SLC2A2,MMP13,MMP2
Bladder Cancer Signaling	MMP20,MMP3,MMP13,MMP2
Granulocyte Adhesion and Diapedesis	MMP20,MMP3,MMP13,MMP2
Agranulocyte Adhesion and Diapedesis	MMP20,MMP3,MMP13,MMP2
Leukocyte Extravasation Signaling	MMP20,MMP3,MMP13,MMP2
Colorectal Cancer Metastasis Signaling	MMP20,MMP3,MMP13,MMP2
Atherosclerosis Signaling	ALOX15,MMP3,MMP13
Oncostatin M Signaling	MMP3,MMP13
Gustation Pathway	TAS1R2,TAS2R38,TAS1R3
IL-17 Signaling	MMP3,MUC5B
Airway Pathology in Chronic Obstructive Pulmonary Disease	MMP2
Axonal Guidance Signaling	MMP3,MMP13,MMP2
Hepatic Fibrosis/Hepatic Stellate Cell Activation	MMP13,MMP2
Maturity Onset Diabetes of Young (MODY) Signaling	SLC2A2
Osteoarthritis Pathway	MMP3,MMP13
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	DEFB1
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	MMP3,MMP13
B Cell Development	HLA-DRB1
Complement System	MBL2
Antigen Presentation Pathway	HLA-DRB1
Intrinsic Prothrombin Activation Pathway	KLK4
Role of IL-17 F in Allergic Inflammatory Airway Diseases	MMP13
Neuroinflammation Signaling Pathway	HLA-DRB1,MMP3
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	MMP3,MMP13
Autoimmune Thyroid Disease Signaling	HLA-DRB1
Graft-versus-Host Disease Signaling	HLA-DRB1
Docosahexaenoic Acid (DHA) Signaling	ALOX15
Nur77 Signaling in T Lymphocytes	HLA-DRB1
Calcium-induced T Lymphocyte Apoptosis	HLA-DRB1
Eicosanoid Signaling	ALOX15
Role of IL-17A in Arthritis	MMP13
Glioma Invasiveness Signaling	MMP2
MSP-RON Signaling Pathway	KLK4
T Helper Cell Differentiation	HLA-DRB1
VDR/RXR Activation	VDR
IL-17A Signaling in Airway Cells	MUC5B
Allograft Rejection Signaling	HLA-DRB1
TGF-β Signaling	VDR
HER-2 Signaling in Breast Cancer	MMP2
Crosstalk between Dendritic Cells and Natural Killer Cells	HLA-DRB1
IL-4 Signaling	HLA-DRB1
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	HLA-DRB1
OX40 Signaling Pathway	HLA-DRB1
Communication between Innate and Adaptive Immune Cells	HLA-DRB1
Antioxidant Action of Vitamin C	SLC2A2
Type I Diabetes Mellitus Signaling	HLA-DRB1
GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	TAS1R3
Neuroprotective Role of THOP1 in Alzheimer's Disease	KLK4
iCOS-iCOSL Signaling in T Helper Cells	HLA-DRB1
Role of Tissue Factor in Cancer	MMP13
Phagosome Formation	MBL2
CD28 Signaling in T Helper Cells	HLA-DRB1
Th1 Pathway	HLA-DRB1
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	MBL2
Ovarian Cancer Signaling	MMP2
IL-12 Signaling and Production in Macrophages	ALOX15
Phagosome Maturation	HLA-DRB1
Th2 Pathway	HLA-DRB1
Type II Diabetes Mellitus Signaling	SLC2A2
PKCθ Signaling in T Lymphocytes	HLA-DRB1
GNRH Signaling	MMP2
Cdc42 Signaling	HLA-DRB1
Acute Phase Response Signaling	MBL2
eNOS Signaling	AQP5
Th1 and Th2 Activation Pathway	HLA-DRB1
Role of NFAT in Regulation of the Immune Response	HLA-DRB1
Regulation of the Epithelial-Mesenchymal Transition Pathway	MMP2
Dendritic Cell Maturation	HLA-DRB1
IL-8 Signaling	MMP2

TAS1R3, AMBN, MBL2, MUC2, DEFB1 and *SLC2A2* form small networks, having less than 10 connections and only some of them interact with the other genes. At the protein level, most proteins are shown to be interacting with each other (Fig. 3). These interactions are restricted to only direct associations formed by four groups, with the biggest group having six proteins, and then four, three and two proteins forming different groups (Fig. 3). The proteins *MMP20, ENAM, AMELX, AMBN* have the highest number of interactions, a total of six, all identified by text mining-based analysis. The protein interaction between *TAS1R3* and *TAS1R2* was the pair having four different methods that confirmed the interaction, the highest between all interactions found.

This study also investigated the biological pathways associated with dental caries and genes, and found several canonical pathways associated with different cellular functions and processes, such as Antigen Presentation Pathway, Inhibition of Matrix Metalloproteases, L-17 Signaling and HIF1a Signaling, which are illustrated in Fig. 4 and described in Table 2.

Most of the dental caries-related genes have also been associated with different pathologies (Table 3), such as amelogenesis (genes *KLK4, MMP20, ENAM, AMBN, AMELX*), abnormal color of incisor (gene *AMBN*), abnormal morphology of enamel and molar crown (*AMBN* and *MMP20*, and *AMBN*, respectively), lack of enamel (gene *AMBN*), dentinogenesis imperfecta (gene *DSPP*) and congenital anomaly of tooth (genes *AMBN, AMELX, DSPP, ENAM, KLK4, MMP20*).

4. Discussion

Genetic association studies have aimed to determine whether genetic factors may affect dental decay, along with other variables. For this study, 51 articles published over a 35-year period were selected and the genetic epidemiology of dental caries was investigated. These studies provide scientific evidence that genetic factors are involved in the development of dental caries and that several genes are associated with the disease.

The included studies were assessed by the NOS scale. This research instrument helps to verify the quality of studies according to certain methodological properties (selection, comparability and exposure). All studies reached the minimum quality score required by the scale.

Some risk factors for dental caries were surveyed in most studies, including visible plaque index, tooth brushing frequency, food intake between meals and the buffering capacity of saliva. Various factors were significantly associated, however there were some studies that only performed bivariate analyses, failing to test the effects of environmental variables along with genetic variables (Anderson & Mandel, 1982; Anderson, Lamberts, & Bruton, 1982).

Sample size estimation is an important method to minimize the

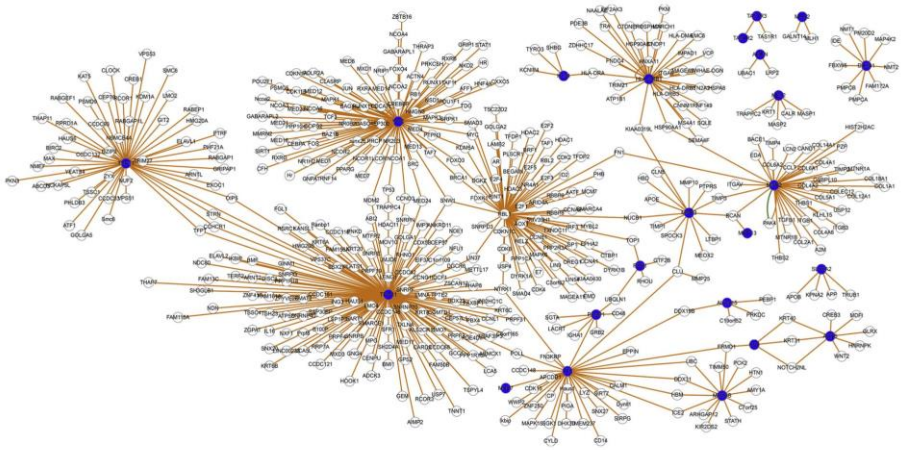

Fig. 2. Genetic interaction of genes associated with dental caries.
Genes associated with caries disease are highlighted in blue. All genetic interactions are restricted to genes having associations previously described in literature by in silico on experimental models.

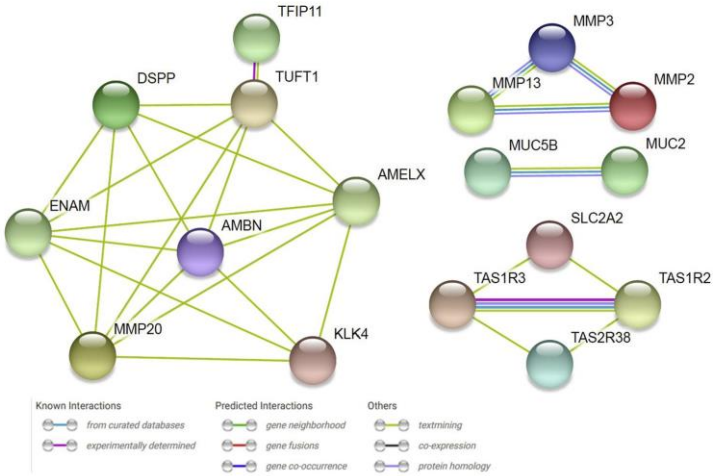

Fig. 3. Protein-protein interaction network of associated genes with dental caries.
Interaction network was restricted to the caries-related genes and did not include indirect correlations.

probability of error. The size of a sample influences in the precision of the estimates and in the power of the study to draw conclusions. There was a variation in the number of participants among the included articles. In this research, only a few studies reported sample size calculation (Table 1). Authors should generally be required to provide detailed information on the sample size calculation approach used when performing population studies.

After a careful analysis of the articles, it was found that 29 genes were replicated by different researches in a multiplicity of populations (*ACE I/D, DB, PRP1, PR, PS, PMF, MBL, AMELX, ENAM, AMBN, TUFT1, TFIP11, KLK4, HLA-DR4, HLA-DQ5, HLA-DQ6, HLA-DQ2, LTF, MBL2, MMP20, MMP2,MMP3, MMP9, TIMP2, GLUT2, TAS1R2, CA-6, DEFB1, VDR-TAQI).* The genes *AMELX* (Slayton et al., 2005; Patir et al., 2008; Deeley et al., 2008; Kang et al., 2011; Olszowski, Adler, Janiszewska-Olszowska, Safranow, & Kaczmarczyk, 2012; Shimizu et al., 2012; Gasse et al., 2013; Abbasoğlu et al., 2015; Yildiz, Ermis, Calapoglu, Celik, & Turel, 2016; Saha, Sood, Sandhu, Diwaker, & Upadhyaye, 2016) and *ENAM* (Slayton et al., 2005; Patir et al., 2008; Deeley et al., 2008; Olszowski et al., 2012; Chaussain et al., 2014) were the most commonly studied genes. It is very important to replicate the findings of genetic studies in different populations to confirm the results. The above replicated genes were studied in different populations, suggesting that the associations with dental caries were not at random. On the other hand, some genes were also searched for replication in other research groups, but the associations were not confirmed, such as for the gene *LTF*. Just to give an example, in a region of Brazil it was found

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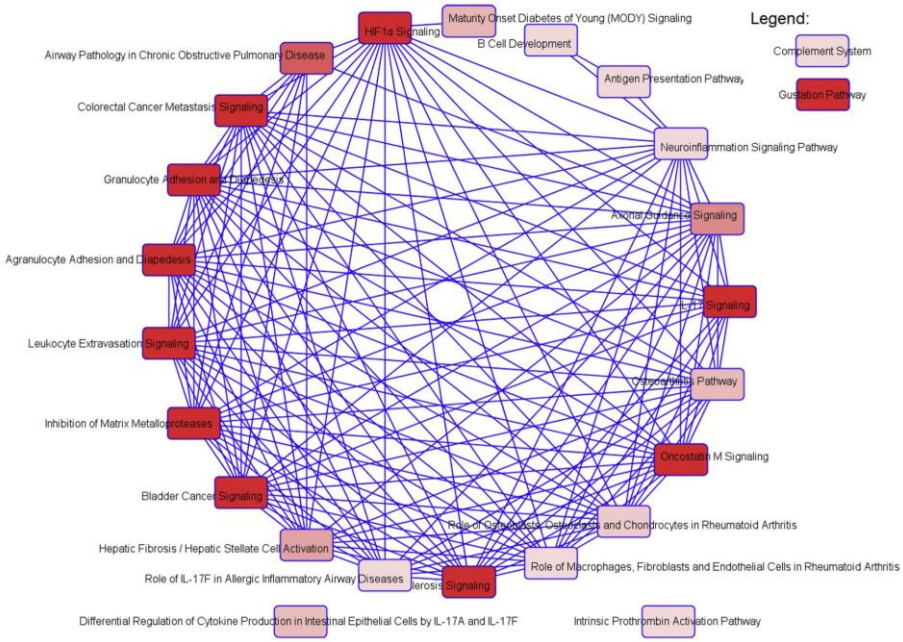

Fig. 4. Canonical pathways of caries-disease associated genes.

an association of the gene LTF (Azevedo et al., 2010; Doetzer et al., 2015), whilst the same gene in the Czech Republic population had no association (Volckova et al., 2014).

It is evident that the most cited candidates genes are more biologically plausible to be tested as caries-related phenotype, since their function is directly associated with enamel formation or saliva composition. The protein encoded by the *ENAM* gene, for example, is involved in the mineralization and structural organization of enamel (Gerreth et al., 2015). The *AMELX* gene encodes a member of the amelogenin family, which is involved in bio mineralization during tooth enamel development (Saha et al., 2016). Furthermore, this study also found that the identified dental caries-related candidate genes are

Table 3
Systematically identified genes with dental caries associated with dental formation and pathologies.

Diseases or Functions Annotation	Molecules
Abnormal morphology of ameloblasts	AMBN,MMP20
Enlargement of dental pulp chamber	DSPP
Tooth development	AMELX,AQP5,ENAM,MMP20
Amelogenesis imperfecta	AMBN,AMELX,ENAM,KLK4,MMP20
Amelogenesis imperfecta hypomaturation type	AMELX,KLK4,MMP20
Amelogenesis imperfecta type IB	ENAM
Amelogenesis imperfecta type IC	ENAM
Amelogenesis imperfecta type IF	AMBN
Amelogenesis imperfecta X-linked 1	AMELX
Autosomal dominant dentin dysplasia, type ii	DSPP
Autosomal recessive amelogenesis imperfecta	AMBN,ENAM,KLK4,MMP20
Hypomaturation type amelogenesis imperfecta type IIA1	KLK4
Hypomaturation type amelogenesis imperfecta type IIA2	MMP20
Hypoplastic amelogenesis imperfecta	AMELX,ENAM
Hypoplastic/hypomaturation amelogenesis imperfecta X-linked 2	AMELX
Pigmented hypomaturation type amelogenesis imperfecta	KLK4,MMP20
Abnormal color of incisor	AMBN
Abnormal morphology of enamel	AMBN,MMP20
Abnormal morphology of molar crown	AMBN
Lack of enamel	AMBN
Autosomal dominant dentinogenesis imperfecta Shields type II	DSPP
Dentinogenesis imperfecta - Shields type III	DSPP
Congenital anomaly of tooth	AMBN,AMELX,DSPP,ENAM,KLK4,MMP20
Abnormal morphology of ameloblasts	AMBN,MMP20

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possibly interacting in genetic and protein levels (Figs. 2 and 3). When the genetic interaction between them had been analyzed, it was discovered that several candidate genes have shown a complex interacting network, such as for the genes *VDR, TF1P11, HLA-DRB1* and *MMP2*.

In this review, the genes *AMELX, KLK4, AMBN, TUFT1* have already been associated in both dentitions. While this appears to be a trend, some studies have warned that the effects of genetic variation may impact primary and permanent dentitions differently, once human deciduous and permanent teeth exhibit different developmental processes, morphologies, histological characteristics and life cycles (Kim et al., 2014; Bayram et al., 2015).

Some of the discussed genes, such as *ENAM* and *AMELX*, are also directly interacting at the protein level, including interactions with several other important genes: *DSPP, MMP20, KLK4, TUFT1* and *AMBN*. At this level, the gene ameloblastin (*AMBN*) is strongly connected to seven other caries-associated protein coding genes, suggesting a key role in dental caries phenotype. These data on genetic and protein interactions suggest that mutations in any of the detected genes could also be interfering in the network formed by these molecules, and then potentially leading to similar phenotypes of dental caries.

Using the IPA approach, several genes have been previously associated with diverse cellular and metabolic functions (Table 3). For instance, a pathway was found the Th1, which is described to be activated by IL-6 to promote local immune system response and might be involved in the formation of edema induced by the progressive intradentinal penetration of bacteria (Farges et al., 2015). Another important pathway was foundthat is involved with dental microorganism activity (the complement system). It is known to play a significant role in the activation of the immune system with the presence of gram-positive oral bacteria (Tsai, Nilsson, McArthur, & Taichman, 1977). The complement system may also contribute to dentin-pulp regeneration by recruiting pulp progenitors, via this pathway (Chmilewsky, Jeanneau, Laurent, & About, 2014). The genes *AMELX, AQP5, ENAM* and *MMP20* were also associated with tooth development (Table 3) and it could be hypothesized that mutations in some of these genes can also affect normal tooth formation and biogenesis, leading to a higher susceptibility to dental caries.

To perform the correlations among genes, protein interactions, metabolic pathways and dental caries, it was initially investigated through the literature the phenotypic characteristics associated with dental caries. Next, it was identified the pathways associated with the mapped characteristics, to finally verify whether the genes found by our literature review could be implicating in the dental caries. Although these data were based on *in silico* experiments using bioinformatics pipelines, it could also be useful to perform a functional analysis to provide insightful information between the genotype-phenotype associations with dental caries. A novel and efficient approach is being used to investigate progression patterns of dental caries, based on longitudinal data that is contrasted to the use of cross sectional assessment scores (Weber et al., 2018). In a recessive model, the approach was able to find a significant innovative association between mutations in the gene Kallikrein 4 (*KLK4*) with different caries sub-phenotypes.

After conducting a multiple single-nucleotide polymorphism (SNP) assay in the genes TAS2R38 and TAS1R2, another study found by transmission disequilibrium test (TDT) analysis, significant associations between these taste pathway genes with caries risk and/or protection (Wendell et al., 2010).

In another study, it was performed a genome-wide investigation to calculate two quantitative measures that combines both the primary and permanent dentition, while adjusting for age effects (Govil et al., 2018). Genes identified in peak linkage regions underline the importance of exploring potential relationships between caries and other traits.

Other studies also correlates the dental caries occurrence with different microorganisms, including Streptococcus mutans, Granulicatella elegans, Veillonella spp., S. mutans and Bifidobacteriaceae spp. (Lof, Janus, & Krom, 2017; Moye, Zeng, & Burne, 2014). These microorganisms within saliva microbiota are able to change metabolic pathways associated with central carbon metabolism or carbohydrate metabolism.

Even though it is still challenging, Other approaches could be used in combination to increase the current knowledge about the interaction of genes and proteins previously associated with dental caries, such as the ones based on UV crosslinking immunoprecipitation for gene and protein interactions (Stork & Zheng, 2016) and two-hybrid for protein-protein interactions (Lin & Lai, 2017). Other methods to correlate genotype-phenotype also involve the use of gain or loss-of-function experiments, such as microRNAs (Williams, Cheng, Blenkiron, & Reid, 2017), plasmid for gene overexpression (Moriya, 2015) and also the CRISPR/cas9 system for both of these previous investigations (Adli, 2018).

Knowledge of inherited genetic variation has a fundamental impact on understanding human disease. New approaches to assess functional significance of inherited genetic variation, to combine molecular genetics, epidemiology and bioinformatics, promise to enhance the reproducibility and plausibility of associations between genotypes and disease phenotype.

One important approach to better identify the genetic impact in dental caries is to look at the individuals with extreme DMFTs. In addition, the ideal would be to select individuals from the same location, having the same access level to fluoridated water, healthcare and with a similar diet. In this SR, most of considered investigations have selected the population study according to similar characteristics, however, only a few investigations have followed the gold standard when considering the criterion of diagnosis and crossed the genetic data with environmental variables. Considering the mentioned aspects, the molecules associated with dental caries were evaluated as controlled risk factors by distinct investigations, and only the following genes were included within studies considering extreme phenotypes: *AMELX, MMP20, GLUT2, TAS1R2, LTF, MMP2, MMP3, CA6, DEFB1, TAS2R38 and CA-VI* (Kang et al., 2011; Tannure, Kuchler et al., 2012; Tannure, Kchler et al., 2012; Kulkarni et al., 2013; Doetzer et al., 2015; Karayasheva et al., 2016; Yildiz et al., 2016; Li et al., 2015). In our review, we also highlighted that distinct genetic factors can play a considerable role in the development of the disease. However, an important consideration is that a gene is no longer studied as an isolated entity but rather part of a complex network, as supported by the interaction network analysis we performed, which showed that distinct genes are interacting and are involved in similar pathways. Together, our investigation suggests that dental caries might not be inherited as a single gene defect, but instead as a result from distinct genetic modifications and by the gene-environment interactions."

Both positive and negative results were presented in this systematic review. Negative results are important for the broader field where they are relevant, since observing unexpected results can always bring us some learning and save time and resources. The reporting of negative results can help other scientists adjust their research.

One limitation of this review is that, while there is clear evidence on the influence of individual and contextual risk factors contributing to the caries experience, some included articles (in compliance with the review protocol) are focused only on genetic polymorphisms associated with susceptibility to dental caries. Some of them are the precursors of this research line and have a relative role in understanding the pathogenesis of dental caries. Nonetheless, the scientific community should build upon an integrated and comprehensive approach to investigating dental caries, since inadequate results from single-variable tests are well established.

Another limitation of this review was the interpretation of diagnostic criteria for dental caries presented by the included articles. Most of the studies included in this SLR used WHO classification, which is widely accepted in epidemiological surveys. Some investigations used the ICDAS criteria (International System for Detection and Evaluation

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of Caries), which can identify the caries lesions, even at the initial stage (Table 1). In the literature, as previously mentioned, it has been well elucidated that a better design to characterize the phenotype consists of the selection of two extreme groups according to the DMFT index for decreasing heterogeneity (Yildiz et al., 2016). In this SLR, some studies classified their samples in high, moderate or low caries lesions presence. These variations in the classification preclude the homogenous comparison between groups. Furthermore, association studies should be well delimited and paired (by gender, age, phenotype or any other relevant information) to minimize biases and confounding factors in the distribution of group.

Furthermore, association studies should be well delimited and paired (by gender, age, phenotype or any other relevant information) to minimize biases and confounding factors in the distribution of group. On the other hand, due to the multifactorial etiology of dental caries, a strength of this review was the use of bioinformatics tools, allowing to advance the first step to better understand the interaction between genes and proteins. The genetic interactions presented here, although based on validated databanks of interacting molecules, still requires further experimental investigation to be confirmed. Therefore, by describing the level of evidence on the subject is still insufficient to determine and correctly rank the gene and/or variant to be more prone to cause the dental caries disease. Future investigations should look for additional evidence of interactions for a better understanding of the genetic associations presented in this study. This research also provides an important resource to create several hypothesis correlating dental caries with specific genes that were previously shown to be involved in potentially disease-causing mutations.

Although several studies have suggested different associations of caries occurrence with genetic modifications or microorganisms, further investigations are necessary to improve the previous correlations found, and also to find for more genetic markers associated with the complex genetic background of the caries condition. The use of high throughput sequencing technologies, combined with interactome, metagenomics, metabolome, metatranscriptome, proteome and several other omics-based approaches can support novel findings in the field of dental caries.

Finally, the knowledge on the genetic basis of dental caries will improve our understanding of the etiopathogenesis of this disease and help to identify risk groups, which could aid in effective screening and disease prevention.

Author contributions

Cavallari, Arima and Werneck: conducted the Systematic Review and drafted the manuscript;

Ferasa and Herai: conducted the protein interaction analysis and genetic interaction analysis;

Moysés and Moysés: designed the study and revised the manuscript

All the authors approved the final version.

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Declaration of Competing Interest

The authors have no conflicts of interest to report.

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CONCLUSÕES GERAIS

A importância da interação entre fatores genéticos e ambientais para determinar o risco no desenvolvimento de doenças complexas, bem como a compreensão de que alguns indivíduos são mais propensos a desenvolver doenças devido a alguns polimorfismos genéticos é essencial. Esta tese apresentou uma relação genética de duas doenças bucais com uma expressiva prevalência: A hipomineralização molar-incisivo e a doença cárie.

A HMI representa um grande desafio devido sua etiologia inconclusiva e complexidade do tratamento, uma vez que esta envolve uma abordagem multidisciplinar. Compreender os fatores causais de doenças com etiologia multifatorial é a premissa básica para realização do diagnóstico e eleição da melhor conduta no tratamento. Dessa forma, o cirurgião-dentista deve estar apto em diagnosticá-la precocemente a fim de estabelecer um plano de tratamento que atenda a necessidade individual em cada caso. Tendo em vista a dificuldade no tratamento desse defeito do esmalte dentário, a HMI merece uma maior atenção por parte da comunidade científica. Neste contexto, essa pesquisa buscou investigar fatores predisponentes no desenvolvimento da HMI e foi encontrado um resultado estatisticamente significativo entre o marcador rs235091 para dominância do alelo A (p=0.002), além de um resultado *borderline* para infecções respiratórias (p=0.099).

Em relação a doença cárie, sabe-se que esta representa o principal agravo a saúde bucal, exercendo um enorme impacto na qualidade de vida dos indivíduos. Os resultados apresentados neste estudo mostraram, por meio de ferramentas da bioinformática, redes de genes e proteínas que se relacionam. Além disso, vias metabólicas foram investigadas e várias vias canônicas associadas à diferentes funções celulares foram encontradas. Esta pesquisa reforçou a magnitude de entender fatores envolvidos no fenômeno da cárie dentária. Uma vez identificada a predisposição é possível planejar estratégias preventivas a fim de proporcionar saúde bucal.

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ANEXOS

TERMO DE ASSENTIMENTO

Você está sendo convidado (a) como voluntário (a) a participar da pesquisa estudo "Análise de associação genética da Hipomineralização Molar- Incisivo".

Para participar deste estudo você precisará passar por um exame clínico odontológico. Esse exame será realizado por meio da observação dos seus dentes. O pesquisador ficará sentado em uma cadeira escolar e você ficará posicionado (a) na frente do pesquisador. O exame será realizado em uma sala de aula, individualmente e com o acompanhamento da sua professora responsável. A duração do exame é em média de 5 minutos. Os seus dentes serão secos com o auxílio de uma gaze. Para afastar a sua bochecha, o pesquisador utilizará um palito de madeira. Após o exame iremos solicitar que você faça um bochecho durante 30 segundos e cuspa dentro de um vidro. Você foi escolhido (a) para participar desta pesquisa, pois está dentro da faixa etária da pesquisa que estamos desenvolvendo para verificar a presença ou a ausência de alguns defeitos na composição do seu dente.

Você será esclarecido (a) em qualquer aspecto que desejar e estará livre para participar ou recusar-se. Para participar deste estudo, o seu responsável deverá autorizar e assinar um termo de consentimento. Você ou o seu responsável poderá retirar o consentimento ou interromper a sua participação a qualquer momento. A sua participação é voluntária e a recusa em participar não acarretará qualquer penalidade.

Os riscos físicos para saúde de participação neste estudo são muito pequenos e limitados ao procedimento de exame clínico. Durante o exame você poderá sentir um leve desconforto temporário devido ao uso do palito de madeira. Em longo prazo, os resultados deste estudo poderão facilitar a detecção um fator etiológico no desenvolvimento de defeitos do esmalte.

Os resultados estarão à sua disposição quando finalizada. Seu nome ou o material que indique sua participação não será liberado sem a permissão do responsável por você. Os dados e instrumentos utilizados na pesquisa ficarão arquivados com o pesquisador responsável por um período de 5 anos, e após esse tempo serão eliminados. Este termo de consentimento encontra-se impresso em duas vias, sendo que uma cópia será arquivada pelo pesquisador responsável, e a outra será fornecida a você.

Eu, _____, fui informado (a) dos objetivos do presente estudo de maneira clara e detalhada e esclareci minhas dúvidas. Sei que a qualquer momento poderei solicitar novas informações, e o meu responsável poderá modificar a decisão de participar se assim o desejar. Tendo o consentimento do meu responsável já assinado, declaro que concordo em participar desse estudo. Recebi uma cópia deste termo assentimento e me foi dada a oportunidade de ler e esclarecer as minhas dúvidas.

Local, ____ de _____ de 2017.

_____ Assinatura do (a) menor

_____ Assinatura do (a) pesquisador (a)

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Seu filho está sendo convidado (a) como voluntário (a) a participar do estudo "Análise de associação genética da Hipomineralização Molar- Incisivo". Esta hipomineralização é uma alteração no dente, que ocorre normalmente em crianças e adolescentes, a qual afeta a estrutura do dente podendo evoluir para dor de dente e cárie. Estamos investigando os possíveis fatores causais no desenvolvimento desta alteração.

PARTICIPAÇÃO NO ESTUDO

Se você concordar que seu filho (a) participe neste estudo, ele (a) será submetido (a) a um exame clínico odontológico e a uma coleta de saliva (por meio de um bochecho), procedimentos estes já consagrados na literatura que terão, em média, 5 minutos de duração. O exame será realizado em uma sala de aula, individualmente e com o acompanhamento da professora responsável.

RISCOS E BENEFÍCIOS

Os riscos serão um provável constrangimento no momento do exame clínico, no entanto, este exame será realizado isoladamente e de maneira reservada pelo pesquisador. Os benefícios desta pesquisa serão o diagnóstico adequado se há ou não alguma alteração no dente e o encaminhamento do meu filho (a), caso eu queira, a um profissional competente na PUCPR-Curitiba.

SIGILO E PRIVACIDADE

Estou ciente de a privacidade de meu representado será respeitada, ou seja, seu nome ou qualquer outro dado ou elemento que possa, de qualquer forma, identificá-lo, será mantido em sigilo. Os pesquisadores se responsabilizam pela guarda e confidencialidade dos dados, bem como a não exposição dos dados de pesquisa. Os dados serão arquivados por um período de 5 anos e depois serão eliminados.

AUTONOMIA

É assegurada a assistência durante toda pesquisa, bem como me é garantido o livre acesso a todas as informações e esclarecimentos adicionais sobre o estudo e suas consequências, enfim, tudo o que eu queira saber antes, durante e depois da minha

participação. Também fui informado de que posso recusar a participar do meu representado no estudo, ou retirar o consentimento a qualquer momento, sem precisar justificar, e de, por desejar sair da pesquisa, este não sofrerá qualquer prejuízo à assistência que vem sendo recebida.

ALTERNATIVA

Se seu (sua) dependente tiver um defeito no esmalte dentário e caso haja a disponibilidade e o interesse, será realizado um encaminhamento para o tratamento na clínica/escola da PUCPR. Portanto, se você decidir não autorizar, ou cessar a participação de seu dependente no estudo a qualquer momento, seu dependente receberá informações sobre a saúde da boca dele, assim como explicações sobre prevenção de doenças da boca.

RESSARCIMENTO E INDENIZAÇÃO

No caso de você decidir autorizar a participação de seu (sua) dependente no estudo, você não terá nenhuma despesa decorrente da participação do estudo e nenhum reembolso, sendo que qualquer dano comprovadamente, referente à esta pesquisa, será ressarcido na forma da lei.

CONTATO

Os pesquisadores envolvidos com o referido projeto são Tayla Cavallari (pesquisadora principal) e Renata Iani Werneck (orientadora da pesquisa) vinculadas a instituição de ensino Pontifícia Universidade Católica do Paraná e com elas poderei manter contato pelo telefone: (41) 3271-1663.

O Comitê de Ética em Pesquisa em Seres Humanos (CEP) é composto por um grupo de pessoas que estão trabalhando para garantir que seus direitos como participante de pesquisa sejam respeitados. Ele tem a obrigação de avaliar se a pesquisa foi planejada e se está sendo executada de forma ética. Se você achar que a pesquisa não está sendo realizada da forma como você imaginou ou que está sendo prejudicado de alguma forma, você pode entrar em contato com o Comitê de Ética em Pesquisa da PUCPR (CEP) pelo telefone (41) 3271-2292 entre segunda e sexta-feira das 08h00 às 17h30 ou pelo e-mail nep@pucpr.br.

DECLARAÇÂO

Declaro que li e entendi todas as informações presentes neste Termo de Consentimento Livre e Esclarecido e tive a oportunidade de discutir as informações deste termo. Todas as minhas perguntas foram respondidas e eu estou satisfeito com as respostas. Entendo que receberei uma via assinada e datada deste documento e que outra via assinada e datada será arquivada nos pelo pesquisador responsável do estudo.

Enfim, tendo sido orientado quanto ao teor de todo o aqui mencionado e compreendido a natureza e o objetivo do já referido estudo, manifesto meu livre consentimento em participar, estando totalmente ciente de que não há nenhum valor econômico, a receber ou a pagar, por minha participação.

Dados do participante da pesquisa	
Nome:	
Idade:	

Dados do represável pelo participante da pesquisa	
Nome:	
Telefone:	

Local, _____ de _____ de _____.

_____ Assinatura do participante da pesquisa

_____ Assinatura do Pesquisador

Questionário

FICHA DO HISTÓRICO DO PACIENTE E ODONTOGRAMA E QUESTIONÁRIO DA PESQUISA

Data:____/____/____ Nº Protocolo:_____
Data de nascimento: ____/____/____
Idade: _____ **Sexo**: ( ) F ( ) M **Cor**:_____

( ) Cesariana ( ) Parto normal
Peso ao nascer: _____kg Comprimento: _____cm
Prematuro: ( ) Sim ( ) Não
Primeiro filho(a)? ( ) Sim ( ) Não, quantos irmão(s): _____

Condição socioeconômica familiar (renda mensal):
( ) Até 01 salário mínimo (até R$ 914,00) ( ) Até 02 salário mínimo (até R$ 1829,64,00) ( ) Até 03 salário mínimo (R$ 2744,46) ( ) Até 04 salário mínimo (R$ 3659,28) ( ) 05 ou mais salário mínimo (R$ 4574,10)

Fatores de risco associados com o desenvolvimento de defeitos de esmalte dentário em dentes permanentes

1. **Alterações durante a gestação:** ( ) febre alta ( ) diabetes ( ) pressão alta ( ) uso de medicamentos (.) exposição a fumaça de cigarro ( ) mãe fumante outros:_____

2. Ingestão de álcool durante a gestação: ( ) Nunca 1) Mensalmente ou menos ( ) De 2 a 4 vezes por mês ( ) De 2 a 3 vezes por semana ( ) 4 ou mais vezes por semana

3. Infecção respiratória 0-3 anos de idade: ( ) Sim ( ) Não

4. Catapora 0-3 anos de idade: ( ) Sim ( ) Não

5. Doença celíaca: ( ) Sim ( ) Não

6. Exposição à fumaça de cigarro (de outros fumantes) 0-3 anos de idade: ( ) Sim ( ) Não

7. Exposição à fumaça de cigarro (da mãe) 0-3 anos de idade: ( ) Sim ( ) Não

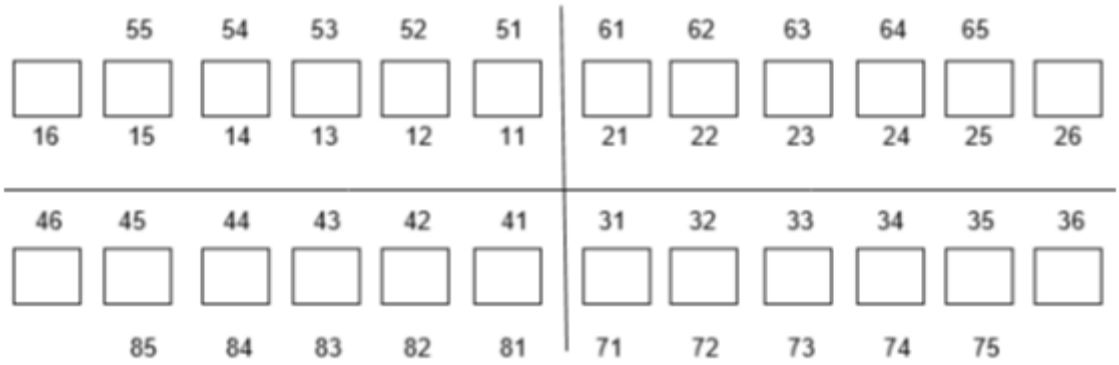
8. Otite 0-3 anos de idade: ( ) Sim ( ) Não

9. Infecção do trato urinário 0-3 anos de idade: ( ) Sim ( ) Não

10. Asma 0-3 anos de idade: ( ) Sim ( ) Não

11. Febre alta 0-3 ano: ( ) Sim ( ) Não

Odontograma



Código	Definição
0	Nenhum defeito de esmalte visível
1	Defeito de esmalte visível não MIH
2	Opacidades demarcadas: branca, amarela ou marrom
3	Fratura pós eruptiva do esmalte
4	Restauração atípica
5	Lesão Cariosa Atípica
6	Ausente devido MIH
7	Não pode ser codificado

Normas para publicação – Caries Reserach (artigo 01)

Caries Research - Author Guidelines

Abstract

A short Abstract should summarize the main points and reflect the content of the article. It should be written in a clear and concise way and be unstructured, set in 1 paragraph. Abbreviations used in the main text may be introduced and used. Use neither bibliographic references nor references to figures or tables in the Abstract.

Refer to the Author Guidelines for more information about the maximum accepted length (word count) of an Abstract in your chosen journal.

Introduction

The Introduction should provide a summary of the background to the relevant field of research and the specific problems addressed and should state the hypotheses being explored as well as the main goal(s) of the study. Conclusions or findings should not appear in the Introduction.

Materials and Methods

The Materials and Methods section should clearly list all inclusion and exclusion criteria, methods of research, and variables evaluated and should state how outcomes were assessed. All terms should be adequately defined and statistical information should be sufficiently detailed so that a study can be repeated.

Results

The Results section should describe the most important findings of the study, analysis, or experiment. The most important results should be indicated, and relevant trends and patterns should be described.

Discussion/Conclusion

The Discussion/Conclusion should provide an evaluation of the results. There should be a clear discussion of the implications, significance, and novelty of the results presented and whether the data support or contradict previous studies.

Statements

All papers must contain the following statements after the main body of the text and before the reference list:

Acknowledgement

In the Acknowledgement section, authors must include individuals and organizations that have made substantive contributions to the research or the manuscript. An exception is where funding was provided, which should be included in Funding Sources. Please refer to the Guidelines issued by the ICMJE to determine non-author contributors that should be included in the Acknowledgement section.

Statement of Ethics

Published research must comply with the guidelines for human studies and should include evidence that the research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. In the manuscript, authors should state that subjects (or their parents or guardians) have given their written informed consent and that the study protocol was approved by the institute's committee on human research. If ethical approval was not required or obtained, please state why.

Disclosure Statement

Authors are required to disclose any possible conflicts of interest. All forms of support and financial involvement (e.g. employment, consultancies, honoraria, stock ownership and options, expert testimony, grants or patents received or pending, royalties) which took place in the previous three years should be listed, regardless of their potential relevance to the paper. Also the nonfinancial relationships (personal, political, or professional) that may potentially influence the writing of the manuscript should be declared. If there is no conflict of interest, please state: "The authors have no conflicts of interest to declare."

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Authors must give full details about the funding of any research relevant to their study, including sponsor names and explanations of the roles of these sources in the preparation of data or the manuscript.

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In the Author Contributions section, a short statement detailing the contributions of each person named as an author should be included. Contributors to the paper who do not fulfil the ICMJE Criteria for Authorship should be credited in the Acknowledgement section.

References (Alphabetical)

References should be listed using the Vancouver style. The reference list should include only those publications which are cited in the text, in alphabetical order. Material submitted for publication but not yet accepted should be referred to as “unpublished data” and should not be included in the reference list. The authors’ surnames should be followed by their initials with no punctuation other than a comma to separate individual authors. A maximum of 6 authors should be listed (followed by “et al.” if there are more than 6 authors). More information on good referencing practice, as well as further examples, can be found in The National Library of Medicine Style Guide for Authors.

Examples

Papers published in journals:

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Papers published only with DOI number:

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Edited Books:

Cohen SR, Gardner TW. Diabetic retinopathy and diabetic macular edema. In: Nguyen QD, Rodrigues EB, Farah ME, Mieler WF, Do DV, editors. Retinal pharmacotherapeutics. Dev Ophthalmol. Basel: Karger; 2016. Vol. 55; p. 137–46.

Websites:

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Figure Legends

Fig. 1. Legend text.

Fig. 2. Legend text.

Please note that the actual figures and all tables should be uploaded separately.

Note regarding Authorship: If an author is removed from or added to the listed authors after submission, an explanation and a signed statement of agreement confirming the requested change are required from all the initially listed authors and from the author to be removed or added.