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Análise da Associação de Variáveis Clínicas e Polimorfismos no Gene da Lactotransferrina (*LTF*) com Condições Bucais Complexas

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Análise da Associação de Variáveis Clínicas e Polimorfismos no Gene da Lactotransferrina (*LTF*) com Condições Bucais Complexas

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.

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Dedicatória

A **Deus**, agradeço pela minha familia, por todas as pessoas presentes em minha vida, pelas oportunidades de melhorar e aprender com meus erros e, enfim, de realizar os meus sonhos.

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Tenho Tanto Sentimento

Tenho tanto sentimento
Que é frequente persuadir-me
De que sou sentimental,
Mas reconheço, ao medir-me,
Que tudo isso é pensamento,
Que não senti afinal.

Temos, todos que vivemos,
Uma vida que é vivida
E outra vida que é pensada,
E a única vida que temos
É essa que é dividida
Entre a verdadeira e a errada.

Qual porém é a verdadeira
E qual errada, ninguém
Nos saberá explicar;
E vivemos de maneira
Que a vida que a gente tem
É a que tem que pensar.

Fernando Pessoa

SUMÁRIO

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RESUMO

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A lactotransferrina (LTF) é uma glicoproteína presente em vários tecidos e fluidos do organismo. Diversos artigos descrevem a sua importância como agente antimicrobiano, antiinflamatório, indutor de crescimento ósseo e envolvido na resposta imunológica do hospedeiro, sendo extremamente conservada entre as espécies. O foco deste estudo foi a análise de polimorfismos (tag SNPs) no gene da LTF (*LTF*), que capturam a informação do gene como um todo em termos de desequilíbrio de ligação (DL), e sua associação com a suscetibilidade a condições buco-dentais complexas: i) doença periodontal crônica (DP), ii) perda de implante e iii) cárie dentária.

A cárie e as doenças periodontais representam as maiores causas de perda dentária no Brasil e no mundo até os dias de hoje, e o tratamento com implantes dentais osseointegráveis é considerado o tratamento de eleição para a reposição de dentes perdidos, em termos funcionais e estéticos. A cárie e as periodontites são doenças infecciosas complexas, e fatores que aumentam o risco às periodontites em adultos podem contribuir para o insucesso de implantes dentais. A LTF apresenta relevante papel na modulação imuno-inflamatória, através da liberação de citocinas a partir de células do sistema imune, além da regulação de osteoblastos e osteoclastos, modulando a formação/destruição óssea, podendo interferir negativamente com a progressão das periodontites e com a osseointegração dos implantes dentais. Na saliva, a LTF é um exemplo de proteína que possui atividade antibacteriana, modulando a microbiota cariogênica e periodontopatogênica bucal.

O objetivo do presente estudo foi investigar a associação de polimorfismos no gene *LTF* (tag SNPs) e variáveis clínicas com a suscetibilidade à doença periodontal crônica, à perda de implantes dentais osseointegráveis e à cárie dentária.

A amostra de pacientes com e sem doença periodontal crônica apresentou 253 pacientes (média=44,9±12,4 anos), 135 pacientes com e 118 sem DP. A amostra de

implantes compôs-se de 278 amostras (média= 51,7±11,3 anos), sendo 184 indivíduos sem perda de implantes e 94 com a perda de pelo menos um implante. A amostra de cárie foi composta por 677 escolares de 12 anos; foram selecionados aproximadamente 30 escolares com experiência de cárie (CPOD≥1) e 30 indivíduos sem experiência de cárie (CPOD=0) de seis escolas públicas e seis particulares de Curitiba. Foram analizados 3 tag SNPs (rs11716497, rs2073495 e rs6441989), utilizando a técnica de *high resolution melting* (HRM), com o uso de sondas híbridas, simultaneamente à reação em cadeia da polimerase (PCR).

As frequências alélicas dos 3 tag SNPs estiveram em Equilíbrio de Hardy-Weinberg. Parâmetros clínicos e genéticos foram comparados entre os grupos por meio de análises univariadas e multivariadas de regressão logística, cujo nível de significância foi $p<0,05$. Na amostra de doença periodontal crônica, as variáveis clínicas associadas com a doença foram: doença renal crônica, proteína C reativa, mobilidade dentária e idade. No entanto, nenhum dos três tag SNPs foi associado à DP. Os parâmetros clínicos associados com a perda de implante dental foram: tratamento médico, reposição hormonal, edentulismo, número de implantes colocados, índice de placa, índice de cálculo e mobilidade dentária. Novamente, nenhum dos três tag SNPs foi associado à perda de implantes dentais. Na amostra de cárie dentária, foi encontrada associação do alelo A do tag SNP rs6441989 com a proteção contra a cárie dentária na presença das variáveis clínicas placa e gengivite.

Concluiu-se que variáveis clínicas específicas foram associadas às três condições bucais complexas; porém, apenas o tag SNP rs6441989 foi associado com a cárie dentária.

ABSTRACT

ABSTRACT

Lactotransferrin (LTF) is a glicoprotein present in several tissues of the organism. Many articles describe its importance as an antimicrobial, antiinflammatory agent, bone growth factor and it is involved in the host immune system, being extremely conserved among the species. The focus on this study is to analyze polymorphisms (tag SNPs) on *LTF* gene, providing information on bins, representing the gene as a whole as means of linkage disequilibrium (DL), and their association with the susceptibility to oral complex conditions: i) chronic periodontal disease (CP), ii) dental implant loss, and iii) dental caries.

Caries and periodontal diseases represent the main cause of tooth loss in Brazil and in the world until nowadays, and osseointegrated dental implants have been considered the treatment of choice for tooth loss, in terms of aesthetics and functionality. Caries and periodontitis are complex infeccious disease, and factors that elevate the risk to periodontitis in adults might contribute to dental implant failure. LTF presents relevant role on immune-inflammatory modulation, influencing cytokine flow through cells of the immune system, besides regulating osteoblasts and osteoclasts modulating bone formation/destruction, and may interfere negatively in periodontitis progression and dental implant osseointegration. In saliva, LTF is an example of multifunctional protein acting as antibacterial, modulating cariogenic and periodontopatogenic microbiota.

The aim of this study was to investigate the association of polymorphisms on *LTF* gene (tag SNPs) and clinical variables with the susceptibility to chronic periodontal disease, dental implants failure and dental caries.

The sample of patients with and without chronic periodontal disease was composed of 253 patients (mean=44.9±12.4 years), 135 patients with and 118 without CP. Dental implant study was composed of 278 samples (mean= 51.7±11.3 years), being 184 individuals without implant loss and 94 with at least one implant loss. Caries

sample was composed of 677 12-yr-old students; selected aproximately 30 scholars with caries experience (DMFT≥1) and 30 individuals without caries experience (DMFT=0) from six public and six private schools in Curitiba. Three tag SNPs (rs11716497, rs2073495 and rs6441989) were analyzed using *high resolution melting* (HRM) technique, with hybrid probe, simultaneously to polymerase chain reaction (PCR).

Allelic frequencies of the 3 tag SNPs were in Hardy-Weinberg Equilibrium. Clinical and genetic parameters were compared between the groups by univariate and multivariate logistic regression analysis, by means of significance of $p<0.05$. In chronic periodontal disease sample, the clinical variables associated with the disease were: chronic renal disease, C-reactive protein, dental mobility and age. However, none of the three tag SNPs were associated with CP. Clinical parameters associated with dental implant failure were: presence of medical treatment, hormonal reposition, edentulism, number of placed implants, plaque index, calculus index and dental mobility. None of the three tag SNPs were associated with the loss of dental implants. There was an association of allele A of tag SNP rs6441989 with protection against dental caries also in the presence of the clinical variables plaque and calculus.

In conclusion, specific clinical variables were associated with the three oral complex conditions; nevertheless, only tag SNP rs6441989 was associated with dental caries.

INTRODUÇÃO

1. INTRODUÇÃO

1.1 Lactotransferrina

1.1.1 A proteína lactotransferrina

A lactotransferrina (LTF) foi descoberta no leite humano em 1960 por Montreuil e colaboradores. É uma proteína ligante de ferro, pertencente ao grupo das transferrinas (Naot et al., 2005). A LTF 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 (Fig. 1). Ambos os lobos possuem a mesma característica estrutural e são subdivididos em dois domínios α / β, separados por um peptídio de conecção (Baker et al., 2004). Quando clivada, a LTF libera um peptídeo chamado lactoferricina (Lfcin) contendo 25 aminoácidos. Este se liga aos lipopolissacarídeos de bactérias Gram- e Gram+, o qual é transportado pela membrana citoplasmática aumentando sua permeabilidade, provocando seu rompimento (Orsi, 2004).

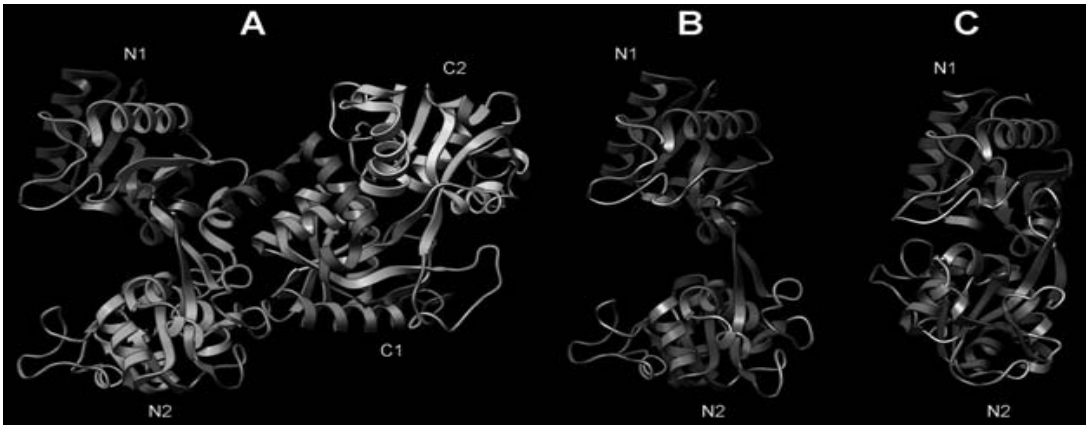

Fig. 1. A- Estrutura da Lactotransferrina humana: o polipeptideo apresenta 2 lobos globulares, o N e C, unidos por uma alfa hélice. B- Conformação da molécula sem ferro. C- Conformação contraída, quando está saturada com ferro.

Fonte: Legrand et al., 2008.

Glândulas exócrinas humanas secretam variada quantidade de LTF (Teng, 2002) em diferentes órgãos e em diferentes condições fisiológicas, sendo também secretada pelos grânulos secundários dos neutrófilos frente a processos inflamatórios (Wong et al., 2009). É encontrada principalmente no colostro (2 a 7 µg/ml) (Ward & Connely, 2004) e, em menor quantidade (0,4 a 2 µg/ml), em fluidos do organismo, como o sangue, a saliva, a lágrima, o sêmen, o suor, o leite e secreções nasais (Teng, 2002; Ward & Conneely, 2004). A concentração de LTF pode chegar a 200 µg/ml, sendo que a partir de 100 µg/ml acarreta fortes alterações nas funções da proteína e interfere em processos complexos, como o cessar da osteoclastogênese (Cornish, 2004).

A LTF tem capacidade de ligar-se, estável, mas reversivelmente, a dois átomos de ferro junto com dois íons bicarbonato através de quatro resíduos presentes em sua estrutura (2 tirosina, 1 asparagina e 1 histidina), os quais fornecem três cargas negativas para balancear com as três positivas do ferro (Legrand et al., 2008). A propriedade de ligar íons ferro faz com que essa proteína possua propriedade bacteriostática, pois limita a disponibilidade de ferro para as células bacterianas (Wong et al., 2009). Os sítios ligantes de ferro, localizados nos lobos N e C, possuem a propriedade de ligar-se mais fortemente ao receptor da bactéria, impedindo a ligação desta com o ferro (Shi et al., 2000). Inibe ainda o crescimento de *Porphyromonas gingivalis*, uma importante bactéria envolvida na doença periodontal e periimplantite, removendo ferro de sua superfície (Shi et al., 2000). Além disso, parece ser uma importante proteína coadjuvante no processo da cárie, pois inibe a adesão do *Streptococcus mutans* no biofilme dental ao aumentar a motilidade deste, uma importante bactéria responsável pela formação de placa dental causativa da cárie (Berlutti et al., 2004).

A LTF é uma proteína multifuncional, sendo, além de antibacteriana/bactericida, antiviral, antifúngica, antiinflamatória (Brock, 2002), antineoplásica (Wolf et al., 2007), também osteogênica (Naot et al., 2005). O efeito antiviral está relacionado à atuação

contra o vírus do herpes simples, inibindo o ciclo de replicação viral (Välimaa et al., 2009) e o vírus da imunodeficiência humana (HIV), aumentando a produção de linfócitos T CD4+, responsáveis por grande parte da resposta imune humana (Zucotti et al., 2006). A LTF inibe o crescimento de *Candida albicans*, que normalmente está presente em pacientes imunodeprimidos e portadores de próteses odontológicas (Lupetti et al., 2003). Está presente na resposta imuno-inflamatória inata, estimulando a produção de citocinas (Fischer et al., 2006), as quais são responsáveis por coordenar a resposta celular humana, atuando na maturação e ativação de neutrófilos e macrófagos, podendo sua deficiência causar supressão no sistema imunológico, e seu excesso, uma exacerbada resposta imune (Son et al., 2002). Influencia a apresentação de antígenos das células bacterianas para o recrutamento dos linfócitos T (Actor et al., 2009) e está envolvida na postergação da apoptose dos neutrófilos de 6 para 9 horas, aumentando o seu tempo de ação (Wong et al., 2009). Os leucócitos polimorfonucleares são ricos em LTF (Wong et al., 2009), que age como fator de proteção contra diversas infecções (Orsi, 2004; Ward & Conneely, 2004), sendo considerada também marcador de inflamação gastrointestinal (Hayakawa et al., 2009) e doença coronariana (Videm et al., 2007). A LTF é considerada um supressor tumoral (Ghosh et al., 2008), prevenindo danos no DNA, influenciando a ativação do gene supressor celular *TP53* (Sacharczuk et al., 2005), sendo considerada um agente quimioterápico (Rodrigues et al., 2009), inibindo o crescimento de células oncogênicas, impedindo sua progressão do ciclo celular na fase G1 (Wolf et al., 2007).

A LTF age nas células mesenquimais da linhagem C2C12, sinalizando a formação de osteoblastos, condroblastos e inibindo a formação de osteoclastos; sendo, portanto, considerada um fator de crescimento ósseo (Yagi et al., 2009). LTF foi encontrada em altas concentrações em locais de periimplatite (Hultin et al., 2002), fluido crevicular em pacientes com gengivite e ausente em tecidos gengivais sadios

(Eberhard et al., 2006), sendo, portanto, um importante marcador de doença periodontal/implantar.

1.1.2 O gene da lactotransferrina

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), como apresentado na figura 2.

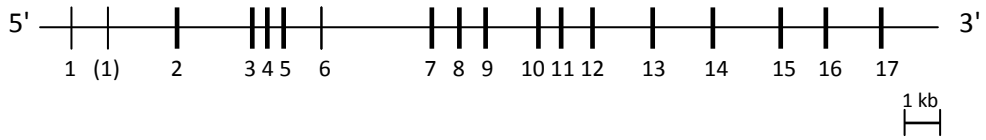

Fig. 2. Gene da LTF com indicação dos 17 éxons. *Fonte*: Teng, 2002.

Devido à sua ampla distribuição no organismo (em fluidos e em tecidos) e ao caráter antiinflamatório, imunológico, antimicrobiano e osteogênico, a LTF está envolvida no metabolismo corporal como um todo. Portanto, polimorfismos (variações na sequência de DNA, resultando em alelos comuns na população) presentes em seu gene podem: i) modificar a codificação de sua proteína (composição dos aminoácidos, no caso de éxons) e ii) alterar a taxa de expressão (se em promotor), influenciando a suscetibilidade ao desenvolvimento da cárie dentária, da doença periodontal e da perda de implantes dentais.

Polimorfismos de único nucleotídeo (SNPs) no gene da *LTF* têm sido descritos e associados à queratite por herpes simples (Keijser et al., 2008) e periodontite agressiva (Velliyagounder et al., 2003; Jordan et al., 2005; Wu et al., 2009). Foi observado que em vários tipos de câncer, a região gênica da LTF esteve subregulada ou tinha sido deletada (Shaheduzzaman et al., 2007; Giuffrè et al., 2007). Um polimorfismo (A/G, rs1126478) no segundo éxon do gene *LTF*, foi identificado como causador da substituição de um aminoácido lisina (Lys) por arginina (Arg) na posição 29, e foi também associado a um fluxo salivar elevado e proteção contra a 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).

1.2 Condições bucais complexas

1.2.1 Doença periodontal crônica

A doença periodontal crônica (DP) ou periodontite representa um grupo de doenças inflamatórias que afetam os tecidos de suporte dos dentes. De acordo com a *American Academy of Periodontology* (2005), 50% dos adultos têm ao menos a forma moderada de doença periodontal. No Brasil, de acordo com dados do Ministério da Saúde (2003) 50% da população entre 35 e 44 anos apresentam algum tipo de doença periodontal.

A DP resulta da interação de espécies bacterianas (presentes no biofilme ou placa dental) e seus subprodutos com componentes da resposta imuno-inflamatória do hospedeiro (Armitage, 1999). A reação inflamatória na periodontite pode resultar na formação de uma bolsa periodontal ao redor do dente afetado, por destruição do ligamento periodontal e osso alveolar, que caracterizam os tecidos de suporte do dente (periodonto de sustentação).

Aspectos da resposta do hospedeiro à presença bacteriana, a qual tem forte envolvimento no início e progressão da periodontite, podem ser geneticamente determinados (Hart, 1994). Nibali (2009), em um estudo sobre infectogenômica (interação entre variações genéticas e composição da microbiota) sugeriu que a doença periodontal esteja sendo modulada por fatores genéticos, alterando a microbiota subgengival causadora da mesma.

A DP tem sido considerada uma complicação em diversas doenças sistêmicas, como é o caso da doença renal crônica (DRC) (Borawski et al., 2007) e foi relatado que a sua prevalência e severidade estão aumentadas nessa população (Kshirsagar et al., 2005). A existência de DP representa foco de infecção aos pacientes renais

crônicos, os quais são extremamente suscetíveis a infecções de modo geral (Sowell, 1982). Pacientes com DRC necessitam de um cuidado mais intensivo com a saúde bucal, pelo fato de a DP ter sido sugerida não apenas como fator de morbidade, mas de mortalidade para esta população. Isso devido a níveis de proteína C reativa, importante mediador de inflamação, terem sido observados significativamente aumentados em pacientes renais com DP, o que pode influenciar o risco de morte por doenças cardiovasculares (de Souza et al., 2007).

1.2.1.1 Doença periodontal e lactotransferrina

A LTF, por ser uma proteína ligada à resposta inflamatória, imunológica e bactericida, está envolvida com a resposta do hospedeiro frente a condições propícias ao desenvolvimento de doença periodontal. Em um estudo com biópsias em gengiva marginal livre de tecidos sadios e inflamados, níveis elevados de RNAm e proteína LTF foram encontrados no fluido crevicular nos indivíduos com inflamação local (Eberhard et al., 2006). Em pacientes apresentando periodontite refratária, os autores suspeitaram de genes que pudessem estar modulando o difícil tratamento da doença; foi realizada extração de RNA nos tecidos gengivais dos pacientes. O gene da lactotransferrina esteve sobreexpresso (Kim et al., 2006).

O polimorfismo rs1126478 (Lys/Arg) no segundo éxon do gene da *LTF* foi associado à periodontite agressiva localizada (Velliyagounder et al., 2003; Wu et al., 2009) e outro polimorfismo na posição +11 foi associado a periodontite agressiva na população afro-americana causando uma substituição do aminoácido treonina/alanina (Jordan et al., 2005).

1.2.2 Implante dental

Com o aumento da longevidade, o número de dentes perdidos tem aumentado (Nishimura & Garrett, 2004), e de acordo com a *National Institute of Dental and*

Cranioafacial Research (Albandar et al., 1999), mais de um quarto dos adultos com mais de 70 anos tem perdas dentárias, o que causa uma grande repercussão na saúde. Com o desenvolvimento de novas tecnologias, surgiu o implante dentário, melhorando a qualidade de vida, o lado emocional e a saúde das pessoas.

A técnica de implante dentário tem uma alta porcentagem de sucesso (89% a 95%). Considerando que em torno de 10 milhões de pessoas são submetidas a cirurgias para colocação de implantes por ano mundialmente, o número de insucessos torna-se significativo (Hospitalar, 2007).

A perda do implante pode ocorrer logo após a realização da cirurgia, enquanto inicia-se a osseointegração, ou após esta etapa, quando o implante está consolidado com o osso (Moy et al., 2005). Várias são as causas que podem ocasionar a perda do implante, entre elas estão as precoces, como fumo (Feloutzis et al., 2003), idade avançada, radiação, terapia com estrogênio na pós-menopausa, diabetes (Moy et al., 2005), e as tardias, como sobrecarga oclusal (Crupi et al., 2004) e periimplantite (Carcuac & Jansson, 2010).

Apesar da identificação de inúmeros fatores relacionados com a perda de implantes dentais osseointegráveis, há casos, nos quais a perda ocorre sem causa clínica aparente. Espósito et al. (1998) relataram que fatores de risco relacionados ao hospedeiro, como qualidade óssea, parafunção, alteração no processo de cicatrização e saúde clínica possam estar envolvidos na suscetibilidade à perda desses implantes.

1.2.2.1 Implante dental e lactotransferrina

Stanford (2010) realizou uma revisão com 10 estudos longitudinais publicados independentemente e avaliou a presença de periimplantite e o sucesso do implante dental em pacientes com e sem história de doença periodontal. O autor concluiu que pacientes com problemas periodontais têm uma chance maior de perder implantes dentários do que indivíduos sem história de periodontite. A perda de implante dental

pode também ter como fator agravante e determinante a resposta do hospedeiro, sendo, portanto, a proposta deste estudo a investigação da associação desta condição complexa com polimorfismos no gene da LTF, uma vez que não há estudos investigando polimorfismos genéticos e sua associação com falhas de implantes.

1.2.3 Cárie dentária

A prevalência da cárie dentária, doença de natureza infecciosa e multifatorial (Fejerskov, 2004), tem reduzido significativamente na América Latina e no Brasil (Bonecker & Cleaton-Jones, 2003; Narvai et al., 2006). Apesar desta redução, em 2006, a previsão de gastos com a saúde bucal no Brasil foi de quase 2 bilhões de reais, os quais triplicaram de um ano anterior (Ministério da saúde, 2006). Os fatores associados ao fenômeno de queda dos níveis da doença estão principalmente relacionados a aspectos socieconômicos (Johnson, 2004; Narvai, 2006), à maior disponibilidade de fluoretos na água de abastecimento (Johnson, 2004), à presença de flúor nos dentifrícios (Johnson, 2004) e ao maior grau de instrução sobre higiene bucal (Milgron, 2001; Reisine & Psoter, 2001). Entretanto, grupos de crianças continuam apresentando alta atividade de cárie. Estima-se que 20 a 25% das crianças e adolescentes concentrem 60 a 80% da prevalência de cárie da população (Kaste et al., 1996).

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 e comportamentais (Peres et al., 2000) também interagem neste 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). Neste 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).

1.2.3.1 Carie dentária e lactotransferrina

Com relação à composição da saliva, diversas proteínas salivares interferem no biofilme, pois possuem ação antimicrobiana (van Nieuw Amerongen et al., 2004). Entre as diversas proteínas salivares, é de especial interesse a LTF.

A LTF presente na saliva desempenha um papel importante com ação antibacteriana: atividade contra o *Streptococcus mutans* (Oho et al., 2002; Berlutti et al., 2004). Ela pode influenciar a etiopatogênese da cárie dentária, uma vez que pode modular a agregação e o desenvolvimento do biofilme dental, inibindo a adesão de *S. Mutans*, um dos principais agentes etiológicos da cárie (Oho et al., 2002; Berlutti et al., 2004).

Uma associação significante entre o índice de superfícies cariadas e a concentração de LTF salivar foi encontrada (Sikorska et al., 2002). Apenas um estudo investigou a associação entre polimorfismo no gene *LTF* e a suscetibilidade à cárie (Azevedo et al., 2010), associando um alelo do polimorfismo rs1126478 no éxon 2 do gene *LTF* com proteção contra a experiência de cárie e aumento do fluxo salivar. Mais estudos para investigar a associação com polimorfismos neste gene poderão elucidar parte da modulação gênica envolvida.

1.3 Análise genética de traços complexos

Um(a) doença/traço complexa(o) é uma condição do hospedeiro que resulta da interação entre fatores de risco ambientais e/ou comportamentais e uma ou mais

variações genéticas, geralmente apresentando múltiplos *loci* associados (Muhle et al., 2004). Geralmente, a análise genética de traços complexos pode abranger estudos observacionais, como: i) agregação familial (Garn et al. 1976); ii) clusterização (Montes et al., 2009); iii) análise em gêmeos (Michalowicz et al., 2000, Bretz et al. 2005), a qual identifica a concordância entre pares de gêmeos monozigóticos e dizigóticos, e iv) análise de segregação complexa (ASC) que identifica por modelos matemáticos o melhor modelo que explica o padrão de herança de um dado traço complexo, através de análise do *pedigree* de famílias multigeracionais (Werneck et al., 2010). Contudo, embora esses estudos consigam identificar um componente genético, são limitados em determinar quantos e quais são os genes envolvidos no controle da suscetibilidade ao traço ou doença em questão, tornando necessários estudos de ligação ou de associação.

Estudos de ligação consistem em estratégias de identificação de regiões cromossômicas, as quais co-segregam com o fenótipo, objetivando a localização física aproximada do gene causal (Weeks & Lange, 1992). Como identificam regiões genômicas extensas, apresentam como limitações: i) a dificuldade de detectar genes com efeito baixo ou moderado sobre o fenótipo, e ii) a complexidade na identificação do gene causal exato.

Estudos de associação baseiam-se na comparação das frequências alélicas de um marcador genético entre indivíduos afetados e não-afetados. Pode ser realizado em famílias (onde o alelo que não é transmitido com o fenótipo representa o alelo controle) ou em nível populacional (caso-controle) (Lander & Schork, 1994). Uma das desvantagens de um estudo caso-controle baseado em população é a presença de estratificação ou miscigenação (Mayeux et al., 2005). Porém, a vantagem principal reside no fato de que alguns traços complexos são prevalentes em populações mais velhas, sendo o recrutamento de famílias um fator limitante. Nesse contexto, é

recomendável o desenho caso-controle baseado em populações; porém, a replicação em uma população independente é mandatória.

Genes candidatos podem ser escolhidos com base funcional e posicional. Genes candidatos funcionais são aqueles que codificam mediadores que modulam aspectos relevantes dos mecanismos fisiopatológicos da doença ou traço complexo. Genes candidatos posicionais são aqueles escolhidos com base nos resultados de análises de ligação.

O presente estudo é transversal de associação caso-controle baseado em populações, que analisa um gene candidato funcional, o gene da lactotransferrina (*LTF*), o qual desempenha papel relevante na fisiopatogênese das três condições complexas acima mencionadas. Em termos da presença de polimorfismos, foi realizada uma análise física, utilizando tag SNPs, que podem capturar a informação do gene como um todo, na qual um tag SNP representa todos os outros polimorfismos em desequibíbrio de ligação (DL) de um *bin*. *Bin*, portanto, representa um conjunto de SNPs herdados em bloco (tag SNPs), por estarem em DL. A estratégia de análise de *bins* representa uma abordagem de satisfatório custo-benefício, diminuindo os recursos financeiros e tempo necessários para genotipagem.

OBJETIVO

2. OBJETIVO

2.1 Objetivo geral

O objetivo do presente estudo foi investigar a associação de variações genéticas (tag SNPs) do gene *LTF*, na presença de variáveis clínicas, com a doença periodontal crônica, a perda de implante dental e a cárie dentária.

2.2 Objetivos específicos

2.2.1 Definir os *bins* (blocos de desequilíbrio de ligação) do gene *LTF* nas amostras das três condições bucais complexas.

2.2.2 Analisar a associação entre tag SNPs e as três condições bucais complexas.

2.2.3 Investigar a associação de variáveis clínicas com as três condições bucais complexas.

ARTIGO 1

3. ARTIGO 1

Chronic kidney disease and age, but not *LTF* gene polymorphisms, are associated with chronic periodontal disease

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Abstract

Chronic periodontal disease (CP) has been considered a worldwide problem that impacts local and systemic aspects of human health. It causes inflammation, decrease of tooth attachment level and may lead to tooth loss. Chronic kidney disease (CKD) is a progressive systemic inflammatory disorder characterized by the destruction of the nephrons (kidney's functional units). CKD patients are more prone to infections and CP may be exacerbated in those patients. Since lactotransferrin (LTF) is a crucial molecule involved in the immune-inflammatory response, the aim of the study is to analyze the potential association between polymorphisms (tag SNPs) in *LTF* gene, which may represent the variability of the whole gene, and susceptibility to CP in patients with and without CKD. Thus, 253 patients were selected: i) control group (n=118) individuals without CP (66 without and 52 with CKD), and ii) test group (n=135) individuals with CP (58 without and 77 with CKD). Along with clinical parameters, three representative *LTF* tag SNPs (rs6441989, rs2073495, rs11716497) were genotyped and results were evaluated using univariate analysis and multivariate logistic regression model ($p<0.05$). No statistically significant association between genetic variations and CP was observed. Chronic kidney disease ($p=0.019$), C-reactive protein ($p=0.023$), dental mobility ($p=0.008$), and age ($p=0.005$) were associated with CP. Clinical, but not genetic variables were found associated with CP. In this context, subjects presenting systemic inflammatory diseases, such as CKD, and older individuals should be carefully handled once they can experience a more intense CP outcome.

Introduction

Chronic periodontitis (CP) is a worldwide health problem, involving not only social, but economical aspects. Billions are directed to buccal treatment each year, as an effort to diminish the hazard it causes on health status. It has been related as one of the main disease prompter of tooth loss (Thorstensson & Johansson, 2010). It is a complex disease depending on the interaction between environmental, microbiologic agents and host factors, being influenced by genetic modulation (Abu-Saleh, 2010). Several epidemiologic studies identified some risk factors that may be aggravating its outcome: smoking (Moimaz et al., 2009), estrogen deficiency (Buencamino et al., 2009), systemic diseases such as diabetes mellitus and renal disease (Kaur et al., 2009; Dag et al., 2010), and aging (Huttner et al., 2009). The main pathogens in CP patients' biofilm are *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* (Ardila et al., 2010). While the biofilm determines the initiation of disease, aspects of host inflammatory response influences its severity and progression (Ohlrich et al., 2009). Moreover, chronic periodontitis has been implicated as a complication for renal diseases´ outcome (Fisher & Taylor, 2009).

Chronic kidney disease (CKD) is a progressive inflammatory disorder characterized by the destruction of the nephrons (kidney's functional units). It might be the result from other diseases as well its consequent metabolic, hydroelectrolytical and immunological disequilibrium might contribute to the severity of other pathologies (Proctor et al., 2005). Patients with CKD are considered more prone to infections than the general population, and its complications (metabolic, immunological and infectious) represent a great challenge in the clinical management of this population. Increased levels of C-reactive protein (CRP), a classical serum marker for systemic inflammatory diseases, were recently observed to be more elevated in CKD individuals presenting periodontitis; being the latter suggested as a predictive mortality risk factor for CKD patients by increasing the risk for cardiovascular complications (de Souza et al, 2007).

Lactotransferrin (LTF) is a multifunctional protein, present in the second granules of neutrophils and most host fluids (Rodrigues et al., 2009) is involved in systemic and local modulatory effects. LTF presents a tumor supression activity (Parodi, 2007), modulates host immune-inflammatory response, displays bactericidal activity (Pierce et al., 2009), controls iron homeostasis (Rodrigues et al., 2009), and acts as a bone growth factor (Yagi et al., 2009) stimulating osteoblast proliferation and inhibiting osteoclast activity (Guo et al., 2009). In local response, LTF binds to seric Fe^{3+} and locks available iron resources, decreasing its disponibility to bacteria, acting as bacteriostatic (Arslan et al., 2009), also playing a role against *Porphyromonas gingivalis*, important bacteria involved in periodontitis progression (Wakabayashi et al., 2010). Elevated levels of LTF were found in smokers periodontal sites (Nishida et al., 2008) and in refractory periodontitis sites (Kim et al., 2006) and has been related as an inflammatory marker in gingival crevicular fluids for periodontitis (Eberhard et al., 2006).

Concerning CKD, Deicher et al. (2000) reported that after stimulation, LTF release was lower in CKD serum/blood tissue than in normal controls, and this activity became normal after kidney transplant. There are not many studies on LTF and CKD, and most are related to renal cancer (Thotathil & Jameson, 2007) in which this gene often presents a deletion of 3p chromosome region and consequently downregulation of LTF functions (Giuffrè et al., 2007).

LTF gene is located on the 3p21 and has 17 exons (NCBI, 2010). Single nucleotide polymorphisms (SNPs) are point nucleotide exchanges in the gene sequences that generate common alleles in the population and might modulate protein expression and function. Sets of inherited linked SNPs are called *bins* (Liu et al., 2010). These bins contain a certain number of SNPs which can be represented by few of them, called tag SNPs (International Hapmap Project, 2010). Genotyping tag SNPs

may save time and reduce costs since this approach is intended to cover the gene as a whole (Liu et al., 2010).

A systemic disease may have an impact in the progression of a local one and vice-versa. Since CKD and CP are chronic immune-inflammatory complex diseases, which share common molecular physiopathological mechanisms, the identification of genes controlling the modulation to both may provide a better understanding of how host response may present an influence. Since LTF is a crucial molecule involved in the immune-inflammatory response, the aim of the study is to analyse the association between polymorphisms (tag SNPs) in *LTF* gene, which represent the variability of the whole gene, and susceptibility to CP in patients with and without CKD.

Methods

Sample selection

A total of 253 unrelated patients, male (120) and female (133), mean age 44.91 ± 12.49 were selected from the dental clinic of Pontifical Catholic University of Paraná (PUCPR) and from dental clinic of Pro-Renal Foundation. All patients were from the Southern region of Brazil. Subjects completed personal, medical, and dental questionnaires, within a protocol approved by the Ethical Committee in Research at PUCPR, protocol 264/10184.

The sample was divided into two groups, based on Armitage criteria, 1999:

Control group (CG): 118 individuals without CP; 52 presenting CKD, glomerular filtration rate < 90 ml/min, estimated according to the Modification of Diet in Renal Disease formula (Levey et al., 1999)

Test group (TG): 135 individuals with CP, presenting clinical attachment loss (CAL) ≥ 5 mm, in at least 3 teeth in at least 2 quadrants. A total of 77 patients in this group presented CKD.

Patients were not included if presented chronic use of anti-inflammatory drugs, HIV infection, immunosupressive chemotherapy, systemic active infection, current pregnancy or lactation, diseases of the oral hard or soft tissues (except caries and CP), use of orthodontic appliances, and presence of necrotizing ulcerative gingivitis.

The clinical parameters gender, chronic kidney disease, C-reactive protein, smoking habits, xerostomy, dental mobility and age were analyzed.

DNA collection

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 (Trevilatto & Line, 2000). The tip of the spatula was then shaken into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm 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℃. 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 amplification and high resolution melting analysis

Tag SNPs markers were selected according to the information available at the International Hapmap Project website, release 24. All selected markers presented a minor allele frequency of at least 0.25. The cutoff parameter to define linkage disequilibrium (LD) between two markers was a pairwise $r^2 > 0.8$. Caucasian population (CEU) was chosen due to the European ancestry of the major South Brazil population. Following these criteria, 3 tag SNPs were included: rs11716497, rs2073495, and rs6441989. The last marker is located in the intergenic region between *LTF* and chemokine (C-C motif) receptor-like-2 (*CCRL2*) genes; however, there was no LD with

the latter. The primers and hybrid probes used to screen the *LTF* gene (ENSG00000012223) were designed to flank and amplify 3 tag SNPs representative of the whole *LTF* gene (Fig. 1). Primer sequences are available in table 1. The 3 tag SNPs were analyzed by high resolution melting (HRM) with a 465-510 SYBR Green I/ HRM Dye. The polymerase chain reaction (PCR) and HRM were performed in a single run on a LightCycler® 480 instrument (Roche Diagnostics, Mannheim, Germany) with a total reaction volume of 10 µl in a 384-well microtiter plate. The LightCycler plate was centrifuged for 5 minutes to eliminate air bubbles that might disturb fluorescence. The reaction mixture contained 2 µl 10 ng genomic DNA, 1.2 µl 2.5 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 0.4 µl 10 *p*mol each primer, 1 µl hybrid probe at 10 *p*mol, and 5 µl LightCycler®480 High Resolution Master Mix (Roche Diagnostics, Mannheim, Germany). The PCR touchdown cycling protocol consisted of an initial heating step at 95℃ for 10 minutes followed by 45 cycles of denaturation at 95℃ for 10 seconds, annealing starting at 68℃ for 15 secon ds (decreasing 1℃ per cycle in the first 10 cycles) and extension at 72℃ for 20 seconds. After amplification, the amplicons were first heated to 95℃ for 1 minute, hybridized with probes at tag SNPs site at 40℃ for 1 minute and then the HMR program went over the range from 65℃ to 95℃ with 25 signal acquisitions per degree. Melting curve analysis of amplicon was performed with Tm Calling module, software version 1.5. The Tm Calling analysis software module calculated for each sample the melting temperature, the melting peak and identified characteristic melting profiles (genotypes) of DNA products or target-probe hybrids. After analysing genotypes results, one sample of each group was sequenced to validate the results.

Statistical Analysis

Nominal variables were expressed as frequencies and percents. To access association between nominal variables, χ^2, Fisher's and Mantel Haenszel exact test, and Odds

Ratio (OR) estimation were used. Continuous variables were accessed by Student t - test. Haploview software (Barret et al., 2005) analysis defined the lack of LD between the three tag SNPs (*bins*). Univariate analysis was used to analyze statistically significant association between the markers and peridontal disease. A multivariate analysis including C-reactive protein, smoking habits, xerostomy, dental mobility and age was performed. The variables were also tested against the three tag SNPs and between themselves. Haplotype association analysis and *p*-values was estimated using THESIAS test. For Odds Ratio (OR) estimation, alleles evidencing positive association were analyzed by logistic regression analysis using SAS software v.9.1.

Results

Clinical findings

No statistically significant differences (NS) were observed in univariate analysis in gender, smoking habits, and xerostomy between TG and CG (Table 2). CKD (p=0.019), C-reactive protein (p=0.023), dental mobility (p=0.008), and age (p=0.005) presented statistically significant differences (SSD) between CG and TG (Table 2). In the multivariate analysis, the association of those variables with CP remained statistically significant.

Genetic findings

The allele frequencies of the 3 tag SNPs were consistent with the assumption of Hardy-Weinberg equilibrium in the control population sample. No significant differences in the 3 allele/genotype/haplotype distributions of *LTF* were observed between the groups for the rs6441989, rs2073495 and rs11716497. The genotype and allele frequencies are shown in table 3. Linkage disequilibrium analysis confirmed independence of all 3 SNPs as shown in figure 2. Thus, the analysis in haplotypes was justified and no association was found between any haplotype and periodontal disease.

Discussion

Periodontal disease has been pointed out as the major dental problem along wih caries that causes tooth loss (Thorstensson & Johansson, 2010). With a worldwide tendency to extend life expectancy, oral health is a great concern to provide a good quality of life, especially for elderly. There are a lot of studies focusing on preventing and treating periodontitis, and the identification of high risk groups might contribute to a better orientation to an intenser treatment over the patients that exhibite an evidence of rapid CP evolution and severity. Pretzl et al (2009) reported that costs to treat periodontitis and prevent tooth loss are minor than using dental implant for tooth replacement besides dental implants are not exactly the same as real teeth.

With twin studies suggesting genetic compound of around 50% for periodontal disease (Michalowicz et al., 2000), it became evident the host contribution to disease development; although not yet clear how many and which genes are involved in the control of CP susceptibility. LTF was shown to have several local and systemic functions and it is present in salivary and other exocrine glands and in almost all body fluids. It is also released from polymorphonuclear neutrophils, modulating immune-inflammatory and bactericidal host response being a reliable inflammatory marker (Eberhard et al., 2006; Nishida et al., 2008). *LTF* can be considered a candidate gene for its several functions. Indeed, *LTF* polymorphisms have been shown associated with herpes simplex keratitis (Keijser et al., 2008), caries (Azevedo et al., 2010), aggressive periodontitis (Velliyagounder et al., 2003; Jordan et al., 2005; Wu et al., 2009). Although *LTF* has pleiotropic activities, a few studies have been focusing on its genetic variability and association with complex diseases.

A recent physical tag SNP approach was used to genotype the sample, which may capture the whole gene information. Several algorithms have been used to calculate LD blocks by selecting a minimum number of SNPs to achieve a maximum gene coverage thus leading to smaller tag SNP sets. Therefore, this physical strategy

revealed to be a better cost-benefit approach than single SNPs, with the advantage of reducing time and costs (Liu et al., 2010).

In this study, there was no genetic association found between CP and *LTF* polymorphisms, therefore further genetic studies may contribute to the enlightenment of host modulatory effects on these diseases.

It was observed a significant association of CKD and CRP with CP. C-reactive protein is an extremely sensitive inflammatory serum marker produced by liver, which is augmented in patients with inflammatory diseases, whose increased level is a predictor for cardiovascular events (Hage & Szalai, 2007), which represent 50% of CKD patients death causes (Foley et al., 1998). This finding should be highlighted once renal patients that also have CP might be at higher morbidity and mortality risk than those with only CKD (Nadeem et al., 2009). Although there has been an association between CKD and CP, it is difficult to determine which one contributes more to the outcome of the other. Thus, the levels of CRP might reflect the inflammatory environment resulting from such a background. Nevertheless, patients with CKD represent a particular susceptible population who must be accompanied for oral health aspects.

Dental mobility and increased age were also associated with CP. Dental mobility is one of the signs of periodontal tissues destruction and bone loss around teeth as a consequence of CP (Dietrich et al., 2009), which may lead to tooth loss. Increased age has also been reported as a clinical risk factor to CP in other previous studies (Kamen, 1996; Horowitz et al., 2001; Wang et al., 2007).

In summary, clinical aspects, such as CKD, CRP, dental mobility, and age are associated with CP, although no genetic susceptibility background concerning *LTF* gene was identified. In this context, subjects presenting systemic inflammatory diseases, such as CKD, and older individuals should be carefully handled once they can experience a more intense CP outcome.

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Table 1. *LTF* gene primers and hybrid probes.

Tag SNP	SNP Alleles	Fragment length (bp)	Primers	sequence 5´->3
rs6441989	A/G	204	F*	Ψ M13-AACTCACGACTGCTCCCACT
			R**	revM13-CTCTTTTGTCTAATCACTACGGAGG
rs2073495	C/G	234	F	M13- CACCACGGCATGATTCGGGGCCATG
			R	revM13- CATTCCACTGACCTCCCAAATGCAC
rs11716497	A/G	183	F	M13- TTCTGAGCCATCCCCTTATG
			R	revM13- CTAGCTGCCATACTCCC

Tag SNP	SNP Alleles	Fragment lenght (bp)	Probe	sequence 5´->3´
rs6441989	A/G	204	P1	CCCTTCTTCCACACCCCCCCTTCTCA
rs2073495	C/G	234	P2	CTTAGCCCATGCCTCATTGTTATTT
rs11716497	A/G	183	P3	CTGAGTCTGCAGAGGTTTGGGGACA

*****F- forward

******R- reverse

Ψ M13- sequencing primer

Table 2. Clinical parameters – univariate analysis (n=253).

Clinical variables	CG n=118 (%)	TG n=135 (%)	*p* value	*p* value Multivariate analysis
Gender (male)	52 (44)	68 (50.3)	0.337*	
Chronic kidney disease	52 (44)	77 (57)	**0.019***	**0.021**
C-reactive protein [£]	19 (22)	37 (31)	**0.023***	**0.023**
Smoking habits[§]	14 (12.1)	22 (16.6)	0.315*	
Xerostomy[§]	28 (24.3)	44 (33.3)	0.118*	
Dental mobility[§]	24 (20.8)	47 (36.1)	**0.008***	**0.005**
Age[¥]	42.59±10.91	46.91±10.96	**0.005****	**0.041**

CG: control group; **TG**: test group (patients with periodontal disease).

*Fisher´s exact test.

**Mantel Haenszel test.

[£] CG, n= 86; CT, n= 99.

[§] CG, n= 115; CT, n= 132.

¥ Mean±standard deviation.

Table 3: Genotype and allele frequencies of tag SNPs rs6441989, rs2073495 and rs11716497.

rs6441989	**CG**	**TG**	
Genotypes	**n (%)**	**n (%)**	***p* value**
AA	21 (17.7)	24 (17.7)	
AG	59 (50)	56 (41.4)	0.324*
GG	38 (32.2)	55 (40.7)	
Alleles			
A	101 (42.7)	104 (38.5)	0.375**
G	135 (57.2)	166 (61.4)	
rs2073495	**CG**	**TG**	
Genotypes	**n (%)**	**n (%)**	***p* value**
CC	55 (46.6)	57 (42.2)	
CG	52 (44)	62 (45.9)	0.704*
GG	11 (11.3)	16 (11.8)	
Alleles			
C	162 (68.6)	176 (65.1)	0.465**
G	74 (31.3)	94 (34.8)	
rs11716497	**CG**	**TG**	
Genotypes	**n (%)**	**n (%)**	***p* value**
AA	31 (26.2)	46 (34)	
AG	58 (49.1)	61 (45.1)	0.390 *
GG	29 (24.5)	28 (20.7)	
Alleles			
A	120 (43.3)	153 (56.5)	0.222**
G	116 (49.1)	117 (50.8)	

*Mantel Haenszel test.

**χ^2 test.

Fig. 1. *LTF* gene and the corresponding bins, whose SNPs present a minimum allele frequency (MAF) of at least 0.25. The SNP rs6441989 (MAF 0.491) was in LD with one SNP inside the 3' downstream *LTF* gene, rs2073495 (MAF 0.333) is a singleton and rs11716497 (MAF 0.249) represents a bin of 12 SNPs inside *LTF* gene.

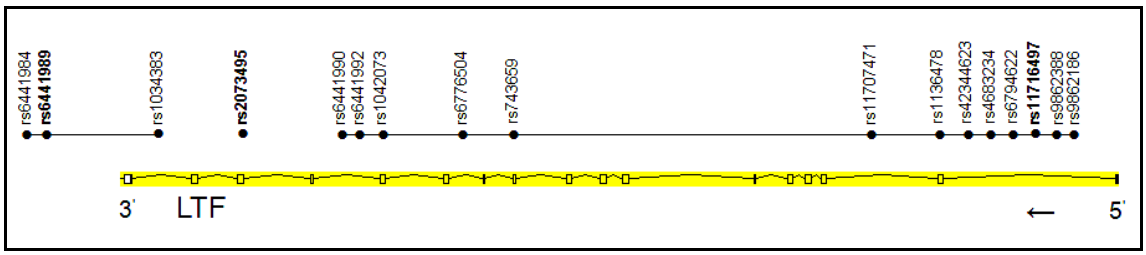
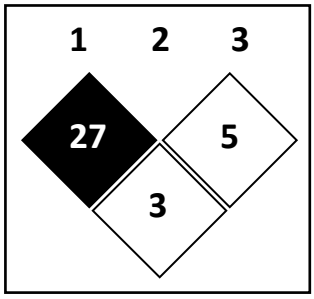


Fig. 2. Analysis of linkage disequilibrium (LD) between *LTF* tag SNPs (r^2<80). The number inside squares indicates the proportion of LD in %. The intensity of the color inside the squares reflects the LD between two loci: the darker intensity represents the highest LD between SNPs.



ARTIGO 2

4. ARTIGO 2

Analysis of the association between *LTF* polymorphisms and implant loss using the tag SNPs approach

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Abstract

Background. Dental implant has been considered the treament of choice for edentulous regions. Due to its large use worldwide, the small percentage of dental implant loss becomes high in numbers of cases. Several studies have aimed to accomplish an adequate osseointegration through improving material, surgery techniques and understanding host response. Lactotransferrin (LTF) is a pleiotropic protein, expressed in various body tissues and fluids, which modulates aspects of the host immune-inflammatory response and bone metabolism. Polymorphisms in *LTF* gene have been associated with several conditions such as caries and periodontal disease. Recently, an evidence for genetic basis to explain the clusterization phenomenon was identified. However, there are few studies investigating genetic susceptibility to implant failure and only in a functional approach. *Aims*. Thus, this study aimed to investigate, in a physical approach, the association of genetic markers (tag SNPs) in *LTF* gene and clinical parameteres with dental implant loss. *Methods.* 278 patients, both sexes, mean age 51 years old, divided into 184 without and 94 with implant loss, were genotyped for three tag SNPs, representative of the whole *LTF* gene. Also, clinical oral and systemic parameters were analyzed. Univariate and Multivariate Logistic Regression model were used to analyze the results ($p<0.05$). *Results*. No association was found between the tag SNPs and implant loss in the study population. Clinical association was found with medical treatment, hormonal reposition, edentulism, number of placed implants, plaque, calculus, and mobility. *Conclusion*. Clinical variables, but not genetic association, were associated with implant loss. Once LTF is highly conserved protein among species, some further investigation over tag SNPs in African populations might reveal more detailed information over the *LTF* gene.

Introduction

With the increase of worldwide life expectancy (Human Mortality Database, 2010), general health needs for elderly people had increased substantially, in terms of psychological and physical perspectives. In this context, health care has improved greatly through the years, including oral treatments, resulting in better life quality. Caries and periodontal diseases have been implicated as the main causes of tooth loss (Thorstensson & Johansson, 2010). Dental implants have been extensively used to replace missing teeth, accomplishing satisfactory aesthetics and function. A dental implant is considered in function when it is loaded, which means, in occlusion and supporting mastigatory forces. Successful loading characterizes an adequate osseointegration, when the implant is strongly bounded to bone (Carlsson et al., 1986). Implant success rate was estimated in 89.43% to 95.32% during 6 to 10-year-follow-up (Jansson et al., 2005; Levin et al., 2008). Although such high records, failure does happen, involving further costs and additional procedures. These failures have been considered multifactorial and might occur before or after loading (Manor et al., 2009), being classified as early or late, respectively. Among the causative factors underlying early failure are smoking (Koldsland et al., 2009), implant design (Olate et al., 2010), surgical complications (Huynh-Ba et al., 2008), uncontrolled diabetes, radiotherapy, and menopausal estrogen therapy (Moy et al., 2005). In the case of late failure factors, it may be caused by abnormal occlusal force (Misch, 2002) and peri-implatitis (Pye et al., 2009). Nevertheless, patients that presented periodontitis are more prone to implant loss (Anner et al., 2010).

There has been evidence that determined groups of individuals are at a higher risk for implant failure, phenomenon called clusterization, suggesting modulatory aspects of the host immune-inflammatory response (Montes et al., 2007). There have been studies associating dental implant loss with polymorphisms (DNA sequence

variations) present in certain genes related to imune-inflammatory host response (Jansson et al., 2005; Montes et al., 2009).

Lactotransferrin (LTF) is an 80 kDa iron-binding protein composing the transferrin family, excreted from exocrine glands and secondary granules of neutrophils in sites of inflammation. It constitutes a single polypeptide chain containing 692 amino acids folded in two symmetric lobes; N and C (Anderson et al., 1989). LTF is part of the innate immune-inflammatory response and presents relevant antifungical and antivirus activities (Kanwar et al., 2009), and has an iron-binding site, exhibiting bacteriostatic activity (Wong et al., 2009). It is also considered a bone growth factor (Naot et al., 2005), showing osteogenic abilities including promoting osteoblast growth, inhibiting osteoclastogenesis, and increasing bone formation. Physiological human concentrations range from 2 µg/ml to 7 µg/ml, but in inflammatory conditions it can reach up to 200 µg/ml. At 100 µg/ml, osteoclastogenesis ceased completely (Cornish, 2004). LTF was found in a much higher concentration around peri-implantitis tissues (Hultin et al., 2002); moreover, it also combats the *Porphyromonas gingivalis* (Wakabayashi et al., 2010), an important bacteria involved in periodontitis, also found in sulcus around dental implants in dentate patients (Sumida et al., 2002).

A single nucleotide polymorphism (SNP) is a common point DNA sequence variation, and is distributed throughout the whole genome. There are millions of SNP and they may confer the susceptibility or protective modulation against diseases (Liu et al., 2010). One SNP in *LTF* gene was associated with aggressive periodontitis and bacteriostatic effects over certain bacterias (Velliyagounder et al., 2003). Our hypothesis is that SNPs in *LTF* gene are associated with implant loss once it modulates aspects of the host imunne-inflammatory, bactericidal and ostegenic response. We used a novel genetic approach, genotyping tag SNPs, which are SNPs often highly linked to others by linkage disequilibrium (LD) forming a bin (a block with strong LD). Tag SNPs are representative of all other SNPs of a given bin, which

reduces genotyping costs and time (Liu et al., 2010). Besides, this physical strategy is intended to capture the information of the whole gene, further than single functional SNPs. Therefore, we aimed to investigate the association of tag SNPs in *LTF* gene with dental implant loss.

Methods

Sample selection

Briefly, the sample selection is described as reported in Alvim et al. (2008-a). All records (n=3,578) of patients treated with dental implants (*Neodent® Implante Osteointegrável*) at Latin-American Dental Research Institute (ILAPEO), Curitiba-PR, between 1996 and 2006, were analyzed (all patients who were treated with implants during this period). From these 3,578 subjects, 126 (3.5%) presented at least one lost implant, most of them (88.2%) occurred in the initial phase of osseointegration (early stage). Out of those 126 individuals, 94 were evaluated, composing the test group (TG, n=94). The other 32 patients either died or were not possible to contact. The control group (CG) was then composed of 184 individuals without any loss, with implant(s) in function for at least six months matched by age, gender, ethnicity and smoking habits (CG, n=184). Thus, the sample was composed of 278 patients (n=278) (Table 1). The sample was collected from the South region of Brazil, in Paraná State. The patients answered a questionnaire on socioeconomic status, medical and dental history, current medication, teeth cleansing methods and dental appointment frequency. Periodontal status was recorded at four points around each tooth using a conventional periodontal probe, Hufriedy tm (Chicago, USA), by a single examiner (F.A.P.). The following parameters were analyzed: gingival index (GI) (Loe & Silness, 1963), plaque index (PI) (Silness & Loe, 1964), calculus index (CI) (Greene & Vermillion, 1964), probing pocket depth (PPD), clinical attachment loss (CAL), and dental mobility (present or absent). All

patients signed a consent form, agreeing to participate in the research, within a protocol approved by the Ethical Committee in Research at PUCPR, south of Brazil, protocol 323.

Aiming to observe the concentration of implant loss in a certain group of patients (clusterization), the sample was redivided into group 1 (G1, n=242), with up to 1 implant loss, and group 2 (G2, n=36), with more than 1 implant loss.

DNA collection

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 (Trevilatto & Line, 2000). The tip of the spatula was then shaken into the retained mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm 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℃. 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 amplification and high resolution melting analysis

Tag SNPs markers were selected according to the information available at the International Hapmap Project website, release 24. All selected markers presented a minor allele frequency of at least 0.25. The cutoff parameter to define LD between two markers was a pairwise $r^2 > 0.8$. Caucasian population (CEU) was chosen due to the European ancestry of the major South Brazil population. Following these criteria, 3 tag SNPs were included: rs11716497, rs2073495, and rs6441989. The last marker is located in the intergenic region between *LTF* and chemokine (C-C motif) receptor-like-2 (*CCRL2*) genes; however, there was no LD with the latter. The primers and hybrid

probes used to screen the *LTF* gene (ENSG00000012223) were designed to flank and amplify 3 tag SNPs representative of the whole *LTF* gene (Fig. 1). Primer sequences are available in table 2. The 3 tag SNPs were analyzed by high resolution melting (HRM) with a 465-510 SYBR Green I/ HRM Dye. The polymerase chain reaction (PCR) and HRM were performed in a single run on a LightCycler® 480 instrument (Roche Diagnostics, Mannheim, Germany) with a total reaction volume of 10 µl in a 384-well microtiter plate. The LightCycler plate was centrifuged for 5 minutes to eliminate air bubbles that might disturb fluorescence. The reaction mixture contained 2 µl 10 ng genomic DNA, 1.2 µl 2.5 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 0.4 µl 10 *p*mol each primer, 1 µl hybrid probe at 10 *p*mol, and 5 µl LightCycler®480 High Resolution Master Mix (Roche Diagnostics, Mannheim, Germany). The PCR touchdown cycling protocol consisted of an initial heating step at 95℃ for 10 minutes followed by 45 cycles of denaturation at 95℃ for 1 0 seconds, annealing starting at 68℃ for 15 seconds (decreasing 1℃ per cycle, in t he first 10 cycles) and extension at 72℃ for 20 seconds. After amplification, the ampli cons were first heated to 95℃ for 1 minute, hybridized with probes at tag SNPs site at 40℃ for 1 minute and then the HMR program went over the range from 65℃ to 95℃ with 25 signal acquisitions per degree. Melting curve analysis of amplicon was performed with Tm Calling module, software version 1.5. The Tm Calling analysis software module calculated for each sample the melting temperature, the melting peak and identified characteristic melting profiles (genotypes) of DNA products or target-probe hybrids. After analysing genotypes results, one sample of each group was sequenced to validate the results.

Statistical Analysis

Nominal variables were expressed as frequencies and percents. To access association between nominal variables, χ^2, Fisher's and Mantel Haenszel exact test, and Odds Ratio (OR) estimation were used. Continuous variables were accessed by Student t-

test. Haploview software (Barret et al., 2005) analysis defined the lack of LD between the three tag SNPs (*bins*). Univariate analysis was used to analyze statistically significant association between the markers and peridontal disease. A multivariate analysis including C-reactive protein, smoking habits, xerostomy, dental mobility and age was performed. The variables were also tested against the three tag SNPs and between themselves. Haplotype association analysis and *p*-values was estimated using THESIAS test. For Odds Ratio (OR) estimation, alleles evidencing positive association were analyzed by logistic regression analysis using SAS software v.9.1.

Results

Clinical findings

No statistically significant differences (NS) were observed in socioeconomic status, general medical condition, and hygiene habits either between TG and CG or between G1 and G2 (Table 3). Medical treatment (p=0.040), hormonal reposition (p=0.040), and edentulism (p=0.050) presented statistically significant differences (SSD) between G1 and G2. Medical treatment and hormonal reposition were more prevalent in G2 group and edentulism, in G1 (Table 3). Number of placed implants presented SSD between CG/TG (p=0.000) and G1/G2 (p=0.000) groups, being increased in groups with implant failure (TG and G2) (Table 3).

PPD was slightly increased in CG (p=0.036). Plaque (p=0.011) and calculus (p=0.012) indexes were higher in TG. There was significantly higher plaque index (p=0.048) and dental mobility (p=0.026) in G2. The other periodontal variables showed NS either between TG and CG or G1 and G2 (Table 4).

Genetic findings

The allele frequencies of the 3 tag SNPs were consistent with the assumption of Hardy-Weinberg equilibrium in the control population sample. The sample was tested against

the 3 tag SNPs with two different sample divisions. No significant differences in the 3 allele/genotype/haplotype distributions of *LTF* were observed between the groups for the rs6441989, rs2073495 and rs11716497. The genotype and allele frequencies are shown in table 5. Linkage disequilibrium analysis confirmed independence of all 3 SNPs as shown in figure 2. Thus, the analysis in haplotypes was justified and no association was found between any haplotype and implant failure. In the multivariate analysis, including the clinical variables significantly associated with implant failure (TG and/or G2), the lack of association of the tag SNPs and haplotypes was maintained.

Discussion

Endosteous dental implant treatment has been considered the gold-standard therapy for absent tooth reposition. Several variables have been considered determinant for implant survival such as implant surface (Wennerberg & Albrektsson, 2009), smoking habits (Abt, 2009), bone type, infection related to surgery (Esposito et al., 1999), occlusal trauma (Fu & Yap, 2007), and host response (Montes et al., 2009). In another study of our group, it was observed that primary stability, edentulism, and implant length/position were also associated with implant loss (Alvim-Pereira et al., 2008-a). In this study, medical treatment was found associated with multiple implant failure, but when current medication was stratified, only hormonal reposition was associated with multiple implant loss. It is well known that estrogen deficiency alters bone and inflammatory metabolism. Some reports associated hormone reposition with protection against tooth loss (Buencamino et al., 2009; Haas et al., 2009); on the other hand, a study has associated hormone treatment with implant failure (Moy et al., 2005). The patients receiving estrogen are treated to this alteration specifically, but other diseases they present may be the reason for the increased medical treatment found in groups with implant loss. Edentulous patients were significantly more frequent in the control group; perhaps the biofilm on the remaining teeth represent a potential risk factor for

implant failure (López-Cerero, 2008). Also, as expected, a higher number of placed implants was found in the test group.

Plaque and calculus index were associated with implant loss; however, only plaque was significant in the group with multiple implant loss. Both are considered to be involved in periodontal disease, which might have been the cause of tooth loss in these patients. PPD was slightly increased in the control group. However, the difference of 0.16 mm between the groups can not be considered clinically relevant. Mobility was higher in the individuals with multiple implant loss. Mobility is a major sign of advanced periodontal disease, which has been suggested as a predictor for implant failure in other studies (Koldsland et al., 2009; Heitz-Mayfield & Huynh-Ba, 2009).

Dental implant breakdown has a multifactorial background and an exacerbated immune-inflammatory response challenges the physiological homeostasis resulting in tissue destruction (Esposito et al., 2008). There are several mediators being studied to date in order to have a more accurate vision of how they play along with other factors predisposing the individual to certain disease or conferring a protective effect. A pleiotropic human metabolism protein is LTF, highly synthesized in saliva and in inflamed tissues (Eberhard et al., 2006). It inhibits biofilm formation, bounds serum iron, promoting bacteriostatic effect (Wakabayashi et al., 2010), besides acting as a front-line immune response mediator in bacterial presence, as an anti-inflammatory agent in inflammatory diseases (Actor et al., 2009), and as a bone growth factor (Naot et al., 2005). LTF was seen to inhibit adhesion of periodontopathic bacteria and biofilm formation (Wakabayashi et al., 2010) and higher levels of LTF were observed in peri-implantitis sites (Hultin et al., 2002). *LTF* gene polymorphisms were observed to be associated with caries (Azevedo et al., 2010), herpes simplex keratitis (Keijser et al., 2008), and aggressive periodontitis (Velliyagounder et al., 2003; Jordan et al., 2005; Wu et al., 2009. So far, data are missing over the study of polymorphisms in *LTF* gene related to implant failure.

Recently, our group identified an evidence for genetic basis to explain the clusterization phenomenon (Montes et al., 2009). With studies pointing at a genetic background as a contributing factor for implant failure, there is a strong need to identify which genes might be interfering with host response contributing to jeopardize osseointegration. To date, there are only a few studies on the investigation of genetic polymorphisms on implant loss in Pubmed database, 2010. However, all studies investigated the association of genetic polymorphisms with implant failure in terms of functionality (Alvim-Pereira et al., 2008-b). Thus, this study aimed to investigate the association of genetic markers (tag SNPs) in *LTF* gene with implant loss in a tag SNP physical approach. It is worth mentioning that *LTF* is considered a functional candidate gene due to its potential involvement in the physiopathologic mechanisms underlying osseointegration process. However, this new physical tag SNP approach, which analyzes the bins, may capture the whole gene information. This physical strategy reveals to be a better cost-benefit approach than single SNPs, reducing time and costs (Liu et al., 2010). To our knowledge, this is the first study with dental implants analyzing genes physically based on tag SNPs. Several algorithms have been used to calculate LD blocks by selecting a minimum number of SNPs to achieve a maximum gene coverage thus leading to smaller tag SNP sets. Nowadays, the algorithm used by the *Hapmap* browser is based on the correlation index (r^2), which calculates LD between tag SNPs and uncollected SNPs, characterizing the *bins*.

No association between *LTF* gene polymorphisms and implant loss was observed in this population. Once LTF is present in the majority of fluids of the organism and plays a vast category of actions in human metabolism, each single alteration in its residue sequence might compromise the host homeostasis. Indeed, it is a highly conserved protein among species (Lambert et al., 2005). In this context, some further investigation over tag SNPs in the African population might reveal more detailed information over the gene.

Stratifying patients by implant loss risk will allow a more precise management of this type of treatment, preventing undesirable failures.

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Table 1. Baseline characteristics of all sampled subjects (n=278).

	CG (n=184)		TG (n=94)	
	n	**%**	**n**	**%**
Ethnic Group				
Caucasian	177	96.1	93	98.9
Non-caucasian	7	3.9	1	1.1
Age years[†]	51.01 ±11.54[†]		53.05 ±10.75[†]	
Gender				
Female	120	65.2	58	61.7
Male	64	34.8	36	38.3
Smoking				
Yes	44	24.0	18	19.2
No	140	76.0	76	80.8

[†]Mean ± standart deviation.

Table 2. *LTF* gene primers and hybrid probes.

Tag SNP	SNP Alleles	Fragment length (bp)	Primers	sequence 5´->3
rs6441989	A/G	204	F*	Ψ M13-AACTCACGACTGCTCCCACT
			R**	revM13-CTCTTTTGTCTAATCACTACGGAGG
rs2073495	C/G	234	F	M13- CACCACGGCATGATTCGGGGCCATG
			R	revM13- CATTCCACTGACCTCCCAAATGCAC
rs11716497	A/G	183	F	M13- TTCTGAGCCATCCCCTTATG
			R	revM13- CTAGCTGCCATACTCCC

Tag SNP	SNP Alleles	Fragment lenght (bp)	Probe	sequence 5´->3´
rs6441989	A/G	204	P1	CCCTTCTTCCACACCCCCCCTTCTCA
rs2073495	C/G	234	P2	CTTAGCCCATGCCTCATTGTTATTT
rs11716497	A/G	183	P3	CTGAGTCTGCAGAGGTTTGGGGACA

*****F- forward

******R- reverse

Ψ M13- sequencing primer

Table 3. Clinical findings in patients with and without dental implant loss (n=278).

	CG	TG		G1	G2	
	n=184 (%)	**n=94 (%)**	***p* value**	**n=242 (%)**	**n=36 (%)**	***p* value**
Socioeconomic status						
A1/A2/B1	95 (51.63)	48 (51.06)	0.895*	122 (50.41)	21 (58.33)	0.374*
B2/C/D	89 (48.36)	46 (48.93)		120 (49.58)	15 (41.66)	
Medical condition						
Systemic diseases	127 (64.65)	69 (73.40)	0.411*	168 (69.42)	28 (77.76)	0.275*
Rheumatoid disease	34 (18.38)	25 (27.17)	0.116*	49 (20.24)	10 (27.77)	0.304*
Osteoporosis	03 (1.62)	02 (2.17)	0.766*	04 (1.65)	01 (2.77)	0.651*
Autoimmune disease	58 (31.52)	26 (27.66)	0.408*	76 (31.40)	08 (22.22)	0.236*
Under medical treatment						
Yes	75 (40.54)	44 (46.80)	0.317*	98 (40.4)	21 (58.33)	**0.040* OR 2.085 CI 1.025-4.243**
Current medication						
Use of medication	78 (42.39)	43 (45.74)	0.568*	101 (41.73)	20 (55.53)	0.109*
NSAID£	06 (3.24)	07 (7.44)	0.115*	10 (41.32)	03 (8.33)	0.245*
SAID§	04 (2.16)	03 (3.26)	0.603*	05 (2.06)	02 (5.54)	0.198*
Hormonal reposition	75 (40.76)	44 (46.80)	0.317*	98 (40.49)	21 (58.33)	**0.040* OR 2.085 CI 1.025-4.243**
Hygiene Habits						
Brushing daily						
3 times or more	135 (73.36)	72 (76.59)	0.513*	179 (73.96)	28 (77.76)	0.573*
Dental floss daily						
Yes	123 (66.85)	62 (65.95)	0.904*	163 (67.35)	22 (61.12)	0.664*
Mouthwashing daily						
Yes	61 (33.15)	30 (31.91)	0.904*	78 (32.23)	13 (36.11)	0.543*
Edentulism¤	16.56±8.82	16.78±7.35	0.849**	17.08±8.54	13.83±7.12	**0.050****
Placed implants¤	4.43±2.82	5.85±3.15	**0.000****	4.58±2.87	7.13±3.19	**0.000****

CG: control group; **TG**: test group (patients who lost implants); **G1**: patients with up to one implant lost; **G2**: patients witn multiple implant losses.
* Fisher´s exact test;
** Student's t-test;
£ Non steroidal antiinflammatory drug;
§ Steroidal antiinflammatory drug;
¤ Mean ± Standard Deviation.

Table 4. Periodontal status of partially edentulous patients (n=186).

Periodontal Status	CG (n=184)	TG (n=94)	*p* value	G1 (n=242)	G2 (n=36)	*p* value
Gingival Index †	0.63 ± 0.33	0.64 ± 0.45	0.868*	0.63±0.36	0.66±0.48	0.718*
Plaque Index †	0.12 ± 0.20	0.22 ± 0.35	**0.011***	0.14±0.25	0.26±0.36	**0.048***
Calculus Index †	0.07 ± 0.10	0.13 ± 0.20	**0.012***	0.09±0.14	0.11±0.20	0.516*
PPD [a] (mm) †	2.71 ± 0.41	2.55 ± 0.40	**0.007 ***	1.83±0.35	1.85±0.26	0.656*
CAL [b] (mm) †	3.61 ± 0.76	3.66 ± 0.92	0.672*	3.62±0.80	3.71±0.97	0.585*
Mobility (presence)	19 (10.33)	16 (17.02)	0.214‡	26 (10.74)	9 (25.0)	**0.026**‡

CG: control group; **TG**: test group (patients who lost implants); **G1**: patients with up to one implant lost; **G2**: patients with multiple implant losses.

[a]Probing pocket depth;

[b]Clinical attachment level;

†Mean±standart deviation;

*Student's t-test;

‡Fisher's exact test.

Table 5. Genotype and allele frequencies of tag SNPs rs6441989, rs2073495, and rs11716497.

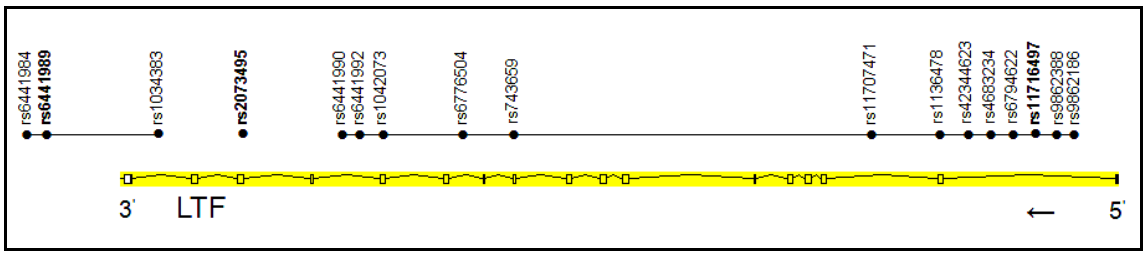
rs6441989	**CG**	**TG**		**G1**	**G2**	
Genotypes	**n (%)**	**n (%)**	***p* value**	**n (%)**	**n (%)**	***p* value**
AA	31 (16.8)	22 (23.4)		47 (19.4)	6 (16.6)	
AG	80 (43.4)	39 (41.4)	0.406**	104 (42.9)	15 (41.6)	0.621**
GG	73 (39.6)	33 (35.1)		91 (37.6)	15 (41.6)	
Alleles						
A	105 (38)	83 (44)		198 (40.9)	27 (37.5)	
G	226 (61)	142 (55)	0.240*	286 (59)	45 (62.5)	0.674*
rs2073495	**CG**	**TG**		**G1**	**G2**	
Genotypes	**n (%)**	**n (%)**	***p* value**	**n (%)**	**n (%)**	***p* value**
CC	63 (34.2)	41 (42.5)		88 (36.3)	16 (44.4)	
CG	93 (50.5)	38 (41.4)	0.894**	117 (48.3)	14 (38.8)	0.933**
GG	28 (15.2)	15 (15.9)		37 (15.2)	6 (16.6)	
Alleles						
C	219 (59)	120 (63)		293 (60.5)	46 (63.8)	
G	149 (40)	68 (36)	0.370*	191 (39.4)	26 (36.1)	0.678*
rs11716497	**CG**	**TG**		**G1**	**G2**	
Genotypes	**n (%)**	**n (%)**	***p* value**	**n (%)**	**n (%)**	***p* value**
AA	73 (39.6)	34 (36.1)		92(38)	15(41.6)	
AG	74 (40.2)	36 (38.2)	0.579**	99 (40.9)	11 (30.5)	0.417**
GG	37 (20.1)	24 (20.1)		51 (21)	10 (27.7)	
Alleles						
A	220 (59.7)	104 (55.3)		283 (58.4)	41 (56.9)	
G	148 (40.2)	84 (44.6)	0.358*	201 (41.5)	31(43)	0.907*

CG: control group; **TG**: test group (patients who lost implants); **G1**: patients with up to one implant lost; **G2**: patients with multiple implant losses.

*Mantel Haenszel exact test;

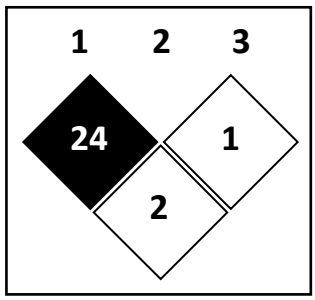
** Fisher's exact test.

Fig. 1. *LTF* gene and the corresponding bins, whose SNPs present a minimum allele frequency (MAF) of at least 0.25. The SNP rs6441989 (MAF 0.491) was in LD with one SNP inside the 3' downstream *LTF* gene, rs2073495 (MAF 0.333) is a singleton and rs11716497 (MAF 0.249) represents a bin of 12 SNPs inside *LTF* gene.



Gene figure: *Hapmap* release 24, 2008.

Fig. 2. Analysis of linkage disequilibrium (LD) between LTF tag SNPs ($r^2 < 80$). The number inside squares indicates the proportion of LD in %. The intensity of the color inside the squares reflects the LD between two loci: the darker intensity represents the highest LD between SNPs.



ARTIGO 3

5. ARTIGO 3

Lactotransferrin polymorphism, gingivitis and plaque are associated with caries experience

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ABSTRACT

Introduction. Dental caries is a common multifactorial disease, resulting from the interaction of biofilm, cariogenic diet and host response over time. Lactotransferrin (LTF) is a main salivary glicoprotein, which modulates host immune-inflammatory and antibacterial response. Although a genetic component for caries outcome has been identified, little is known over genetic aspects underlying its susceptibility. Thus, the aim of this study was to verify if there is an association between *LTF* polymorphisms and caries susceptibility. *Methods*. 677 12-yr-old students were selected from 6 public and 6 privated schools: 346 with (DMFT≥1) and 331 without caries experience (DMFT=0). Also, individuals concentrating higher levels of disease (polarization group, DMFT≥2, n=253) were tested against those with DMFT≤1 (n=424). Along with clinical parameters three representative *LTF* tag SNPs (rs6441989, rs2073495, rs11716497) were genotyped and results were evaluated using univariate analysis and multivariate logistic regression model (p<0.05). *Results*. Allele A for tag SNP rs6441989 was found to be significantly less frequent in the polarization group, confering a protective effect against caries experience [AA+AG x GG (OR: 0.710, IC: 0.514-0.980, p=0.045)], and remained significantly associated with caries protection in the presence of gingivitis (p=0.020) and plaque (p=0.035). *Conclusion. LTF* tag SNP rs6441989 was associated with protection against caries. These results might contribute for the unveiling towards caries genetic background. Further genetic research should be carried out in order to define what other variances might be contributing to caries phenotype.

Introduction

One of the most important and common diseases, that until nowadays involves the population in several socioenvironmental and biological levels is dental caries. It is an infectious, multifactorial disease caused by an imbalance in the interaction of specific microbiota along with cariogenic diet and host response over time (Newbrun, 1978; Bratthall et al., 2005; Nordlund et al., 2009). It has been well recognized the contribution of behavioral (Chandra et al., 2009), environmental (Peres et al., 2009) and genetic (Werneck et al., 2010) risk aspects in caries development. With the melioration of dental treatments and preventive techniques, dental caries prevalence has been decreasing worldwide. There has been reported a global reduction in the prevalence of dental caries in Germany (Schulte et al., 2006), Sweden (Hugoson & Koch, 2008), Norway (Mjör et al., 2008), Hungary (Madlena et al., 2008), and Brazil (Brasil, 2004; Carvalho et al., 2009). This decrease may be due to socioeconomical aspects (Oliveira et al., 2008), presence of fluor in toothpastes (Twetman, 2009), water supply (Evans et al., 2009), and better hygiene instruction (Longbottom et al 2009). In spite of such decrease, a great deal of investment has still been employed in dental treatments, instead of in preventive techniques. Only in Brazil, the estimative to be spent with oral health in 2006 was approximately of 1 billion dollars, which had tripled from one year before (Ministry of health, 2006).

There has been evidence of caries concentration in small groups (phenomenon termed polarization), who seem to be more susceptible to disease outcome (Peres et al., 2008).

Although a significant genetic component has been suggested in twin studies, (Bretz et al., 2005), very little upon the genetic background underlying the susceptibility to dental caries is known (Werneck et al., 2010). Recently, a genomic scan was performed for dental caries and genomic regions were identified, where genes related

with saliva properties and diet preference (host response) would be present and could be functional candidate genes for susceptibility to caries (Vieira et al., 2008).

Saliva is one of the host factors that interact with the biofilm, avoiding enamel demineralization caused by acids resulting from cariogenic bacteria metabolism (Wolff & Larson., 2009). Saliva influences cariogenicity of the biofilm through its flow (Mese & Matsuo, 2007), composition and buffer capacity (Lenander-Lumikari & Loimaranta, 2000; Dawes, 2008). One of the glicoproteins that composes saliva, involved in immune-inflammatory response is lactotransferrin (LTF) (Bournazou et al., 2009). It is present in several fluids of the body and is present in neutrophil second granules and exocrine glands (Pierce et al., 2009). LTF plays an important role in antibacterial activities against *Streptococcus mutans*, the prior bacteria that initiates caries lesions (Berlutti et al., 2004); binding to seric Fe+++, it decreases its availability to bacteria, working as bacteriostatic (Arslan et al., 2009). *LTF* gene is located on the 3p21 and presents 17 exons (NCBI, 2010). Polymorphisms in the *LTF* gene were reported to be associated with herpes simplex keratitis (Keijser et al., 2008), aggressive periodontitis (Velliyagounder et al., 2003; Jordan et al., 2005; Wu et al., 2009), and caries (Azevedo et al., 2010). However, there are few studies investigating the association of polymorphisms in salivary protein genes with caries susceptibility (Jonasson et al., 2007; Peres et al., 2010).

Single nucleotide polymorphisms (SNPs) are nucleotide exchanges in the gene sequences that generate common alleles in the population and may modulate the protein expression rate and function. Nearby sets of SNPs are inherited in blocks or haplotypes. These blocks usually contain a large number of SNPs, and can be represented by few of them. The SNPs, which represent sets of others, are called tag SNPs (International Hapmap Project, 2010). These SNPs represent the gene as a whole and may influence individual susceptibility to diseases (Maniatis, 2007; Liu et al., 2010).

To the authors' knowledge, there is only one report analyzing the association of *LTF* gene polymorphism (rs1126478) with caries susceptibility (Azevedo et al., 2010). Moreover, that study analyzes only the second exon of *LTF*. Thus, the aim of this study was to investigate the association of tag SNPs in the *LTF* gene, through a physical approch, which intends to capture the whole gene, with susceptibility to dental caries in 12-year-old students.

Methods

Sample Selection

The sample was composed of 677 unrelated, 12-year-old, students from 6 private and 6 public schools of Curitiba-PR, Brazil. The scholars were not included if smokers, using orthodontic appliances or taking chronic antiinflammatory and antibiotics in the last three months. 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), Resolution 96/96 of the Health National Council, register n. 487.

Clinical exams were conducted in the school, with natural light, mirror, probe, wooden spatula, gauze and gloves. The students were diagnosed according to the decayed, missing and filled teeth (DMFT) index in accordance WHO (WHO, 1997). All evaluations were performed by two calibrated examiners. Inter- and intra-examiner reproducibility was taken on 10% of the sample and the Kappa test was used to measure reliability. The obtained values for Kappa test were 0.93 for inter- and 0.99 for intra-examiner. Teeth were considered decayed when presenting either cavities or white lesions. Individuals were divided into two groups, according to caries experience:

Control Group (CG): 331 individuals without caries experience (DMFT=0);

Test Group (TG): 346 individuals with caries experience (DMFT≥ 1).

The sample was also analyzed focusing on polarization aspects:

Group 1 (G1): 424 individuals with low caries experience (DMFT≤1);

Group 2 (G2): 253 individuals with higher caries experience (DMFT≥ 2).

The following clinical variables were evaluated: fluorosis, salivary flow, buffer capacity, gingivitis, and presence of plaque.

The presence of any degree of fluorosis was established according to Dean's index (WHO, 1997).

The stimulated salivary flow rate (SSFR) was measured as previously described (de Almeida et al., 2008). To classify the SSFR the following numerical scores were attributed: 0 for low caries risk >0.5 ml/min and 1 for high caries risk ≤0.5 ml/min, because the cut line for dichotomization was based on the data distribution. The buffering capacity (BC) was performed as described previously (Ericsson, 1959). Final pH of the mixture was determined using a pH-meter and BC was considered good (score 0) if the final pH was ≥5.0 and deficient (score 1) for pH<5.0 (Kitasako et al., 2005).

Individuals were considered positive for gingivitis when all teeth from at least one sextant were affected, with bleeding and inflammation.

The biofilm accumulation was verified by the Plaque Index (PI) (Loe & Silness, 1963) modified, which adopted the same criteria, but evaluating 6 teeth surfaces: 16 [buccal (B)], 12 B, 26 [lingual (L)], 36 B, 32 L, and 46 L. According to data distribution, code 0 was considered as no plaque accumulation (PI=0), code 1 for regular plaque accumulation (PI>0 and <1) and code 2 for high plaque accumulation (PI≥1).

DNA collection

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 (Trevilatto & Line, 2000). The tip of the spatula was then shaken into the retained

mouthwash solution. Buccal epithelial cells were pelleted by centrifugation at 2000 rpm 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℃. 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 amplification and high resolution melting analysis

Tag SNPs markers were selected according to the information available at the International Hapmap Project website, release 24. All selected markers presented a minor allele frequency of at least 0.25. The cutoff parameter to define LD between two markers was a pairwise $r^2 > 0.8$. Caucasian population (CEU) was chosen due to the European ancestry of the major South Brazil population. Following these criteria, 3 tag SNPs were included: rs11716497, rs2073495, and rs6441989. The last marker is located in the intergenic region between *LTF* and chemokine (C-C motif) receptor-like-2 (*CCRL2*) genes; however, there was no LD with the latter. The primers and hybrid probes used to screen the *LTF* gene (ENSG00000012223) were designed to flank and amplify 3 tag SNPs representative of the whole *LTF* gene (Fig. 1). Primer sequences are available in table 1. The 3 tag SNPs were analyzed by high resolution melting (HRM) with a 465-510 SYBR Green I/ HRM Dye. The polymerase chain reaction (PCR) and HRM were performed in a single run on a LightCycler® 480 instrument (Roche Diagnostics, Mannheim, Germany) with a total reaction volume of 10 µl in a 384-well microtiter plate. The LightCycler plate was centrifuged for 5 minutes to eliminate air bubbles that might disturb fluorescence. The reaction mixture contained 2 µl 10 ng genomic DNA, 1.2 µl 2.5 mM $MgCl_2$ (Roche Diagnostics, Mannheim, Germany), 0.4 µl 10 *p*mol each primer, 1 µl hybrid probe at 10 *p*mol, and 5 µl LightCycler®480 High Resolution Master Mix (Roche Diagnostics, Mannheim, Germany). The PCR

touchdown cycling protocol consisted of an initial heating step at 95℃ for 10 minutes followed by 45 cycles of denaturation at 95℃ for 10 seconds, annealing starting at 68℃ for 15 seconds (decreasing 1℃ per cycle, in the first 10 cycles) and extension at 72℃ for 20 seconds. After amplification, the amplicons were first heated to 95℃ for 1 minute, hybridized with probes at tag SNPs site at 40℃ for 1 minute and then the HMR program went over the range from 65℃ to 95℃ with 25 signal acquisitions per degree. Melting curve analysis of amplicon was performed with Tm Calling module, software version 1.5. The Tm Calling analysis software module calculated for each sample the melting temperature, the melting peak and identified characteristic melting profiles (genotypes) of DNA products or target-probe hybrids. After analysing genotypes results, one sample of each group was sequenced to validate the results.

Statistical Analysis

Nominal variables were expressed as frequencies and percents. To access association between nominal variables, χ^2, Fisher's test, Mantel Haenszel exact test, and Odds Ratio (OR) estimation were used. The continuous variables were accessed by Student's t-test. Haploview software (Barrett et al., 2005) analysis defined the lack of LD between the three tag SNPs (bins). Univariate analysis was used to analyze statistically significant association of the tag SNPs and baseline and clinical variables with caries (TG and G2). The baseline variables were ethnic group and gender. The analyzed clinical variables were: fluorosis, salivary flow, buffer capacity, gingivitis, and presence of plaque. A multivariate analysis by logistic regression model tested the clinical variables and the 3 tag SNPs against caries disease (TG and G2). Haplotype association analysis and *p*-values were estimated using THESIAS test. The univariate and multivariate analyses were performed with SAS software v.9.1.

Results

Clinical findings

The demographic characteristics of the sample are observed in table 2. No statistically significant differences (NS) were observed in fluorosis, salivary flow, and buffer capacity either between TG and CG or between G1 and G2 (Table 3). Gingivitis presented statistically significant differences (SSD) between TG and CG (p=0.010), and between G1 and G2 (p=0.001), being more prevalent in TG and G2. Presence of plaque was significantly more prevalent in G2 group (p=0.001) (Table 3).

Genetic findings

The allele frequencies of the 3 tag SNPs were consistent with the assumption of Hardy-Weinberg equilibrium in the control population sample. The sample was tested against the 3 tag SNPs with two different sample divisions. No significant differences in the allele/genotype distributions of *LTF* were observed between the groups for the rs2073495 and rs11716497. In relation of rs6441989 tag SNP, when G1 was tested against G2 (polarization group) allele A was associated with protection against caries experience [AA + AG x GG (OR: 0.710, IC: 0.514 - 0.980, p=0.045)]. In the multivariate analysis, the tag SNP rs6441989 remained significantly associated with caries protection in the presence of gingivitis (p=0.020) and plaque (p=0.035). The genotype and allele frequencies are shown in table 4. Linkage disequilibrium analysis confirmed independence of all 3 SNPs as shown in figure 2. Thus, the analysis in haplotypes was justified and no association was found between any haplotype and caries experience.

Discussion

It has been noted that not every patient with poor dental hygiene or unadequate diet may develop dental cavitation, indicating a modulation of host response determining

the severity and progression of the disease. Several predictive factors have been proposed for caries outcome: previous caries experience, tooth morphology, and bacterial cascade of adhesion (Sánchez-Pérez et al., 2009). Those risk factors have been used by some clinicians as predictor risk markers, but notwithstanding, there is not a predictive test that may precisely indentify host predisposing conditions (Twetman & Fontana, 2009). Therefore, it is important the identification of groups at higher risk to work more intensively on patients preventive conduct.

Presence of plaque and gingivitis was associated with caries and they are directly related to the presence of cariogenic biofilm. Plaque index represents a quantitative measure of biofilm and has been extensively described as implicative factor for caries development (Marsh, 2010). The presence of gingivitis may be caused by endotoxin excreted from cariogenic bacteria and was already associated with caries disease (Johanson et al., 2009). However, a qualitative evaluation was not carried out in this study to identify which bacteria are present in the biofilm.

Caries studies on monozigotic twins revealed a genetic component of 50% - 70% (Bretz et al., 2005a; Bretz et al., 2005b), which is considered high, even though only latterly this genetic compound has begun to be dissected. Studies investigating the association of genetic polymorphisms and caries susceptibility are rare (Slayton, et al., 2005; Jonasson et al., 2007; Azevedo et al. 2010) and might bring light for the elucidation of the complex nature of dental decay. A genome-wide scan recently performed for caries identified some loci that may be involved in caries susceptibility. Genes related to diet preference and salivary aspects may be found in those loci (Vieira et al., 2008).

Lactotransferrin is a protein shown to be present in salivary and other exocrine glands and in almost all body fluids. Therefore, *LTF* is a possible functional candidate gene that might be predisposing to dental caries. It is found in polymorphonuclear

neutrophils, modulating immune-inflammatory and bactericidal host response being a reliable inflammatory marker.

Some studies reported the association of LTF protein and caries protection, but to the authors' knowledge, there is only one study investigating the association of polymorphisms in *LTF* gene with caries (Azevedo et al., 2010). However, that study analyzed only one restricted part of the gene (second exon). Therefore, a novel physical approach using tag SNPs was carried out, in which a small subset of SNPs may capture information of others, exploiting linkage disequilibrium (Liu et al., 2010). This approach allows the whole gene information to be capture without having to genotype all restrained SNPs.

There was an association found between rs6441989 tag SNP and caries experience in polarization group (G2); allele A confering a protective effect against caries disease. When multivariate analysis was carried out, the SNP presented a stronger association with the higher caries concentration group (G2) in the presence of plaque and gingivitis. That means that in the presence of such variables, the protective effect could be better detected. This tag SNP is located outside and downstream *LTF* gene, but it is in high linkage disequilibrium with rs1034383, located between exon 16 and 17, possibly modifying DNA secondary structure and altering affinity to transcription factors; besides, intron polymorphisms effects are not completely understood. Caries is a multifactorial disease and other genes might be involved with its susceptibility, but the association with *LTF* gene is of great importance once not much of caries genetic background has been elucidated.

Eventhough most populations have access to fluoride, there are still groups at higher risk that require special attention and appropriate oral health promotion programs must be employed. There is a need for identifying those groups in order to achieve a better cost-effective prevention and treatment. It is understood that caries is a polygenic complex disease, leaving a great spectrum of possible host response

modulators to be identified. Further studies are needed to identify other genetic risk markers for this disease, in order to work on better predictive and therapeutic actions.

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Table 1. *LTF* gene primers and hybrid probes.

Tag SNP	SNP Alleles	Fragment length (bp)	Primers	sequence 5´->3
rs6441989	A/G	204	F*	Ψ M13-AACTCACGACTGCTCCCACT
			R**	revM13-CTCTTTTGTCTAATCACTACGGAGG
rs2073495	C/G	234	F	M13- CACCACGGCATGATTCGGGGCCATG
			R	revM13- CATTCCACTGACCTCCCAAATGCAC
rs11716497	A/G	183	F	M13- TTCTGAGCCATCCCCTTATG
			R	revM13- CTAGCTGCCATACTCCC

Tag SNP	SNP Alleles	Fragment lenght (bp)	Probe	sequence 5´->3´
rs6441989	A/G	204	P1	CCCTTCTTCCACACCCCCCCTTCTCA
rs2073495	C/G	234	P2	CTTAGCCCATGCCTCATTGTTATTT
rs11716497	A/G	183	P3	CTGAGTCTGCAGAGGTTTGGGGACA

*****F- forward

******R- reverse

Ψ M13- sequencing primer

Table 2. Baseline characteristics of all sampled subjects (n=677)

Variables	CG (n=331)	TG (n=346)	*p* value*	G1 (n=424)	G2 (n=253)	*p* value*
Ethnic group	**n (%)**	**n (%)**		**n (%)**	**n (%)**	
Caucasian	302 (91.2)	298 (86.1)		383 (90.3)	217 (85.7)	
Afro-American	21 (6.3)	38 (10.9)	0.131	33 (7.7)	26 (10.2)	0.129
Asian	8 (2.4)	10 (2.8)		8 (1.8)	10 (3.9)	
Gender	**n (%)**	**n (%)**	***p* value***	**n (%)**	**n (%)**	***p* value***
Female	185 (55.8)	189 (54.6)	0.799	235 (55.4)	139 (54.9)	0.939
Male	146 (44.1)	157 (45.3)		189 (44.5)	114 (45.1)	

*χ^2 test.

Table 3. Clinical parameters (n=677).

Clinical variables	CG (n=331) n (%)	TG (n=346) n (%)	*p* value *	G1 (n=424) n (%)	G2 (n=253) n (%)	*p* value *
Fluorosis	93 (26.8)	94 (28.3)	0.787	126 (29.7)	61 (24.1)	0.107
Salivary flow ≤0.5 ml/min	82 (24.7)	82 (23.6)	0.744	106 (25.1)	58 (22.9)	0.490
Buffer capacity pH<5.0	7 (2.1)	10 (2.8)	0.268	11 (2.5)	6 (2.3)	0.847
Gingivitis	120 (36.2)	159 (45.9)	**0.010**	155 (36.5)	124 (49.0)	**0.001**
Plaque Index			***p* value ****			***p* value ****
No plaque	51 (15.4)	41 (11.8)		64 (15.2)	28 (11.1)	
Regular Plaque	230 (69.4)	241 (69.6)	0.101	306 (72.1)	165 (65.2)	**0.001**
High plaque	50 (15.1)	64 (18.4)		54 (12.7)	60 (23.7)	

CG: control group; **TG**: test group (patients with 1 or more caries); **G1**: patients with up to one carie; **G2**: patients with 2 or more caries.

*Fisher´s exact test;

**Mantel Haenszel test.

Table 4. Genotype and allele frequencies of tag SNPs rs6441989, rs2073495, and rs11716497.

rs6441989	**CG**	**TG**		**G1**	**G2**	
Genotypes	**n = 331 (%)**	**N=346 (%)**	***p* value**	**N=424 (%)**	**N=253 (%)**	***p* value**
AA	46 (13.8)	62 (17.9)		66 (15.5)	42 (16.6)	
AG	172 (51.9)	156 (45)	0.153**	219 (51.6)	108 (42.6)	**0.045****
GG	113 (34.1)	128 (36.9)		139 (32.7)	103 (40.7)	
Alleles						
A	264 (38.1)	280 (42.3)	0.870*	192 (37.9)	351 (41.3)	0.232*
G	398 (61.8)	412 (57.7)		314 (62)	497 (58.6)	
rs2073495	**CG**	**TG**		**G1**	**G2**	
Genotypes	**n (%)**	**n (%)**	**p value**	**n (%)**	**n (%)**	**p value**
CC	122 (36.8)	141 (40.7)		163 (38.4)	99 (39.2)	
CG	159 (48)	153 (44.2)	0.550**	199 (46.9)	114 (44.8)	0.866**
GG	50 (15.1)	52 (15)		62 (14.6)	40 (15.8)	
Alleles						
C	403 (60.8)	435 (62.8)	0.486*	312 (61.6)	525 (61.9)	0.973*
G	259 (39.1)	257 (37.1)		194 (38.3)	323 (38)	
rs11716497	**CG**	**TG**		**G1**	**G2**	
Genotypes	**n (%)**	**n (%)**	***p* value**	**n (%)**	**n (%)**	***p* value**
AA	134 (40.4)	139 (40.1)		173 (40.8)	100 (39.5)	
AG	148 (44.7)	153 (44.2)	0.958**	191 (45)	110 (43.3)	0.608**
GG	49 (14.8)	54 (15.6)		60 (14.1)	43 (17)	
Alleles						
A	416 (62.8)	431 (62.2)	0.876*	310 (61.2)	537 (63.3)	0.484*
G	246 (37.1)	261 (37.7)		196 (38.7)	311 (36.6)	

CG: control group; **TG**: test group (patients with 1 or more caries); **G1**: patients with up to one caries; **G2**: patients with 2 or more caries.

*Fisher´s exact test;

**Mantel Haenszel test.

Fig. 1. *LTF* gene and the corresponding bins, whose SNPs present a minimum allele frequency (MAF) of at least 0.25. The SNP rs6441989 (MAF 0.491) was in LD with one SNP inside the 3' downstream *LTF* gene, rs2073495 (MAF 0.333) is a singleton and rs11716497 (MAF 0.249) represents a bin of 12 SNPs inside *LTF* gene.

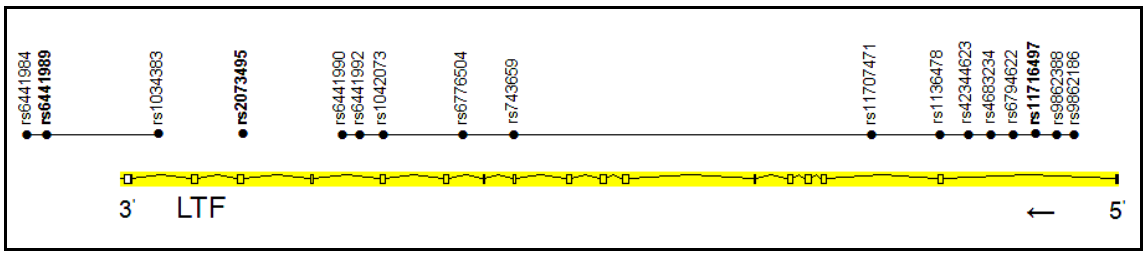
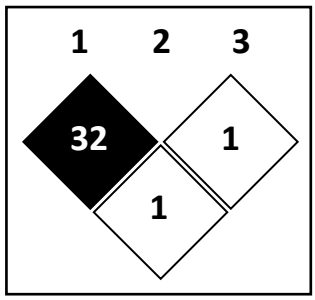


Fig. 2. Analysis of linkage disequilibrium (LD) between *LTF* tag SNPs (r^2<80). The number inside squares indicates the proportion of LD in %. The intensity of the color inside the squares reflects the LD between two loci: the darker intensity represents the highest LD between SNPs.



DISCUSSÃO E PERSPECTIVAS FUTURAS

6. DISCUSSÃO E PERSPECTIVAS FUTURAS

A lactotransferrina é uma glicoproteina multifuncional da família das transferrinas, com ampla distribuição no organismo (em fluidos e em tecidos) e devido ao caráter antiinflamatório, imunológico, antimicrobiano e osteogênico, está envolvida no metabolismo corporal como um todo. É encontrada também nos grânulos secundários dos neutrófilos, estando presente em inflamação aguda e influenciando a modulação de inflamações crônicas. Polimorfismos (variações na sequência de DNA) presentes em seu gene podem provocar uma diferente codificação de sua proteína, podendo contribuir para a suscetibilidade ao desenvolvimento de diversas condições complexas, como a doença periodontal, perda de implantes dentais e a cárie dentária. Já foram descritos polimorfismos no gene *LTF* associados com periodontite agressiva localizada (Vellyagounder et al., 2003; Wu et al., 2009) e cárie dentária (Azevedo et al., 2010). Para esta pesquisa, foi realizado um estudo físico com tag SNPs do gene *LTF*, e foi testada a associação dos genótipos encontrados com as amostras independentes de doença periodontal, perda de implante dental e cárie dentária. Os tag SNPs foram selecionados no site www.hapmap.org (2008), onde se encontram os SNPs de cada *bin* de desequilíbrio de ligação já calculados. Comparando a identificação dos polimorfismos dos sites *Hapmap* e *NCBI* (www.ncbi.nlm.nih.gov/gene/4057-2010) do gene da LTF, apenas 5 SNPs tinham numeração em comum. No *Hapmap* o gene continha 43 SNPs, entre eles, 16 tag SNPs. No site *NCBI*, 41 SNPs estavam descritos para o mesmo gene. Esta diferença na numeração dos polimorfismos tornou difícil a identificação de quais polimorfismos já foram descritos como funcionais. O tag SNP rs2073495 deste estudo foi o único descrito no site *NCBI* como funcional e validado (missense C/G). Há um estudo com este SNP e estress oxidativo no processo de envelhecimento, mas não foi encontrada associação (Kachiwala et al., 2005). Outro SNP descrito em ambos os *sites* foi o rs1042073, o qual está em desequilibrio de ligação com o tag SNP rs11716497, o qual

foi genotipado para esta pesquisa. Até a presente data, não há nenhum estudo com este SNP no www.pubmed.com.

Além do fator genético, foi avaliada a possível associação com outros fatores clínicos do hospedeiro. Fatores ambientais e comportamentais foram investigados em todas as amostras. Os alelos e genótipos dos polimorfismos rs6441989, rs2073495, rs11716497 não estiveram associados à perda de implantes dentais osseointegráveis e à doença periodontal crônica. Foi encontrada associação do alelo A do tag SNP rs6441989 com a proteção contra a cárie dentária. Apesar de este tag SNP estar próximo ao gene, ele está em desequilíbrio de ligação com o SNP rs1034383, o qual está situado dentro do gene *LTF*, entre os éxons 16 e 17. A associação do polimorfismo rs6441989 gene da LTF com a cárie dentária contribui para a elucidação da etiopatogênese da doença, mas estudos genéticos futuros são mandatórios para um entendimento mais aprofundando da mesma.

O gene da LTF tem-se apresentado como um gene conservado entre as espécies, talvez devido às suas importantes funções multifuncionals, pois alterações na proteína poderiam influenciar em diversas funções metabólicas. Estudos de polimorfismos no gene *LTF* com doenças sistêmicas e locais são raros, necessitando, portanto, investigações futuras para determinar o papel de alterações deste gene em doenças humanas. As condições bucais neste estudo são doenças complexas, ou seja, poligênicas. Com a possibilidade de melhor identificação destes grupos que apresentam um risco elevado para o desenvolvimento destas condições bucais, poderá ser possível um manejo individualizado adequado.

Em pesquisa futura, uma análise das amostras, considerando bins na população afro-americana do gene LTF complementaria esta pesquisa, para uma cobertura gênica mais detalhada. Será utilizada a população afro-americana (YRI: Yoruba in Ibadan, Nigeria), e não mais a caucasiana (CEU: CEPH, Utah residents with ancestry from northern and western Europe), para a escolha dos Tag SNPs no gene de estudo.

A justificativa é que, em termos de ancestralidade, a YRI representa uma população mais antiga e, assim, o número de tag SNPs é maior (bins menores). Dessa forma, esta abordagem tornará a captura de informação do gene mais completa, com um mínimo (se houver) de perda de informação. Isso potencializará a identificação de SNPs associados a condições bucais complexas e ampliará a investigação do gene candidato funcional de forma integral. Com essa mudança de abordagem, o número de tag SNPs (aqueles que capturam a informação de outros SNPs em desequilíbrio de ligação - bins) que representam os genes candidatos funcionais integralmente, aumentará para 19, pois consideraremos a frequência alélica mínima a partir de 0,05, e não mais de 0,25. Assim, espera-se que as chances de perder alguma informação, em termos de variabilidade, associada à suscetibilidade condições bucais complexas, serão reduzidas ao mínimo.

CONCLUSÃO

7. CONCLUSÃO

Neste estudo, somente o rs6441989 (alelo A) esteve associado à proteção contra a cárie dentária. Nenhum dos três tag SNPs foi associado à doença periodontal crônica e à perda de implantes dentais. Porém, variáveis clínicas foram associadas às três condições complexas, como segue:

i) Doença Periodontal Crônica: As variáveis clínicas doença renal crônica, proteína C reativa, mobilidade dentária e idade associaram-se com a doença periodontal crônica. No entanto, nenhum dos três tag SNPs foi associado à doença periodontal crônica.

ii) Perda de Implantes Dentais: As variáveis clínicas associadas com a perda de implante dental foram tratamento médico, reposição hormonal, edentulismo, número de implantes colocados, índice de placa, índice de cálculo e mobilidade dentária. Entretanto, nenhum dos três tag SNPs foi associado à perda de implantes dentais.

iii) Cárie Dentária: Além da associação do tag SNP rs6441989 com a proteção contra a cárie dentária, placa e gengivite foram associadas positivamente com esta doença complexa.

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8. REFERÊNCIAS

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ANEXO

ANEXO

Outros artigos publicados e submetidos à publicação durante o doutorado:

Int J Cardiol. 2010 Apr 7

J Neurol Neurosurg Psychiatry. 2009 Dec;80(12):1402-4.

Frequency and phenotype of SPG11 and SPG15 in complicated hereditary spastic paraplegia.

Schüle R, Schlipf N, Synofzik M, Klebe S, Klimpe S, Hehr U, Winner B, Lindig T, Doetzer A, Riess O, Winkler J, Schöls L, Bauer P.

Mov Disord. 2010 Sep 15;25(12):1982-6.

Prevalence of THAP1 sequence variants in German patients with primary dystonia.

Söhn AS, Glöckle N, Doetzer AD, Deuschl G, Felbor U, Topka HR, Schöls L, Riess O, Bauer P, Müller U, Grundmann K.

Orthognathic surgery without blood transfusion.

Luis Eduardo Almeida, Andrea Duarte Doetzer, Juliano Perotto, Paula Cristina 3999Trevilatto, Marco Antonio de Oliveira Filho

Myositis ossificans traumatic of the Lateral Pteygoid Muscle in a 12-year-old child: Case report and description of an alternative surgical access.

Luis Eduardo Almeida, Andrea Duarte Doetzer, Paula Cristina Trevilatto, Marco Antonio de Oliveira Filho