UNESP - Universidade Estadual Paulista
"Júlio de Mesquita Filho"
Faculdade de Odontologia de Araraquara

Ingra Gagno Nicchio

Investigação da associação de polimorfismos em genes do metabolismo lipídico com o estado periodontal e perfil bioquímico do paciente

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Dissertação apresentada à Universidade Estadual Paulista (UNESP), Faculdade de Odontologia de Araraquara, para obtenção do título de Mestre em Odontologia, na Área de Periodontia.

Orientadora: Profa. Dra. Raquel Mantuaneli Scarel Caminaga

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Dissertação para obtenção do grau de Mestre em Odontologia

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DADOS CURRICULARES

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“A educação é a arma mais poderosa que você pode usar para mudar o mundo”

Nelson Mandela

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RESUMO

Globalmente continua-se a buscar evidências de suscetibilidade genética à periodontite (P), pois as variações genéticas entre as diferentes etnias costumam influenciar em tal suscetibilidade. Também há grande interesse em compreender melhor as inter-relações da periodontite com patologias como o Diabetes Mellitus tipo 2 (T2DM, *type 2 Diabetes Mellitus*) e a Dislipidemia. Devido a similaridades na etiopatiogenia dessas doenças, levantamos a hipótese que genes do metabolismo lipídico estão associados com o desenvolvimento da P e do T2DM. O objetivo deste estudo foi investigar polimorfismos em genes do metabolismo lipídico relacionando-os com o estado periodontal e perfil glicêmico e lipídico do paciente para avaliar se estes estão associados com a P na presença ou não de T2DM. Considerando o cálculo amostral, foram investigados: pacientes com T2DM e periodontite severo ou moderada (Grupo T2DM_P, n=205 pacientes); pacientes sem T2DM com periodontite severo ou moderada (Grupo Periodontitis, n=345); e pacientes sem T2DM e sem periodontite, ou seja, sistemicamente e periodontalmente saudáveis (Grupo Healthy, n= 343). Assim, o Grupo Periodontitis foi considerado controle para o T2DM e o Grupo Healthy foi considerado controle para a T2DM e P. Após o exame periodontal completo, aos participantes deste estudo foi oferecida a realização de exames bioquímicos (perfil glicêmico e lipídico), mesmo para os pacientes diagnosticados pelo seu médico como afetados pelo T2DM. Foram obtidas células da mucosa bucal de cada paciente para extração do DNA pelo método de *salting-out*. Foram investigados nove polimorfismos de nucleotídeo único (SNP) nos genes *LPL, HNF4A, APOE, ABCC8, HNF1A* por meio do sistema de genotipagem *TaqMan* utilizando PCR em tempo real. Os dados periodontais e genéticos foram comparados entre os grupos, realizando análises de regressão logística múltipla buscando-se ajustar os resultados a variáveis como idade, sexo e tabagismo. Correlações do estado periodontal com o perfil glicêmico e lipídico foram feitas. Como principais resultados, foi observado que pacientes com genótipo CT para o SNP rs1800961 (gene *HNF4A*) tiveram maior suscetibilidade ao T2DM, principalmente mulheres.Evidenciou-se que indivíduos do sexo masculino carregando o genótipo CC do SNP rs13702 (gene *LPL*) apresentaram maior suscetibilidade a P na presença de T2DM, enquanto que aqueles carregando o SNP rs6544718-CT (gene *ABCC8*) mostraram menor chance de desenvolvimento de ambas as doenças. Também homens carregando o genótipo CT do SNP rs285 (gene *LPL*) foram menos suscetíveis ao desenvolvimento de P isoladamente. Conclui-se que na população estudada foram observados SNPs em importantes genes do metabolismo lipídico, como rs285 (gene *LPL*) que foi associado à *P*, o rs1800961 (gene *HNF4A*) que foi associado ao T2DM, enquanto que rs6544718 (gene *ABCC8*) e rs13702 (gene *LPL*) foram associados à P na presença de T2DM.

Palavras chave: Polimorfismo Genético. Diabetes Mellitus. Periodontite. Metabolismo dos lipídeos.

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ABSTRACT

Globally, we continue to seek detection of genetic susceptibility to periodontitis (P), as genetic changes between different ethnicities tend to influence susceptibility. Also, there a strong interest from the scientific community to better understand interrelationships between periodontitis and conditions such as Diabetes Mellitus Type 2 (T2DM) and Dyslipidemia. Due to similarities in the etiopathogenesis of these diseases, we hypothesized that genes of lipid metabolism are associated with the development of P and T2DM. This study aimed to investigate polymorphisms in the lipid metabolism genes, relating them to the periodontal state and the patient's glycemic and lipid profile to assess whether these associated are associated with a presence or not of T2DM. For sample calculation, we investigated: patients with T2DM and severe or moderate periodontitis (T2DM_P Group, n = 205 patients); patients without T2DM with severe or moderate periodontitis (Periodontitis Group, n = 345); and patients without T2DM and without periodontitis, or periodontally healthy (Group Healthy, n = 343), which are systemically and periodontally healthy. Thus, the Periodontitis Group was considered control for T2DM and the Healthy Group was considered control for T2DM and P. After a complete periodontal exam of the contributors, they were offered biochemical exams (glycemic and lipid profiles) even for that patients that were diagnosed by their doctor as affected by T2DM. Oral mucosa cells were obtained from each patient for DNA extraction using the method salting out. Single Nucleotide polymorphisms (SNP) of the *LPL, HNF4A APOE, ABCC8, HNF1A* genes were investigated by Taqman genotyping using real-time PCR. Periodontal and genetic results were compared, and multiple logistic regression analysis was performed using the PLINK and STATA programs to adjust the results to variables such as age, gender, smoking and biochemical profile. Correlations among lipid and glycemic profile with the periodontal status were made. As a result, it was observed that patients with CT genotype for the SNP rs1800961 (gene *HNF4A*) showed a higher chance of T2DM, mainly in women. . It became evident that for male patients who carried the CC genotype for SNP rs13702 (gene *LPL*) showed susceptibility for P+T2DM whereas that patients with rs6544718 (gene *ABCC8)* showed less chance to develop both diseases. Also, male patients carrying CT genotype in SNP rs285 (gene *LPL*) were less susceptible to develop P Therefore, it was concluded that important SNPs related to lipid and glycemic metabolism, such as rs285 (*LPL*) was related to P, rs1800961 (*HNF4A*) was related to T2DM, and rs6544718 (*ABCC8)* and rs13702 *(LPL)* were associated to P in the presence of T2DM.

Keywords: Polymorphisms. Genetic. Diabetes Mellitus. Periodontitis. Lipid metabolism.

SUMÁRIO

1 INTRODUÇÃO

Esta introdução foi dividida em seções abordando a Periodontitie, Diabetes tipo 2, Dislipidemia, Inter-relação de todas estas doenças e Polimorfismos Genéticos.

1.1 Periodontite

A doença periodontal (DP) denominada neste trabalho como Periodontite (P) possui natureza multifatorial que envolve a interação da resposta imunoinflamatória do hospedeiro em conjunto a um eventual desequilíbrio bacteriano e influência de fatores ambientais. Em resposta a isso ocorre a migração apical do epitélio juncional, perda das fibras do ligamento periodontal e osso alveolar[1,2]. Dessa forma, o fator etiológico primário da P é a infecção do periodonto por microrganismos do biofilme dental[3].

As perdas dentárias em adultos estão altamente associadas à periodontite, perfazem aproximadamente 10% da população sendo que 30% dos indivíduos acima dos 50 anos manifestam periodontite severa citado por Brown, Oliver, & Loe, 1990; Gjermo, 1998. A DP é iniciada por uma gengivite, ou seja, inflamação gengival induzida por bactérias do biofilme dentário, que quando não tratada pode progredir para uma P. Somente a resposta ao biofilme bacteriano costuma não ser suficiente para a patogênese da P; ou seja, a P ocorre quando é perdido o equilíbrio entre biofilme bacteriano e resposta imunoinflamatória do hospedeiro, manifestando um dano tecidual ao periodonto por uma reação exagerada do sistema imunológico à presença microbiana [4].

A função primordial da cascata inflamatória é proteger o organismo contra agentes estranhos; porém, quando a resposta imune não é suficiente para eliminar os microrganismos invasores, a persistência de mediadores inflamatórios pode levar a injúrias ao tecido, com consequente formação de bolsa periodontal, perda de tecido conjuntivo, destruição do ligamento periodontal e reabsorção de osso alveolar, culminando com a perda do elemento dentário[5].

Embora fatores microbianos e outros fatores ambientais iniciem e modulem a P, sabe-se que ocorre uma resposta diferente entre os indivíduos frente aos mesmos estímulos. Essa resposta diferencial é influenciada por diversos fatores relacionados ao hospedeiro, como o tabagismo, doenças sistêmicas e herança genética, que tem sido extensivamente estudados para compreender sua participação para o início e progressão da P[6, 7]. Evidências apontam para a existência de uma base genética para a maioria das doenças, incluindo a P [8]. Estudos de genética clássica incluindo gêmeos monozigóticos e dizigóticos mostram a importância dos

efeitos e fatores genéticos em doenças comuns. Um dos principais estudos na área desenvolvido por Michalowicz e colaboradores[9] que avaliaram a influência genética e ambiental da P usando dados clínicos de uma amostra independente de gêmeos adultos, incluindo variáveis como higiene oral, tabagismo e outros comportamentos que poderiam influenciar a ocorrência da P. Foi constatado que a P possui aproximadamente 50% de hereditariedade, já considerando o ajuste para variáveis comportamentais. Ainda são necessários mais estudos para compreender a influência genética na P para permitir um melhor entendimento da etiologia, diagnóstico e tratamento[10] da doença. Pode explicar, em parte também, porque a P pode se manifestar de diferentes formas clínicas na população[11].

1.2 Diabetes Mellitus Tipo 2 (T2DM) e Dislipidemia

O Diabetes Mellitus (DM) é uma doença metabólica que envolve primariamente os carboidratos, seguido dos lipídeos e proteínas, sendo caracterizado pela hiperglicemia resultante de defeitos na secreção de insulina, em sua ação ou em ambos[12]. Como resultado direto da hiperglicemia e do desequilíbrio osmótico, uma tríade clínica clássica de sintomas é desenvolvida, e inclui polifagia, polidipsia e poliúria[6] .O DM possui causa multifatorial e pode ser agravada por diversos fatores, como: fatores genéticos, ambientais[12] e comportamentais [13].

A prevalência do DM para todas as faixas etárias em 2000 foi de 2,8%. Essa doença acomete cada vez mais pacientes a cada ano, o que é justificado também pelo aumento de pessoas com 65 anos ou mais [14]. É previsto em 2030 que o número de pessoas com essa doença aumente de 171 milhões para 366 milhões.[15] .

Aproximadamente 90% da população que possuem DM, tem o tipo 2 (T2DM) [16]. Fatores importantes para a severidade do T2DM são a falta de atividade física, obesidade, consumo excessivo de álcool e estresse, os quais são modificáveis pelo paciente.

Estima-se também que 2/3 dos pacientes que possuam T2DM mostrem alterações nas taxas em lipídios e lipoproteínas[17]. O T2DM pode ocorrer em conjunto com outras alterações sistêmicas, como a Dislipidemia, uma vez que existe uma relação direta entre os índices glicêmicos e alterações quantitativas e qualitativas de lipídeos no sangue[18]. Lipoproteínas são moléculas capazes de transportar esses lipídeos, visto que eles não se misturam facilmente com o plasma sanguíneo. O excesso de colesterol no sangue prejudica a captação das lipoproteínas levando a uma oxidação e formação de placas lipídicas, também conhecida como ateroma[19]. Essa desordem quando não tratada pode levar a obstrução de vasos sanguíneos e consequentemente à chance de ataques cardíacos, infartos, acidente vascular

cerebral, aterosclerose e outras. [19-21] Como algumas das principais características clínicas da Dislipidemia estão a elevada taxa de triglicérides em jejum, redução de lipoproteína de alta densidade (HDL) e elevada taxa de lipoproteínas de baixa densidade (LDL)[18].

Assim como o T2DM, a Dislipidemia pode ter causa genética e/ou ambiental. Entre as causas genéticas estão: hiperlipedimia combinada, defeito na Apolipoproteína B100, hipercolesterolemia familiar, entre outras. Dentre as causas ambientais pode-se incluir: dietas pouco saudáveis, falta de prática de atividades físicas, estresse, entre outros[22]. A Dislipidemia representa uma séria ameaça à saúde global; portanto o seu controle promove diretamente a diminuição na taxa de mortalidade e morbidade das doenças cardiovasculares [23].

1.3 Inter-relação entre Periodontite, T2DM e Dislipidemia

As doenças inflamatórias, como a P tem sido associada a distúrbios do metabolismo lipídico e glicêmico. Pacientes com DM tem maior chance de desenvolver a P e aumentar sua severidade quando estes não controlam os níveis de hemoglobina glicada (HbA1c)[24]. Atualmente a P, assim como o T2DM, tem sido consideradas doenças complexas ou multifatoriais, em que fatores genéticos e ambientais exercem papel essencial na sua patogênese[9]. Uma característica importante das doenças complexas, como a T2DM e a P, é o fato de serem poligênicas, ou seja, inúmeros genes estão envolvidos na suscetibilidade e na severidade da doença, cada qual garantindo uma pequena contribuição.

A periodontite pode ser reconhecida como a sexta maior complicação associada ao DM [25], sendo que foi detectada maior prevalência e severidade da doença periodontal em pessoas diabéticas do que naqueles não portadores da doença [26-28]. A inter-relação bi-direcional entre P e T2DM tem sido estudada há vários anos, sendo que a premissa para esta é a resposta inflamatória exarcebada e desregulada [29,30]. Acredita-se que a relação bidirecional entre a P e o T2DM resulta de: 1) uma relação causal direta, na qual o estado hiperglicêmico e as complicações do T2DM impactam os tecidos periodontais e agem como modificadores da expressão da P; 2) a presença de citocinas inflamatórias da P possam levar a alterações no metabolismo da glicose e de lipídios [29,31,32,33]. Isso é demonstrado pela bacteremia e endotoxemia associadas à P que elevam os níveis séricos de citocinas, como TNF-α, PGE2, IL-1 e IL-6, fomentando a DM. O tecido conjuntivo periodontal inflamado pode atuar como reservatório para esses mediadores, o que acarretaria em alterações no metabolismo da glicose e dos lipídios, induzindo à resistência insulínica[34].

A inter-relação entre P e Dislipidemia tem sido alvo de intensas pesquisas nos últimos

anos. O tratamento periodontal pode reduzir a inflamação sistêmica e melhorar o perfil lipídico. Uma meta-análise demonstrou que pacientes com P apresentaram níveis significativamente mais altos de LDL e Triglicérides, e níveis séricos reduzidos de HDL [35].

A Dislipidemia e T2DM são causas importantes para a morbidade e mortalidade em pacientes com doenças cardiovasculares[36], que causaram mais de 18 milhões de mortes em todo o mundo, sendo que 44% dessas ocorreram em pessoas com menos de 60 anos de idade, e 80% em países de baixa renda[25]. A dislipidemia contribui para o desenvolvimento de doenças cardiovasculares, tendo em vista que o ateroma é formado pela acúmulo focal de lipídeos, através do processo de peroxidação, modificando as paredes dos vasos [27,28]. A P permite a entrada de bactérias na corrente sanguínea ativando a resposta imune do hospedeiro por múltiplos mecanismos. Essa resposta favorece a formação, maturação e exacerbação de ateroma

1.4 Polimorfismos Genéticos

Polimorfismos genéticos são formas variantes (alelos) de um lócus específico do cromossomo, que coexistem naturalmente na população humana. São, portanto, variações normais do genoma humano, no qual o alelo mais raro ocorre com uma frequência maior que 1% na população[37]. Os polimorfismos surgem como resultado de mutações, como inserções, deleções ou substituições de bases nucleotídicas, podendo gerar uma proteína não funcional ou alterar a expressão da referida proteína. O tipo de polimorfismo mais comumente relatado na literatura é o Polimorfismo de Nucleotídeo Único (*Single Nucleotide Polymorphism - SNP*), que consiste em uma variação da identidade de um nucleotídeo singular num sítio particular do genoma [37].

O primeiro estudo demonstrando a associação entre P e um polimorfismo genético, no caso no gene *IL1*, foi de Kornman e colaboradores[38] em 1997. A partir de então, centenas de estudos vêm sendo realizados com o objetivo de verificar se há associação de polimorfismos em diversos genes candidatos com a P, principalmente genes do sistema imunoinflamatório [5, 39-42]. Na última década vem sendo realizados estudos englobando todo o genoma, o Genome-Wide Association Study (GWAS), que mostrou da P associação com genes com funções metabólicas pouco exploradas. Considera-se esse tipo de estudo como uma estratégia livre de hipóteses[43,]

Esses estudos de GWAS indicaram que muitos outros genes, além dos envolvidos na resposta imunoinflamatória, podem ter papel relevante para a ocorrência da P. Considerando evidências como a meta-análise que demonstrou associação da P com níveis alterados do

perfil lipídico [35], observamos que há importantes genes do metabolismo lipídico que foram pouco ou nunca explorados com relação à P, como: *APOE (OMIM: 107741 - Apolipoprotein E*), *LPL (OMIM: 609708 – Lipoprotein Lipase), ABCC8 (OMIM: 600509 – ATP- Binding Casset, Subfamily C, Member 8), HNF4A (OMIM: 600281 – Hepatocyte Nuclear Factor 4-Alpha)* e *HNF1A (OMIM: 14210 - Hepatocyte Nuclear Factor 1-Alpha).*

A *Apolipoprotein E* (*APOE)* pertence a uma classe de apolipoproteínas encontradas em quilomicron e em lipoproteínas de densidade intermediária (LDLs), sendo essencial para o catabolismo normal de lipoproteínas ricas em triglicerídeos ("Entrez Gene: APOE apolipoprotein E"). *APOE* é produzida principalmente pelo fígado e macrófagos, mediando o metabolismo de lipoproteínas, que envolve sua ligação ao receptor do LDL (LDLR), tendo também importância reconhecida na doença cardiovascular [44]. O gene *APOE* localiza-se no braço longo do cromossomo 19, sendo que mutações resultam em disbetalipoproteinemia familiar ou hiperlipoproteinemia tipo III (HLP III), em que o aumento do colesterol plasmático e triglicerídeos são a consequência do metabolismo prejudicado de quilomícrons, VLDL e LDL[45].

O gene *LPL* atua como um ligante de *LDLR* para a endocitose de VLDL e LDL. O gene *LPL* localiza-se no cromossomo 8, sendo que mutações mais graves nesse gene causam hiperlipoproteinemia tipo I, enquanto mutações menos extremas em LPL estão ligadas a muitas doenças do metabolismo de lipoproteínas[46].

A proteína ABCC8 (OMIM: 600509 - ATP Binding Cassette Subfamily C Member 8) atua como um modulador de ATP-sensível a canais de potássio liberando insulina, interagindo com *PPARG (Peroxisome Proliferator Activated Receptor Gamma*) e *HNF4A* (Hepatocyte Nuclear Factor 4 Alpha)[47]. Mutações e deficiências nesta proteína têm sido observadas em pacientes com hipoglicemia hiperinsulinêmica da infância, uma doença autossômica recessiva relacionada à secreção desregulada de insulina. Polimorfismos no gene *ABCC8* têm sido associados com a T2DM em vários estudos [48-51].

O gene *HNF4A* codifica um fator de transcrição que atua em células beta pancreáticas regulando a expressão do gene da insulina, bem como de genes que codificam proteínas envolvidas no transporte de glicose e metabolismo mitocondrial, todos os quais estão ligados a secreção de insulina [52]. Barroso et al., [53] (2003) identificou um haplótipo no gene *HNF4A* significativamente associado com o risco reduzido de T2DM. É plausível que variantes neste gene aumentem os níveis de expressão da proteína *HNF4A* levando ao aumento da capacidade de secreção de insulina e a proteção contra o desenvolvimento do T2DM [53]. Um SNP

rs1884614 na região promotora do gene *HNF4A* pode influenciar a secreção de insulina em indivíduos japoneses não-obesos com T2DM [54], bem como em Chineses [55] e Ingleses do Reino Unido[53]. O gene *HNF4A* também controla a expressão de vários genes, como o *HNF1A* que tem como função codificar proteínas ligadas principalmente ao fígado. Polimorfismos nesse gene estão associados ao desenvolvimento de DM em idades jovens e a diminuição de expressão de Apoliproteina M (ApoM), um importante componente do HDL[56]. Steele et al. associou que pacientes que tinham DM associadas ao gene *HNF1A* poderiam ter maiores chances de problemas cardiovasculares[57].

Mediante a importância dos genes do metabolismo lipídico *LPL*, *HNF4A, HNF1A, APOE* e *ABCC8,* do fato de existem milhares de genes com funções metabólicas diferentes que podem ter influência ainda não conhecida com a P, e com o intuito de compreender melhor a inter-relação da P com doenças sistêmicas como a T2DM, nossa hipótese é que SNPs nesses genes possam estar associados com a suscetibilidade ou predisposição à P, seja esta como uma doença isolada, ou conjunta ao T2DM (T2DM_P).

Dessa forma, acreditamos que poderemos contribuir para o estudo da inter-relação de T2DM e P, e da possível relação destas com a Dislipidemia. Para isso é necessário incluir a avaliação rigorosa do controle glicêmico e lipídico à avaliação periodontal.

2 PROPOSIÇÃO

O objetivo deste estudo foi investigar polimorfismos em genes do metabolismo lipídico relacionando-os com o estado periodontal e perfil glicêmico e lipídico do paciente para avaliar se estes estão associados com a P na presença ou não de T2DM.

3 PUBLICAÇÃO

Nesta seção apresentamos o artigo científico em língua inglesa referente à pesquisa desenvolvida nesta dissertação e que será submetido à publicação.

3.1 Artigo 1*

Polymorphisms in genes of lipid metabolism are associated with the periodontal status and glycemic and lipid profiles of the patients

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Abstract

Background: There is a strong interest of the scientific community to better understand interrelationships between Periodontitis (P) and systemic conditions such as Type 2 Diabetes Mellitus (T2DM) and Dyslipidemia. This study aimed to investigate polymorphisms in genes of the lipid metabolism, to determine whether they are associated with the susceptibility to P, or with simultaneous P and T2DM, and whether they are correlated with the patient's glycemic, lipid and periodontal statuses. .

Material and Methods: We investigated patients with T2DM also affected by Periodontitis (T2DM_P group, n = 205), patients affected by only P (Periodontitis group, n = 345) and patients without any T2DM or P (Healthy group, n = 343). The patients underwent complete periodontal examination and biochemical evaluation. DNA from buccal cells of each patient was utilized for investigating 9 Single Nucleotide polymorphisms (SNP) in the *HNF1A*, *HNF4A*, *APOE*, *ABCC8*, and *LPL* genes. Periodontal and genetic results were analyzed among groups by multiple logistic regression analysis, adjusting for covariates and also considering stratifications by sex and smoking habits. Correlations among lipid and glycemic profile with the periodontal status were made.

Results: We observed that women carrying the rs1800961-CT (*HNF4A* gene) were more susceptible to T2DM. Men carrying the rs13702-CC (*LPL* gene) were more susceptible to T2DM in the presence of P (T2DM+P), while those carrying the rs6544718-CT (*ABCC8* gene) were less prone to the development of both diseases. Regarding the isolated P, men showed again lower susceptibility when carrying the rs285-CT (*LPL* gene). Positive (directly proportional) or negative (inversely proportional) significant correlations of biochemical profile and periodontal parameters were found for each group.

Conclusions: Relevant gene-phenotype associations were found in the studied population, some of them demonstrating gene-sex interaction, such as the rs285 (LPL gene) with P, the rs1800961 (*HNF4A* gene) with T2DM, in addition to both the rs6544718 (*ABCC8* gene) and rs13702 (*LPL* gene) with the T2DM together with P (T2DM+P). Significant correlations showing a relevant relationship between biochemical profile and periodontal parameters were found.

Keywords: Genetic Polymorphisms, Diabetes Mellitus, Periodontitis, Dyslipidemia, Lipids, lipid metabolism

Introduction

Periodontitis is a multifactorial disease which involves an interaction by the host immunoinflammatory response and bacteria unbalance. In response to that will occur apical migration of the junctional epithelium, loss of periodontal ligament fibers and alveolar bone [1-3].Dysbiosis in the microbiota it's the primary factor of periodontitis, constant changes in the interaction between this and lifestyle habits (diet, smoking, stress, etc), leads to constants changes in the host physiology[4]. Individuals respond in different ways influenced by environmental and genetic factors. Genes play an important role when comes to predisposition and progression of P, evidence shows that exist an important genetic base for most diseases, including P[4].

Diabetes mellitus (DM) is a metabolic disorder that involves persistent hyperglycemia, resulting from defects in insulin production, action or both[5]. The classification of DM has been based on its etiology, by Type 1 and Type 2. Diabetes Mellitus Type 2 (T2DM) occurs when there is a progressive loss of insulin secretion combined with insulin resistance[6]. Dyslipidemia occurs when there is an increase of blood fat, in other words a qualitative and quantitative change in lipoproteins and lipids[7]. Lipoproteins are responsible to transport lipids since they do not mix easily with blood in the plasma[8]. T2DM can occur in conjunction with other systemic changes such as dyslipidemia, because this will allow a direct relationship between glycemic levels and alterations of quantitative and qualitative levels of lipids and lipoproteins. Excess cholesterol in the blood impairs the uptake of lipoproteins, leading to oxidation and the formation of lipid plaques, also known as atheroma. Dyslipidemia in association with T2DM are important diseases that are closely related to the consequences of cardiovascular problems, increasing the morbity and mortality[9].

Polygenicity is an important factor in complex diseases, as T2DM and P, since uncounted genes are involved in the susceptibility and severity of the disease, each one performing a short contribution[10]. P, T2DM and dyslipidemia are complex and multifactorial diseases, in which genetic and environmental factors play an essential role in the pathogenesis[11].

Evidences are increasing demonstrating that P, T2DM and dyslipidemia are complex and have a multifactorial etiology in which each disease will occur a systemic hyper-inflammatory state [12]. In the multifactorial diseases, genetic and environmental factors play an essential role in their pathogenesis[4, 13, 14]. Individuals with T2DM show more prevalence and gravity of P when compared to individuals that do not carry the disease [15], being P the sixth biggest complication of T2DM[16]. Reinforcing the evidences regarding the association

between P and dyslipidemia, a meta-analysis demonstrated that P is significantly associated with reduced levels of HDL, but elevated LDL and triglyceride concentrations[17]. In spite of some similarities in the etiopathogenic factors among P, T2DM and dyslipidemia, more studies are necessary to achieve better understand in their potential connection.

This study aimed to investigate polymorphisms in the lipid metabolism genes, relating them to the patient's periodontal status in the presence or not of T2DM, and whether there are correlations with the glycemic and lipid profiles of the patients.

Materials and Methods

Patient´s Selection

The study was approved by the Ethics in Human Research Committee of the School of Dentistry at Araraquara (UNESP – São Paulo State University, Araraquara, Brazil; Protocol number 50/06). The patients were recruited at the Periodontics Clinic of the same Institution. All volunteers were informed about the aims and methods of this study, and they provided their written consent to participate.

Basic inclusion criteria were patient´s at minimum age of 30 years old who had at least 15 natural teeth. Patients were excluded based on the following criteria: history of antibiotic therapy in the previous 3 months and/or nonsteroidal anti-inflammatory drug therapy in the previous 6 months and pregnancy or use of contraceptives or any other hormone.

All patients answered a structured questionnaire about demographic characteristics, personal and family medical history, and use of medications.

Biochemical evaluation of glycemic and lipid profiles

All blood samples were collected after 12 hours overnight fast by the Laboratory of Samples Analysis (NAC) from UNESP. Were evaluated insulin levels by the chemiluminescence method (U/L), blood count (ABX Micros 60), fasting plasma glucose (mg/dL) by modified Bondar & Mead method, glycated hemoglobin (HbA1c) by enzymatic immunoturbidimetry (Roche kit), triglycerides (TGs), Total Cholesterol and HDL by enzymatic methods and VLDL and LDL fractions was determined by the Friedewald formula. To avoid the inclusion of individuals with transitory dyslipidemia, the cutoff points used were the highest values according European Association of Atherosclerosis[18]: TC $\geq$ 240 mg/dL, LDL $\geq$ 160 mg/dL, HDL $\leq$ 40 mg/dL, and TGs $\geq$ 200 mg/dL.

Non-Diabetics was considered as adequate when HbA1c < 6.4% and fasting glucose levels < 99 mg/dl and patients diagnosed by an endocrinologist with T2DM, were divided in

two groups: with poorly glycemic control (HbA1c ≥ 7.1%) or well-controlled demonstrating a metabolic compensation (HbA1c ≤ 7.0% and ≥5.8%)[19]. For the results of the lipid profile, patients with at least one of the above-mentioned clinical parameters are considered as dyslipidemic[18].

Periodontal clinical examination profiles

The periodontal examination was performed assessing the following clinical signs and parameters at four interproximal sites around each tooth: probing pocket depth (PPDi) and clinical attachment level (CALi), measured to the nearest millimeter by a UNC-15 periodontal probe. The presence of visible plaque, marginal bleeding and bleeding on probing (BOP)[20] were recorded as a percentage of the total number of interproximal sites assessed. All fully erupted teeth, except the third molars and teeth with extensive destruction of the clinical crown, were examined. A history of smoking was defined according to Miyake et al. (2014) as follows: (i) never smoked or (ii) ever smoked (including patients who are current smokers and former smokers)[21].

According to the criteria defined by the CDC/AAP (Center for Disease Control and Prevention/American Academy of Periodontology)[22, 23] the patients were diagnosed with their periodontal condition in periodontitis as periodontally healthy or mild periodontitis (moderate-severe) (Tables 2 and 3). Such criteria have already been used in genetic studies[24, 25].

The needed sample size was calculated by the G* Power Calculator, version 3.1.9 (Faul et al., 2007) software, as detailed in the Supplementary material.

After the assessment of the glycemic and periodontal profiles and the periodontal status, all the 893 selected patients were divided into 3 groups: T2DM_P group containing 205 individuals with T2DM and Periodontitis; Periodontitis group containing 345 individuals affected by only moderate-severe Periodontitis; and Healthy group containing 343 individuals systemically and periodontally healthy.

DNA Extraction and Genotyping

The subjects did a mouthwash for 2 minutes with 3ml of 3% of dextrose solution to obtain buccal epithelial cells, which were centrifuged at 2000rpm for 10 minutes and stored in a buffer solution (TrisHCl 10mM, EDTA 0,1 M, SDS 0,5% - pH 8.0). After the proteinase K digestion, the DNA was extracted using 8M of ammonium acetate[26]. The genomic DNA of

each sample was evaluated for concentration (ng/µl) and purity (260/280nm; 260/230nm) in the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA), followed by quantification using the Qubit 2.0 fluorescence system (Fisher Scientific, USA).

All samples showed $A_{260/230}$ and $A_{260/280}$ ratios between 1.7 and 2.0, and concentration was adjusted to 50 ng/µL to genotype the SNPs in the genes presented in the Table 1. Each of the 9 SNPs were analized by Real Time Polimerase Chain Reaction (PCR) using TaqMan® (Thermo Fischer Scientific, Foster City, CA, EUA) systems. The amplification was performed in final volume of 12.5 µL, containing 0.63 µl of the specific TaqMan assay for genotyping each referred SNPs (including forward and reverse primers and probes marked with VIC and FAM, as presented in Table 1); 6.25 µl of TaqMan® Genotyping Master Mix(2X TaqMan®Genotyping Master Mix, Thermo Fisher Scientific), 20ng of genomic DNA and DNAse/RNAse free water in enough quantity to complete the final genotyping reaction. The following conditions were used for cycling the SNPs on a qPCR thermocycler (StepOnePlus Real-Time PCR System - Thermo Fisher Scientific): 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 min. The genotyping analysis was performed with an online software (Applied Biosystems™ Analysis Software, Genotyping Analysis Module, version 3.2.) using autocalling for the automated grouping of sequences, deemed by the analysis software to have similar High Resolution Melt (HRM) curve shapes and temperature melting (Tm). The quality value of the data points for the genotyping was determined by a threshold above 95%.

Statistical Analysis

The general characteristics of each group were submitted to a descriptive and analytical statistics using IBM SPSS Statistics 20.0 and GraphPad Prism 6.0 softwares. First, all data was evaluated by normality tests (D'Agostino & Pearson), then were applied parametric (multiple comparison with the One-way ANOVA followed by Tukey test) or nonparametric (multiple comparison by Kruskal-Wallis test followed by Dunn's test) tests. Continuous demographic variables were compared among the groups using the Student's-t test (for age), while the chi-squared test was used for categorical variables (sex, ethnicity and smoking). To evaluate differences in the periodontal parameters among the groups we used the Kruskal-Wallis followed by Dunn's test, or the Mann-Whitney test.

The Hardy–Weinberg equilibrium (HWE) formula was applied to the genotype frequencies, separately for each group, using chi-square goodness-of-fit for any deviations

from the expected genotype equilibrium, as well the minor allele frequency (MAF) was calculated separately for each group. These analyses and to detect differences in distributions for each SNP among the groups were made using the PLINK software [27].

Multiple logistic regression models assuming additive allelic effects, adjusting for age, sex and smoking habits (as independent variables) were used for testing associations between SNPs and: the occurrence of only P (when comparing Healthy and Periodontitis groups); the occurrence of only T2DM (when comparing Periodontitis and T2DM_P groups), and the occurrence of T2DM together with P (when comparing Healthy and T2DM_P groups). These gene-phenotype associations were estimated by odds ratio (OR) and Confidence Interval (CI) of 95%.

Furthermore, the same analysis was stratified by sex to identify the sex-specific effects of the SNPs on periodontitis, by the additive model using multiple logistic regression adjusted by age and smoking. Similarly, to identify the effects of smoking habits with those SNPs on periodontitis, we stratified the population by smoking status and executed the multiple logistic regression adjusting by age and sex. All the multiple logistic regressions were performed by using the STATA software version 12.0 for Windows (Statistics/Data Analysis, Stata Corporation, College Station, TX, USA). Spearman's correlations among glycemic and lipid profile with periodontal parameters were made for each group. Differences were considered significant when $p<0.05$.

Results

It could be observed in Table 1, that for most of the SNPs investigated here, the alleles with the minor frequency found in the groups Healthy, Periodontitis and T2DM_P were in agreement with the minor allele frequency recorded in the Admixed American population (according to the 1000 Genome database, accessed in February, 25, 2020). Excepting for the rs6544718 in the Admixed American population the MAF is 0.19 (T allele), but we found in the groups the MAF in the C allele. The alleles and genotypes of the same SNPs distributed in the groups, as well the respective Hardy–Weinberg equilibrium (HWE) can be found in the Supplementary Table 1 (performed by chi-square test).

A total of 1158 patients were screened for the study, being selected 893 individuals according to the inclusion and exclusion requirements. The general characteristics of the studied population as presented in the Table 2 is the presence of older patients in the T2DM group, balanced representation of men and women in each group, excepting for the Healthy,

due to higher percentage of women, and higher percentage of Never-smokers. It is possible to note differences by the Student's-t test in the age among the groups. By the chi squared test, we found significant differences among the groups for sex: $p < 0.0001$ (Healthy *versus* Periodontitis, and Periodontitis *versus* T2DM_P) and $p< 0.025$ (Healthy *versus* T2DM_P); for smokers, former and never smokers ($p< 0.002$ Healthy *versus* Periodontitis; $p< 0.0001$ Healthy *versus* T2DM_P; $p< 0.0001$ Periodontitis *versus* T2DM_P), for ethnicity ($p< 0.0044$ Healthy *versus* Periodontitis, and Periodontitis *versus* T2DM_P and $p< 0.0006$ Healthy *versus* T2DM_P).

Table 2 showed the absence of periodontitis and T2DM diseases in the Healthy group, as expected. The T2DM_P group showed the highest percentage in Visible Plaque Index (VPI), Marginal Bleeding (MB), Bleeding on Probing (BOP), Probing pocket Depth (PDi) ≥ 5 mm, Clinical Attachment Level (CALi) 4-5 mm and CALi ≥ 6, representing worst periodontal conditions when compared with Periodontitis group. Better metabolic control and biochemical levels were observed in the Healthy and Periodontitis groups. Fasting glucose and HbA1C levels were higher in T2DM_P group, confirming the poor glycemic control. Total Cholesterol, LDL-cholesterol, and Triglycerides levels were higher in the T2DM_P group. To assess the obesity in patients, we calculated the Body Mass Index (BMI) and Waist-to-hip-ratio, which were also higher in the T2DM_P group in comparison with the other groups.

In Table 3, when we subdivide T2DM_P by the severity of periodontitis, and we compare well-controlled (HbA1c ≤ 7.0% and ≥5.8%) patients with those poorly glycemic controlled (HbA1c ≥ 7.1%), it was possible to observe the patients with severe periodontitis presented, in general, a higher glycemic and lipid levels. For example, poor-controlled with severe periodontitis T2DM_P patients showed the highest levels of fasting glucose and HbA1c. When we considered T2DM_P moderate versus severe periodontitis patients compared by their lipid parameters, we observed in Dyslipidemic T2DM patients with severe P statistically significant higher levels of Total Cholesterol, LDL- cholesterol and Triglycerides, suggesting that the severity of P could be related with the worst lipid profile of the patients.

We performed multiple logistic regression analyses, using the additive model, to assess the association of each SNP with the pathological phenotype: P, T2DM or T2DM together with P (T2DM+P) (Table 4). Considering all patients, those carrying the CT genotype for the rs180096 SNP (*HNF4A* gene) were almost twice more susceptible to the development of T2DM + P (OR = 1.96; CI 95% = 1.01 – 3.80; p = 0.04), and showed more

than three times increased susceptibility to only T2DM (OR = 3.75; CI 95%= 1.65 – 8.60; p = 0.002).

In addition, all analyzes of multiple logistic regressions were made isolating “sex” and “smoking habits” characteristics to know their influence in the gene-phenotype association (Table 4). Stratifying patients by sex, it was observed significant association of rs180096-CT SNP (*HNF4A* gene) with the development of T2DM + P for each the analysis considering men and that considering women. Also, men affected by T2DM + P demonstrated significant genetic association, being carriers of rs6544717-CT (*ABCC8* gene) showing 63% less chance to develop T2DM+P (OR=0.37; CI 95%= 0.16-0.83; p=0.01). Similarly, for the rs285 (*LPL* gene), we found that heterozygous (CT) men showed 56% lower chance to develop only P (OR= 0.44, CI 95%=0.23–0.85, p=0.01). Again, in men, those homozygous (CC) for the rare allele in the rs13702 (*LPL* gene) were more susceptible to T2DM + P. Stratifying patients by smoking habits, the interesting result found was that never smokers carrying the rs180096-CT (*HNF4A* gene) demonstrated higher susceptibility to only T2DM.

Positive (directly proportional) or negative (inversely proportional) significant correlations of biochemical profile and periodontal parameters were found for each group and highlighted in Tables 5-7. For Healthy group we observed that Fasting Glucose was directly proportional to Insulin and HbA1c levels, as well with BMI, while the HDL Cholesterol and triglycerides levels were inversely proportional. Even being low, there were correlations between the Waist-to-Hip ratio and BMI and the periodontal parameters of visual plaque index (VPI) and marginal bleeding (MB) (Table 5).

Patients belonging to the Periodontitis group showed the same level of correlation between the periodontal parameters VPI and MB, bleeding on probing (BoP), probing depth (PoD)≥ 5 mm and clinical attachment level (CAL)≥ 6 mm. In regard, patients with probing depth ≤ 4mm had an antagonistic correlation when compared to bleeding on probing (Table 6).

Discussion

This case-control study demonstrated the association of polymorphisms in candidate genes of lipid metabolism with the development of P, T2DM, or T2DM together with P, in Brazilian patients with detailed information of periodontal clinical, and glycemic and lipid profiles. The found significant correlations for the Healthy group even at low levels agreed with the expected physiological context. The more interesting were the correlations between

the Waist-to-Hip ratio and BMI and the periodontal parameters of visual plaque index (VPI) and marginal bleeding (MB) (Table 5). The correlations found for the Periodontitis group, were in accordance with the aggravation of the periodontal disease.

The pathogenesis of diabetes and dyslipidemia are complex. Insulin contributes to the absorption of fatty acids (FFA) mainly in fat and muscle tissues. Consequently, insulin resistance can result in increased levels of FFA giving rise to increased activity of low-density lipoprotein (VLDL)[28]. In the same way, with the impairment of lipoprotein lipase (LPL) and increased hepatic lipase (HL) activity, there will be a decrease in the number of HDL particles and the formation of smaller and denser LDL particles[3]. Increasing the complexity of both diabetes and dyslipidemia, there is the increased chance of those affected patients to develop periodontitis. Whether the lipid status can influence susceptibility to T2DM and this, in turn, increases the risk of developing periodontitis, patients who do not have adequate biochemical and metabolic control are more likely to develop an exacerbated response to bacterial dental biofilm, and, consequently, periodontitis. This fact could be observed in this study from the analysis of data on glycemic and lipid profiles, which showed worse levels of fasting glucose, HbA1c, total cholesterol, HDL, LDL and triglycerides in the T2DM_P group in comparison to the Healthy group (systemically and periodontal healthy) (Tables 2 and 3). These present findings also reinforce the relationship between T2DM and dyslipidemia, mainly by the evidence of the significant dyslipidemia phenotype in T2DM patients with severe periodontitis (Table 3). Periodontitis is more severe and prevalent in patients with T2DM[29 30, 31]. This is in line with the results found in our study since the T2DM_P group showed higher rates of probing depth, bleeding on probing and clinical insertion level (≤6), when compared with the Periodontitis group. Other studies have also correlated the severity of Periodontitis with the decompensation of HbA1c and fasting glycemia[32, 33]. Recently, Polak & Shapira[34] suggested that elevated inflammatory factors in periodontal tissues of T2DM patients created a biological pathway that worsened Periodontitis. In the same way, patients with P and its inflammatory state might manifest greater difficulty in controlling T2DM.

The multiple logistic regression analyses which we performed demonstrated for the rs13702 SNP in the *LPL* gene, that male patients in the T2DM_P group with the CC genotype had a greater chance of developing T2DM+P. The relationship between exactly this CC genotype with a greater susceptibility to developing T2DM was reported by Hatefi et al[35]. The *LPL* gene (OMIM *609708*) was selected here as candidate gene because the association to lipid metabolism. Hatefi et al[35] suggested that the rs13702-CC probably influence the greater entry of fatty acids into peripheral tissues, such as liver, adipocytes and skeletal muscle,

which leads to the development of insulin resistance and later T2DM. It was also possible to observe that in this same *LPL* gene, the rs285 SNP, patients with CT genotype had a lower chance of developing P, still this SNP was not previously studied with any of the pathologies investigated here.

Polymorphisms in the Hepatocyte Nuclear Factor-4α (*HNF4A)* (OMIM: *60028*) and HNF1 homeobox A (*HNF1A)* (OMIM *14210)* genes were included in the present study because both genes encode important transcription factors involved in the regulation of lipid and glycemic metabolism[36]. *HNF4A* has been extensively associated to maturity-onset diabetes of the Young (MODY) characterized by pancreatic beta-cell-deficient insulin secretion[37, 38]. *HNF1A* it's a transcription factor that interact with *HNF4A* and leads to subtypes of MODY and consequently increase T2DM susceptibility. This gene is in the T2DM linked region on chromosome and as a result a positional candidate gene for the disease[37]. Important regulated proteins in *HNF4A* are present in T2DM pathogenesis[39]. Also, it has been demonstrated that patients with this type of Diabetes have lower levels of very low density lipoprotein – C[40].

We identified significant association for the rs1800961 of the *HNF4A* gene, whose patients with the CT genotype were almost twice more likely to develop T2DM together with P (T2DM+P). These patients are heterozygotes for the rare nonsynonymous mutation T130I (rs1800961). Other SNPs in the *HNF4A* gene were associated with elevated serum lipid levels and the metabolic syndrome, as well as with elevated glucose parameters in dyslipidemic Finnish and Mexican families (Weissglas-Volkov et al.)[41].

The *Apolipoprotein E (ApoE*) gene (OMIM: *107741)* is one of the most investigated genes involved in the lipid metabolism [42, 43, 44, 45]. It was found that *ApoE* is highly expressed in adipocytes and plays a key role in the metabolic control of cholesterol and triglycerides[46, 47]. However, there is no concordant data on its relationship with T2DM and P. Here, we investigated two of the most explored SNPs in this gene, but no associations were found in our population with T2DM and P and dyslipidemic parameters. This did not mean that this gene is not important in the pathologic pathway of T2DM and P. The *ApoE* gene harbors many other SNP than the two investigated here, therefore the true association may be missed, or the true causal SNPs may be in other regions of the gene, including SNPs in its vicinity.

Genetic variants in the *ABCC8* gene (OMIM: *600509)* have also been widely explored in candidate genes case-controls studies due to its function of encoding the sulfonylurea receptor, which plays an important role in insulin secretion. Pharmacogenetics studies usually investigate this gene because its involvement with the efficacy of

hypoglycemic drugs, since the failure in oral drug therapy leads to poorly regulated glycemia and dyslipidemia[48,49]. Whether polymorphisms in the *ABCC8* gene may influence the efficacy of drugs used in the control of hyperglycemia, it seems plausible to suppose that this influence can alter the glycemic levels of T2DM patients, thus favoring the development of P. From the two SNPs investigated in the *ABCC8* gene, the rs6544718 showed a positive association on the direction of a lower chance to develop T2DM+P, and influenced by the sex, since men carrying the rs6544718-CT were 63% less likely to develop T2DM together with P.

Genetic studies in last years have explored several interactions between sex-genes. Although there is no consensus regarding the different susceptibility between males and females, in general, men shows the worst periodontal conditions [50]. Some other studies reports a sex-specific susceptibility to complex diseases in humans, including periodontitis [51-53], which were supported by the sexual dimorphism of some polymorphisms. Moreover a gender influence in gene transcription is necessary, as males and females use a similar genome to achieve distinct phenotypes [54]. It has been suggested that gender-biased differences in gene expression may particularly occur in tissues with multifarious physiological roles, and gene-ontology analysis identified that immune response genes were differentially expressed between males and females [55]. Clinical experience [56], as well in vitro [57] and in vivo [58] studies, indicate the influence of steroid hormones on inflammation, immune response and bone metabolism, demonstrating existence of an association of periodontitis with sex hormone levels.

This study has some limitations. Although when we considered all the investigated patients, the number of enrolled subjects have enough statistical power, unfortunately, in stratifying the population by sex or by smoking habits (Table 4) we had a decreased sample size. This fact is reflected in the observed large CIs, making the genetic association less plausible. Therefore, it is suggested to investigate the same genetic variants in larger Brazilian populations from different regions of the country, and in multiple ethnic populations throughout the world, in agreement with Laine et al. (2014). In addition, increasing the number of male and female patients, it could be made a study design to better assess the sex influence in the genetic susceptibility of the diseases. Similarly, this could be made regarding the influence of smoking habits. Importantly, we did not applied in our statistical analyses the Bonferroni´s correction, as we made in a family-based MIH study, because it would reduce the α-value to 0.0055 (0.05/9 SNPs)[59]. This would increase the type II error which would lead us to reject a hypothesis of a true biological association. The use of these corrections may prevent the identification of discrete biological effects. In 2008, Vieira et al. showed that true

associations may be lost once a correction for multiple testing is implemented. Thus, our results should be interpreted with caution, since some of the p values lower than 0.05 may be due to chance.

Another limitation of all genetic studies enrolling a Brazilian population is the nature of its admixed ethnic origin, since Brazilians form one of the most heterogeneous populations in the world, resulting of the 5 centuries of inter-ethnic crossing between Europeans, Africans, Asians and autochthonous Amerindians[60]. Despite we showed here the data on the skin color of the studied patients, the ethnically admixed Brazilian population is a characteristic that prevented stratification of the sample into ethnic groups or skin color. Parra et al., 2003 do not recommend grouping Brazilians into ethnic groups based on skin color and other physical characteristics associated with racial divisions. Brazilian individuals classified as 'white' or 'black' have significantly overlapping genotypes considered as race-associated loci for Caucasians, Africans and Indigenous people[61]. Because this fact, we did not explore this characteristic in any of our genetic analysis.

In conclusion, relevant gene-phenotype associations were found in the studied population, some of them demonstrating gene-sex interaction, such as the rs285 (*LPL* gene) with P, the rs1800961 (*HNF4A* gene) with T2DM, in addition to both the rs6544718 (*ABCC8* gene) and rs13702 (*LPL* gene) with the T2DM together with P (T2DM+P). Also, correlation analysis showed important associations between biochemical profile and periodontal disease. Further studies are necessary to confirm the association of lipid metabolism genes in the occurrence of T2DM and P (together with, or not), and the potential influence of sex in this context, to finally investigate the potential functional role of those SNPs in the transcriptional or translational levels of the genes, unravelling the biological interconnected pathway of the diseases.

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Tables

Table 1. Polymorphisms information and Minor Allele Frequency (MAF) data of the 1000 Genomes (1000G) database and each studied group.

SNP	Gene Symbol	Assay Code	MAF 1000G Admixed American‡	MAF Healthy	MAF Periodontitis	MAF T2DM_P
rs6544718	*ABCC8*	C__25642779_10	T= 0.196	*C = 0.15*	C= 0.16	C = 0.17
rs6544713	*ABCC8*	C__26135636_10	T= 0.309	T= 0.55	T= 0.43	T = 0.14
rs285	*LPL*	C__12104266_10	C= 0.483	C = 0.48	C = 0.45	C= 0.47
rs3735964	*LPL*	C__25800209_10	A= 0.135	A= 0.43	A= 0.35	A = 0.22
rs13702	*LPL*	C___9639448_10	C= 0.320	C = 0.33	C = 0.71	C = 0.40
rs2650000	*HNF1A*	C__15874272_10	A= 0.360	A= 0.32	A= 0.31	A = 0.32
rs429358	*ApoE*	C___3084793_20	C= 0.155	C = 0.13	C = 0.15	C = 0.12
rs7412	*ApoE*	C____904973_10	T= 0.063	T = 0.06	T = 0.05	T = 0.07
rs1800961	*HNF4A*	C___7591528_10	T= 0.037	T = 0.02	T = 0.02	T = 0.05

† Genome Reference Consortium Human Build 37 (GRCh38.p13) or hg19

‡1000 Genome database, accessed in February, 25, 2020)

Table 2. Demographic characteristic of the studied groups, periodontal clinical parameters, glycemic and lipid profile and physical exams of all groups

	HEALTHY	**PERIODONTITIS**	**T2DM_P**
Demographic Characteristics	**n= 343**	**n= 345**	**n= 205**
Age, Mean (Std. Deviation)	43(±10.88)[a]	49.06(±9.29)[b]	56.03(±9.49)[c]
Sex, n (%)			
Male	110 (32%)[a]	204 (59%)[b]	86 (42%)[c]
Female	233 (68%)[a]	141 (41%)[b]	119 (58%)[c]
Ethnic group, n (%)			
White	230 (67%)[a]	231 (67%)[a]	136 (66.3%)[b]
Pardo	22 (6.5%)[a]	40 (11.6%)[b]	32 (15.7 %)[a,b]
Black	90 (26.5%)[a]	73 (21.4%)[b]	37 (18%)[c]
Smoke, n (%)			
Smoker	29 (8.45%)[a]	34 (9.85)[a]	20 (10%)[a]
Former-Smoker	47 (13.7%)[a]	89 (25.8%)[a]	64 (31%)[a]
Never-Smoker	267 (77.85%)[a]	222 (64.35%)[b]	121 (59%)[b]
Periodontal Parameters - Mean (±SD)			
Number of teeth	25.5(±3.43)[a]	22.84 (±4.48)[b]	20.54 (±5.51)[c]
Visible Plaque Index (% site)	24.75(±20.0)[a]	54.82 (±28.13)[b]	56.53(±26.96)[c]
Marginal Bleeding (% site)	8.79(±11.72)[a]	20.07(±23.25)[b]	40.63 (±23.91)[c]
BOPi (% site)	3.30 (±4.98)[a]	39.98 (±23.17)[b]	51.03 (±27.16)[c]
PPDi ≤ 4mm (% site)	99.93 (±0.30)[a]	86.87 (±14)[b]	79.11 (±22.69)[c]
PPDi ≥ 5mm (% site)	0.07 (±0.30)[a]	13.13 (±14.01)[b]	20.89 (±22.89)[c]
CALi ≤ 3mm (% site)	99.49 (±0.78)[a]	68.40 (±21.39)[b]	56.22 (±27.32)[c]
CALi = 4-5mm (% site)	0.46 (±0.75)[a]	20.07 (±11.92)[b]	24.99 (±15.23)[c]
CALi ≥ 6mm (% site)	0.04 (±0.23)[a]	11.53 (±13.68)[b]	18.79 (±20.79)[c]
Biochemical and Physical data Median (mininum –maximum)			
Fasting Glucose	91.50 (80.0 – 99)[a]	92 (60.0 – 99)[a]	152.2 (83.0 – 467.9)[b]
HbA_{1c}	5.4 (4.8-5.6)[a]	5.6 (4.4 -5.60)[a]	7.9 (4.5-14.30)[b]
Insulin	173.0 (123.0-321.0) [a]	176.0(137.0-273.0) [ab]	192.5 (39.0 - 306.0)[ab]
Total Cholesterol	177.5 (134 – 218) [a]	176 (137 – 273) [a]	192.5 (39 – 306) [ab]
HDL-Cholesterol	56 (42 - 84) [a]	53 (30 – 88) [a]	44 (24 – 350.6) [b]
LDL-Cholesterol	96.40 (50.6 -155.8) [a]	96.20 (17.0- 732.0) [a]	109.0 (7.5-523.0) [a]
Triglycerides	7.4 (3.6-37.8) [a]	9.90 (4.1-32.40) [a]	13.70 (3.2-161.0) [b]
BMI (m/kg^2)	27.81 (18.0-41.6) [a]	26.9 (18.21-37.9) [a]	29.82(18.5-44.9) [b]
Waist-to-Hip-Ratio (cm)	0.88 (0.09-1.09) [a]	0.91 (0.74-1.07) [a]	0.95 (0.76-1.25) [b]

BOPi= Interproximal Bleeding on Probing; P= Periodontitis; PPDi= Interproximal Probing Pocket Depth; CAL= Level of Interproximal Clinical Attachment. Chi Squared Test was used to determine differences among groups for sex, smoking habits and ethnicity. Periodontal parameters = [a,b,c] Different letter means p value <0.05 by comparison with One-way ANOVA, followed by Tukey´ test. Biochemical and lipid levels=[a, b, c] Different letters means p< 0.05 (Kruskal-Wallis Test, followed by the Dunn´s test).

Table 3 -Median (mininum -maximum) of data related to the results of glicemic and lipid

profile and physical exams of patients of the T2DM_P group

	T2DM_P						
	MODERATE PERIODONTITIS			*SEVERE PERIODONTITIS*			
	*Well-Controlled * (n= 22)*	*Poor-Controlled * (n= 27)*	*p value**	*Well-Controlled ** (n= 34)*	*Poor-Controlled ** (n= 72)*	*p value***	*p value [ab] among the 4 groups*
Fasting Glucose	111.0 (90.0 – 149.0) [a]	176.0 (84.0 – 400.0) [b]	**0.0001**	118.0 (83.00 - 215.0) [a]	198.0 (87.00 - 467.9) [b]	**0.0001**	**0.0001**
HbA1c	6.50 (4.50 - 7.00) [a]	8.50 (7.10 - 13.40) [b]	**0.0001**	6.20 (4.70 - 6.90) [a]	9.35 (7.10 - 14.30) [b]	**0.0001**	**0.0001**
Insulin	11.70 (6.03 - 47.30) [a]	15.00 (4.80 - 161.0) [a]	0.2770	13.90 (3.20 - 52.60) [a]	13.50 (5.10 - 101.9) [a]	0.8809	0.7036
Total Cholesterol	155.6 (111.0 - 221.0) [a]	204.0 (104.0 - 275.0) [b]	**0.0021**	216.0 (131.9 - 306.0) [b]	188.0 (118.0 - 304.0) [b]	**0.0283**	**0.0005**
HDL Cholesterol	45.50 (25.00 - 69.00) [a]	41.00 (29.00 - 72.00) [a]	0.4373	45.00 (30.00 - 75.00) [a]	42.30 (24.00 - 87.00) [a]	0.3124	0.5568
LDL Cholesterol	75.60 (30.80 - 142.4) [a]	121.5 (45.40 - 190.4) [a]	**0.0022**	128.6 (33.26 - 209.6) [b]	102.8 (7.50 - 523.0) [a]	0.1955	**0.0409**
Triglycerides	140.0 (47.00 - 283.0) [a]	174.5 (57.00 - 440.0) [a]	0.1207	171.0 (61.00 - 439.0) [a]	155.0 (58.90 - 765.0) [a]	0.7153	0.4249
BMI (m/kg2)	27.28 (22.48 - 44.98) [a]	30.85 (23.42 - 47.27) [a]	**0.0361**	30.85 (21.93 - 47.27) [a]	29.34 (21.64 - 41.37) [a]	0.3240	0.1647
Waist-to-Hip-Ratio (cm)	0.91 (0.76 - 1.17) [a]	0.96 (0.87 - 1.15) [a]	0.1272	0.94 (0.84 - 1.06) [a]	0.99 (0.86 - 1.25) [a]	0.0327	0.0843

∗P<0.05 between well-controlled and poor-controlled patients with moderate periodontitis (Mann Whitney Test, α=5%);
∗∗P<0.05 between well-controlled and poor-controlled patients with severe periodontitis (Mann Whitney Test, α=5%); [a,b,c] Different letter means p value <0.05 considering the comparison among the four groups (Kruskal-Wallis, followed by Dunn´s Test, α= 5%)

Table 4 – Multiple logistic regression analyses for all patients (adjusted by age, sex and smoking) and for patients stratified by sex ‡ (adjusted by age and smoking) and by smoking habits (adjusted by age and sex)

	HEALTHY *versus* *Periodontitis*		**HEALTHY *versus* T2DM_P**		***Periodontitis versus* T2DM_P**	
***HNF1A* (C>A) rs2650000**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**
All patients						
CC	Ref		Ref			
AC	0.82 (0.59 – 1.15)	0.26	1.18 (0.78 – 1.79)	0.42	0.80 (0.51 – 1.24)	0.32
AA	0.87 (0.49 – 1.52)	0.63	0.96 (0.49 – 1.85)	0.90	1.14 (0.56 – 2.35)	0.70
Male ‡	Adjusted OR‡		Adjusted OR‡		Adjusted OR‡	
CC	Ref		Ref		Ref	
AC	1.10 (0.62 – 1.94)	0.72	1.68 (0.85 – 3.32)	0.13	0.96 (0.45 – 2.04)	0.91
AA	1.76 (0.67 – 4.58)	0.24	0.55 (0.18 – 1.62)	0.28	0.71 (0.19 – 2.65)	0.61
Female ‡						
CC	Ref		Ref		Ref	
AC	0.71 (0.47 – 1.08)	0.11	0.95 (0.55 – 1.61)	0.85	0.71 (0.41- 1.22)	0.22
AA	0.56 (0.27 – 1.16)	0.12	1.43 (0.61 – 3.33)	0.40	1.33 (0.57 -3.10)	0.50
	Adjusted OR¶		Adjusted OR¶		Adjusted OR¶	

Never Smoking						
CC	Ref		Ref		Ref	
AC	0.68 (0.46- 1.01)	0.61	1.56 (0.91 -2.68)	0.10	1.00 (0.58 – 1.69)	0.99
AA	0.96 (0.49 – 1.86)	0.90	1.38 (0.62 – 3.07)	0.42	1.70 (0.73 – 3.94)	0.21
Ever Smoking †¶						
CC	Ref		Ref		Ref	
AC	1.34 (0.70 – 2.57)	0.36	0.80 (0.41 – 1.57)	0.53	0.60 (0.26 – 1.37)	0.23
AA	0.73 (0.26 – 2.03)	0.55	0.48 (0.14 – 1.61)	0.24	0.58 (0.15 – 2.25)	0.43

HNF4A **(C>T)rs1800961**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**
All patients						
CC	Ref		Ref			
CT	0.90 (0.45 – 1.82)	0.78	**1.96 (1.01 – 3.80)**	**0.04**	**3.75 (1.64 – 8.60)**	**0.002**
TT	1	-	1	-	1	-
Male ‡	Adjusted OR‡		Adjusted OR‡		Adjusted OR‡	
CC	Ref		Ref		Ref	
CT	1.50 (0.46 – 4.92)	0.49	1.48 (0.52 – 4.21)	0.45	**6.08 (1.25 – 29.48)**	**0.02**
TT	-	-	-	-	-	-
Female ‡						
CC	Ref		Ref		Ref	
CT	0.67 (0.27 – 1.66)	0.39	2.36 (1.01 – 5.56)	0.04	**3.23 (1.22 – 8.58)**	**0.01**
TT	1	-	1	-	1	-
Never Smoking ¶	Adjusted OR¶		Adjusted OR¶		Adjusted OR¶	
CC	Ref		Ref		Ref	
CT	0.97 (0.43 – 2.19)	0.95	1.84 (0.77 – 4.35)	0.16	**2.96 (1.16 – 7.53)**	**0.02**
TT	1	-	-	-	1	-
Ever Smoking † ¶						
CC	Ref		Ref		Ref	
CT	0.72 (0.18 – 2.83)	0.64	2.14 (0.76 – 6.01)	0.14	6.23 (0.74 – 52.09)	0.09
TT	1	-	1	-	1	-

ApoE **(C>T) rs7412**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**
All patients						
CC	Ref		Ref			
CT	0.91 (0.55 – 1.50)	0.72	1.15 (0.63 – 2.08)	0.64	1.30 (0.69 – 2.46)	0.41
TT	1	-	1	-	3.41 (0.50 – 23.19)	0.20
Male ‡	Adjusted OR‡		Adjusted OR‡		Adjusted OR‡	
CC	Ref		Ref		Ref	
CT	1.13 (0.45 – 2.82)	0.78	1.87 (0.71 – 4.92	0.20	2.92 (0.88 – 9.63)	0.07
TT	1	-	1	-	1.29 (0.01 – 142.0)	0.91
Female ‡						
CC	Ref		Ref		Ref	
CT	0.83 (0.45 -1.52)	0.56	0.84 (0.39 – 1.84)	0.68	0.92 (0.41 – 2.03)	0.83
TT	1	-	1	-	4.03 (0.46 – 34.93)	0.20
Never Smoking ¶	Adjusted OR¶		Adjusted OR¶		Adjusted OR¶	
CC	Ref		Ref		Ref	
CT	0.97 (0.53 – 1.76)	0.93	1.40 (0.69 – 2.83)	0.34	1.64 (0.79 – 3.40)	0.17

TT	1	-	1	-	2.35 (0.16 – 33.59)	0.52
Ever Smoking †¶						
CC	Ref		Ref		Ref	
CT	0.77 (0.30 – 1.95)	0.58	0.71 (0.23 – 2.17)	0.55	0.83 (.020 – 3.33)	0.79
TT	1	-	1	-	8.56 (0.42 – 174.7)	0.16
***ApoE* (T>C) rs429358**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**
All patients						
TT	Ref		Ref			
CT	1.06 (0.73 – 1.54)	0.73	0.84 (0.52 – 1.36)	0.48	0.84 (0.45 – 1.12)	0.15
CC	1.79 (0.63 – 5.05)	0.27	0.43 (0.11 – 1.64)	0.21	0.64 (0.15 – 2.7)	0.54
Male ‡	Adjusted OR‡		Adjusted OR‡		Adjusted OR‡	
TT	Ref		Ref		Ref	
CT	1.22 (0.64 – 2.33)	0.54	0.73 (0.34 – 1.53)	0.41	0.86 (0.36 – 2.05)	0.73
CC	4.76 (0.56 – 40.48)	0.15	1	-	1	-
Female ‡						
TT	Ref		Ref		Ref	
CT	0.98 (0.62 – 1.56)	0.96	0.91 (0.48 – 1.72)	0.79	0.69 (0.36 – 1.31)	0.26
CC	1.06 (0.28 – 3.95)	0.92	0.78 (0.18 – 3.36)	0.74	2.28 (0.31 – 16.53)	0.41
Never Smoking ¶	Adjusted OR¶		Adjusted OR¶		Adjusted OR¶	
TT	Ref		Ref		Ref	
CT	1.15 (0.74 – 1.79)	0.52	0.86 (0.47 – 1.60)	0.65	0.87 (0.47 – 1.61)	0.67
CC	0.93 (0.28 – 5.36)	0.77	0.60 (0.11 -3.31)	0.56	5.04 (0.39 -63.54)	0.21
Ever Smoking †¶						
TT	Ref		Ref		Ref	
CT	0.88 (0.43 – 1.77)	0.72	0.76 (0.34 – 1.66)	0.49	0.40 (0.14 – 1.09)	0.07
CC	2.61 (0.51 – 12.90)	0.24	0.27 (0.03 – 2.49)	0.25	0.23 (0.01 – 3.00)	0.36
***ABCC8* (C>T)rs6544718**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**
All patients						
CC	Ref		Ref			
CT	0.87 (0.60 – 1.25)	0.45	0.71 (0.45 – 1.12)	0.15	0.93 (0.57 – 1.52)	0.79
TT	1.21 (0.43 – 3.39)	0.71	0.64 (0.15 – 2.70)	0.54	0.42 (0.10 – 1.73)	0.23
Male ‡	Adjusted OR‡		Adjusted OR‡		Adjusted OR‡	
CC	Ref		Ref		Ref	
CT	0.75 (0.41 – 1.37)	0.35	**0.37 (0.16 – 0.83)**	**0.01**	0.74 (0.29 – 1.87)	0.52
TT	0.75 (0.10 – 5.45)	0.77	0.89 (0.12 – 6.19)	0.90	1.96 (0.16 – 22.84)	0.59
Female ‡						
CC	Ref		Ref		Ref	
CT	0.95 (0.60 – 1.50)	0.84	1.01 (0.57 – 1.77)	0.96	1.06 (0.60 – 1.89)	0.82
TT	1.43 (0.42 – 4.76)	0.55	0.40 (0.04 – 3.99)	0.44	0.17 (0.02 – 1.55)	0.11
Never Smoking ¶	Adjusted OR¶		Adjusted OR¶		Adjusted OR¶	
CC	Ref		Ref		Ref	
CT	0.90 (0.59 – 1.38)	0.65	0.69 (0.38 – 1.27)	0.24	0.66 (0.36 – 1.20)	0.17
TT	0.59 (0.14 – 2.39)	0.46	1.36 (0.08 – 22.70)	0.83	0.15 (0.01 – 1.35)	0.09
Ever Smoking †¶						
CC	Ref		Ref		Ref	
CT	0.78 (0.39 – 1.54)	0.47	0.72 (0.36 – 1.44)	0.35	1.88 (0.71 – 4.98)	0.20
TT	3.94 (0.46 – 33.82)	0.21	0.51 (0.89 – 2.91)	0.45	2.46 (0.29 – 20.84)	0.40

ABCC8	Adj OR (95% CI)	*p*-value	Adj OR (95% CI)	*p*-value	Adj OR (95% CI)	*p*-value
(T>C) rs6544713						
All patients †	Adjusted OR†		Adjusted OR†		Adjusted OR†	
CC	Ref		Ref			
CT	1.06 (0.76 – 1.49)	0.69	0.93 (0.61 – 1.41)	0.74	1.04(0.66 – 1.62)	0.86
TT	1.10 (0.61 – 1.98)	0.74	1.30 (0.63 – 2.67)	0.46	1.21(0.59 – 2.4)	0.59
Male ‡	Adjusted OR‡		Adjusted OR‡		Adjusted OR‡	
CC	Ref		Ref		Ref	
CT	1.04 (0.59 – 1.81)	0.84	0.75 (0.39 – 1.45)	0.40	0.73 (0.34 – 1.56)	0.42
TT	0.67 (0.22 – 2.03)	0.48	0.61 (0.16 – 2.30)	0.47	1.32 (0.29 – 6.06)	0.71
Female ‡						
CC	Ref		Ref		Ref	
CT	1.06 (0.69 – 1.62)	0.78	1.07 (0.62 – 1.83)	0.80	1.25 (0.71 – 2.18)	0.42
TT	1.34 (0.66 – 2.71)	0.40	1.90 (0.79 – 4.57)	0.15	1.23 (0.55 – 2.74)	0.59
Never Smoking ¶	Adjusted OR¶		Adjusted OR¶		Adjusted OR¶	
CC	Ref		Ref		Ref	
CT	0.89 (0.60 -1.33)	0.58	0.88 (0.51 – 1.50)	0.64	0.84 (0.49 – 1.44)	0.54
TT	1.02 (0.51 – 2.02)	0.94	1.46 (0.57 – 3.71)	0.41	0.86 (0.36 – 2.04)	0.73
Ever Smoking ¶						
CC	Ref		Ref		Ref	
CT	1.70 (0.88 – 3.26)	0.10	1.00 (0.51 – 1.94)	0.99	1.52 (0.66 – 3.53)	0.32
TT	1.29 (0.39 – 4.20)	0.67	1.06 (0.34 – 3.27)	0.91	2.84 (0.73 – 10.95)	0.12
CC	Ref		Ref		Ref	
AC	0.83 (0.50 – 1.39)	0.48	1.07 (0.62 – 1.83)	0.80	1.02 (0.51 – 2.06)	0.93
AA	0.62 (0.15 – 2.57)	0.51	1.90 (0.79 -4.57)	0.15	1	-
Never Smoking ¶	Adjusted OR¶		Adjusted OR¶		Adjusted OR¶	
CC	Ref		Ref		Ref	
AC	0.69 (0.42 – 1.14)	0.15	1.77 (0.94 – 3.33)	0.07	1.29 (0.71 – 2.35)	0.39
AA	0.60 (0.14 – 2.48)	0.48	1	-	1	-
Ever Smoking †¶						
CC	Ref		Ref		Ref	
AC	0.93 (0.44 – 1.96)	0.84	0.52 (0.21 -1.27)	0.15	0.60 (0.19 – 1.89)	0.38
AA	1	-	-	-	1	-

LPL **(C>T) rs13702**	Adj OR (95% CI)	*p*-value	Adj OR (95% CI)	*p*-value	Adj OR (95% CI)	*p*-value
All patients						
TT	Ref		Ref			
CT	0.88 (0.62 – 1.25)	0.49	0.97 (0.62 – 1.52)	0.98	0.74 (0.46 – 1.20)	0.23
CC	1.07 (0.62 – 1.83)	0.80	1.20 (0.62 – 2.34)	0.57	1.44 (0.70 – 2.98)	0.31
Male ‡	Adjusted OR‡		Adjusted OR‡		Adjusted OR‡	
TT	Ref		Ref		Ref	
CT	0.77 (0.42 – 1.39)	0.39	1.92 (0.92 – 3.99)	0.08	0.86 (0.36 – 2.04)	0.73
CC	1.26 (0.51 – 3.11)	0.61	**4.38 (1.52 – 12.58)**	**0.006**	2.17 (0.69 – 6.81)	0.18
Female ‡						
TT	Ref		Ref		Ref	
CT	0.94 (0.60 -1.46)	0.79	0.64 (0.36 – 1.13)	0.68	0.68 (0.38 – 1.20)	0.18
CC	0.98 (0.50 – 1.93)	0.96	0.47 (0.18 – 1.20)	0.11	1.10 (0.40 – 2.97)	0.85
Never Smoking ¶	Adjusted OR¶		Adjusted OR¶		Adjusted OR¶	

TT	Ref		Ref		Ref	
CT	0.74 (0.49 – 1.12)	0.16	0.98 (0.56 – 1.73)	0.95	0.81 (0.46 – 1.42)	0.47
CC	0.79 (0.43 – 1.46)	0.46	0.74 (0.30 – 1.85)	0.53	0.92 (0.37 – 2.26)	0.86
Ever Smoking †¶						
TT	Ref		Ref		Ref	
CT	1.35 (0.70 – 2.59)	0.36	1.02 (0.49 – 2.11)	0.94	0.56 (0.23 – 1.39)	0.21
CC	3.47 (0.87 -13.80)	0.07	2.29 (0.80 – 6.53)	0.11	4.00 (0.81 – 19.69)	0.08
***LPL* (T>C) rs285**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**	**Adj OR (95% CI)**	***p*-value**
All patients						
TT	Ref		Ref			
CT	0.87 (0.60 – 1.26)	0.48	0.71 (0.44 – 1.14)	0.16	0.88 (0.53 – 1.43)	0.61
CC	0.71 (0.46 – 1.11)	0.13	0.86 (0.50 – 1.46)	0.58	0.95 (0.55 – 1.66)	0.88
Male ‡	Adjusted OR‡		Adjusted OR‡		Adjusted OR‡	
TT	Ref		Ref		Ref	
CT	**0.44 (0.23 – 0.85)**	**0.01**	0.62 (0.29 – 1.33)	0.22	0.91 (0.39 – 2.11)	0.83
CC	0.64 (0.29 – 1.37)	0.25	0.49 (0.20 – 1.22)	0.13	0.71 (0.26 – 1.93)	0.51
Female ‡						
TT	Ref		Ref		Ref	
CT	1.22 (0.76 – 1.94)	0.39	0.76 (0.41 – 1.39)	0.38	0.83 (0.45 – 1.54)	0.45
CC	0.74 (0.43 – 1.29)	0.29	1.17 (0.60 – 2.29)	0.62	1.06 (0.54 – 2.08)	0.85
Never Smoking ¶	Adjusted OR¶		Adjusted OR¶		Adjusted OR¶	
TT	Ref		Ref		Ref	
CT	0.94 (0.60 – 1.46)	0.78	0.79 (0.38 – 1.30)	0.26	0.95 (0.52 – 1.74)	0.88
CC	0.90 (0.53 – 1.50)	0.69	0.93 (0.48 – 1.79)	0.83	1.16 (0.60 – 2.21)	0.64
Ever Smoking †¶						
TT	Ref		Ref		Ref	
CT	0.70 (0.34 – 1.44)	0.34	0.69 (0.33 – 1.47)	0.34	0.53 (0.20 – 1.39)	0.20
CC	**0.38 (0.16 – 0.89)**	**0.02**	0.75 (0.29 – 1.93)	0.55	1.04 (0.18 – 1.78)	0.33

OR = Odds Ratio; CI = Confidence interval † Ever smoking, patients who are current smokers together with those former smokers. **Bold** font indicates $p < 0.05$

Table 5 – Correlations between biochemical profiles and periodontal parameters for Healthy group

	FG	Insulin	HBA1C	Triglyc	Total Chol	HDL - Chol	LDL - Chol	WtoHR	BMI	VPI	MB	BoP	PoD≤4	PoD≥5	CALi≥3_	CALi4-5_P	CALi≥6
Fasting Glucose		0.034	0.027	0.595	0.564	0.296	0.922	0.253	0.044	0.302	0.14	0.291	0.861	0.861	0.697	0.85	0.104
Insulin	0.355		0.255	0.172	0.102	0.72	0.477	0.369	0.04	0.061	0.345	0.667	0.165	0.165	0.983	0.983	
HBA1C	0.3	-0.195		0.554	0.9	0.234	0.945	0.779	0.912	0.52	0.415	0.371	0.053	0.053	0.865	0.647	0.275
Triglycerides	0.092	0.236	-0.102		0.103	0.004	0.724	0.739	0.922	0.258	0.347	0.918	0.285	0.285	0.556	0.556	
Total Cholesterol	0.099	0.281	0.022	0.268		0.952	0	0.627	0.297	0.1	0.386	0.758	0.564	0.564	0.736	0.736	
HDL - Cholesterol	0.179	0.063	0.204	-0.454	-0.01		0.129	0.133	0.897	0.356	0.085	0.704	0.929	0.929	0.431	0.431	
LDL - Cholesterol	-0.017	0.124	-0.012	0.059	0.868	-0.251		0.99	0.172	0.073	0.7	0.674	0.987	0.987	0.774	0.774	
Wais to Hip Ratio	0.196	0.184	0.048	-0.072	-0.105	-0.316	-0.003		0.001	0.008	0.034	0.098	0.66	0.66	0.484	0.945	0.098
BMI	0.347	0.423	0.02	-0.022	0.227	-0.029	0.295	0.497		0.121	0.079	0.07	0.626	0.626	0.023	0.127	0.106
VPI	-0.143	0.311	0.089	0.188	-0.271	-0.154	-0.294	0.407	0.252		0	0.082	0.014	0.014	0.001	0.017	0.035
MB	-0.204	-0.16	-0.113	-0.157	-0.145	-0.283	0.065	0.331	0.284	0.477		0	442,372	442,372	8,740	843,906	0.002
BoP	-0.146	-0.073	0.124	-0.017	-0.052	-0.064	-0.071	0.262	0.293	0.285	0.35		0.001	0.001	0	0	0.01
PoD≤4	-0.024	-0.233	0.265	-0.178	-0.097	-0.015	-0.003	0.071	-0.081	-0.133	-0.189	-0.181		0	0	0.027	280,204
PoD≥5	0.024	0.233	-0.265	0.178	0.097	0.015	0.003	-0.071	0.081	0.133	0.189	0.181	-1,000		0	0.027	280,204
CALi≤3	-0.054	0.004	0.024	-0.099	-0.057	-0.132	0.048	-0.113	-0.362	-0.174	-0.262	-0.457	0.372	-0.372		0	0.095
CALi4-5	-0.026	-0.004	-0.064	0.099	0.057	0.132	-0.048	0.011	0.249	0.129	0.211	0.433	-0.314	0.314	-0.954		0.744
CALi≥6	0.224		0.151					0.262	0.262	0.114	0.169	0.139	-0.224	0.224	-0.283	0.018	

FG = Fasting Glucose; Triglyc = Triglycerides; Total Chol = Total Cholesterol; HDL Chol = HDL Cholesterol; WtoHR = Wais to Hip Ratio; BMI = Body mass index; VPI = Visible Plaque index; MB = Marginal Bleeding; BoP = Bleeding on Probing; PoD = Probing on depth; CAL = Clinical Attachment Loss. Values above light gray meant p values. The green colors meant that there was a positive significant correlation, and the red color meant negative significant correlation. Upper

diagonal = p values. Lower diagonal values = ρ=Spearman's correlations coefficients.

Table 6 – Correlations between biochemical profiles and periodontal parameters for the Periodontitis group

	FG	Insulin	HBA1C	Triglyc	Total Chol	HDL - Chol	LDL - Chol	WtoHR	BMI	VPI	MB	BoP	PoD≤4	PoD≥5	CALi≥3_	CALi4-5_P	CALi≥6
Fasting Glucose		0.869	0.000	0.004	0.097	0.016	0.969	0.002	0.558	0.021	0.093	0.192	0.003	0.003	0.177	0.116	0.049
Insulin	0.017		0.571	0.895	0.293	0.883	0.805	0.352	0.218	0.630	0.545	0.604	0.768	0.768	0.487	0.175	0.695
HBA1C	0.754	0.057		0.216	0.482	0.324	0.893	0.011	0.885	0.320	0.402	0.697	0.037	0.037	0.571	0.095	0.218
Triglycerides	0.241	0.013	0.103		612.108	2.917	0.057	0.052	0.599	0.010	0.064	0.007	0.003	0.003	0.001	0.069	0.004
Total Cholesterol	0.138	0.105	0.058	0.306		0.932	0.000	0.889	0.428	0.018	0.479	0.110	0.002	0.002	0.380	0.593	0.133
HDL - Cholesterol	-0.203	-0.015	-0.084	-0.398	0.007		0.406	27.767	0.028	0.118	0.290	0.011	0.149	0.149	0.001	0.063	0.005
LDL - Cholesterol	-0.003	0.025	0.012	0.156	0.782	-0.069		0.489	0.024	0.335	0.740	0.622	0.030	0.030	0.645	0.868	0.367
Wais to Hip Ratio	0.321	-0.105	0.264	0.199	0.014	-0.462	0.073		0.028	0.030	0.079	0.114	0.029	0.029	0.064	0.051	0.246
BMI	0.069	0.160	-0.017	0.060	0.090	-0.252	0.257	0.251		0.638	0.842	0.870	0.361	0.361	0.831	0.859	0.541
VPI	0.190	-0.049	0.082	0.202	0.186	-0.127	0.081	0.207	-0.051		0.000	0.000	0.001	0.001	0.035	0.002	0.013
MB	0.139	-0.062	0.069	0.146	0.056	-0.086	0.028	0.168	0.022	0.585		0.563	0.141	0.141	0.538	0.029	0.812
BoP	0.108	-0.053	0.032	0.212	0.126	-0.207	0.041	0.151	-0.018	0.478	0.349		0.000	0.000	0.000	0.000	0.000
PoD≤4	-0.246	0.030	-0.170	-0.232	-0.247	0.118	-0.180	-0.208	-0.099	-0.418	-0.392	-0.765		0.000	0.000	4.015	0.000
PoD≥5	0.246	-0.030	0.170	0.232	0.247	-0.118	0.180	0.208	0.099	0.418	0.392	0.765	-1000.000		0.000	4.015	0.000
CALi≤3	-0.112	0.071	-0.046	-0.261	-0.069	0.269	-0.039	-0.177	-0.023	-0.407	-0.350	-0.901	0.728	-0.728		0.000	0.000
CALi4-5	-0.130	-0.138	-0.136	0.144	-0.042	-0.151	-0.014	0.187	-0.019	0.221	0.156	0.639	-0.353	0.353	-0.694		0.382
CALi≥6	0.163	-0.040	0.101	0.227	0.118	-0.228	0.075	0.112	0.066	0.417	0.313	0.792	-0.755	0.755	-0.880	0.381	

FG = Fasting Glucose; Triglyc = Triglycerides; Total Chol = Total Cholesterol; HDL Chol = HDL Cholesterol; WtoHR = Waist to Hip Ratio; BMI = Body mass index; VPI = Visible Plaque index; MB = Marginal Bleeding; BoP = Bleeding on Probing; PoD = Probing on depth; CAL = Clinical Attachment Loss. Values above light gray meant p values. The green colors meant that there was a positive significant correlation, and the red color meant negative significant correlation. Upper diagonal = p values. Lower diagonal values = ρ=Spearman's correlations coefficients.

Table 7 – Correlations between biochemical profiles and periodontal parameters for the T2DM_P group

	FG	Insulin	HBA1C	Triglyc	Total Chol	HDL - Chol	LDL - Chol	WtoHR	BMI	VPI	MB	BoP	PoD≤4	PoD≥5	CALi≥3_	CALi4-5_P	CALi≥6
Fasting Glucose		0.039	0.119	0.173	0.541	0.087	0.997	0.004	0.229	0.117	0.051	0.262	0.827	0.827	0.820	0.594	0.829
Insulin	0.289		0.396	0.006	0.287	0.021	0.633	0.135	0.344	0.598	0.746	0.776	0.707	0.707	0.333	0.052	0.602
HBA1C	0.221	0.121		0.729	0.760	0.502	0.991	0.373	0.629	0.523	0.803	0.665	0.412	0.412	0.933	0.969	0.644
Triglycerides	0.194	0.383	-0.050		0.050	0.048	0.782	0.169	0.349	0.645	0.345	0.831	0.512	0.512	0.875	0.656	0.379
Total Cholesterol	0.088	0.152	-0.044	0.270		0.297	0.003	0.663	0.865	0.957	0.041	0.016	0.647	0.647	0.056	0.058	0.177
HDL - Cholesterol	-0.242	-0.323	0.096	-0.273	0.146		0.144	0.118	0.188	0.499	0.567	0.919	0.829	0.829	0.840	0.955	0.591
LDL - Cholesterol	0.001	0.069	-0.002	0.039	0.708	-0.203		0.964	0.165	0.800	0.128	0.016	0.612	0.612	0.035	0.031	0.159
Wais to Hip Ratio	0.481	0.261	0.158	0.242	0.078	-0.274	0.008		0.056	0.411	0.840	0.278	0.019	0.019	0.504	0.758	0.577
BMI	0.212	0.168	0.086	0.166	-0.030	-0.231	-0.243	0.326		0.607	0.201	0.259	0.420	0.420	0.114	0.243	0.096
VPI	-0.222	-0.076	-0.092	0.065	0.008	0.095	0.036	-0.139	0.085		0.000	1.580	0.015	0.015	390.000	0.001	0.002
MB	-0.275	0.046	-0.036	0.132	0.282	0.080	0.212	0.034	0.209	0.426		1470.000	0.479	0.479	0.239	0.303	0.303
BoP	-0.160	-0.041	-0.062	0.030	0.329	0.014	0.331	-0.183	-0.185	0.278	0.203		0.000	0.000	0.000	0.000	0.000
PoD≤4	-0.031	-0.054	-0.117	0.092	0.064	-0.030	0.071	0.384	-0.133	-0.131	-0.038	-0.601		0.000	0.000	0.000	0.000
PoD≥5	0.031	0.054	0.117	-0.092	-0.064	0.030	-0.071	-0.384	0.133	0.131	0.038	0.601	-1000.000		0.000	0.000	0.000
CALi≤3	0.033	0.138	0.012	0.022	-0.264	0.028	-0.291	0.113	0.257	-0.190	-0.064	-0.866	0.641	-0.641		0.000	0.000
CALi4-5	-0.076	-0.273	-0.006	-0.063	0.262	0.008	0.296	-0.052	-0.191	0.171	0.056	0.777	-0.476	0.476	-0.883		0.000
CALi≥6	0.031	0.075	0.066	0.123	0.188	-0.076	0.196	-0.095	-0.271	0.169	0.056	0.695	-0.629	0.629	-0.838	0.539	

FG = Fasting Glucose; Triglyc = Triglycerides; Total Chol = Total Cholesterol; HDL Chol = HDL Cholesterol; WtoHR = Waist to Hip Ratio; BMI = Body mass index; VPI = Visible Plaque index; MB = Marginal Bleeding; BoP = Bleeding on Probing; PoD = Probing on depth; CAL = Clinical Attachment Loss. Values above light gray meant p values. The green colors meant that there was a positive significant correlation, and the red color meant negative significant correlation. Upper diagonal = p values. Lower diagonal values = ρ=Spearman's correlations coefficients.

SUPPLEMENTARY MATERIAL

1. Material and Methods

1.1. Study subjects

To calculate the sample size needed for the study to have power to attest the association between the alleles and periodontitis, the G * Power Calculator, version 3.1.9 (Faul et al., 2007) program was used, considering the parameters: logistic regression; two tail, OR: 1.7; Pr (Y = 1 / X = 1) H0 = 0.2; alpha of 0.0055 and 80% power, R2 = 0. It is important to note that alpha = 0.05 was obtained from p = 0.05 divided by 9 SNPs. This calculation resulted in a total sample of 270 patients, being 90 patients per group. Therefore, the genetic analyses in this study have a power greater than 80%, since each studied group comprises more than 90 patients.

2. Results

In the Supplementary Table 1, it is possible to note the allele and genotype frequencies of each SNP. The results were distributed in The Groups Healthy, Periodontitis and T2DM_P. The Hardy – Weinbeirg Equilibrium was calculated in each of the three groups. Its also possible to observe that in the SNP rs285, in the T2DM_P group, was not in the hardy - Weinberg Equilibrium, showing a possible association the genotype with a disease. However, if the Bonferroni correction is considered, these associations will lack the statistical significance.

Supplementary Table 1. Alleles and genotype distribution of the 9 SNPs compared among the groups (chi-square test), HWE

ABCC8	**Healthy N= 342**	***Periodontitis* n= 343**	**T2DM_P n= 202**
(C>T) rs6544718	n (%)	n (%)	n (%)
Alleles C/T	574 (83.80) /110 (16.20)	574 (83.90) / 112 (16.10)	332 (82.20) / 72 (17.80)
MAF	0.15	0.16	0.17
Genotypes	n (%)	n (%)	n (%)
CC	239 (70.0)	242 (70.55)	133 (65.85)
CT	96 (28.0)	90 (26.25)	66(32.70)
TT	7 (2.0)	11(3.20)	3 (1.45)
HWE	0.55	0.43	0.14

ABCC8 **(C>T) rs6544713**	**Healthy N= 342** n (%)	***Periodontitis*** **n= 344** n (%)	**T2DM_P N= 201** n (%)
Alleles C/T	505 (73.83) /179 (26.17)	496 (76.14) / 192 (23.86)	287 (77.33) / 115 (22.67)
MAF	0.26	0.27	0.28
Genotypes	n (%)	n (%)	n (%)
TT	190 (55.55)	182 (56.29)	104 (51.72)
CT	125 (36.55)	132 (39.71)	79 (38.90)
CC	27 (7.90)	30(4.00)	18 (9.38)
HWE	0.32	0.42	0.60
LPL **(T>C) rs285**	**Healthy N= 341** n (%)	***Periodontitis*** **n= 339** n (%)	**T2DM_P N= 193** n (%)
Alleles T/C	351 (51.46) /331 (48.54)	372 (56.86) / 306 (45.14)	201 (52.75) / 185 (47.25)
MAF	0.48	0.45	0.47
Genotypes	n (%)	n (%)	n (%)
TT	94 (55.55)	108 (56.29)	60 (51.72)
CT	163(36.55)	156 (39.71)	81 (38.90)
CC	84 (7.90)	75 (4.00)	52 (9.38)
HWE	0.44	0.18	**0.03**
LPL **(T>C) rs285**	**Healthy N= 341** n (%)	***Periodontitis*** **n= 339** n (%)	**T2DM_P N= 193** n (%)
Alleles T/C	351 (51.46) /331 (48.54)	372 (56.86) / 306 (45.14)	201 (52.75) / 185 (47.25)
MAF	0.48	0.45	0.47
Genotypes	n (%)	n (%)	n (%)
TT	94 (55.55)	108 (56.29)	60 (51.72)
CT	163(36.55)	156 (39.71)	81 (38.90)
CC	84 (7.90)	75 (4.00)	52 (9.38)
HWE	0.44	0.18	**0.03**
LPL **(C>A) rs3735964**	**Healthy N= 340** n (%)	***Periodontitis*** **n= 340** n (%)	**T2DM_P N= 187** n (%)
Alleles C/A	600 (88.23) /80 (11.77)	613 (90.14) / 67 (9.86)	337 (90.10) / 37 (9.10)
MAF	0.11	0.09	0.09
Genotypes	n (%)	n (%)	n (%)
CC	266 (78,23)	278 (81.77)	150 (80.20)
AC	68(20.00)	57 (16.76)	37 (19.80)
AA	6 (1.77)	5 (1.47)	0 (0.0)
HWE	0.43	0.35	0.22
LPL **(T>C) rs13702**	**Healthy N= 313** n (%)	***Periodontitis*** **n= 321** n (%)	**T2DM_P N= 176** n (%)
Alleles T/C	393 (63.80) /233 (36.20)	418 (65.10) / 224 (35.90)	232 (65.90) / 120 (34.10)

MAF	0.37	0.34	0.34
Genotypes	n (%)	n (%)	n (%)
TT	119(38.02)	134 (41.75)	79 (44.85)
CT	155(49.52)	150 (46.72)	74 (42.04)
CC	39 (12.46)	37 (11.53)	23 (13.10)
HWE	0.33	0.71	0.40
HNF1A **(C>A) rs2650000**	**Healthy N= 341** n (%)	***Periodontitis*** **n= 340** n (%)	**T2DM_P N= 199** n (%)
Alleles C/A	457 (67.01) /225 (32.09)	467 (68.70) / 217 (31.30)	270 (67.83) / 128 (32.17)
MAF	0.32	0.31	0.32
Genotypes	n (%)	n (%)	n (%)
CC	148 (43.40)	160 (47.05)	92 (46.23)
AC	161 (47.20)	147 (43.23)	86 (43.21)
AA	32 (9.40)	35 (9.72)	21 (10.57)
HWE	0.26	0.90	0.87
ApoE **(T>C) rs429358**	**Healthy N= 343** n (%)	***Periodontitis*** **n= 341** n (%)	**T2DM_P N= 202** n (%)
Alleles T/C	594 (86.30) /92 (13.70)	579 (84.89) / 103 (15.11)	354 (87.62) / 50 (12.38)
MAF	0.13	0.15	0.12
Genotypes	n (%)	n (%)	n (%)
TT	257 (74.92)	249 (73.02)	155(76.73)
CT	80(23.32)	81 (23.75)	44 (21.78)
CC	6 (1.76)	11 (3.23)	3 (1.49)
HWE	1	0.20	1
ApoE **(C>T) rs7412**	**Healthy N= 342** n (%)	***Periodontitis*** **n= 345** n (%)	**T2DM_P N= 201** n (%)
Alleles C/T	637 (93.10) /47 (6.90)	655 (94.90) / 35 (5.10)	371 (92.30) / 31 (7.70)
MAF	0.06	0.05	0.07
Genotypes	n (%)	n (%)	n (%)
CC	298 (87.13)	310 (89.9)	173 (86.10)
CT	41(12.00)	35 (10.10)	25 (12.45)
TT	3 (0.87)	0 (0.00)	3 (1.45)
HWE	0.20	1	0.09
HNF4A **(C>T) rs1800961**	**Healthy N= 338** n (%)	***Periodontitis*** **n= 341** n (%)	**T2DM_P N= 204** n (%)
Alleles C/T	656 (97.00) /20 (3.0)	664 (97.36) / 18 (2.64)	385 (94.36) / 23 (5.64)
MAF	0.02	0.02	0.05
Genotypes	n (%)	n (%)	n (%)
CC	319 (94.37)	323 (94.72)	181 (88.72)
CT	18 (5.32)	18 (5.28)	23 (11.28)

TT	1 (0.31)	0 (0.00)	0 (0.00)
HWE	0.25	1	1

HWE: Equilíbrio de Hardy Weinberg; MAF: Minor Allele Frequencies; $p \leq 0.05$ in bold

3. Discussion

A gender influence in gene transcription is necessary, as males and females use a similar genome to achieve distinct phenotypes [54]. It has been suggested that gender-biased differences in gene expression may particularly occur in tissues with multifarious physiological roles, and gene-ontology analysis identified that immune response genes were differentially expressed between males and females [55]. Moreover, epigenetic mechanisms have been investigated in the association with periodontitis, as demonstrated by the finding that female patients exhibited lower methylation levels of MMP-9 but higher methylation levels of TIMP-1 compared with male patients with periodontitis [62]. Clinical experience [56], as well in vitro [57] and in vivo [58] studies, indicate the influence of steroid hormones on inflammation, immune response and bone metabolism, demonstrating existence of an association of periodontitis with sex hormone levels.

Ten SNPs in strong linkage disequilibrium, upstream from the *NPY* gene, suggested gene–sex interaction with respect to the risk of periodontitis on a genome-wide scale in German/Austrian population. A different SNP (rs198712) was studied and showed the strongest association in interaction ($p = 5.4 \times 10^{-6}$) with odds ratios in males and females of 1.63 and 0.69, respectively [63].

There is only one study that investigated the gingival crevicular fluid (GCF), salivary and plasma levels of IL-37 in individuals with periodontitis in comparison with periodontally healthy controls [64]. There was no difference in the concentration of IL-37 in GCF, saliva and plasma between control and periodontal disease patients. This suggested that IL-37 is not involved in periodontal disease; however, polymorphisms in this gene, like the rs3811046, may be involved in the regulation of the IL-37 protein expression or function, but there are no functional studies focusing on this SNP.

The ethnically admixed Brazilian population is a characteristic that prevented stratification of the sample into ethnic groups or skin color. Parra et al., 2003 do not recommend grouping Brazilians into ethnic groups based on skin color and other physical characteristics associated with racial divisions. Brazilian individuals classified as ‘white’ or ‘black’ have significantly overlapping genotypes considered as race-associated loci for Caucasians, Africans and Indigenous people [61]. Brazilians form one of the most

heterogeneous populations in the world, resulting of the 5 centuries of inter-ethnic crossing between Europeans, Africans, Asians and autochthonous Amerindians [60]

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4 CONCLUSÃO

Conclui-se que polimorfismos relacionados a importantes genes do metabolismo lipídico e glicêmico como *HNF4A* (rs1800961), *LPL* (rs13702), *LPL* (rs285), *LPL* (rs3735964) e *ABCC8* (rs6544718), estão associados à periodontite na presença ou não ao DM2 após análise de regressão logística múltipla ajustada para idade, sexo e tabagismo. Correlações significativas foram encontradas mostrando associações entre perfil bioquímico e paramêtros periodontais. Quando feita a estratificação da amostra, em relação ao grau de doença periodontal, do controle metabólico e dislipidêmico foi visto resultados significativos, confirmando a associação entre SNPs e as doenças na população estudada.

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APÊNDICE A - Material e Métodos

Seleção da amostra

Este estudo tem a aprovação do comitê de Ética em Pesquisa em humanos da FOAr – UNESP Protocolo 50/60 (Anexo 1). Pacientes foram recrutados na Clínica de Periodontia da Faculdade de Odontologia de Araraquara, após esclarecer a natureza e os objetivos desta pesquisa, estes, foram encaminhados para realizar exames periodontais em pesquisadores calibrados e exames bioquímicos apresentados no item 3.3.

Considerando os dados do exame periodontal, de acordo com os critérios definidos pela CDC/AAP (*Centers for Disease Control and Prevention/ American Academy of Periodontology*)[58], os indivíduos foram classificados em grupos conforme sua condição periodontal e DM: Grupo Healthy: pacientes periodontalmente e sistemicamente saudáveis; Grupo *Periodontitis* (P= Periodontite): pacientes com periodontite moderada ou severa sem DM; e Grupo T2DM_P composto por pacientes com periodontite (moderada ou severa) e que apresentam Diabetes Mellitus tipo 2.

Os portadores de T2DM tiveram tal diagnóstico do seu médico que o acompanha. Os paramêtros para classificar os pacientes com T2DM estão no item 3.3. Serão considerados metabolicamente descompensados os pacientes com T2DM que apresentarem HbA1c ≥7,1%, e metabolicamente compensados os que apresentarem HbAc1 ≤ 7,0% e ≥ 5,8%. Os pacientes sem T2DM apresentaram HbA1c < 6,4 % [59]. Todos os pacientes receberam informação dos resultados dos seus perfis glicêmico, lipídico e periodontal.

Os pacientes foram divididos conforme seus níveis glicêmicos e seu perfil periodontal nos seguintes Grupos:

- **T2DM_P** (n=205) – Indivíduos com diabetes mellitus tipo 2 e com periodontite crônica (moderada e severo)
- ***Periodontitis*** (n=345) – Indivíduos sem diabetes mellitus tipo 2 e com periodontite crônica severo ou moderada
- ***Healthy*** (n=343) - Indivíduos sem diabetes mellitus tipo 2 e sem periodontite crônica

Para calcular o tamanho da população necessária para que o estudo tenha poder suficiente de atestar a associação do(s) alelo(s) com a P foi utilizado o programa G*Power Calculator, versão 3.1[60], considerando os parâmetros: regressão logística; two tail, OR: 1.6;

Pr(Y=1/X=1) H0 = 0,2; alfa de 0,00066 e poder de 80%, R2 = 0. Este cálculo resultou em uma amostra total de 538 pacientes (Figura 1). Com o "Total sample size" de 538, considerando 3 grupos teríamos o valor por grupo de 179,3. Arredondando para 180 pacientes por grupo, a amostra total seria de 540 pacientes. Deste modo, como cada um dos grupos estudados aqui é maior que 180, o nível de confiança do nosso estudo ficou maior que 80%.

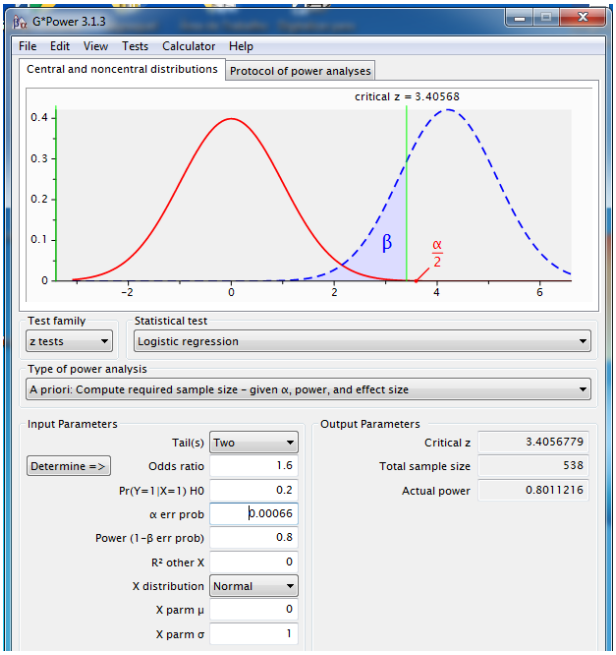

Figura 1. Resultado do G*Power utilizando os parâmetros descritos.

Anamnese, Exame Físico e Periodontal

Todos os pacientes foram submetidos à anamnese completa para avaliar o histórico de saúde geral e bucal. Além disso também foram submetidos a exame físico para avaliar: circunferência abdominal (cm), proporção cintura/quadril, peso (kg) e altura (m) para obtenção do índice de massa corporal (IMC), em m/kg^2 de cada indivíduo.

O exame periodontal foi realizado com sonda periodontal (tipo Williams - Trinity® – Campo Mourão, Paraná, Brazil), e foi composto de índice de placa visível (IPV), índice de sangramento marginal (ISM), sangramento à sondagem (SS), mensuração da profundidade de sondagem (PS), posição da margem gengival e nível de inserção clínica (NIC) examinados em seis sítios (mésio-vestibular, vestibular, disto-vestibular, mésio-lingual, lingual e disto-lingual) de cada dente.

- Índice de placa visível: presença ou não de placa bacteriana visível a olho nu, após secagem da superfície dentária com jato de ar, em todas as faces de todos os dentes[61];
- Índice de sangramento marginal: presença ou ausência de sangramento marginal após percorrer o espaço do sulco de uma proximal a outra, com a sonda periodontal milimetrada inclinada em 60 graus em relação ao dente[61];
- Posição da margem gengival: distância da margem gengival à junção cemento-esmalte;
- Mensuração da profundidade de sondagem: distância da margem gengival ao fundo do sulco gengival, medida com sonda periodontal milimetrada em 6 sítios por dente: disto-vestibular, vestibular, mésio-vestibular, disto-lingual, lingual e disto-lingual;
- Sangramento à sondagem: presença ou ausência de sangramento, decorrido um tempo de 30 segundos depois de mensurada a profundidade de sondagem[62];
- Avaliação do nível clínico de inserção: corresponde à somatória das medidas da posição da margem gengival e profundidade de sondagem, para cada sítio de cada elemento dentário.

Após o exame periodontal, os indivíduos foram classificados em dois diferentes Grupos conforme sua condição periodontal. Tais critérios periodontais já foram utilizados em estudos de polimorfismos genéticos, incluindo estudos do tipo GWAS para Periodontite [63, 64].

Tabela 1. Classificação da Periodontite, de acordo com os critérios definidos pela CDC/AAP

		Definição Clínica		
Classificação		**NI**		**PS**
Grupo Healthy	Periodontite leve e Saudáveis	Não se enquadram nos critérios de periodontite severo ou moderada		
Grupo *Periodontitis*	Periodontite moderada	≥ 2 sítios com NIi ≥ 4mm	E	≥ 2 sítios com PSi ≥ 5mm
	Periodontite Severa	≥ 2 sítios com NIi ≥ 6mm	E	≥ 1 sítio com PSi ≥ 5mm

*Exclusão de terceiros molares. P: periodontite; PSi: profundidade de sondagem interproximal; NIi: nível de inserção interproximal.

Critérios de Inclusão e Exclusão para cada grupo estudado

Referente ao Grupo **T2DM-P** :

- CRITÉRIOS DE INCLUSÃO: indivíduos com T2DM acima de 30 anos, de ambos os sexos, com pelo menos 15 dentes na cavidade bucal e sem distinção grupo étnico-racial (pois a tentativa de isolar determinadas características raciais é difícil em um país altamente miscigenado). Apresentar periodontite conforme descrito na página anterior.
- CRITÉRIOS DE EXCLUSÃO: indivíduos com história de hepatite e infecção por HIV, com anemia, com pré-medicação antibiótica nos últimos 6 meses ou uso crônico de drogas anti-inflamatórias, além de pacientes grávidas ou em lactação.

Referente ao Grupo ***Periodontitis*:**

- CRITÉRIOS DE INCLUSÃO: indivíduos sem T2DM acima de 30 anos, de ambos os sexos, com pelo menos 15 dentes na cavidade bucal e sem distinção étnico-racial (pois a tentativa de isolar determinadas características raciais é difícil em um país altamente miscigenado). Apresentar periodontite conforme descrito na página anterior.
- CRITÉRIOS DE EXCLUSÃO: pacientes com história de diabetes, hepatite e infecção por HIV, com pré-medicação antibiótica recente ou uso crônico de drogas anti-inflamatórias, além de pacientes grávidas ou em lactação.

Referente ao Grupo ***Healthy***:

- CRITÉRIOS DE INCLUSÃO: indivíduos sem T2DM acima de 30 anos, de ambos os sexos, com pelo menos 15 dentes na cavidade bucal e sem distinção étnico-racial (pois a tentativa de isolar determinadas características raciais é difícil em um país altamente miscigenado). Sem presença de periodontite crônica conforme descrito na página anterior.
- CRITÉRIOS DE EXCLUSÃO: pacientes com hepatite e infecção por HIV, com pré-medicação antibiótica recente ou uso crônico de drogas anti-inflamatórias, além de pacientes grávidas ou em lactação.

Avaliação Exames Bioquímicos

Os pacientes foram encaminhados ao Laboratório de Análises Clínicas da UNESP (NAC) para coleta do sangue por punção venosa para realização dos exames: Hemograma (ABX Micros 60), Insulina (método quimiluminescência), Glicemia de Jejum (método Bondar e Mead modificado) (Kit Labtest), HbA_{1c} (método de imunoensaio de inibição turbidimétrica) (Kit Roche), Triglicérides (método enzimático-Trinder) (Kit Labtest), Colesterol Total (método enzimático-Trinder) (Kit Labtest) e HDL-colesterol (método enzimático) (Kit Labtest). Está sendo utilizado o equipamento COBAS MIRA plus. As frações do VLDL e LDL-colesterol foram calculadas segundo a equação de Friedewald (válida se Triglicérides <400mg/dl) (Sociedade Brasileira de Cardiologia):

- *Colesterol VLDL = triglicérides / 5*
- *Colesterol LDL= Colesterol Total – (HDL+VLDL)*
- *Perfil Glicêmico*
    - Sem T2DM = HbA1C < 6.4%
    - T2DM = HbA1C ≥ 7.1%
    - T2DM compensado = ≤ 7.0% e ≥ 5.8%

Para os resultados do perfil lipídico, são considerados como dislipidêmicos os pacientes que apresentarem alterados pelo menos um dos seguintes parâmetros clínicos, de acordo com a Sociedade Europeia de Aterosclerose e Federação Europeia de Química e Medicina Laboratorial[65]:

- Colesterol Total (CT): Considerado alto para valores ≥ 240 mg/dl.
- LDL (Low density lipoprotein): Considerado alto para valores ≥ 160 mg/dl.
- HDL (High density lipoprotein): Considerado baixo para valores:
    - Homens ≤ 40 mg/dl;
    - Mulheres ≤ 45 mg/dl.
- Triglicérides (TG): Considerado alto para valores ≥ 200 mg/dl.

OBSERVAÇÃO: Os pacientes com T2DM que foram incluídos neste estudo receberam tal diagnóstico pelo seu médico. Referente aos pacientes participantes com periodontite, ao recebermos os resultados do perfil glicêmico e lipídico do paciente (e lhe encaminharmos cópia do seu exame), quando porventura observamos parâmetros suspeitos da presença de T2DM e/ou dislipidemia, instruíamos o mesmo a buscar orientação médica.

Coleta de material biológico e extração de DNA

Foi solicitado a cada indivíduo que realizasse um bochecho com solução de dextrose a 3% auto clavada por 2 min. Os tubos contendo o bochecho foram mantidos resfriados (em caixa de isopor com gelo) do local da coleta até o momento do processamento em laboratório. Cada tubo foi centrifugado a 2000 rpm por 10 min para separar o *pellet* de células que se despregam da mucosa oral. A este *pellet* de células foi adicionado uma solução Tampão (TrisHCl 10mM, EDTA 0,1 M, SDS 0,5% - pH 8.0), sendo armazenado em freezer (–80°C) até a finalização das coletas de todos os indivíduos.

Para extração do DNA, como foi um grande número de amostras, e é necessária alta qualidade do DNA, foi utilizado o método do acetato de amônio a 8M [66]. Nesta técnica as amostras foram descongeladas, adicionado 5 μL de proteinase K e incubado a 55ºC por 2h. Em seguida adicionou-se 500μL de acetato de amônio 8M e centrifugou-se a amostra a 14.000 rpm por 10 mim a 25ºC. Ao sobrenadante foi adicionado 540 μL de isopropanol e posteriormente centrifugado a 14.000 rpm por 5 mim. Foram feitas 2 lavagens do pellet obtido com etanol 70% para remover o isopropanol e centrifugou-se a 14.000 rpm por 5 mim. O pellet foi seco à temperatura ambiente e ressuspendido em 50 μL de tampão Tris-EDTA (TE).

O DNA genômico de cada amostra foi avaliado quanto à concentração (ng/μl) e pureza (260/280nm; 260/230nm) no aparelho NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, USA), e foi armazenado a -20°C até o momento da genotipagem. Em seguida, as amostras também foram quantificadas pelo sistema de fluorescência Qubit 2.0 (Thermo-Fisher).

Seleção dos SNPs

Para a seleção dos SNPs foi utilizado o programa HAPLOVIEW[67] a partir de dados do banco do *International HapMap Project* (www.hapmap.org), um consórcio internacional contendo informações de milhares de polimorfismos de indivíduos de diferentes etnias. Utilizamos dados de SNPs validados nos blocos haplotípicos considerando as populações CEU+TSI (combinação de populações caucasianas: [CEU = indivíduos residentes de Utah com ascendência Europeia Ocidental e Norte] e [TSI = indivíduos da Toscana na Itália]) do Projeto HapMap. Por exemplo, verificamos nesse banco de dados a frequência do alelo mais raro (*Minimum Allele Frequency* – MAF) de cada SNP de interesse, de modo que fosse pelo menos 0,05 de preferência (equivale a 5%). Foram investigados 9 SNPs no gene presentes na Tabela 2

Tabela 2 Informações dos ensaios e SNPs avaliados, incluindo dados do Minor Allele Frequency (MAF)

Genes relacionados ao metabolismo lipídico						
Código Assay	**NCBI SNP Reference**	***Gene Symbol***	**Alelos**	**CEU(branco) %**	**TSI(italianos) %**	**MAF do estudo**
C___9639448_10	rs13702	*LPL*	**C**|**T**	**C**|**C**: 0.091	**C**|**C**: 0.159	**C**|**C**: 0,118
C___7591528_10	rs1800961	*HNF4A*	**C**|**T**	**C**|**T**: 0.081	**C**|**T**: 0.037	**T**|**T**:0,001
C__15874272_10	rs2650000	*HNF1A/ TCF1*	**A**|**C**	**A**|**A**: 0.152	**A**|**A**: 0.112	**A**|**A:** 0,101
C__12104266_10	rs285	*LPL*	**C**|**T**	**T**|**T / C**|**C**: 0.242	**C**|**C** 0.206	**C**|**C**:0,237
C__25800209_10	rs3735964	*LPL*	**A**|**C**	**A**|**A**: 0.020	**A**|**A**: 0.028	**A**|**A:**0,016
C___3084793_20	rs429358	*Apo E*	**C**|**T**	**C**|**C**: 0.020	**C**|**T**: 0.206	**C**|**C**: 0,026
C__26135636_10	rs6544713	*ABCG8*	**C**|**T**	**T**|**T**: 0.091	**T**|**T**: 0.093	**T**|**T:**0,085
C__25642779_10	rs6544718	*ABCG8*	**C**|**T**	**T**|**T**: 0.051	**T**|**T**: 0.028	**T**|**T**: 0,026
C____904973_10	rs7412	*Apo E*	**C**|**T**	**C**|**T**: 0.131	**C**|**T**: 0.093	**T**|**T:**0,004

Genotipagem

Os 9 SNPs foram investigados por meio de reações de PCR (*Polimerase Chain Reaction*) em Tempo Real com o sistema de genotipagem TaqMan® (Thermo-Fischer Scientific, Foster City, CA, EUA). Os alelos de cada SNP foram discriminados com um ensaio específico TaqMan® conforme a Tabela 2, que possui primers e sondas específicas para cada alelo marcadas com os fluoróforos VIC e FAM, no qual, o alelo 1 é marcado com VIC e o alelo 2 é marcado com FAM.

Assim, temos os resultados em VIC/VIC, VIC/FAM ou FAM/FAM. Desta forma, um indivíduo homozigoto para um alelo tem a emissão de somente um comprimento de onda (referente à sonda que se anelou no gene alvo), enquanto para um indivíduo heterozigoto há a emissão de ambos os fluoróforos das duas sondas empregadas para detecção do polimorfismo.

Esse método baseia-se na atividade 5'- endonuclease da *Taq* DNA Polimerase para clivar uma sonda anelada durante a extensão do DNA. O sinal de fluorescência é detectado quando as subunidades "*quencher*" e *repórter*" da sonda se separam. A vantagem dos ensaios com TaqMan é a facilidade, rapidez na obtenção e confiabilidade dos resultados.

O DNA genômico de cada paciente foi acrescentado ao TaqMan Genotyping Master Mix (Thermo-Fischer Scientific, Foster City, CA, EUA) em uma placa de 96 poços e esta foi inserida no termociclador StepOne Real Time PCR System (Thermo-Fischer Scientific, Foster City, CA, EUA) para realização da reação de PCR em tempo real.

Cada reação de genotipagem foi realizada com: 0,63 µl do ensaio TaqMan específico para genotipagem de cada referido SNPs (incluindo os iniciadores direto e reverso e as sondas marcadas com VIC e FAM); 6,25 µl de TaqMan® Genotyping Master Mix e 20ng de DNA genômico e água DNAse /RNAse free em quantidade suficiente para completar 12,5 µL finais. As seguintes condições foram empregadas para ciclagem dos SNPs: 95°C por 10min, seguidos de 40 ciclos de 95°C por 15 segundos, 60°C por 1 min.

A visualização gráfica dos resultados foi dada pelo programa StepOne System SDS Software que faz a correlação entre a fluorescência emitida com cada alelo em específico.

Análise estatística dos dados

Os dados demográficos e clínicos foram submetidos a análise estatística descritiva e analítica pelo programa IBM SPSS Statistics 20.0 ou com o auxílio do programa GraphPad Prism 6.0. Inicialmente os dados foram avaliados quanto à normalidade pelo teste de D'Agostino & Pearson, e em seguida, aplicados testes paramétricos ou não paramétricos para comparação entre grupos. As variáveis demográficas entre casos e controles foram comparadas pelo teste t de Student (idade e parâmetros periodontais) para variáveis contínuas, e pelo teste do $\chi 2$ para variáveis categóricas (sexo e tabagismo). O teste de Kruskal-Wallis foi utilizado para avaliar os parâmetros periodontais (número de dentes, placa visível, sangramento marginal, sangramento à sondagem, profundidade de bolsas periodontais e perda de inserção clínica interproximal) e genótipo individual de cada SNP independentemente.

A fórmula de equilíbrio de Hardy-Weinberg (HWE) foi aplicada às frequências genotípicas entre os sujeitos de controle para testar quaisquer desvios do equilíbrio genotípico esperado usando a adequação do qui-quadrado.

As diferentes variáveis independentes binárias (Grupo Healthy vs. *Periodontitis*; Grupo Healthy vs. Grupo *Periodontitis* moderada ; Grupo Healthy vs. Grupo *Periodontitis* severa) foram usadas para determinar a associação entre cada SNP e o diagnóstico de periodontite e da T2DM. Todas as análises de associação de caso-controle foram realizadas, as quais incluíram associações alélicas e genotípicas. Os genótipos foram categorizados em três Grupos (alelo homozigoto principal – 11. Heterozigoto – 12. E variante homozigoto – 22). Diferentes graus de gravidade da doença (moderada ou grave) foram avaliados pelo teste do qui-quadrado em quatro modelos: i) alelo 1 *versus* 2; ii) genótipo 11 *versus* 12 *versus* 22 (modelo aditivo); iii) genótipo 11 + 12 *versus* 22 (modelo dominante); e iv) genótipo 11 *versus* 12 + 22 (modelo recessivo).

A associação entre cada SNP com periodontite (moderada e severa) ou T2DM (compensada ou descompensada) foi estimada como odds ratio (OR) e intervalo de confiança (IC) de 95% por meio de regressão logística múltipla ajustada por idade, sexo e tabagismo. Além disso, os polimorfismos genéticos foram avaliados, através do modelo de regressão logística, separadamente pelo sexo dos pacientes, para investigar a suscetibilidade à P, ajustada por idade e tabagismo.

O software PLINK foi usado para detectar diferenças nas distribuições para cada SNP entre os grupos. Todas as outras análises foram realizadas pelo software STATA (Statistics / Data Analysis. Stata Corporation. College Station, Texas, EUA. Correlações de Spearman's entre paramêtros periodontais, níveis glicêmicos e lipídicos foram feitos para cada grupo. Significância estatística foi considerada quando $p<0.05$.

ANEXO A - Certificado do Comitê de Ética

UNIVERSIDADE ESTADUAL PAULISTA " JÚLIO DE MESQUITA FILHO"

FACULDADE DE ODONTOLOGIA DE ARARAQUARA

Comitê de Ética em Pesquisa

Certificado

Certificamos que o projeto de pesquisa intitulado *"DIABETES MELLITUS E DOENÇA PERIODONTAL: ANÁLISE CLÍNICA, GENÉTICA, MICROBIOLÓGICA, IMUNOLÓGICA E BIOQUÍMICA"*, sob o protocolo nº **50/06**, de responsabilidade do Pesquisador (a) *SILVANA REGINA PEREZ ORRICO*, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa–FOAr, com validade de 04 (quatro) anos, quando será avaliado o relatório final da pesquisa.

Certify that the research project titled *"DIABETES MELLITUS AND PERIODONTAL DISEASE: CLINICAL, GENETICAL, MICROBIOLOGICAL, IMMUNOLOGICAL AND BIOCHEMICAL ANALYSIS"*, protocol number **50/06**, under Dr *SILVANA REGINA PEREZ ORRICO*, responsability, is under the terms of Conselho Nacional de Saúde/MS resolution # 196/96, published on May 10, 1996. This research has been approved by Research Ethic Committee, FOAr-UNESP. Approval is granted for 04 (four) years when the final review of this study will occur.

Araraquara, 23 de abril de 2007.

Profª Drª *Miriam Aparecida Onofre*
Coordenadora

Não autorizo a publicação deste trabalho pelo prazo de 2 anos.

Araraquara, 27 de março de 2020

Ingra Gagno Nicchio