JOÃO ARMANDO BRANCHER

ANÁLISE DE VARIABILIDADE NA REGIÃO PROMOTORA DO GENE DA LACTOTRANSFERRINA (*LTF*) EM INDIVÍDUOS COM E SEM CÁRIE DENTÁRIA

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde (PPGCS) do Centro de Ciências Biológicas e da Saúde (CCBS) da Pontifícia Universidade Católica do Paraná (PUCPR), como parte dos requisitos para a obtenção do título de Doutor em Ciências da Saúde, Área de Concentração Medicina e Áreas Afins.

Orientadora: Profa. Dra. Paula Cristina Trevilatto

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Sumário

SUMÁRIO

Resumo

RESUMO

Cárie dentária é uma doença infecciosa, crônica, complexa e multifatorial assim como câncer, diabetes e doenças cardiovasculares. É causada por ácidos orgânicos oriundos da fermentação de carboidratos da dieta por parte das células bacterianas. Condições socioeconômicas, hábitos de higiene, o fluxo e a composição da saliva também podem influenciar a susceptibilidade do indivíduo ao desenvolvimento da cárie. Com respeito ao hospedeiro, existe uma forte evidência para um componente genético na etiologia da doença cárie. A proteína salivar lactotransferrina (LTF) exibe atividade bactericida e bacteriostática contra bactérias bucais gram-positivas e gram-negativas, mas não existem estudos investigando a associação de polimorfismos na região promotora do gene da *LTF* com cárie dentária. O objetivo deste estudo foi analisar a região promotora do gene da *LTF*, buscando polimorfismos, e investigar a associação de polimorfismos nesta região com cárie dentária em estudantes de 12 anos de idade. Para tanto, cinquenta (n=50) indivíduos de ambos os sexos, com 12 anos de idade, estudantes de escolas públicas e privadas de Curitiba-PR, com fenótipos extremos, foram examinados e divididos em dois grupos, de acordo com a experiência de cárie: Grupo 1 (G1): 25 estudantes sem cárie dentária (CPOD=0); Grupo 2 (G2): 25 estudantes com cárie dentária (CPOD≥4). Após a purificação do DNA, a região promotora do gene da *LTF* foi analisada por *High Resolution Melting* (HRM). Destes, quinze (15) estudantes, 8 sem cárie (CPOD=0) e 7 com experiência de cárie (CPOD médio=6,28), apresentaram diferenças nas curvas padrões obtidas por HRM e foram sequenciados. Os resultados obtidos foram analisados utilizando o programa DNASTAR. Não foram identificados polimorfismos na região promotora do gene da LTF (região +39 / -1143). Estudos futuros deverão ser conduzidos, analisando *bins*, que podem capturar a informação do gene da *LTF* como um todo, para melhor entender a contribuição deste gene na etiopatogênese da cárie dentária.

Abstract

ABSTRACT

Dental caries is an infectious, chronic, complex and multifactorial disease such as cancer, diabetes and cardiovascular illnesses. Caries disease is caused by organic acids that originate from microbial fermentation of carbohydrates from the diet. Other risk factors that may influence individual susceptibility to caries development are socioeconomic status, hygiene habits and saliva flow and composition. Regarding host aspects, there is strong evidence for a genetic component in the etiology of caries disease. The salivary protein LTF exhibits bactericidal and bacteriostatic activity against a wide range of gram-negative and gram-positive bacteria, but there is no study investigating the association of polymorphisms in the promoter region of *LTF* gene with caries. The aim of this study was search the promoter region of the human lactotransferrin gene (*LTF*) for polymorphisms and investigate the association of the identified *LTF* gene polymorphisms in this region with dental caries in 12-year-old students. Fifty (50) unrelated, 12-year-old, both sexes, students, with extreme phenotypes, of private and public schools of Curitiba-PR were examined and divided into two groups, according to caries experience: *Group 1 (G1)*: 25 students without caries experience (DMFT=0); *Group 2 (G2)*: 25 students with caries experience (DMFT≥4). After DNA purification, *LTF* gene promoter region of the 50 students was analyzed by high resolution melting (HRM) and samples whose results did not follow the standard curves were sequenced and analyzed using DNASTAR program. Fifteen (15) students, 8 without (DMFT=0) and 7 with caries experience (mean DMFT=6.28), were classified as cases and sequenced. No polymorphisms were identified in the putative promoter region (+39 to -1143) of *LTF* gene. Further studies should be

conducted, analyzing *bins* which may capture the whole gene information, to better understand the contribution of this gene for the caries ethiopathogenesis.

Introdução

INTRODUÇÃO

Nas últimas décadas, a pesquisa em saúde pública voltou sua atenção para os determinantes sociais da saúde e da doença. Isso tem levado a questões que realçam a importância do ambiente social, no qual os indivíduos portadores de enfermidades vivem (Watt, 2002). Com relação à saúde bucal, não tem sido diferente (Soetiarto, 1999; Peres et al., 2000).

A prevalência da cárie dentária, doença de natureza infecciosa e multifatorial, tal como o câncer, doenças cardíacas e a diabete (Fejerskov, 2004), tem reduzido significativamente na América Latina e no Brasil (Narvai et al., 1999; Bonecker & Cleaton-Jones, 2003). Os fatores associados ao fenômeno de queda dos níveis da doença estão principalmente relacionados a aspectos socieconômicos (Reisine & Psoter, 2001; Johnson, 2004), à maior disponibilidade de fluoretos na água de abastecimento (Krasse, 1996; König, 2004), à presença de flúor nos dentifrícios (Milgron, 2001; Marthaler, 1996) e ao maior grau de instrução sobre higiene bucal (Milgron, 2001; Reisine & Psoter, 2001). Apesar disso, grupos de crianças continuam apresentando alta atividade de cárie. Estima-se que 20 a 25% das crianças e adolescentes americanos concentrem 60 a 80% da prevalência de cárie da população (Kaste et al., 1996). Em amostra brasileira, 17% das crianças apresentaram 46% do total de lesões de cárie da população avaliada, fenômeno denominado polarização (Mattos-Graner et al., 1996; Baldani et al., 2002).

A complexidade etiológica da cárie dentária é indiscutível. Historicamente eram considerados três fatores necessários para a sua iniciação: microrganismos

cariogênicos, carboidratos fermentáveis e tecido mineralizado (Keyes, 1962), com a adição de um quarto fator: o tempo (Newbrun, 1978). Atualmente é reconhecido que fatores de risco genéticos (Shuler, 2001), ambientais (Soetiarto, 1999) e comportamentais (Peres et al., 2000) também interagem nesse processo.

Biologicamente, o biofilme cariogênico é determinante para o início e progressão da doença (van Houte, 1994). Além disso, uma alta exposição a carboidratos fermentáveis pode modificar a composição do biofilme, favorecendo sua cariogenicidade (Cury, 2000; Nobre dos Santos et al., 2002). Nesse contexto, a saliva desempenha um papel importante, pois o fluxo, a capacidade tampão e a composição salivar podem influenciar a cariogenicidade do biofilme (Lenander-Lumikari & Loimaranta, 2000). Com relação à composição da saliva, diversas proteínas salivares interferem na cariogenicidade do biofilme, pois possuem ação antimicrobiana (van Nieuw Amerongen et al., 2004).

Entre as diversas proteínas salivares, é de especial interesse a lactotransferrina (LTF). Ela é uma proteína ligante de ferro, pertencente ao grupo das metaloproteínas (Fine et al., 2002) e das transferrinas (Baker & Baker, 2004).

A lactotransferrina é uma glicoproteína com massa molecular de aproximadamente 80 kDa e com cadeia polipeptídica composta por 692 aminoácidos (Ward & Conneely, 2004). Estruturalmente, a cadeia polipeptídica contém duas dobras internas repetidas, denominadas Lobo N e Lobo C. Ambos os lobos possuem a mesma característica estrutural e são subdivididos em dois domínios α / β, separados por um peptídio de conexão, conforme ilustra a figura 1 (Karthikeyan et al, 1999; Baker et al., 2002).

O tecido normal humano expressa diferentes quantidades de LTF (Teng, 2002; Liu et al., 2003) em diferentes órgãos e em diferentes condições fisiológicas (Panella et al., 1991). A maior concentração de LTF é encontrada no colostro (Ward & Connely, 2004) e, em menor quantidade, em fluidos do organismo, como a saliva, a lágrima, o sêmen, o suor, o leite e secreções nasais (Teng, 2002; van Veen et al., 2002; Ward & Connely, 2004).

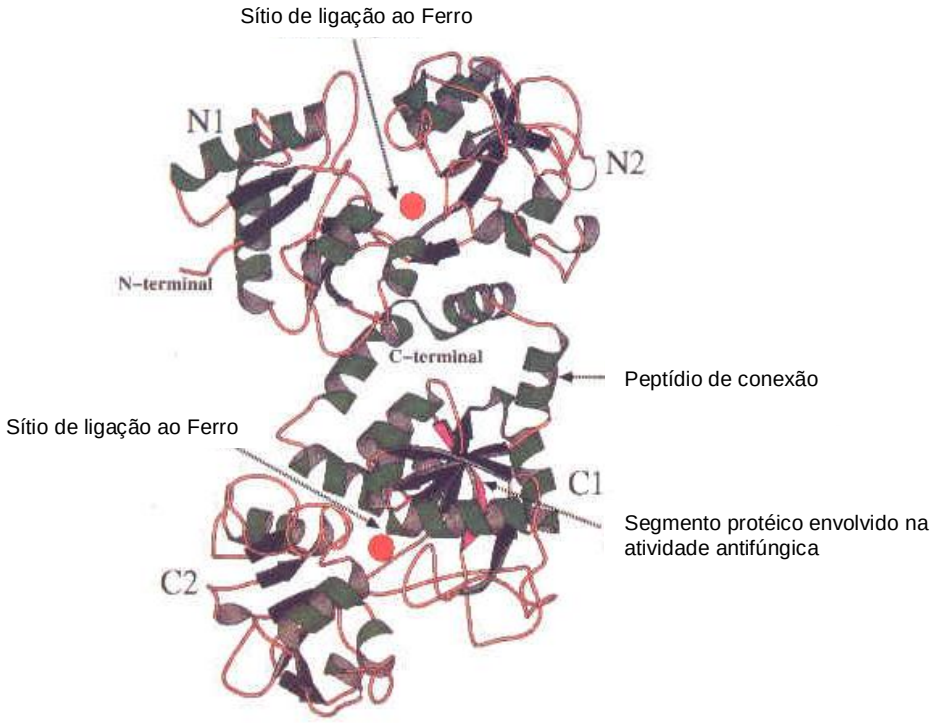

Fig. 1. Estrutura tridimensional da lactotransferrina. Em destaque os lobos N e C, sítio de ligação do ferro e a estrutura helicoidal do peptídio de conexão. *Fonte*: Karthikeyan et al., 1999.

A LTF tem capacidade de se ligar, reversivelmente, a dois átomos de ferro junto com dois íons bicarbonato. A propriedade de ligar íons ferro faz com essa

proteína possua propriedade bacteriostática, pois limita a disponibilidade de ferro para as células bacterianas (Singh, 2002; Baker & Baker, 2004; van Nieuw Amerongen et al., 2004). Os sítios ligantes de ferro, localizados nos lobos N e C, contêm dois aminoácidos tirosina (Tyr 92, Tyr 192), um ácido aspártico (Asp 60) e uma histidina (His 253) (Baker & Baker, 2004).

Bellamy et al. (1992) atribuem à LTF uma grande atividade bactericida. Segundo esses autores, existe um domínio bactericida muito bem caracterizado no lobo N em um local diferente do sítio de ligação do ferro. Esse domínio, chamado *lactoferricina*, é altamente catiônico e corresponde ao segmento proteico relativo aos aminoácidos 1 - 47 da proteína. Quando liberado do lobo N, esse segmento possui uma atividade bactericida maior do que a proteína nativa (Bellamy et al., 1992; Tomita et al., 1994). Estudos *in vitro* e *in vivo* demonstraram que a lactoferricina contém dois domínios catiônicos separados, o primeiro compreende um *cluster* de 4 argininas carregadas positivamente, que correspondem aos aminoácidos 1 - 5 (Nibbering et al., 2001), e o segundo contém um domínio correspondente aos aminoácidos 20 - 37, similar ao encontrado na LTF de outras espécies (Vorland et al., 1998).

Além de apresentar atividade antibacteriana, a LTF apresenta diversas funções biológicas: antiviral, antifúngica e antiinflamatória (Brock, 2002). O efeito antiviral está relacionado à atuação contra o citomegalovírus, o vírus da herpes simples e o vírus da imunodeficiência humana (HIV) (Berkhout et al., 2002). Outro estudo demosntrou que a LTF possui atividade de serina protease, a qual pode prevenir a colonização de *Haemophilus influenzae* pela inativação da membrana externa (Qiu et al., 1998). A LTF inibe o crescimento de *Candida albicans*, que

normalmente está presente em pacientes portadores de próteses odontológicas (Lupetti et al., 2003). Ela também estimula a produção de citocinas (van Veen et al., 2002), que são responsáveis por coordenar a resposta celular humana, atuando na maturação e ativação de macrófagos e neutrófilos, podendo sua deficiência causar supressão no sistema imunológico, e seu excesso, uma exacerbada resposta imune (Son et al., 2002). Os leucócitos polimorfonucleares são ricos em LTF (Panella et al., 1991), que age como fator de proteção contra diversas infecções (Orsi, 2004; Ward & Conneely, 2004). A LTF pode regular diretamente a resposta inflamatória (Singh et al., 2002), ligando-se a endotoxinas, como o lipopolissacarídeo (LPS), mediador central da resposta inflamatória em infecções bacterianas (Brock, 2002; van Veen et al., 2002). Foi observado que a LTF pode se ligar à membrana externa de bactérias gram-negativas e causar uma rápida liberação de LPS, aumentando a permeabilidade das membranas (Ellison & Giehl, 1991).

Com relação ao efeito antibacteriano da LTF, a atividade contra o *Streptococcus mutans* pode fornecer subsídios para a importância dessa molécula na etiopatogênese da cárie dentária, uma vez que esta proteína pode modular a agregação e o desenvolvimento do biofilme dental, inibindo a adesão de *S. mutans* (Oho et al., 2002; Francesca et al., 2004), provavelmente devido às pontes dissulfeto existentes entre os aminoácidos cisteínas localizados nas posições 481 e 532 no lobo C (Mitoma et al., 2001).

Uma associação significante foi encontrada entre o índice de superfícies cariadas e a concentração de LTF salivar (Sikorska et al., 2002).

O gene da LTF (*LTF*) apresenta 17 éxons, com comprimento total de 24,5 kb. Está localizado no cromossomo humano 3, na posição 3p21 (Kim et al., 1998) (Fig. 2).

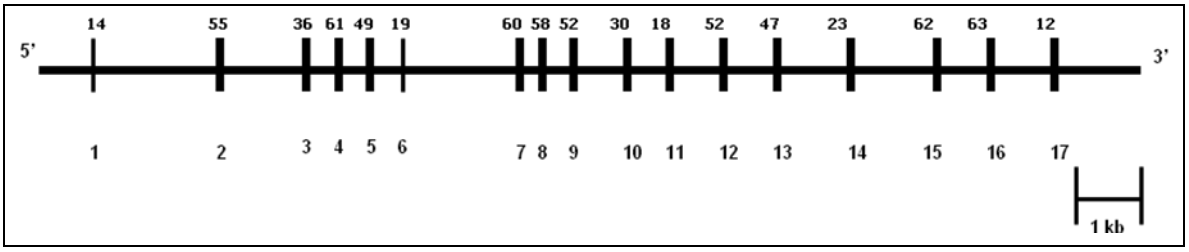

Fig. 2. Gene da LTF com indicação dos 17 éxons. Os algarismos indicados acima representam o número de aminoácidos codificados por cada éxon.

Polimorfismos no gene da LTF têm sido descritos e relacionados a doenças tais como periodontite agressiva (Velliyagounder et al., 2003, Karasneh et al., 2010; Wu et al., 2010), queratite por herpes simples (Keijser et al., 2008) e cárie dentária (Azevedo et al., 2010). Polimorfismo genético é uma variação gerada por uma mutação, cuja freqüência do alelo mais raro é maior ou igual a 1% (Chiba-Falek & Nussbaum, 2001). Um polimorfismo (A/G) (rs1126478), no segundo éxon do gene da LTF, é responsável pela substituição de um aminoácido lisina (Lys) por arginina (Arg), na posição 29, e foi associado recentemente com variações no volume de fluxo salivar e suscetibilidade à cárie (Azevedo et al., 2010). A variante contendo o resíduo Lys esteve associada a uma maior atividade antibacteriana em *S. mutans* (Vellyagounder et al., 2003).

O promotor *putative* do gene LTF contém aproximadamente 1.000 pb e várias caixas de transcrição, que estão envolvidas na regulação da expressão da proteína. Os primeiros 85 pares de bases que flaqueiam a região 5' do promotor

contêm diversas caixas de transcrição, incluindo TATA, Myb, C/EBP, ET e SP1, todos envolvidos na regulação da expressão do gene. Tais sítios de transcrição são muito conservados entre as espécies (Pugh & Tjian, 1990; Liu & Teng, 1991; Zheng et al., 2005). Na região compreendida entre -340/-394 localiza-se um segundo bloco com importantes elementos regulatórios do gene da *LTF*: ERE, COUP-TF e GATA, responsivos ao hormônio estrogênio, também conservados entre as espécies (Liu & Teng, 1992; Teng, 2006). Entretanto, a região promotora do gene da *LTF* humana contém uma caixa de transcrição adicional denominada SFRE que aumenta ainda mais a resposta da região promotora ao hormônio estrogênio (Teng, 2006). Finalmente, na região -774/-778, existe um elemento silenciador do gene, denominado TAACC. Quando fatores de transcrição ligam-se neste sítio, a transcrição cessa (Khanna-Gupta et al., 1997). O estudo de Zheng et al. (2005) mostrou que, para a atividade basal do promotor, são necessárias as sequências contidas até a região -543, o que inclui todas as caixas de transcrição citadas, mas que para a atividade máxima do promotor, são necessárias as sequências contidas até a posição -1029.

Poucos polimorfismos foram relatados na região promotora do gene da *LTF*. Existem apenas 5 descritos (NCBI, 2010): rs67994108 (posição -41), rs28365893 (posição -232), rs4637321 (posição -420), rs35869674 (posição -489), rs5848800 (posição -696), embora nenhum deles tenha sido validado nem mesmo por frequência. Teng & Gladwell (2006), em um estudo com 91 indivíduos de diferentes etnias, descreveram 7 polimorfismos nas posições -261, -374, -401, -421, -1010, -1119 e -1261; entretanto, somente um mostrou-se frequente (-1010, ATAT/-), e nenhum deles foi validado ou registrado com rs no NCBI.

Com relação a estudos genéticos da cárie dentária em humanos, Bretz et al. (2005 a) analisaram 388 pares de gêmeos mono e dizigóticos e identificaram um componente genético alto (cerca de 70%) para o desenvolvimento da cárie dentária. O estudo de Werneck et al. (2010 b) identificou um gene de efeito principal por meio de análise de segregação complexa. Apesar disso, ainda não se sabe quais e quantos genes estão envolvidos na determinação da suscetibilidade a essa doença complexa.

O primeiro estudo de scan genômico de ligação foi realizado por Vieira e colaboradores (2008), com 392 marcadores, objetivando identificar regiões cromossômicas que contêm genes relacionados com a cárie dentária. A população estudada foi composta de 46 famílias (624 indivíduos), que co-habitavam uma mesma região nas Filipinas. Cinco prováveis *loci* foram identificados: três para baixa susceptibilidade de cárie (5q13.3, 14q11.2 e Xq27.1) e dois para alta susceptibilidade de cárie (13q31.1 e 14q24.3). Os autores sugeriram que genes associados ao controle de fluxo salivar e à preferência de dieta podem ser genes candidatos (13q31.1, 14q24.3 e 14q11.2).

Genes candidatos investigados, envolvidos no desenvolvimento e formação do esmalte dentário, foram: amelogenina (*AMELX*), ameloblastina (*AMBN*), tuftelina (*TUFT1*), enamelina (*ENAM*), proteína que interage com tuftelina (*TFIP11*) e calicreína 4 (*KLK*4) (Slayton et al., 2005). Polimorfismos nesses genes candidatos foram testados para associação, seguindo um modelo caso-controle em uma amostra de crianças com 3 a 5 anos de idade. Não foram encontradas evidências para a associação entre cárie dentária e nenhum gene investigado independentemente. Embora, quando foi realizada uma análise multivariada, o

gene *TUFT1*, combinado com o efeito de altos níveis de *S. mutans*, mostrou aumento da susceptibilidade à cárie dentária (Slayton et al., 2005). Outro estudo também investigou *AMELX*, *AMBN*, *TUFT1*, *ENAM* e *TFIP11* para associação com cárie dentária em uma população da Guatemala. Uma forte evidência (p=0,0000001) para associação foi encontrada para um marcador do gene *AMELX* com alto CPOD (CPOD≥20) e aumentou a experiência de cárie ajustada pela idade (Deeley et al., 2008). O mesmo gene foi estudado em uma amostra da população turca e os resultados confirmaram o estudo prévio (Patir et al., 2008). Os autores concluíram que o melhor modelo que explica o aumento do CPOD foi uma maior frequência de alelos específicos de um marcador da *TUFT1* e um marcador da *AMELX* no grupo estudado.

Genes candidatos associados à resposta imune também foram analisados e um genótipo do gene *CD14* foi significativamente associado com a presença de 4 ou mais lesões de cárie (De Soet et al., 2008). Adicionalmente, associação entre HLA-DRB1*04 e cáries em crianças foi encontrada (Bagherian et al., 2008).

No que diz respeito a genes que afetam a composição da saliva, alguns estudos têm investigado proteínas salivares, associando-as à formação e adesão de bactérias ao biofilme e também à sua capacidade tamponante (Almstahl et al., 2001; Van Nieuw Amerongen & Veerman, 2002; Peres et al., 2010). Yu et al. (1986) encontraram associação entre o aumento do CPOS e o gene da proteina rica em prolina salivar (PRP), uma proteina da saliva que influencia a agregação de bactérias ao biofilme dentário. Um estudo subsequente investigando genes relacionados com as PRPs demonstrou associação entre a cárie dentária e o alelo

Db, um dos três alelos do gene *PRH1* (Zakhary et al., 2007). O mesmo estudo mostrou que Caucasianos negativos para o alelo Db tinham signficativamente mais cáries do que pacientes Afro-Americanos, também negativos para o alelo Db, demonstrando a importância da etnia associada com a informação genética. Outro estudo enfocou a anidrase carbônica VI (*CA6*), que catalisa a formação do dióxido de carbono na saliva e em outros fluidos biológicos. Os autores não encontraram associação entre os alelos e os genótipos dos três polimorfismos na região codificante do gene *CA6* e a experência de cárie, embora uma associação positiva entre a capacidade de tamponamento e o rs2274327 (C/T) tenha sido encontrada (Peres et al., 2009). Mais recentemente, um alelo do gene da *LTF* foi associado com proteção contra a cárie e aumento do fluxo salivar (Azevedo et al., 2005).

Até o momento, existem poucos estudos investigando a associação de polimorfismos em genes candidatos funcionais com a cárie dentária. Com relação ao gene da lactotransferrina, apenas um estudo mostrou associação de um alelo presente no segundo éxon com a proteção contra a cárie. No entanto, não existem estudos investigando a associação de polimorfismos na região promotora do gene *LTF* com a cárie dentária.

Proposição

PROPOSIÇÃO

Os objetivos deste estudo foram: i) analisar a variabilidade genética da região promotora do gene da LTF (*LTF*), investigando a presença de polimorfismos e ii) validar a existência de polimorfismos previamente descritos e registrados no NCBI e iii) investigar a associação dos polimorfismos identificados com a experiência de cárie dentária em estudantes de 12 anos de idade.

Artigo

ANALYSIS OF POLYMORPHISMS IN LACTOTRANSFERRIN GENE (*LTF*) PROMOTER AND DENTAL CARIES

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ABSTRACT

Background: Dental caries is an infectious, chronic, complex and multifactorial disease such as cancer, diabetes and cardiovascular illnesses. Caries disease is caused by organic acids that originate from microbial fermentation of carbohydrates from the diet. Other risk factors that may influence individual susceptibility to caries development are socioeconomic status, hygiene habits and saliva flow and composition. Regarding host aspects, there is strong evidence for a genetic component in the etiology of caries disease. The salivary protein LTF exhibits bactericidal and bacteriostatic activity against a wide range of gram-negative and gram-positive bacteria, but there is no study investigating the association of polymorphisms in the promoter region of *LTF* gene with caries. The aim of this study was search the promoter region of the human lactotransferrin gene (*LTF*) for polymorphisms and investigate the association of the identified *LTF* gene polymorphisms in this region with dental caries in 12-year-old students. **Methods:** 50 unrelated, 12-year-old, both sexes, students, with extreme phenotypes, of private and public schools of Curitiba-PR were examined and divided into two groups, according to caries experience: *Group 1 (G1)*: 25 students without caries experience (DMFT=0); *Group 2 (G2)*: 25 students with caries experience (DMFT≥4). After DNA purification, *LTF* gene promoter region of the 50 students was analyzed by high resolution melting (HRM) technique and samples whose results did not follow the standard curves were sequenced and analyzed using DNASTAR program. **Results:** Fifteen (15) students, 8 without (DMFT=0) and 7 with caries experience (mean DMFT=6.28), were classified as cases and

sequenced. **Conclusion:** No polymorphisms were identified in the putative promoter region (+39 to -1143) of *LTF* gene. Further studies should be conducted, analyzing *bins* which may capture the whole gene information, to better understand the contribution of this gene in the caries ethiopathogenesis.

Introduction

Dental caries is a multifactorial infectious disease that may result in loss of mineral from affected teeth (Fejerskov, 2004). The prevalence of the disease has reduced significantly, including in Latin America and Brazil (Bonecker & Cleaton-Jones, 2003). Nevertheless, groups of children have still been showing high levels of caries activity. It is estimated that 20 to 25% of children and adolescents in USA concentrate 60 to 80% of the caries prevalence (Kaste et al., 1996) and 17% of children concentrate 46% of the caries prevalence in Brazil (Mattos-Graner et al., 1996; Baldani et al., 2002). This phenomenon of dental caries concentration in small groups is termed *polarization* and represents one of the epidemiological disease aspects, in which a portion of the population has focused most needs for treatment (Antunes et al., 2002; Narvai et al., 2006). Treatment of caries is extremely costly, representing the fourth most expensive disease to treat in most of the third world countries (Yee & Sheiham, 2002).

Caries disease is caused by organic acids that originate from microbial fermentation of carbohydrates from the diet (Burt & Pai, 2001; Ajdi et al., 2002). Besides the microflora (Vogel et al., 2002; Orsi, 2004), cavities may appear whether cariogenic microorganisms and carbohydrates are present in a susceptible individual during a certain time in the mouth (Conrads, 2002; Zero, 2004). Other risk factors that may influence individual susceptibility to caries development are socioeconomic status (Peres et al., 2000), oral health behavior (Jones & Worthinton, 2000; Stecksen-Blicks et al., 2004), gender (Antunes et al., 2006) and ethnicity (Pattussi et al., 2006). In addition, it seems that host response,

represented by teeth and saliva, contributes to caries outcome (Featherstone, 2004).

Saliva presents various innate and acquired defense factors capable of inhibiting bacterial invasion, growth and metabolism by different mechanisms (Atkison & Baum, 2001; Van Nieuw Amerongen & Veerman, 2002; Kidd & Fejerskov, 2004) such as bacterial adherence and streptococci acid production (Tenovuo, 2002). So far, researches have investigated several biological determinants, which can influence the biofilm cariogenicity (Burt & Pai, 2001; Singh et al., 2002), such as saliva flow and composition (Kidd & Fejerskov, 2004; Nariyama et al., 2004). A constant salivary flow efficiently eliminates microorganisms from oral cavity; thus, a reduced flow may easily take to microbial growth, followed by teeth deterioration (Van Nieuw Amerongen & Veerman, 2002; Fejerskov, 2004). Some salivary proteins have an antibacterial effect, like lysozime, lactoperoxidase, immunoglobulins, aglutinines, mucins and lactotransferrin (Jentsch et al., 2004; Kidd & Fejerskov, 2004). At the molecular level, there is a functional overlapping among several salivary proteins (Iacono, 1980; Atkison & Baum, 2001).

Lactotransferrin (LTF) is a multifunctional metalloprotein (Van Nieuw Amerongen et al., 2004), belonging to the transferrin family (Leone & Oppenheim, 2001; Daly et al., 2006), with a molecular weight of about 80 kDa and 670-690 amino acid residues organized in two lobes: N and C (Karthikeyan et al., 1999). It is expressed in several cells, such as glandular epithelial tissues and human neutrophils (Leone & Oppenheim, 2001; Liu et al., 2003) and present in diverse organism fluids, such as tears, semen, sweat, colostrum, milk, nasal secretion and

saliva (Teng, 2002; Liu et al., 2003). LTF is considered a cytokine that plays a role in the protection against several infections (Van Nieuw Amerongen et al., 2004; Teng, 2002) such as by fungi (Bellamy et al., 1993), protozoa (Orsi, 2004) and viruses (Bellamy et al., 1992b; Valenti et al., 2000; Orsi, 2004). LTF can modulate dental biofilm aggregation and development, inhibiting *Streptococcus mutans* adhesion (Panella et al., 1991; Ward & Conneely, 2004).

Regarding host aspects, there is strong evidence for a genetic component in the etiology of caries disease (Boraas et al., 1988; Nariyama et al., 2004). However, little is known concerning how many and which are the genes influencing caries genetic predisposition.

The *LTF* gene is localized on the human chromosomal 3p21 (Teng et al., 1987; Berlutti et al., 2004), organized into 17 exons, with 24.5 kb in humans (Liu et al., 2003). Polymorphisms are gene sequence variations whose minimum allele frequency is higher than 1% in the population, and are distributed throughout the entire genome (Chiba-Falek & Nussbaum, 2001). Catalogued single nucleotide polymorphisms (SNPs) in public databases have been growing from 1.4 million in 1999 (Sachidanandam et al., 2001) to 2.1 million in 2001 (Venter et al., 2001) up to approximately 4.1 million markers (NCBI, 2010). Functional polymorphisms are variations, which may: i) alter amino acid sequence in the protein sometimes affecting the function of the protein and ii) modify the levels of transcripts and protein. Polymorphisms in regulatory sequences of the gene promoter can affect the protein function indirectly by altering its expression and RNA processing (Teng & Gladwell, 2006). *LTF* gene polymorphisms have been described (Teng & Gladwell, 2006; Liu et al., 2002) and associated with aggressive periodontitis

(Velliyagounder et al., 2003, Karasneh et al., 2010; Wu et al., 2010), herpes simplex keratitis (Keijser et al., 2008) and dental caries (Azevedo et al., 2010). However, to the authors' knowledge, there is only one report investigating the association between polymorphisms in *LTF* gene and dental caries (Azevedo et al., 2010) and there is no study investigating the association of polymorphisms in the promoter region of *LTF* gene with caries.

Thus, the aims of this study were to i) search the promoter region of the human lactotransferrin gene (*LTF*) for polymorphisms and ii) investigate the association of the identified *LTF* gene polymorphisms in this region and dental caries in 12-year-old students.

Materials and Methods

Sample selection

The study sample was composed of fifty (n=50) 12-year-old, both sexes, students of private and public schools of Curitiba-PR, with extreme phenotype (Table 1):

Group 1 (G1): 25 students without caries experience (DMFT=0);

Group 2 (G2): 25 students with caries experience (DMFT≥4).

The students were selected for study only if the parent/caregiver returned the informed consent form, according to norms of the Ethical Committee on Research of the Center for Health and Biological Sciences of Pontifical Catholic University of Paraná (PUCPR), according to Resolution 196/96 of the Health National Council, register n. 487. Twelve schools were randomizely chosen, one public and one private school from each health district of the city. Students were

not included if smokers, using orthodontic appliances, taking chronic anti-inflammatory and antibiotics in the last three months, or with history of any disease known to compromise immune function.

The students were diagnosed according to the decayed, missing and filled teeth index (DMFT). All examinations were conducted by two examiners. To assess the consistency of each examiner (inter and intra-examiner reproducibility), duplicate examinations were conducted on 10% of the sample and the Kappa test was used to measure reliability and the value of 0.93 was obtained, which indicated almost perfect reproducibility of the data. Examinations were conducted in schoolrooms in accordance with international standards established by the WHO (WHO, 2008).

DNA collection

The sampling of epithelial buccal cells was performed as previously described (Trevilatto & Line, 2000). Briefly, the individuals undertook a mouthwash after 1 min, containing 5 mL 3% glucose. Following mouthwash, a sterile wood spatula was used to scrape oral mucosa. The tip of the spatula was then shaken into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000g for 10 min. The supernatant was discarded and the cell pellet resuspended in 1.300 mL of extraction buffer [10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5 % SDS]. Ten µL proteinase K (20 mg/mL) were added to the solution, being left overnight at 65°C. DNA was purified by adding ammonium acetate 10 M, precipitated with isopropanol and resuspended with 50 µL Tris 10mM (pH 7.6) and EDTA 1 mM (Aidar & Line, 2007).

LTF gene promoter region amplification by high resolution melting (HRM)

For the PCR analysis, fifty (50) students with extreme phenotype for caries (25 DTMF=0 and 25 DTMF≥4) were selected. For the analysis, 15 μL final volume of reaction was prepared with 2 μL (10 ng) genomic DNA, 7.5 μL LightCycler®480 High Resolution Master Mix (Roche Diagnostics, Mannheim, Germany), 0.4 μL (10 *pmol*) each oligonucleotide primer, 1.2 μL $MgCl_2$ (Roche Diagnostics, Mannheim, Germany) and 3.5 μL deyonizated water. Five primer pairs were used to amplify a promoter sequence in the *LTF* gene containing transcription boxes (Table 2).

The polymerase chain reaction (PCR) and melting acquisition were performed in a single run on a LightCycler® 480 instrument (Roche Diagnostics, Mannheim, Germany). According to the manufacturer's instructions, it was transferred 10 μL PCR product to 384-well plates suitable for HRM analysis. A centrifugation was performed as specified by the manufactured to eliminate air bubbles that might disturb fluorescence curves.

The PCR cycling protocol consisted of an initial heating step at 95°C for 10 minutes followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing starting at 68°C for 15 seconds and extension at 72°C for 20 seconds. After amplification, the amplicons were first heated to 95°C for 1 minute and then the HMR program went over the range from 65°C to 95°C with 25 signal acquisitions per degree. Melting curve analysis was performed on the Lightscanner with Lightscanner Software and on the LightCycler 480 with the Gene Scanning module. The software program employ a 3-step analysis: 1) normalization by selecting linear regions before (100% fluorescence) and after (0% fluorescence)

the melting transition, 2) temperature shifting by moving the curves along the x-axis, facilitating grouping, and 3) use of the Autogroup function. To analyze sample melting temperature profiles, the fluorescence of the samples was monitored while the temperature of the LightCycler® 480 Instrument thermal blockcycler had steadily increased. As the temperature increased, sample fluorescence decreased. The reaction conditions are shown in table 3.

PCR and DNA Sequencing of "Cases"

Samples, whose results did not follow the standard curves, needed to be checked for polymorphisms and were termed "cases". With the intention of sequencing the cases, PCR was carried out in a final reaction volume of 45 µL, containing 1.8 µL each primer (R and F), 1.8 µL DNA and 39.6 µL PCR Supermix - Invitrogen™. Amplification was performed with an initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, with a final extension for 7 min at 72 °C on a Touchgene Gradient Thermocycler (Techne, Cambridge, UK).

The PCR products were evaluated following electrophoresis through a 1.5% agarose gel (Promega, Madison, WI, USA), stained with ethidium bromide (Sigma) and visualized using an AlphaImager (Alpha Innotech, San Leandro, CA, USA). Each PCR product was purified using a Genomed JETquick, PCR Product Purification spin kit (Poststr. 22, D-32582 Lo¨hne, Germany). The sequencing reactions were performed by MWG-Biotech forward and reverse twice, and the sequence data were analyzed using DNASTAR suite of programs (DNASTAR, Inc., Madison WI, USA).

Results

Fifty (50) students with extreme phenotype, 25 without caries experience (DMFT=0) and 25 with caries experience (DMFT≥4) were analyzed by HRM technique, whose amplification patterns can be seen in figure 1.

Fifteen (15) students, being 8 without and 7 with caries experience (mean DMFT=6.28), were classified as “cases”, being further sequenced (Table 4). All the five primer pairs showed good quality results in the sequencing. An example of one sequenced sample using primer pair 5 can be observed in figure 2.

No polymorphisms in the study promoter region of *LTF* gene (+39/-1143 bp) were identified.

Discussion

Although dental caries has been declining recently (Peterson, 2005), it is still a major public health problem concerned worldwide, affecting from 60 to 90% of children at school age and the majority of the adult population (WHO, 2004). It has an impact on individuals and communities by leading to tooth loss and dental pain, resulting in suffering, impairment of function, reduced quality of life, and absenteeism at school and work (Fejerskov, 2004; WHO, 2004).

The etiology of dental caries has been studied for many years. Multiple factors may be contributing to a person’s risk to caries, including three essential interactive factors: host, such as saliva properties and tooth enamel surface, biofilm, and diet (Keyes, 1962), with addition of another factor: time (Newbrun, 1978). More recently, environmental, such as socioeconomic status (Peres et al.,

2000), and oral health behavior (Stecksen-Blicks et al., 2004) and genetic aspects (Vieira et al., 2008) have been also related to caries etiology.

In spite of all that has been known about this disease, there are still individuals who appear to be more susceptible to caries and those who are extremely resistant, regardless the environmental risk factors to which they are exposed (Slayton et al., 2005). Recently, our group showed for the same study sample that the DMFT index was significantly higher (2.88) among the students with caries experience than that for the whole sample (1.46). This finding evidenced the polarization phenomenon in the study sample and points to an individual host response modulation influencing caries outcome.

Based on the multifactorial nature of dental caries, it has been suggested that susceptibility or resistance to caries would be the result of one or more gene-environment interactions (Slayton et al., 2005). Studies have identified a strong genetic component controlling susceptibility to caries (see Werneck et al., 2010a, for revision). Hereditary aspects of caries have been discussed since the 20's (Bachrach & Young, 1927). Firstly, the studies investigated genetic aspects related to cariogenic bacteria (Macrina et al., 1990). Nowadays, genetic analyses report aspects associated with the individual susceptibility to dental decay development (Boraas et al., 1988; Conry et al., 1993). Since the 30's, there have been pieces of evidence associating hereditary aspects with dental caries, such as familial aggregation (Klein & Palmer, 1938). Gold standard studies aiming to dissect the genetic component underlying a given complex disease, such as caries, are i) twin studies (Townsend et al., 2003; Bretz et al., 2005 a; b), and ii) complex segregation analysis (CSA) (Werneck et al., 2010b). Twin studies, which compare concordance

rates between monozygous and dyzigous twins, have shown that between 50 and 70% of the phenotype variation are explained by genes (Bretz et al., 2005a; 2005b), while the CSA detected a dominant major gene effect which best explained the phenotype. However, these kinds of analyses fail to identify how many and which the genes underlying the controlling of susceptibility to diseases are (Werneck et al., 2010 b).

Candidate genes underlying host susceptibility to caries could range from: 1) genes contributing to enamel formation (Patir et al., 2008); 2) to those for saliva composition (Azevedo et al., 2010), and 3) immune response (De Soet et al., 2008). Concerning saliva, several studies have been investigating salivary proteins involved in modulating biofilm aggregation and adhesion, buffer capacity and other qualitative aspects of saliva (Almstahl et al., 2001; Van Nieuw Amerongen & Veerman, 2002; Peres et al., 2010).

The salivary protein LTF exhibits bactericidal and bacteriostatic activity against a wide range of gram-negative and gram-positive bacteria due to its ability to chelate iron, which is essential for microbial growth and metabolism (Arnold et al., 1981). Specifically, LTF may interfere with *Streptococcus mutans* aggregation, adhesion, and biofilm development (Berlutti et al., 2004; Sikorska et al., 2002). In addition, LTF exhibits non-iron-dependent antibacteria properties (Orsi, 2004; Ward & Conneely, 2004), antifungal, antiviral, antitumor, anti-inflammatory and immunoregulatory activities (Baveye et al., 1999; Vorland, 1999; Conneely, 2001; Elass et al., 2002).

Results involving *LTF* gene and dental caries are scarce. To the authors' knowledge, there is only one report investigating the association between

polymorphisms in *LTF* gene and dental caries (Azevedo et al., 2010). This study found an association of a polymorphism in the second exon of *LTF* gene with lower values of DMFT, as well as with higher levels of salivary flow. The same polymorphism failed to associate with localized aggressive periodontitis, but did associate with antibacterial activity against *S. mutans*, a main cariogenic bacterium (Velliyagounder et al., 2003).

To understand the molecular mechanisms of *LTF* gene expression and regulation, it is necessary to characterize its genetic regulatory regions in the promoter. The human *LTF* putative gene promoter presents nearly 1000 bp and several transcription factors binding sites have been involved in the positive or negative regulation of *LTF* gene expression and transcriptional activity (Fig. 3).

Previous analyses revealed that the first 85 bp 5' flanking region contain diverse transcription boxes, including TATA, Myb, CCAAT enhancer binding protein (C/EBP), estrogen receptors (ETs), and two sites called specific protein 1 (SP1), which influence *LTF* gene expression (Khanna-Gupta et al., 2000) and are almost perfectly conserved among several species (Zheng, et al., 2005). Sequence analysis of the *LTF* gene promoter region revealed that TATA box (position -28) is next to a high GC region and two SP1 binding sites (position -35/-85), consistent with that found in other species (Liu & Teng, 1991). Two previous studies suggested that TATA box in the mouse *ltf* gene promoter (Shi & Teng, 1999) and a SP1 binding site in porcine *ltf* promoter (Wang et al., 1998) may play a role in the regulation of gene expression. Deletion of the TATA box abolishes the mouse *ltf* promoter activity, whereas analysis of SP1/GC element suggested that they play a significant role in the basal activity of gene promoter. These two elements were

highly conserved across species and participate in the recruitment of the general transcription machinery (Pugh & Tjian, 1990). The C/EBP site, flanked by two SP1, has been implicated in myelopoiesis. Several members of the *C/EBP* family of transcription factors have been shown to be indispensable for normal development of the myeloid lineage and have been implicated in regulating gene expression in a variety of cell types (Darlington et al., 1998; Diehl, 1998). Additionally, the *C/EBP* family of proteins was found to interact functionally with SP1 to activate the *LTF* promoter at the transcriptional level (Khanna-Gupta et al., 2000). Putative binding sites for ETs were recognized within the first 85 bp of the *LTF* promoter, but preliminary analysis demonstrated that the ETs site bind an unidentified member of the *Ets* family of transcription factors and suggested that it does not play an important role in regulating *LTF* gene expression (Khanna-Gupta et al., 2000). Although the regulation of *LTF* gene expression has not been fully elucidated, it is known that *LTF* gene promoter is differentially regulated through multiple signaling pathways including steroid hormone. These regulatory elements are located in the region -340/-372. A composite Estrogen Response Element (ERE) is present and overlapping with a chicken ovalbumin upstream promoter tanscription factor (*COUP-TF*) and both are responsible for estrogen induced gene expression (Liu and Teng, 1992; Teng, 2006). In addition, human *LTF* gene contains a steroidogenic factor binding element (SFRE) located in -377/-394, that is an extended estrogen response element, which renders the human *LTF* gene extremely responsive to estrogen stimulation (Teng, 2006). It is suggested a role for GATA, that is a binding site for the erythroid growth factor, in the control of *LTF* expression during hematopoiesis (Johnston et al., 1992). Finally, TAACC, that

binds the CCAAT displacement protein (*CDP/cut*), located between -774/-778 is a silencer element involved in *LTF* expression during myeloid differentiation (Khanna-Gupta et al., 1997). The study by Zheng et al. (2005) showed that the sequences up to -543 are sufficient for *LTF* basal gene promoter activity, but the maximal promoter activity requires the sequences up to -1029 in mammary epithelial cells.

The purpose of this study was to characterize the putative promoter region of *LTF* gene aiming to identify polymorphisms which could affect on expression and biological functions, such as the iron-binding and bacteria-killing abilities of LTF, and then be associated with dental caries.

In this study, five oligonucleotide primer pairs were made to amplify all putative promoter region (+39 to -1143), which presents an abundance of identified transcription factor binding sites, in subjects with and without caries experience, intending to further associate variations in this region with caries susceptibility. Fifty samples were analyzed by high resolution melt (HRM) (LightCycler 480), which was able to detect different melting profiles in the sample. HRM appears to be a sensitive, robust mutation scanning technique that could significantly reduce the time and cost of screening for mutations/polymorphisms (Whittall et al., 2010). For the 50 students analyzed, 15 individual curves from 8 without and 7 with caries experience subjects were identified as outstanding by HRM and needed to be sequenced by MWG-Biotech. The sequences analyses revealed that no polymorphisms in the promoter region of *LTF* gene (+39/-1143 bp) were identified.

We examined the GenBank database (NCBI, 2010) for polymorphisms within the study promoter region and five gene sequence variations were found

[rs67994108 (position -41), rs28365893 (position -232), rs4637321 (position -420), rs35869674 (position -489), rs5848800 (position -696)]. However, no of them is validated by frequency. These findings are reinforced by Teng & Gladweel (2006) study, which reported a total of 7 SNPs in the human *LTF* gene promoter: at -261, -374, -401, -421, -1010, -1119 and -1261 positions, being only polymorphism -1010 (ATAT/-) frequent. In that study, 91 healthy donors of different ethnicities were used to search for polymorphisms in the exons and promoter region of *LTF* gene. In the position -261, the C to T change might affect the methylation status at the CpG dinucleotides. Furthermore, the SNPs at -374, -401, and -421 are clustered around hormone response elements and the GATA element and might affect transcription-factor interaction at these sites, influencing the expression levels of the LTF.

LTF gene is highly conserved among different species (Teng, 2002). The number of aminoacids encoded by 15 of the 17 exons in these species is identical, and in 12 intron–exon splice junctions, they have identical codon interruptions. Comparing the *LTF* gene promoters from different species, common characteristics are observed. The human, mouse, bovine, porcine, and bubaline promoters are very similar in terms of number and position of transcription boxes, especially between humans and mice (Teng, 2002). The fact of being extremely conserved among species and widely expressed in diverse human body tissues (Siebert & Huang, 1997) and body fluids (Teng et al., 1989) highlights LTF as an important functional protein involved in several aspects of body homeostasis. These aspects related to LTF properties may partially explain the failure in identifying gene variations in the hotspot region of the *LTF* regulation, in spite of the significant

sample size and genetic admixture of the Brazilian population, which could impact significantly biological functions. In this context, the regulation might be controlled more by different transcriptional factors (depending on the tissue) than by gene variations.

Common diseases are usually interpreted to be caused by the additive effects of several common variances. However, rare variations also could be playing a role in modulating the susceptibility of those complex diseases. Thus, if this is the case for caries, 100 chromosomes, which in general is considered a good opportunity to identify common variations (termed polymorphisms), may not be sufficient and sample should be significantly augmented.

Dental caries is a complex, multifactorial disease and many gene variations and gene-environment interactions may contribute to its outcome (Slayton et al., 2005). Thus, as LTF is considered a pleiotropic protein involved in different aspects of caries ethiopathology, the investigation of polymorphisms capturing the information of the gene as a whole may be desirable. In this context, future studies should include the analyses of tag SNPs, are a small number of polymorphisms in linkage disequilibrium (LD), which capture the information of other polymorphisms present in the same *bins* (LD blocks).

In summary, no polymorphisms were identified in the putative promoter region (+39 to -1143) of *LTF* gene. Further studies should be conducted, analyzing *bins* which may capture the whole gene information, to better understand the contribution of this gene in the caries ethiopathogenesis.

Table 1. Characteristics of the study population. The sample was composed of 50 unrelated, 12-year-old, both sexes.

Variables	G1 (n=25)	G2 (n=25)	*p* value*
Ethnic group	**n (%)**	**n (%)**	
Caucasian (46)	25 (100.0)	21 (84.0)	0.145
Afro-American (3)	0 (0)	3 (12.0)	
Asian (1)	0 (0)	1 (4.0)	
Gender	**n (%)**	**n (%)**	
Female (28)	16 (64.0)	12 (48.0)	0.254
Male (22)	9 (36.0)	13 (52.0)	

*Chi-square, $p<0.05$

Table 2. Sequence of oligonucleotide primers used for DNA amplification.

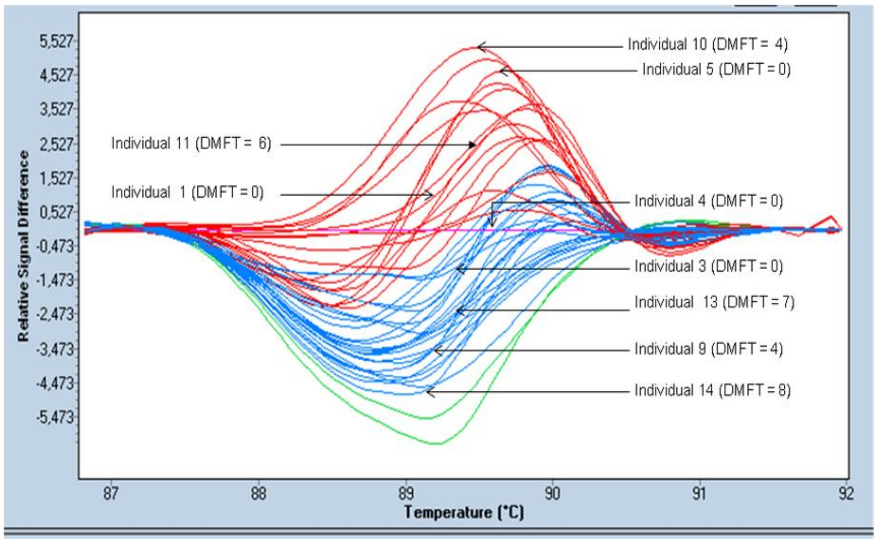
Primer	Primers' sequences	Region	Transcription Boxes
1	Sense 5-GAGGAACAGCAGGACGAG-3 Antisense 5-AGAGGAAAGCCAGCCTGC-3	+70 / -188	*TATA, Myb, SP1, C/EBP, Ets, SP1*
2	Sense 5-AGGCAGGACAGGACTCCAC-3 Antisense 5-AAGGTGCCTAGGAGCCAGTT-3	-142/-412	*ERE, COUP, GATA-1*
3	Sense 5-ATCGCCTTGACCTGTGAGAC-3 Antisense 5-CAAGGCTGGTTCCATGTTCT-3	-346/-653	*SFRE, COUP*
4	Sense 5-AGGGACCTCAGAGGGGAAT-3 Antisense 5-CGTAAATACATTCCCATGACACA-3	-605/-878	*TAACC*
5	Sense 5-AACAATCGCCATAAATGTCAG-3 Antisense 5-TGGATGTGAACTTAGCCCAAGAG-3	-810/ -1100	*TAACC*

Table 3. Reaction conditions for melting acquisition performed by Lightscanner and Light-Cycler 480.

Program / Cycles	**Temperature**
Pre-incubation / 01	Initial Heating - 95°C
Amplification / 45	Denaturation - 95°C Annealing – 68°C Final Extension - 72°C
High Resolution Melting / 1	Heating - 95°C Hybridation - 40°C Melting Acquisition - 65°C to 95°C
Cooling / 1	40°C

Table 4. Baseline characteristics of the fifteen (15) students classified as “cases” being further sequenced, being 8 without and 7 with caries experience (mean DMFT=6.28).

Variables	**G1 (n= 8)** **DMFT= 0**	**G2 (n=7)** **DMFT= 6.28±1.7)**
Ethnic group	**n (%)**	**n (%)**
Caucasian	8 (100)	7 (100)
Gender	**n (%)**	**n (%)**
Female	7 (87.5)	4 (57.14)
Male	1 (12.5)	3 (42.86)



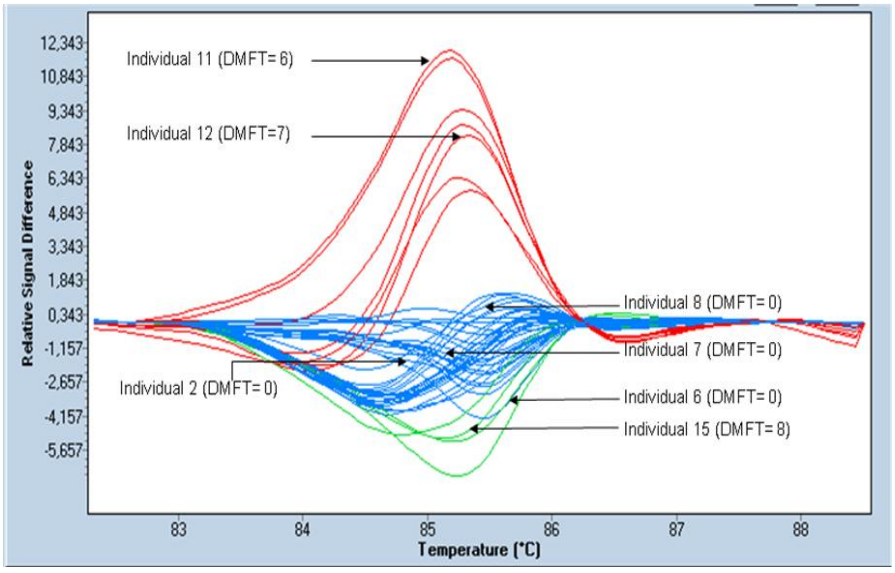


Fig. 1. Comparative sequence analysis of the promoter region amplified by polymerase chain reaction (PCR) whose melting outcome was performed in a single run on a LightCycler® 480 instrument (Roche Diagnostics, Mannheim, Germahy). Fifteen (15) individuals, being 8 without and 7 with caries experience (mean DMFT=6.28), were classified as “cases”, being further sequenced.

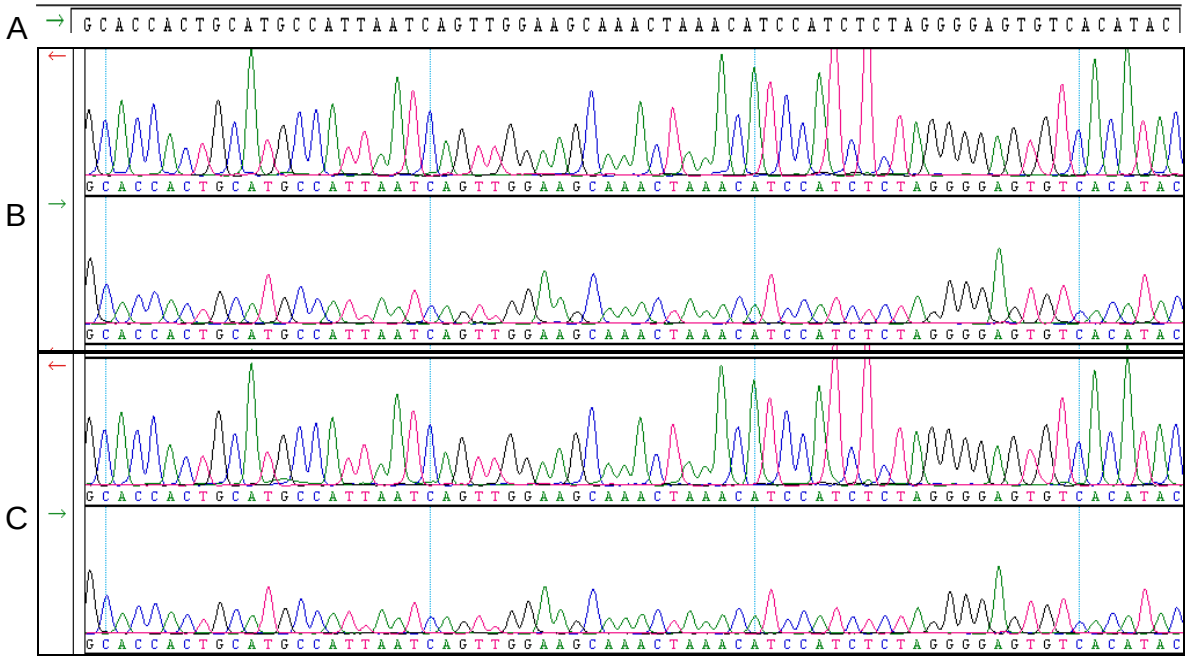


Fig. 2. Comparative sequence analysis of the promoter region amplified by primer 5. (A) Consensus sequence of the *LTF* gene, (B) individual 6 (DMFT=0), and (C) individual 11 (DMFT=6). There was not difference between the individual sequences of the promoter region.

ATGTCTGCGGTCTGGAGGCGACTTGGCAAACGAAGGCTC**T**GCCACTTGCGCCTGCCCTGCGCCCC**TTTATT**CAGG**GCTTTG**[1]**CCCCGCC** **-50**

TATA

CTG[2]**TTGCCCA**[3]**ATAGACACCCCTTCCC**[4]**TCCCCACTCCCC**[5]GCGGCCAGGTCTACTTGTTCCTTGAGGATCCAGGCTCCGAAAAGCCCTGA **-137**

A

GGCAGGACAGGACTCCACACCGGCTGCGAGAGGAAAGCCAGCCTGCACCTCACCTGTCCTGGTTCTGCCTGGCTGCTGCGATGTTCTTT **-226**

CTCTCCCACTAGTCTGCAAGCCCCTAGGAGTCGCAGCCCCCTGAGCCCTGTCCCAGCTCTGGTCTCAGTGCCTTCTAGAGGGCAGGACAC **-316**

AGAGCAGAGGGTCTTTACTTGA**AGATCGCCTTGACCT**[6]**GTGAGACT**[7]**GCTATCTTC**[8]TTCAGC**AGATGACCTTGA**[9]**AGGTG**[10]CCTAGGAG **-401**

B C

CCAGTTAGGGCAGGTGGATTTGCTTTCACCTTGGCTCAGCAGTGAGCTGAGAGTTGAAAAGATGACCCGACCACCCCCAGCAGCAGCCT **-490**

AGGGACAGCTGACAGAGAAAAGAGAGGCTGCCCAGAGTCTCAGGCAAGCTGCCCATGGGGTATCTCCCTGCCCTGCTGAGACCCACAA **-578**

TACAGCTCCCAGAAAACAGACAGGGACCTCAGAGGGGAATGGCCACCACCCAAGGCTGGTTCCATGTTCTTATTTGCCAATGAGAAAGAT **-668**

GCCCTATGGCTAAGATGCCCTACCACTTTTTTTTTTTTTTGTCTTTTTTTTCTCCATCTCCTCCTTCTTCTTTTTTGGTGTTGATAGTATTCAGTA **-765**

GGAAATGG**TAACC**[11]TCATTTTAACAATCGCCATAAATGTCAGATTTTAAAATTCAAAAGCTAGAATACTGCTTCTAAGAATACATCTCGTAA **-855**

D

ATACATTCCCATGACACACAGCACCCCAGGTACAAATGTTTCCAGGCACCACTGCATGCCATTAATCAGTTGGAAGCAAACTAAACATCCAT **-947**

CTCTAGGGGAGTGTCACATACAGCATGGTGCCTGTGGGACACATTGGGGGCCTCAGAAAGAATATCCTGTGTGTATGGATGTGAACTTAG **-1037**

CCCAAGAGTAACTAGGCAGGGCACAGTGGCTCATGCCTGTAATCCCAGCACTTTGGGAGGCCAAGATGGGAGGATCGCTTGAGCCCAGG **-1126**

AGTTCAAGACTAGCCTG **-1143**

Fig. 3. Promoter region (+39 to - 1143 bp) of the human *LTF* gene and transcription boxes: **A) Myb[1], SP1[2], C/EBP[3], ETs*[4]*** and **SP1*[5]*** (region –35 to –85) are involved in LTF expression during myeloid differentiation (Khanna-Gupta et al. 2000). **B) ERE[6], COUP[7]** and **GATA-1[8]** (region –340 to –372), a highly conserved estrogen response element (ERE) overlapping with a chicken ovalbumin upstream promoter (COUP) element (Liu and Teng 1992; Teng et al. 1992). **C) SFRE[9]** and **COUP[10]** (region – 377 to –394), an extended estrogen response element half site in addition to the *ERE*, which renders the human *LTF* gene extremely responsive to estrogen stimulation (Teng, 2006). **D) TAACC[11]**, a highly conserved silencing factor (–774 to –778) that binds the CCAAT displacement protein (CDP/cut) (Khanna-Gupta et al. 1997).

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Conclusão

CONCLUSÃO

Não foram identificados polimorfismos na região promotora do gene da LTF (região +39 / -1143). Estudos futuros deverão ser conduzidos analisando *bins* que capturem a informação do gene como um todo para melhor entender a contribuição deste gene na etiopatogênese da cárie dentária.

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Anexos

SALIVARY PARAMETERS, ORAL HEALTH HABITS, AND SOCIOECONOMIC ASPECTS AS RISK FACTORS FOR DENTAL CARIES IN 12-YEAR-OLD CHILDREN FROM A PRIVATE SCHOOL OF THE CITY OF CURITIBA, BRAZIL

Parâmetros salivares, hábitos de saúde bucal e aspectos socioeconômicos como fatores de riscos para cárie dental em crianças de 12 anos de idade em uma escola particular na cidade de Curitiba, Brasil.

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Abstract

OBJECTIVES: The aim of this study was to compare socioeconomic aspects, oral health habits and salivary parameters between 12-year-old students with and without caries experience. MATERIAL AND METHODS: A sample of 113 non-related 12-year-old subjects was selected in a private school of Curitiba-PR, Brazil, for a case-control study. They were divided into groups with and without caries experience through the DMFT index. The frequency of tooth brushing, use of dental floss, diet, frequency of dentist visit, fluoride use, dental plaque index, socioeconomic aspects and salivary parameters were evaluated. Data were analyzed by qui-square and Mann-Whitney. RESULTS: Results showed no statistical difference between the groups in relation to oral hygiene habits and socioeconomic status. It was observed an association between dental plaque presence and caries experience, although not significant ($p = 0.08$). A reduced salivary flow was highly associated with decay experience ($p = 0.00$). CONCLUSION: It was concluded that the salivary flow was an important factor to determine dental caries experience in a homogeneous group of children from a private school of Curitiba-PR. Studies on host response aspects may be desirable in this kind of populations.

Keywords: Dental caries; Risk factors; Salivary flow.

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Analysis of the association between lactotransferrin (LTF) gene polymorphism and dental caries

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ABSTRACT

Objective: The present study evaluated the association between lactotransferrin (LTF) gene polymorphism (exon 2, A/G, Lys/Arg) and dental caries. Material and Methods: A convenience sample of 110 individuals, 12 years old, was divided into: *group 1*, 48 individuals without caries experience (DMFT=0), and *group 2*, 62 subjects with caries experience (DMFT≥1). DNA was obtained from a mouthwash with 3% glucose solution, followed by a scrapping of the oral mucosa. After DNA purification, polymerase chain reaction (PCR), single strand conformation polymorphism (SSCP) was performed to access the study polymorphism. The LTF A/G (Lys/Arg) polymorphism had been previously reported as located in exon 1. Results: Allele 1 of the study polymorphism was associated with low DMFT index and showed a protective effect against caries experience (OR=0.16, IC=0.03-0.76, *p*=0.01). Conclusion: Lactotransferrin A/G (exon 2, Lys/Arg) polymorphism was associated with susceptibility to dental caries in 12-year-old students.

Key words: Dental caries, ocurrence. LTF. Gene polymorphism. Exon 2.

Artigo Submetido

Research Report

Multilevel Modelling of social and biological factors in dental caries

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Abstract

Background: Dental caries is a complex disease which has a multifactorial nature. Thus, an approach, considering factors that may influence disease at different levels and their integration, is desirable. Multilevel Modelling (MM) is a clustered analysis that considers variables involved in the disease outcome from the individual to the populational level. The aim of this study is to investigate, through MM, the combination of social and biological factors, grouped into hierarchical levels, with dental caries in 12 year-old students. **Methods:** The study evaluated 687 students, both sexes, from six public and six private schools from six different health districts of Curitiba, Brazil. The following parameters were evaluated: i) *individual level*: sex, ethnic group, socioeconomic status, oral health behavior (toothbrushing, flossing, fluoride use, diet, dental services access, frequency of dental visits), dental biofilm accumulation (plaque index), gingivitis, fluorosis, salivary parameters (flow rate, buffer capacity); ii) *school level*: type of school (public or private), oral health education, permission for sweeties consumption, and iii) *district level*: concentration of fluoride in water supply, and district socioeconomic conditions. The multilevel software MLwiN version 2.01 (Centre for Multilevel Modelling, Bristol, UK) was used to estimate the effects of individual, school and district variables on caries experience. **Results:** the main findings were that individual variables had a highly significant association with caries experience even in the presence of school and district levels (contextual variables). It was found that male sex negatively associated with caries experience. However, the interaction between male sex and no fluoride use was positively associated with caries experience. Lower socieconomic status, dental plaque accumulation, and fluorosis were significantly associated with caries experience. Nevertheless, the interaction between dental biofilm accumulation and fluorosis was negatively associated with caries experience. In addition, the interaction between no flossing and use of public dental services were associated with caries outcome. **Conclusion:** Considering the MM, it was observed that individual factors further than contextual variables were associated with caries experience in the evaluated students.

Keywords: dental caries, Multilevel Modelling, individual factors, contextual factors

PUCPR

Código do voluntário:

Ficha de Exame Clínico

Data:	Duplicata INTER:	Duplicata INTRA:	Cód examinador:
Nome:			Série e turma:
Cidade de nascimento:		Sexo:	Grupo Étnico (B, N, M, A, I):
Data de nascimento:			Idade em anos:
Endereço:			Bairro:
Telefone(s) para contato:			Cidade:
Escola: D.S.:			Tipo:

Índice de Placa (IP) Modificado

16V 12V 26P

46L 32L 36V

IP=

Características:

0 – **ausência** de placa
1 – **visualização** da placa com **uso de sonda** periodontal
2 – placa **clinicamente visível**
3 – placa **abundante**

CPO-D

17 16 15 14 13 12 11 21 22 23 24 25 26 27

47 46 45 44 43 42 41 31 32 33 34 35 36 37

CPO-D:

Códigos Diagnóstico CPO-D:

0 – Hígido
1- Cárie
2- Restaurado com cárie
3- Restaurado sem cárie
4- Perdido por cárie
5- Perdido por outras razões
6- Selado
7- Suporte para prótese, coroa protética ou faceta / implante
8- Dente não irrompido
9- Dente excluído
10- Mancha branca *ativa*
11- Mancha branca *paralisada*
12- Erosão
T – Trauma (fratura)

FLUOROSE

( ) SIM – grau e região:_____ ( ) NÃO

GENGIVITE

( ) SIM – grau e região:_____ ( ) NÃO

PERIODONTITE

( ) SIM – grau e região: _____ ( ) NÃO

ANÁLISE BIOQUÍMICA

Fluxo salivar:_____

Capacidade tampão:_____

pH salivar:_____

Análise em multinível de fatores sociais e biológicos associados à cárie

Data:	
Nome do responsável pelo aluno:	
Estado civil:	
Nome do aluno (a):	
Endereço:	
Bairro:	Cidade:
Escola onde o aluno(a) estuda:	

Questionário a ser Respondido Pelos Responsáveis dos Alunos

Número de pessoas que moram na sua casa (inclusive você): _____

Posse de itens (quantos)

ITEM	QUANTIDADE
Televisão em cores	
Rádio (vale também *microsystem* ou rádio tipo *walkman*)	
Banheiro	
Automóvel	
Empregada mensalista (que trabalha pelo menos 5 dias por semana)	
Aspirador de pó	
Máquina de lavar roupa	
Videocassete e/ou DVD	
Geladeira duplex (com duas portas)	
Geladeira simples (não duplex)	
Freezer	

Assinale com um "X" o <u>maior</u> grau de instrução da mãe e do pai do aluno

	MÃE	PAI
Analfabeto		
Primário incompleto		
Primário completo		
Ginasial incompleto		
Ginasial completo		
Colegial incompleto		
Colegial completo		
Superior incompleto		
Superior completo		

Primário – 1ª a 4ª série do 1º grau (ensino fundamental)
Ginasial – 5ª a 8ª série do 1º grau (ensino fundamental)
Colegial – 1ª, 2ª e 3ª séries do 2º grau (ensino médio)
Superior – faculdade

Perguntas sobre a saúde bucal do seu filho (a)

1) Seu filho (a) apresenta algum problema de saúde?
sim ( ) não ( ) Qual?_____

2) Seu filho (a) toma algum remédio?
sim ( ) não ( ) Qual?_____

3) Seu filho (a) tomou antibiótico nos últimos três meses ? (exemplos de antibióticos: penicilina, amoxicilina)
sim ( ) não ( )

4) Quantas vezes seu filho (a) escova os dentes por dia?
nenhuma vez ( ) uma vez ( ) duas vezes ( ) três vezes ( ) quatro ou mais vezes ( )

5) Seu filho (a) usa fio dental?
sim ( ) não ( )

5.1) Caso a resposta anterior tenha sido **sim**, relate quando seu filho (a) usa fio dental:
todos os dias ( ) de vez em quando ( )

6) Quantas vezes seu filho (a) ingere açúcar (salgadinhos, bolachas, lanchinhos), por dia, fora das refeições?
nenhuma vez ( ) até 3 vezes ( ) mais de 3 vezes ( )

7) Quantas vezes ao ano seu filho (a) vai ao dentista?
nenhuma vez ( ) 1 vez ( ) 2 vezes ou mais ( )

8) Seu filho (a) freqüenta:
dentista particular ( ) dentista do Posto de Saúde ( )

9) Seu filho (a) usa flúor?
sim ( ) não ( )

9.1) Caso sua resposta anterior tenha sido **sim**, relate quando seu filho (a) utiliza flúor:
todos os dias ( ) de vez em quando ( ) só no dentista ( )

9.2) Qual forma seu filho (a) utiliza o flúor:
bochecho ( ) gel ( ) verniz ( )

10) Seu filho (a) bebe água **mineral** com freqüência?
sim ( ) não ( )