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Rafael Nepomuceno Oliveira

Investigação de polimorfismos genéticos e metabolismo lipídico com doença periodontal crônica: metanálise e estudo caso-controle

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Orientadora: Profa. Dra. Raquel Mantuaneli Scarel Caminaga

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DADOS CURRICULARES

Rafael Nepomuceno Oliveira

NASCIMENTO: 17 de novembro de 1988 – Fortaleza – CE

FILIAÇÃO: Jose Alberto de Oliveira Filho
Sueli Nepomuceno Oliveira

2007 - 2011 Curso de Graduação em Odontologia – Faculdade de Farmácia, Odontologia e Enfermagem (FFOE) – Universidade Federal do Ceará (UFC).

2012 - 2014 Curso de Pós-Graduação em Odontologia, Área de concentração em Periodontia – Nível Mestrado - Faculdade de Odontologia de Araraquara (FOAr) - Universidade Estadual Paulista (UNESP).

2013 - 2014 Curso de Especialização em Periodontia – Faculdade de Odontologia de Araraquara (FOAr) - Universidade Estadual Paulista (UNESP).

2014 - 2018 Curso de Pós-Graduação em Odontologia, Área de concentração em Periodontia – Nível Doutorado - Faculdade de Odontologia de Araraquara (FOAr) - Universidade Estadual Paulista (UNESP).

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Oliveira RN. Investigação de polimorfismos genéticos e metabolismo lipídico com doença periodontal crônica: metanálise e estudo caso-controle [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2018.

RESUMO

Diversos fatores relacionados ao hospedeiro, como fatores ambientais (tabagismo), doenças sistêmicas (diabetes e dislipidemia) e herança genética vêm sendo estudados quanto à influência no início e progressão da doença periodontal. Embora muitos estudos tenham investigado a relação entre dislipidemia e periodontite crônica (PC), examinando os níveis séricos de lipídeos, os resultados ainda são contraditórios, indicando a demanda da realização de uma metanálise. Alguns *Genome-Wide Association Studies* (GWAS), estudos de bioinformática e diversos estudos caso-controle evidenciaram variantes genéticas associadas à suscetibilidade à periodontite, mas os resultados para alguns desses polimorfismos são escassos ou contraditórios entre as diferentes populações. Assim, nota-se a importância de realizar metanálises e estudos caso-controle para buscar validar ou identificar a associação de polimorfismos com a PC na população brasileira. O objetivo deste estudo foi dividido em 3 capítulos: (1) investigar se os pacientes com PC apresentam diferentes níveis séricos de parâmetros lipídicos (HDL, LDL, colesterol total e triglicérides) em comparação com indivíduos saudáveis; (2) avaliar se polimorfismos de base única (SNPs, *single nucleotide polymorphisms*) nos genes *LDLR* (rs5925 e rs688) e *APOB* (rs676210 e rs693) contribuem para a suscetibilidade à PC, uma vez que PC está associada a níveis plasmáticos de LDL mais elevados, e os polimorfismos nestes genes podem aumentar as concentrações plasmáticas de LDL; (3) validar na população brasileira a associação de polimorfismos genéticos previamente associados à PC em outras populações, por meio de estudos que utilizaram técnicas sofisticadas de bioinformática e GWAS. No capítulo (1), foi realizada uma revisão sistemática com metanálise e meta-regressão. Nos capítulos (2) e (3) foram realizados estudos caso-controle com genotipagem de SNPs em pacientes com e sem PC para investigar a associação destes SNPs entre os diferentes genótipos em relação a PC e parâmetros periodontais, além de avaliar a associação genética sexo-específica, e a da interação destas variações genéticas com fatores ambientais (tabagismo). Na metanálise, foram incluídas 19 publicações, verificando-se como resultado que os participantes com PC apresentaram níveis séricos significativamente elevados de LDL e triglicerídeos ($p = 0,003$ e $p < 0,0001$, respectivamente) e níveis significativamente mais baixos de HDL ($p = 0,0005$), comparando-se a indivíduos sem PC. Como resultado do capítulo (2), não foi verificada associação significativa entre os parâmetros clínicos periodontais e as frequências de genótipos de qualquer

dos SNPs nos genes *APOB* e *LDLR* avaliados. Comparando-se pacientes com e sem PC, não houve diferenças significativas em nenhuma das análises multivariadas, interações multiplicativas e aditivas entre cada SNP e tabagismo e frequência de haplótipos nos dois genes. Referente ao capítulo (3), constatou-se que os SNPs rs2521634 (próximo ao gene *NPY*) e rs3811046 (no gene *IL37*) foram validados na população brasileira estudada como associado à PC. Em conclusão, observou-se que a PC foi associada a níveis mais altos de LDL e triglicérides, e níveis mais baixos de HDL; enquanto que não foi associada aos SNPs estudados nos genes *LDLR* e *APOB;* mas houve validação na população brasileira estudada dos SNPs próximo ao gene *NPY* e no gene *IL37,* previamente identificados por GWAS, com associação de maior risco à PC severa e moderada.

Palavras chave: Periodontite crônica. Dislipidemias. Metanálise. Polimorfismo genético. Genótipo.

Oliveira RN. Investigation of genetic polymorphisms and lipid metabolism with chronic periodontal disease: meta-analysis and case-control study [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2018.

ABSTRACT

Several factors related to the host, such as environmental factors (smoking), systemic diseases (diabetes and dyslipidemia) and genetic inheritance have been studied regarding the influence on the onset and progression of periodontal disease. Although several studies have investigated the relationship between dyslipidemia and chronic periodontitis (CP), focusing on serum lipid levels, the results are still contradictory, indicating the necessity of a meta-analysis. Some genome-wide association studies (GWAS), bioinformatics studies and several case-control studies have shown genetic variants associated with susceptibility to CP, but the results of some of these polymorphisms are sparse and contradictory among different populations. Thus, it is important to conduct meta-analysis and case-control studies in order to validate or identify an association of polymorphisms with CP in a Brazilian population. The objective of this study was divided into three chapters: (1) to investigate whether patients with CP present different serum levels of lipid parameters (HDL, LDL, total cholesterol and triglycerides) compared to healthy individuals; (2) to assess whether single nucleotide polymorphisms (SNPs) in the *LDLR* (rs5925 and rs688) and *APOB* (rs676210 and rs693) genes contribute to CP susceptibility, since CP is associated with higher plasma LDL levels and the polymorphisms of these genes may increase plasma LDL concentration; (3) to validate, in the Brazilian population, the association of genetic polymorphisms previously associated with CP in other populations, through studies using sophisticated bioinformatics techniques and GWAS. A systematic review with meta-analysis and meta-regression was performed in chapter (1). Case-control studies with genotyping of SNPs in patients with and without CP were performed in chapters (2) and (3). These studies was conducted to investigate the association of different SNPs genotypes respecting to CP and periodontal parameters, as well as to evaluate sex-specific genetic association, and gene-environmental interaction. In the meta-analysis, 19 publications were included. It was found that participants with CP had significant higher LDL and triglycerides serum levels ($p = 0.003$ and $p < 0.0001$, respectively) and lower HDL levels ($p = 0.0005$) compared to healthy patients. As a result of chapter (2), no significant association was found between the periodontal clinical parameters and the frequencies of genotypes of any of the SNPs in the APOB and LDLR genes evaluated. Comparing patients with and without CP, there were no significant differences in any multivariate analysis, multiplicative and additive interactions between each SNP and smoking and haplotype frequency in the two genes. Regarding chapter

(3), the rs2521634 (near the NPY gene) and rs3811046 (in the IL37 gene) SNPs were validated in the Brazilian population studied in association with CP. In conclusion, CP was associated with higher levels of LDL and triglycerides and lower levels of HDL; while it was not associated with the SNPs studied in the LDLR and APOB genes; but there was validation in the studied Brazilian population of the SNPs near to the NPY gene and in the IL37 gene with a higher risk association to severe and moderate CP, previously identified by the GWAS.

Keywords: Chronic periodontitis. Dyslipidemias. Meta-analysis. Genetic polymorphism. Genotype.

SUMÁRIO

1 INTRODUÇÃO

O workshop internacional para a classificação de doenças e condições periodontais da Academia Americana de Periodontologia em 1999 definiu a periodontite crônica (PC) como uma "doença infecciosa que resulta na inflamação das estruturas de sustentação do dente, com progressiva destruição do ligamento periodontal, cemento e osso alveolar. É caracterizada por formação de bolsa periodontal e / ou recessão gengival. É reconhecida como a forma mais frequente de periodontite" [1,2]. As características da periodontite crônica listadas no referido workshop são: maior prevalência em adultos, mas pode ocorrer em crianças e adolescentes; a quantidade de destruição é consistente com a presença de fatores locais; o cálculo subgengival é um achado frequente, associado a um padrão microbiano variável; taxa de progressão lenta a moderada, podendo ter períodos de rápida progressão; pode ser associado a fatores predisponentes locais (por exemplo, fatores iatrogênicos); podem ser modificados e / ou associados a doenças sistêmicas (por exemplo, diabetes mellitus); podem ser modificados por fatores ambientais (como tabagismo e estresse emocional) [2-4].

Doenças complexas são causadas por uma combinação de fatores genéticos, ambientais e de estilo de vida, sendo a grande maioria das doenças enquadrada nesta categoria, incluindo doenças como diabetes mellitus (DM), dislipidemia e doença periodontal [5]. Embora herdemos genes associados a essas doenças, os fatores genéticos representam apenas parte do risco associado ao fenótipo das doenças complexas. Além disso, essas doenças são poligênicas, pois vários genes estão envolvidos na suscetibilidade e na severidade da doença, cada qual garantindo uma pequena contribuição. Uma predisposição genética significa que um indivíduo tem uma suscetibilidade genética ao desenvolvimento de uma determinada doença, mas isso não significa que tal pessoa desenvolverá obrigatoriamente a doença. Embora não possamos mudar nossos genes, podemos alterar nosso estilo de vida e alterar fatores ambientais para prevenir ou retardar o aparecimento de tal transtorno. De fato, a interação entre fatores genéticos e ambientais em doenças complexas continua a desafiar a compreensão dos pesquisadores [6].

Isso contrasta com as doenças mendelianas simples, nas quais a alteração de um só gene determina o fenótipo da doença [7-9]. Para revelar as influências genéticas e ambientais em uma dada doença, são realizados estudos investigando gêmeos monozigóticos (MZ - gerados a partir da divisão de um único zigoto) e dizigóticos (DZ - gerados a partir de zigotos independentes). Gêmeos MZ, além dos genes completamente compartilhados, quando criados juntos, têm também influências ambientais muito similares. Nos estudos envolvendo pares de gêmeos, é

avaliado se uma característica (inclusive uma doença) ocorre em ambos os gêmeos (concordância) ou em somente um deles (discordância). Quanto maior a frequência da mesma característica em ambos os membros dos pares MZ, em comparação aos DZ, maior a probabilidade de que a característica tenha um componente genético. Os resultados de estudos em famílias e em gêmeos indicam que há herança genética para suscetibilidade à PC [10,11]. Comparando-se gêmeos MZ e DZ, foi observada maior concordância da ocorrência da PC entre os gêmeos MZ criados juntos do que a encontrada nos gêmeos DZ, significando que o fator genético tem forte contribuição para o acometimento da doença [12]. Os autores estimaram que a PC possui aproximadamente 50% de hereditariedade, mesmo após ajustes para variáveis comportamentais, como o tabagismo [12].

Como mencionado anteriormente, não apenas fatores genéticos influenciam na doença periodontal. Diversos outros fatores relacionados ao hospedeiro, como fatores ambientais (tabagismo), estresse emocional e doenças sistêmicas (diabetes mellitus e dislipidemia) passaram a serem estudados para compreender como contribuem para o início e progressão da PC [13,14]. O termo Medicina Periodontal, como sugerido pela primeira vez por Offenbacher [15] (1996), pode ser visto como um termo amplo que define uma relação entre saúde/doença periodontal e saúde/doença sistêmica. Acredita-se em uma relação bidirecional, ou seja, a doença periodontal pode influenciar na saúde sistêmica de um indivíduo, bem como doenças sistêmicas podem influenciar a saúde ou doença periodontal de um indivíduo [15]. Dentre estas doenças, vem crescendo o número de estudos investigando a relação entre a doença periodontal e o diabetes mellitus e também a dislipidemia [16-20].

FATORES GENÉTICOS E DOENÇA PERIODONTAL

A PC pode se manifestar de diferentes formas clínicas na população e a influência genética pode explicar isso em parte[21]. Polimorfismos genéticos são formas variantes (alelos) de um lócus específico do cromossomo, que coexistem naturalmente na população humana. Polimorfismos genéticos são também entendidos como variações normais do genoma humano, no qual o alelo mais raro ocorre com uma frequência maior que 1% na população. 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 de base

única, conhecido como Polimorfismo de Nucleotídeo Único (*Single Nucleotide Polimorphism - SNP*), que consiste em uma variação da identidade de um nucleotídeo singular num sítio particular do genoma [22].

Os polimorfismos genéticos são muito úteis em estudos da área de Genética de Populações. As frequências dos genótipos e alelos podem variar entre grupos de pacientes doentes e saudáveis. Subsequentemente, quando um determinado alelo está associado com a doença, estudos enfocando a genética funcional podem ser realizados para investigar a possível influência do referido polimorfismo na expressão do gene e seu papel na etiologia e patogênese da doença [23,24]. É muito importante essa investigação da funcionalidade de um polimorfismo, pois tais variações alélicas podem estar situadas em regiões promotoras do gene e isso pode influenciar a transcrição do mesmo; ou seja, um SNP pode influenciar na produção de maior ou menor quantidade de proteína, provocando variações nas respostas imunológica e inflamatória individuais frente a uma agressão bacteriana [25]. Além disso, um SNP também pode estar localizado em exons, de forma que, dependendo do nucleotídeo variante e de sua posição, um determinado aminoácido pode ser alterado para outro, levando à mudança da sequência original da proteína, o que pode comprometer sua função [26].

Nota-se uma tendência mundial em identificar marcadores genéticos de suscetibilidade à PC, principalmente focando-se em genes relacionados ao sistema imunoinflamatório, particularmente das citocinas. Citocinas são proteínas secretadas por células do sistema imune, importantes na regulação imune e comprovadamente relacionadas ao processo de inflamação periodontal e sistêmica [27,28]. Estudos relatam que variações alélicas, como os polimorfismos em genes que codificam citocinas, podem afetar a suscetibilidade e a progressão da PC [8,28-31]. O primeiro estudo demonstrando a associação entre PC e um polimorfismo genético, no caso no gene *IL1*, foi de Kornman e colaboradores [32] em 1997. A partir de então, muitos estudos vêm sendo realizados com o objetivo de associar diversos polimorfismos em vários genes com a PC [33-37].

Existem milhões de SNPs distribuídos no genoma que têm sido investigados em associação a doenças, mas apenas uma pequena parcela destes foi estudado em relação à PC [38]. Os primeiros estudos investigando a suscetibilidade genética à PC na população brasileira são de 2002, utilizando a técnica da Reação em Cadeia da Polimerase (PCR) aliada à de Polimorfismo de Comprimento de Fragmento de Restrição (RFLP), ou seja, a PCR-RFLP [39]. A partir de então, várias outras pesquisas têm sido realizadas na população brasileira a fim de

identificar marcadores genéticos que possam estar associados com a suscetibilidade ou predisposição à PC [40-43].

Para otimizar a análise de vários SNPs, atualmente tem sido empregadas tecnologias de alto desempenho, como *microarrays*. A partir de 2009, surgiram as primeiras publicações utilizando-se um protocolo baseando em uma tecnologia de alto desempenho e rendimento de genotipagem através de PCR em tempo real, conhecido como Plataforma de Genotipagem TaqMan ™ OpenArray® da empresa Life Technologies [44].

A tecnologia OpenArray ™ utiliza um chip do tamanho de uma lâmina para microscopia com 3.072 orifícios. Cada placa contém 48 subarranjos (*subarray*), cada uma com 64 poços de 300 μm de diâmetro e 300 μm de profundidade. A superfície da placa tem propriedades hidrofóbicas, enquanto o interior dos poços (onde as sondas e primers já estão inseridos) são de natureza hidrofílica. Os reagentes são retidos nos orifícios através da tensão superficial. Estas propriedades físicas permitiram que um pequeno volume (33 nL) da amostra possa ser inserido com grande acurácia e precisão [45].

Uma placa OpenArray pode conter tantas amostras como oito placas tradicionais de 384 poços. Tal tecnologia atua por meio de um chip contendo sondas para detecção de alelos de diferentes SNPs, assim esse chip de genotipagem permite a análise simultânea de no mínimo 16 e máximo 256 SNPs. Uma das principais vantagens desse método é que o pesquisador escolhe os SNPs que deseja investigar, comunicando-se com a empresa que desenvolverá o chip de modo "customizado". O chip contém os ensaios em nano-cavidades, requerendo apenas a adição da amostra quantificada de DNA dos pacientes. A manipulação e distribuição das amostras no chip de OpenArray® é realizada por um pipetador automático, o que aumenta a precisão e diminui erros de pipetagem [45].

Apesar de aparentemente mais caro, na verdade, em comparação a técnicas de genotipagem convencionais (onde cada SNP é investigado de cada vez), o uso da plataforma OpenArray® é mais econômica quando pretende-se analisar diversos polimorfismos [46]. Em um estudo recente realizado em uma população brasileira, a plataforma de genotipagem OpenArray® provou ser altamente eficaz (taxa de precisão – *call rate* de 96,99%) para a genotipagem de SNPs suspeitos de estarem relacionados à perda auditiva [46]. O resultado de genotipagem com o OpenArray foi validado por meio de outras técnicas como sequenciamento direto, PCR multiplex e PCR-RFLP.

A limitação da abordagem de genes candidatos é que centenas de genes não são

estudados porque suas funções são desconhecidas. Portanto, estudos com estratégia livres de hipótese e abordando todo o genoma são novas estratégias que estão sendo utilizadas para diversas doenças [47]. Dentre estas estratégias, destacam-se *Genome-Wide Association Study* (GWAS) e análises envolvendo técnicas sofisticadas de bioinformática.

O número de publicações usando bases de dados genéticos e métodos de bioinformática para identificar genes candidatos a doenças vem se expandindo. Uma combinação de ferramentas computacionais vem sendo utilizada para a identificação de genes candidatos para a periodontite. Esses resultados teóricos fornecem novas pistas para os pesquisadores planejarem experimentos específicos para cada gene identificado. Há poucos estudos publicados na literatura utilizando as técnicas de bioinformática para identificar os genes candidatos mais promissores para PC. Apenas um estudo usou as múltiplas fontes de dados associado a diferentes métodos computacionais para identificar genes em todo o genoma humano que fossem candidatos ao desenvolvimento da periodontite [48]. Por meio desse método integrativo de ranqueamento de genes, alguns destacam-se: (i) a *interleucina 18* (*IL18*) que apresenta um papel importante na regulação da resposta imunoinflamatória, modulando atividade de macrófagos e neutrófilos [49], cuja proteína encontra-se em concentração aumentada na saliva de pacientes com PC [50]; e (ii) membro 9 da família de *Receptores Toll-like* (TLR), ou seja o *TLR9*, cuja expressão mostrou-se aumentada em pacientes com periodontite comparado a pacientes com gengivites e pacientes saudáveis [51].

GWAS são estudos de associação do tipo caso-controle envolvendo uma grande amostra populacional que, ao invés da abordagem de gene candidato, investigam simultaneamente milhares de polimorfismos genéticos para identificar variantes genéticas associadas à uma doença [52]. Assim, GWAS permitem uma análise em larga escala e imparcial do genoma, utilizando grande número de indivíduos [53]. Os GWAS são capazes de analisar aproximadamente 650.000 SNPs e são capazes de mostrar aproximadamente 22% da variância fenotípica observada na PC [54]. Uma série de GWAS relataram novos achados para doenças complexas e, curiosamente, a maioria desses genes identificados não foram previamente estudados como genes candidatos. Isso também foi observado em relação à PC, uma vez que diversos GWAS evidenciaram variantes genéticas associadas à susceptibilidade à PC. Pode-se destacar polimorfismos no gene *IL37* e próximo ao gene *NPY* [54-56].

O neuropeptídio Y (NPY) é um potente vasoconstritor que é co-localizado com noradrenalina no sistema nervoso simpático [57]. O NPY mostrou ter vários papéis, incluindo a

modulação da resposta imune e angiogênese, sugerindo um papel no metabolismo e reparo tecidual [58-61]. O NPY é o neuropeptídio mais abundante no osso e recentemente demonstrou ter um papel na manutenção do equilíbrio entre a formação e reabsorção óssea, processos que são relevantes na periodontite [62,63]. Além disso, o NPY está presente no fluido crevicular gengival (FCG), apresentando níveis significativamente maiores em sítios periodontalmente saudáveis em comparação aos sítios com periodontite [64]. Isso indica um possível papel do *NPY* no processo de saúde / doença periodontal.

A interleucina-37 (IL-37), também conhecida como IL-1F7, é o membro mais recentemente descrito da família de citocinas IL-1 [65]. É uma potente citocina anti-inflamatória, produzida por monócitos, células dendríticas, células plasmáticas e células epiteliais [66,67]. Em condições normais, a IL-37 atenua a resposta imune inata pela supressão de citocinas pró-inflamatórias (IL-1ß, TNF-α, IL-6, IL-17 e IFN-γ) [67]. Nas infecções bacterianas, a IL-37 é capaz de inibir o processo inflamatório [66]. Existe apenas um estudo que investigou o FCG, níveis salivares e plasmáticos de IL-37 em indivíduos com PC e em indivíduos sistemicamente e periodontalmente saudáveis [68]. A IL-37 foi detectada em FCG, saliva e plasma de todos os grupos, porém as quantidades totais de IL-37 foram semelhantes nos sítios de pacientes controle e com doença periodontal.

DISLIPIDEMIA E DOENÇA PERIODONTAL

Evidências crescentes mostram a relação da PC com doenças sistêmicas [69]. Estudos epidemiológicos comprovam que a PC confere maior risco à doença cardiovascular (DCV) [70]. A base patológica da DCV é a aterosclerose [71], que geralmente se desenvolve como resultado de um desequilíbrio no perfil lipídico, uma vez que a placa ateromatosa pode ser iniciada pela acúmulo localizado de lipídeos [72].

Anormalidades nos níveis de lipídeos séricos caracterizam a dislipidemia, que é uma disfunção metabólica caracterizada por mudanças qualitativas e quantitativas das lipoproteínas no sangue, além de transtornos no metabolismo de lipídeos [73]. Os lipídeos não são solúveis no plasma, em vez disso são transportados em partículas conhecidas como lipoproteínas [74]. Esta doença pode surgir e ser agravada devido a descompensação metabólica gerada pelo quadro de diabetes mellitus (DM) e / ou devido a uma dieta rica em gorduras [75].

Uma das principais características da dislipidemia é o aumento na concentração de colesterol total, lipoproteínas de baixa densidade (LDL) e triglicérides, além da redução de lipoproteínas de alta densidade (HDL) no sangue [75-77]. De acordo com *National Cholesterol Educational Program* (NCEP) *Adult Treatment III* (ATP III) [73], os seguintes pontos de corte foram usados para definir dislipidemia: (i) Hipercolesterolemia: nível de colesterol total ≥ 200 mg/dL; (ii) Hipertrigliceridemia: níveis de triglicérides ≥ 150 mg/dL; (iii) Baixos níveis de HDL: níveis de HDL < 40 mg/dL e (iv) Altos níveis de LDL: níveis de LDL ≥ 130 mg/dL.

As causas da dislipidemia podem ser genética, ambiental, ou ambos. Dentre as causas genéticas, há a hipercolesterolemia familiar (HF), apolipoproteína B100 defeituosa, hiperlipidemia combinada (familiar), entre outras. Dentre as causas secundárias (ambientais), incluem a dieta, uso de alguns medicamentos, além de doenças e desordens metabólicas, como obesidade e DM tipo 2 (DM2) [74].

A hipercolesterolemia, como já comentado, pode ser ocasionada por fatores genéticos. Entre as mutações e polimorfismos envolvidos no aumento sérico de colesterol, é possível destacar os que ocorrem no gene *Apolipoproteína B* (*APOB*) [78,79] e, principalmente, no gene do Receptor de LDL (*LDLR*) [80,81]. ApoB é uma Apolipoproteína primária da LDL, sendo a principal transportadora natural de colesterol e fosfolípideos, atuando como um suprimento constante de colesterol para tecidos periféricos e células [82]. ApoB contém múltiplas regiões associadas a lipídeos em sua estrutura que são necessários para ligar-se ao LDL-R [83,84]. O LDL-R desempenha um papel fundamental na regulação do metabolismo do colesterol, removendo o excesso de LDL do sangue [85]. Além disso, estudos sugerem que *LDLR* é expresso em células epiteliais do tecido gengival [86], e que a proteína ApoB pode ser encontrada no FCG, estando em concentração aumentada em sítios com PC [87]. Estes estudos mostram uma possível participação desses dois genes na patogênese da PC. Apesar de existirem estudos comprovando a relação de polimorfismos nos genes *LDLR* e *APOB* com dislipidemia, não existem estudos publicados sobre a associação destes polimorfismos com a PC.

A dislipidemia em associação com DM2 são as principais causas da morbidade e mortalidade devido ao elevado índice de doenças cardiovasculares severas decorrentes [88,89]. Quando a PC está também associada a estas duas patologias, o quadro pode ser ainda mais grave, pois a PC também tem sido indicada como um fator de risco independente para doenças cardiovasculares [90,91].

Desde 1999, a relação entre PC e níveis lipídicos plasmáticos tem sido investigada, considerando a hipótese de que a periodontite pode alterar os níveis séricos de lipídeos [16-20,92].

Atualmente, vários trabalhos investigaram a relação entre periodontite e parâmetros lipídicos [93]. Já foi observado que indivíduos com PC têm maiores níveis séricos de colesterol total, LDL e de triglicérides quando comparados com indivíduos periodontalmente saudáveis [94]. Estes resultados levaram pesquisadores a considerar a inter-relação entre PC e dislipidemia como um exemplo de uma doença sistêmica predispondo à infecção por via oral, e uma vez estabelecida a infecção oral, ela agrava a doença sistêmica (assim como ocorre com a DM). Assim, acredita-se que a dislipidemia e a PC estão envolvidas em um relacionamento bidirecional [95].

Estudos in vitro fornecem as bases biológicas para tal associação. Existem evidências científicas de que a PC, como uma doença infecciosa crônica, produz altos níveis de citocinas pró-inflamatórias; que, por sua vez, levam à alteração dos parâmetros lipídicos séricos, promovendo assim a hiperlipidemia [96,97]. Níveis elevados de citocinas pró-inflamatórias ocorrem como consequência da infecção por microrganismos periodontopatogênicos, cujos componentes de superfície (muitas vezes descritos como endotoxinas), particularmente lipopolisacarídeos (LPS), provocam uma reação do sistema imune [98,99]. Por outro lado, quando os níveis séricos de lipídeos são elevados além do limite do intervalo fisiológico aceitável, como consequência, as funções das células imunes são alteradas. Os lipídeos podem promover hiperatividade de leucócitos, além de alterar o perfil de expressão gênica dos macrófagos, aumentando a produção de citocinas pró-inflamatórias, como o fator de necrose tumoral alfa (TNF-alfa) e a interleucina 1 beta (IL-1β), aumentando-se a produção de espécies reativas de oxigênio em pacientes hiperlipidêmicos [98,100-104]. A liberação de citocinas pró-inflamatórias comprometem a resposta do tecido periodontal e afeta a cicatrização de feridas, aumentando assim a suscetibilidade e severidade da PC [102,105-107].

No entanto, existem relatos contraditórios na literatura quanto à existência de associação entre a PC e os níveis séricos de lipídeos. Em um estudo que investigou 8.028 indivíduos, verificou-se que os níveis séricos de lipídeos não estavam associados à infecção periodontal (definida como a presença de dentes com bolsas periodontais profundas) [108]. Alternativamente, foi indicado que a dislipidemia não está associada com a perda de nível clínico de inserção periodontal, mas que DM2 seja um fator mais importante para determinar a porcentagem de sítios com destruição periodontal [109]. Acredita-se que a DM seja um preditor mais forte para a PC do que a dislipidemia [76]. Contrapondo-se a isso, foi demonstrado que a maior produção de citocinas pró-inflamatórias por macrófagos ocorra em decorrência da hiperlipidemia, e não da hiperglicemia [100,110].

Tem sido cada vez mais comum encontrar pacientes com DM2 que também são afetados por dislipidemia e periodontite crônica, mas contraditoriamente, existem limitados estudos sobre a associação conjunta dessas doenças. Em vista disso, nosso grupo de pesquisa tem estudado nos últimos anos a expressão genética em pacientes com DM2 compensados e descompensados e em indivíduos normoglicêmicos, associadas a dislipidemia e periodontite crônica [111,112]. Foi possível obter evidências que a dislipidemia foi a principal doença associada ao aumento de *IL10* e *IFNA* e à diminuição da expressão dos genes *IFNG*, *IP10*, *IRF1*, *JAK1*, *STAT3;* porém sem ignorar a participação da periodontite crônica nessa expressão gênica, uma vez que os parâmetros periodontais se correlacionaram significativamente com os níveis de mRNA dos citados genes [111].

Além disso, resultados de Oliveira (2014) [113] ainda não publicados, mostraram que os parâmetros lipídicos se correlacionaram com os parâmetros periodontais (Tabela 1). Aumento nos níveis de colesterol total, triglicérides e LDL correlacionaram-se com aumento na porcentagem dos parâmetros clínicos periodontais (placa visível, sangramento marginal, sangramento à sondagem, profundidade de sondagem ≥ 6 mm, perda de inserção ≥ 5 mm e supuração). Estes resultados indicam que a maior gravidade da PC se correlaciona com pior controle lipídico, caracterizado pelo aumento nos níveis lipídicos (colesterol total, triglicérides e LDL).

Esses achados fornecem evidências de que o estado inflamatório se associe principalmente à dislipidemia, porém a regulação da resposta imune sistêmica é complexa, sendo correlacionada também à PC. Além disso, observamos que pacientes dislipidêmicos apresentaram maior gravidade da periodontite, afetando negativamente a saúde e a qualidade de vida.

Tabela 1 - Correlação ajustada entre parâmetros periodontais e lipídicos

	Colesterol total	HDL	LDL	Triglicérides
IPV (% sítios)	0.38	-0.02	0.26	0.32
ISM (% sítios)	0.32	-0.05	0.23	0.28
SS (% sítios)	0.31	0.00	0.24	0.19
PS ≤ 3mm (% sítios)	-0.21	0.10	-0.16	-0.23
PS 4-5mm (% sítios)	0.06	-0.11	0.02	0.14
PS ≥ 6mm (% sítios)	0.28	-0.06	0.22	0.25
NI ≤ 2mm (% sítios)	-0.18	0.04	-0.14	-0.15
NI 3-4mm (% sítios)	-0.09	0.04	-0.09	-0.09
NI ≥ 5mm (% sítios)	0.30	-0.07	0.25	0.24
Supuração (nº sítios)	0.20	-0.11	0.20	0.13

Os coeficientes de correlação de Spearman são apresentados (r; α = 5%) ajustados para idade e sexo. Correlações significativas (p <0,05) foram destacadas: cinza escuro significa correlação significativa negativa e cinza claro significa correlação significativa positiva. IPV: índice de placa visível; ISM: índice de sangramento marginal; SS: sangramento à sondagem; PS: profundidade de sondagem; NI: nível de inserção.
Fonte: Elaboração próprio com dados extraídos de Oliveira [113].

Embora muitos estudos tenham investigado a relação entre dislipidemia e PC, examinando os níveis séricos de lipídeos, os resultados ainda são contraditórios, indicando a demanda da realização de uma metanálise. Metanálise é "a análise estatística de uma grande coleção de resultados oriundos de diferentes estudos com a finalidade de integrar os achados" [114]. Os resultados de uma metanálise são mais precisos em determinar o efeito de um tratamento ou o risco de um fator para uma doença, ou outros resultados, do que qualquer estudo individual que contribua para a análise conjunta [115]. Portanto, a metanálise desempenha um papel central na medicina baseada em evidências, sendo considerada a melhor forma de evidência científica e, portanto, colocada no topo da hierarquia da evidência[115,116].

Considerando que alguns GWAS e diversos estudos caso-controle evidenciaram variantes genéticas associadas à suscetibilidade à periodontite, mas os resultados para alguns desses polimorfismos são escassos ou são contraditórios entre as diferentes populações, nota-se a importância de realizar metanálises e estudos caso-controle para buscar validar ou identificar a associação de polimorfismos com a PC na população brasileira.

2 PROPOSIÇÃO

Proposição geral

Investigar a associação do metabolismo lipídico e de polimorfismos genéticos na periodontite crônica por meio de metanálise e estudos caso-controle.

Tal proposição está dividida em três capítulos, tendo sido o primeiro já publicado, e os capítulos seguintes já formatados para futura publicação.

Capítulo 1: Serum lipid levels in patients with periodontal disease: A meta- analysis and meta-regression

O objetivo deste estudo de revisão sistemática e metanálise foi investigar se os pacientes expostos / afetados pela doença periodontal crônica apresentam diferentes níveis séricos de parâmetros lipídicos (HDL, LDL, colesterol total e triglicérides) em comparação com indivíduos sem doença periodontal.

Capítulo 2: Lack of association of Low-Density Lipoprotein Receptor and Apolipoprotein B genes polymorphisms with chronic periodontitis in the Brazilian Population

O objetivo deste estudo foi avaliar se os polimorfismos nos genes *LDLR* (rs5925 e rs688) e *APOB* (rs676210 e rs693) contribuem para a suscetibilidade à periodontite crônica (PC), uma vez que PC está associada a níveis plasmáticos de LDL mais elevados, e os polimorfismos nestes genes podem aumentar as concentrações plasmáticas de LDL.

Capítulo 3: Validation of the Neuropeptide Y and Interleukin 37 polymorphisms as associated with moderate or severe chronic periodontitis in a Brazilian population

O objetivo deste estudo foi validar na população brasileira a associação de polimorfismos genéticos previamente associados à periodontite crônica em outras populações, por meio de estudos que utilizaram técnicas sofisticadas de bioinformática e *genome-wide association studies* (GWAS).

3 CAPÍTULOS

3.1 Capítulo 1*

Serum lipid levels in patients with periodontal disease. A meta-analysis and meta-regression

Running title: Serum lipid and periodontal disease

Keywords: Periodontal Disease, Dyslipidemia, Cholesterol, High-Density Lipoprotein Cholesterol, Low Density Lipoprotein Cholesterol, Triglycerides.

Rafael Nepomuceno[1], Suzane C Pigossi[1], Livia S Finoti[2], Silvana R P Orrico[1], Joni A Cirelli[1], Silvana P Barros[3], Steven Offenbacher[3], Raquel M Scarel-Caminaga[4]

1. Department of Diagnosis and Surgery, São Paulo State University – UNESP, School of Dentistry at Araraquara, Araraquara, SP, Brazil
2. Department of Periodontics, University of Pennsylvania School of Dental Medicine, Philadelphia, PA, USA
3. Department of Periodontology, University of North Carolina at Chapel Hill School of Dentistry, Chapel Hill, NC, USA
4. Department of Morphology, São Paulo State University – UNESP, School of Dentistry at Araraquara, Araraquara, SP, Brazil

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ORIGINAL ARTICLE

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Serum lipid levels in patients with periodontal disease: A meta-analysis and meta-regression

Rafael Nepomuceno[1] | Suzane C. Pigossi[1] | Livia S. Finoti[2] | Silvana R. P. Orrico[1] | Joni A. Cirelli[1] | Silvana P. Barros[3] | Steven Offenbacher[3] | Raquel M. Scarel-Caminaga[4]

[1]Department of Diagnosis and Surgery, School of Dentistry at Araraquara, São Paulo State University – UNESP, Araraquara, SP, Brazil

[2]Department of Periodontics, University of Pennsylvania School of Dental Medicine, Philadelphia, PA, USA

[3]Department of Periodontology, University of North Carolina at Chapel Hill School of Dentistry, Chapel Hill, NC, USA

[4]Department of Morphology, School of Dentistry at Araraquara, São Paulo State University – UNESP, Araraquara, SP, Brazil

Correspondence
Raquel M. Scarel-Caminaga, Department of Morphology, School of Dentistry at Araraquara, UNESP – São Paulo State University, Araraquara, SP, Brazil.
Email: raquel@foar.unesp.br

Rafael Nepomuceno, Department of Diagnosis and Surgery, School of Dentistry at Araraquara, São Paulo State University – UNESP, Araraquara, SP, Brazil.
Email: rafaelnepomuceno@gmail.com

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Abstract

Aim: Several papers have considered the potential relationship between periodontitis and lipid parameters. The present systematic review, meta-analysis and meta-regression studies focused on investigating whether serum lipid parameter levels were elevated in patients with periodontal disease (PD; without altered systemic conditions) in comparison with periodontally healthy subjects.

Materials and Methods: Eligible studies were those with data about serum lipid parameter levels in non-smoking subjects with and without chronic periodontitis, who are generally healthy and not taking any medication for dyslipidaemia. Mean differences and 95% confidence intervals for total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol and high-density lipoprotein (HDL) cholesterol were obtained from all the selected studies.

Results: A total of 19 publications were included for meta-analysis. Participants with chronic periodontitis presented significantly higher serum levels of LDL and triglycerides (p = .003 and p < .0001, respectively). The total cholesterol was higher in the PD group, but without significant difference in comparison with healthy participants. Significantly (p = .0005) lower HDL serum levels were found in patients with chronic periodontitis than in healthy subjects.

Conclusions: Even considering the limitations of this meta-analysis, it is suggested that PD is significantly associated with reduction in HDL and elevation of LDL and triglyceride concentrations. This analysis supports the rationale that periodontal disease is associated with lipid metabolic control.

KEYWORDS
cholesterol, dyslipidaemia, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, periodontal disease, triglycerides

1 | INTRODUCTION

Periodontal disease (PD) is a common, chronic, multifactorial, infectious disease of the gingiva and supporting structures of the teeth (Loe, Theilade, & Jensen, 1965; Persson, 2006). Near 10% of the adult population and 30% of individuals over the age of 50 suffer from severe periodontitis (Brown, Oliver, & Loe, 1990; Gjermo, 1998). Increasing evidence linking periodontitis to systemic diseases has been shown (Kuo, Poison, & Kang, 2008). There is consistent and strong epidemiologic evidence that periodontitis imparts increased risk of future cardiovascular disease (CVD) (Tonetti & Van Dyke, 2013). The pathological basis of CVD is atherosclerosis (Kinane & Lowe, 2000), which usually develops as a result of an imbalance in the lipid profile, as atheromatous plaque can be initiated by the focal accumulation of lipids (Ross, 1999).

Abnormalities in the serum lipid levels characterize dyslipidaemia (DLP), which includes high levels of total cholesterol (TC),

triglycerides (TG) and low-density lipoprotein (LDL), as well as decreased levels of high-density lipoprotein (HDL) (Lee, Yi, & Bae, 2013). Since 1999, the relationship between periodontitis and plasma lipid levels (TC, TG, LDL and HDL) has been investigated, considering the hypothesis that periodontitis could alter serum lipid levels (Ebersole et al., 1999). Nowadays, several papers have investigated the relationship between periodontitis and lipid parameters (Bullon et al., 2009).

It was suggested that dyslipidaemia and PD are involved in a two-way relationship, in which dyslipidaemia increases the risk of PD, and periodontal inflammation negatively affects serum lipid control (Awartani & Atassi, 2010). The biological rationale for this association is in Appendix S7: Introduction. However, there are conflicting reports in the literature as to whether there is an association between PD and serum lipid levels. For example, Saxlin et al. (2008), who investigated a huge population, found that serum lipid levels were not associated with periodontal infection (defined as the presence of teeth with deepened periodontal pockets).

Although many studies have investigated the relationship between dyslipidaemia and periodontitis, examining serum lipid parameter levels, no meta-analysis has been carried out to date. We hypothesize that serum lipid levels in periodontitis patients, with no other systemic disease conditions, are significantly altered compared to periodontally healthy patients. Therefore, following PICO/PECO format (Moher, Liberati, Tetzlaff, & Altman, 2009), the purpose of this systematic review and meta-analysis study was to investigate whether patients exposed/affected by destructive periodontal disease present different serum lipid parameters levels (HDL, LDL, TG and TC) in comparison with subjects without periodontitis.

Clinical Relevance

Scientific rationale for the study: There is strong evidence that individuals with chronic periodontitis (CP) produce high levels of pro-inflammatory cytokines, which may lead to alteration of serum lipids. Therefore, it is important to assess the association between lipid profile and CP.

Principal findings: Our analyses showed that CP was significantly associated with reduction of HDL and elevation of LDL and triglycerides.

Practical implications: Given the emerging evidence of the association between CP and lipid metabolism, healthcare providers should be aware of the interplay between lipid level imbalance and exacerbated inflammatory responses. Future investigations can further elucidate the potential impact of periodontal therapy in the improvement of lipid levels.

2 | MATERIALS AND METHODS

A systematic review was conducted in accordance with the guidelines of the Transparent Reporting of Systematic Reviews and Meta-Analyses PRISMA statement and also of Meta-analysis of Observational Studies in Epidemiology (MOOSE) (Moher et al., 2009; Stroup et al., 2000).

2.1 | Focused question

The focused question was proposed by following PECO principles to summarize the differences in serum lipid parameters of patients with and without chronic periodontitis: "Do individuals with chronic periodontal disease with no other systemic disease condition have different serum lipid parameter levels (HDL, LDL, triglycerides and total cholesterol) when compared with healthy subjects?"

2.2 | Search strategy

Electronic searches of the PubMed, Web of Science, Scopus, EMBASE and Cochrane Controlled Trials Registry databases, complemented by a search through the reference lists of included studies, were conducted from 1968 until the end of February 2016. Relevant papers were identified through a search in databases following the search words:

- MESH terms in all trees/subheadings: "Periodontal Diseases" and "Lipids".
- Keywords for periodontal diseases: "Periodontal Disease" or "Periodontitis" or "Alveolar bone loss"; AND
- Keywords for lipids: "HDL" or "High Density Lipoprotein" or "LDL" or "Low Density Lipoprotein" or "Triglycerides" or "Total Cholesterol" or "Dyslipidemia" or "Hyperlipidemia" or "Hypertriglyceridemia" or "Hypercholesterolemia" or "Lipids".

The literature search in all electronic databases was exported to EndNote Program™ X7 version (Thomson Reuters, New York, NY, USA) in order to eliminate duplicate references.

2.3 | Study selection

We selected original research articles according to the following inclusion criteria: (i) longitudinal studies and cross-sectional studies (cohort and case–control studies); (ii) reporting data about serum lipid parameters (HDL, LDL, triglycerides and total cholesterol) of adults with and without chronic periodontitis; (iii) subjects with no systemic disorders, including no history of CVD and diabetes; (iv) non-smoking patients; (v) studies showing the use of statistical methods and the numerical values of mean and standard deviation, including the units for quantifying serum lipid levels; (vi) articles published in the English language.

Studies specifically designed to investigate pregnant women and subjects with atherosclerosis, rheumatoid arthritis, haemodialysis treatment and/or with systemic treatment for dyslipidaemia were not included in the review. Studies that included patients selected among

TABLE 1 Characteristics of studies and participants included in the meta-analysis

Author(s) Year	Country	Study design	Sample size	% Males	Males ratio (PD:C)	Mean age (year)	Age Ratio (PD:C)	Diagnostic Criteria for PD	Diagnostic thresholds (Secure or Insecure)	Blood collection (serum or plasma)	Blood test (fasting or non-fasting)	BMI control	Study Quality (NOS score)
Akalin et al. (2008)	Turkey	Case–control	34	50.00	1.12	46.74	1.12	At least 30% periodontal bone loss and at least three teeth with PPD ≥5 mm	Insecure	Plasma	Fasting	No	4
Andrukhov et al. (2013)	Austria	Case–control	143	54.55	1.29	39.22	1.23	Subjects with severe (loss of supporting bone ≥1/3 of the root length) and generalized (≥30% affected sites) periodontal disease with at least five sites with a PPD ≥5 mm	Secure	Serum	Fasting	No	4
Banu et al. (2015)	India	Case–control	60	38.33	1.18	NI	NI	Radiographic evidence of interproximal bone loss (≥50% alveolar bone loss in ≥2 quadrants of the dentition). Clinically ≥4 teeth should be involved in each jaw, ≥5 mm PPD, ≥4 mm CAL and 80% BOP of the proximal sites	Secure	Plasma	Fasting	No	5
Cutler et al. (1999)	United States	Case–control	13	23.08	0.43	36.12	1.52	At least four or more PPD ≥6 mm with BOP and radiographic evidence of >50% bone loss at those same teeth.	Secure	Serum	Non-fasting	No	5
Dhotre et al. (2011)	India	Case–control	200	60.00	1	51.50	1.05	Patients with CAL of ≥ 4 mm, PPD ≥ 4 mm and BOP	Insecure	Plasma	Fasting	No	4
Duzagac et al. (2016)	Turkey	Longitudinal	30	43.33	1.17	40.36	1.04	Patients with PPD ≥4 mm in ≥30% of periodontal sites; BOP in ≥50% of sites; interproximal CAL >2 mm in ≥20% of periodontal sites; radiographic evidence of bone loss	Secure	Serum	Fasting	Yes	8
Fentoglu et al. (2015)	Turkey	Case–control	74	48.64	1	42.55	0.97	At least four teeth with PPD ≥5 mm and CAL ≥2 mm at the same time	Insecure	Serum	Non-fasting	No	8
Golpasand Hagh et al. (2014)	Iran	Case–control	90	46.67	0.91	34.74	0.96	At least one site with PPD of 4 mm in every quadrant and showed bone destruction in their radiography	Insecure	Serum	Fasting	Yes	7
Joseph et al. (2011)	India	Case–control	176	35.80	0.86	33.73	1.02	At least two interproximal sites with ≥4 mm CAL, not on the same tooth or ≥2 interproximal sites with PPD ≥5 mm, not on the same tooth	Insecure	Plasma	Fasting	Yes	4

(Continues)

TABLE 1 (Continued)

Author(s) Year	Country	Study design	Sample size	% Males	Males ratio (PD:C)	Mean age (year)	Age Ratio (PD:C)	Diagnostic Criteria for PD	Diagnostic thresholds (Secure or Insecure)	Blood collection (serum or plasma)	Blood test (fasting or non-fasting)	BMI control	Study Quality (NOS score)
Joshipura et al. (2004)	United States	Case–control	468	100.00	1	57.58	1.05	Person who reported being professionally diagnosed with periodontal disease	Insecure	Plasma	Non-fasting	Yes	1
Kumar et al. (2014)	India	Case–control	50	NI	NI	NI	NI	Patients with radiographic evidence of marginal alveolar bone loss at ≥30% of sites affecting at least 50% of the dentition and CAL of ≥4 mm at a minimum of six sites	Secure	Serum	Fasting	No	5
Leite et al. (2014)	Brazil	Longitudinal	55	32.73	0.97	33.78	1.04	According to Armitage & Cullinan (2010)	Insecure	Serum	Fasting	No	6
Liu et al. (2010)	China	Case–control	80	52.50	1.21	49.30	1.07	A mean CAL ≥1.6 mm, sites with interproximal CAL ≥3 mm distributed through at least three quadrants or at least six teeth	Secure	Serum	Non-fasting	No	6
Lösche et al. (2000)	Germany	Case–control	79	41.77	1.39	55.10	0.99	More than three pockets with PPD ≥4 mm	Insecure	Plasma	Fasting	No	6
Monteiro et al. (2009)	Brazil	Case–control	80	42.50	0.89	45.00	1.01	At least 10 sites with PPD >5 mm	Insecure	Plasma	Fasting	Yes	7
Penumarthy et al. (2013)	India	Case–control	60	78.33	NI	33.92	1.46	Patients with PPD of ≥6 mm involving more than 30% of sites or CAL of 3 to 4 mm and radiographic evidence of bone loss	Insecure	Serum	Fasting	No	6
Pushparani et al. (2014)	India	Case–control	300	51.67	0.94	38.56	1.17	Patients with mean PPD of ≥5 mm and CAL of ≥3 mm in at least 40% of teeth	Insecure	Serum	Fasting	No	6
Sridhar et al. (2009)	India	Case–control	60	43.44	1.17	44.77	1.09	CAL of >1 mm and / or PPD of >4 mm in around 30% of the existing natural teeth	Insecure	Serum	Fasting	No	3
Taleghani et al. (2010)	Iran	Cohort	52	34.62	1	46,5	0.94	At least one site within 4 mm PPD in every quadrant and bone destruction	Insecure	Serum	Fasting	Yes	6

PD:C periodontal disease group: control group ratio; NOS score, Newcastle-Ottawa Scales score (minimum = 0; maximum = 9); PPD, probing pocket depth; CAL, clinical attachment level; BOP, bleeding on probing; NI, not informed.

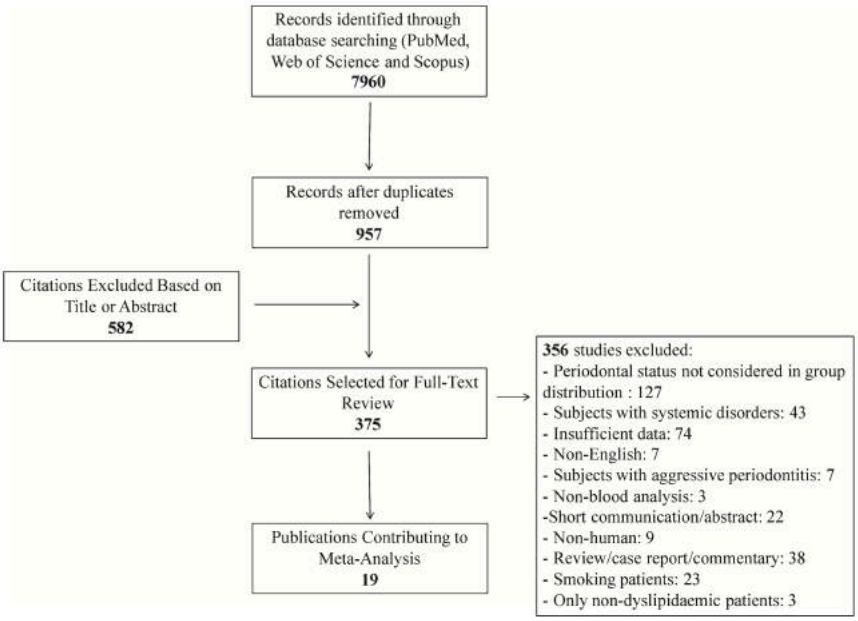


FIGURE 1 Flow chart of the search strategy of the study

subjects who did not have hyperlipidemia (related to outcome) were excluded, as that limited the lipid to low levels. Papers without abstracts with titles suggesting relation to the objectives of this review were also selected, for full-text screening. However, experimental studies (animal and cellular models), letters to the editor, historical reviews and unpublished articles were excluded. Original research articles that failed to follow all the criteria described above were also excluded from this meta-analysis.

Due to the lack of a general consensus for diagnosis of PD, and to the use of different PD definitions in the searched articles, we set diagnostic thresholds as part of an assessment of methodological quality and external validity. Selection criteria regarding this secure diagnosis of PD were based on a previous meta-analysis (Nibali et al., 2013) as follows:

Clinical Definitions of Periodontal Disease

a. Secure periodontitis:
   - At least two sites on different teeth with clinical attachment level (CAL) ≥6 mm and at least one site with probing pocket depth (PPD) ≥4 mm, or
   - At least two sites in non-adjacent teeth with proximal attachment loss ≥3 mm, or
   - Community periodontal index (CPI) score of 4 in at least one quadrant, or
   - In cases where no CAL or PPD is reported, radiographic alveolar bone loss ≥30% of root length or 5 mm in at least two teeth.

b. Insecure periodontitis:

Parameters for disease classification, that is probing depths, clinical attachment level and alveolar bone loss were not clearly defined or not so rigorously categorized.

Quality Assessment and Data Extraction—Detailed descriptions can be found in Appendix S7: Materials and Methods.

2.4 | Statistical analysis

The effect size was estimated and reported as the mean difference (MD). The 95% confidence interval (CI) was calculated for each lipid parameter, and the pooled effect was considered significant if two-sided p values <.05 were reached. Statistical software (Review Manager [RevMan], Version 5.1. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011) was used to pool the data and produces the forest plots. We performed subgroup analyses for studies with and without Secure PD diagnosis.

Meta-regressions were undertaken to explore the impact of risk of bias of included studies using a random-effects model. Univariate and multivariate regression analyses were performed. As demonstrated in Table 1, for the univariate analysis, we added the year in which the study was published, total sample size, quality of studies (considering Newcastle-Ottawa Scales score), periodontal diagnosis (secure or insecure), age (centred on mean of all patients), age ratio (centred on 1:1—PD Group: Control group ratio), gender (centred on per cent of men), gender ratio (centred on 1:1—PD Group: Control group ratio), studies matched for BMI between groups (yes or no), blood serum or plasma collection, fasting or non-fasting blood test as covariates. If the regression coefficient of the covariates was significant at the level of .1, then the covariates were entered into the multivariate meta-regression. We used 95% CI and considered a p-value below .05 to be statistically significant in multivariate meta-regression.

If any of the parameters had influenced the outcome of the meta-analysis, a new statistical analysis was performed to exclude bias. Articles

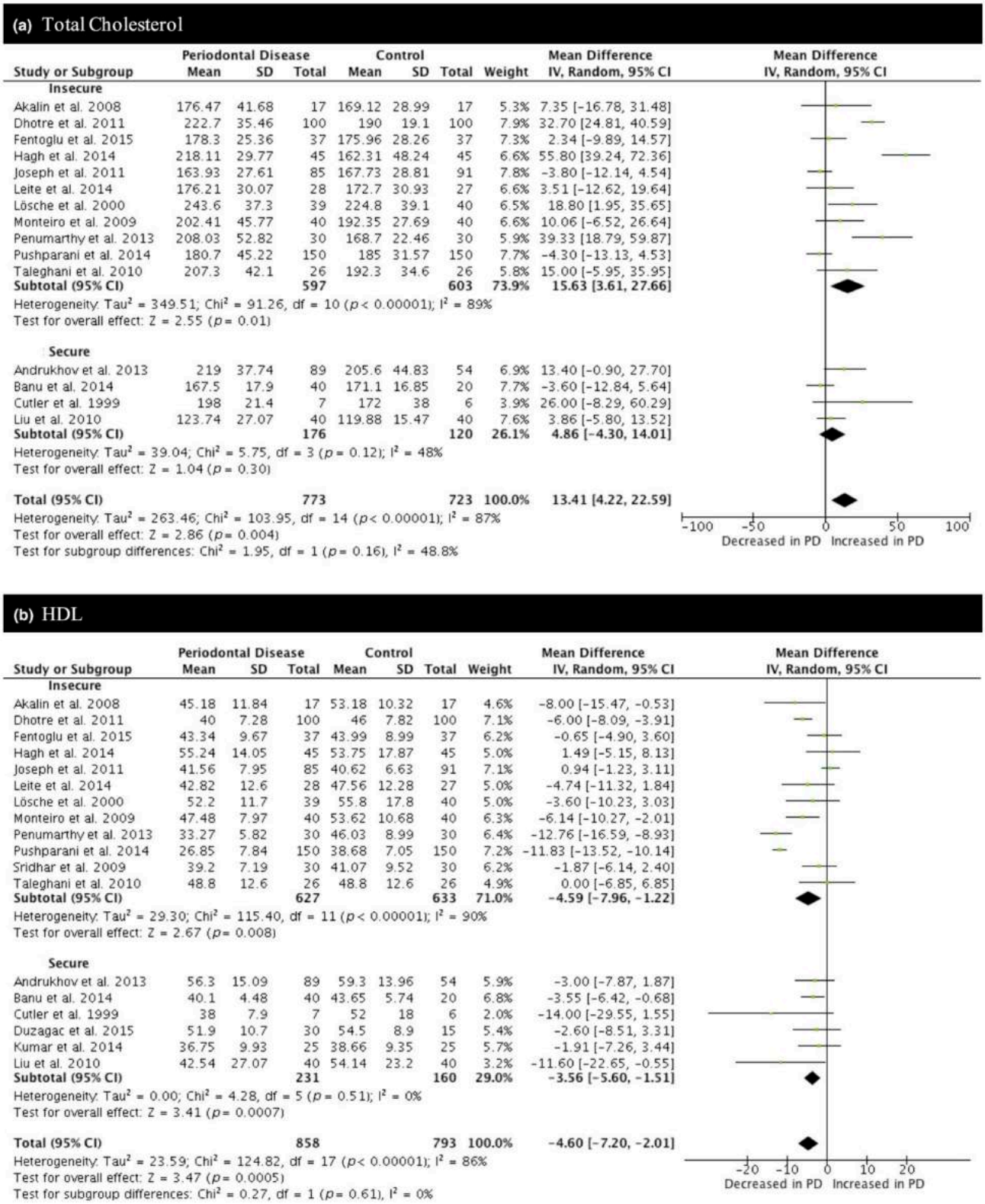
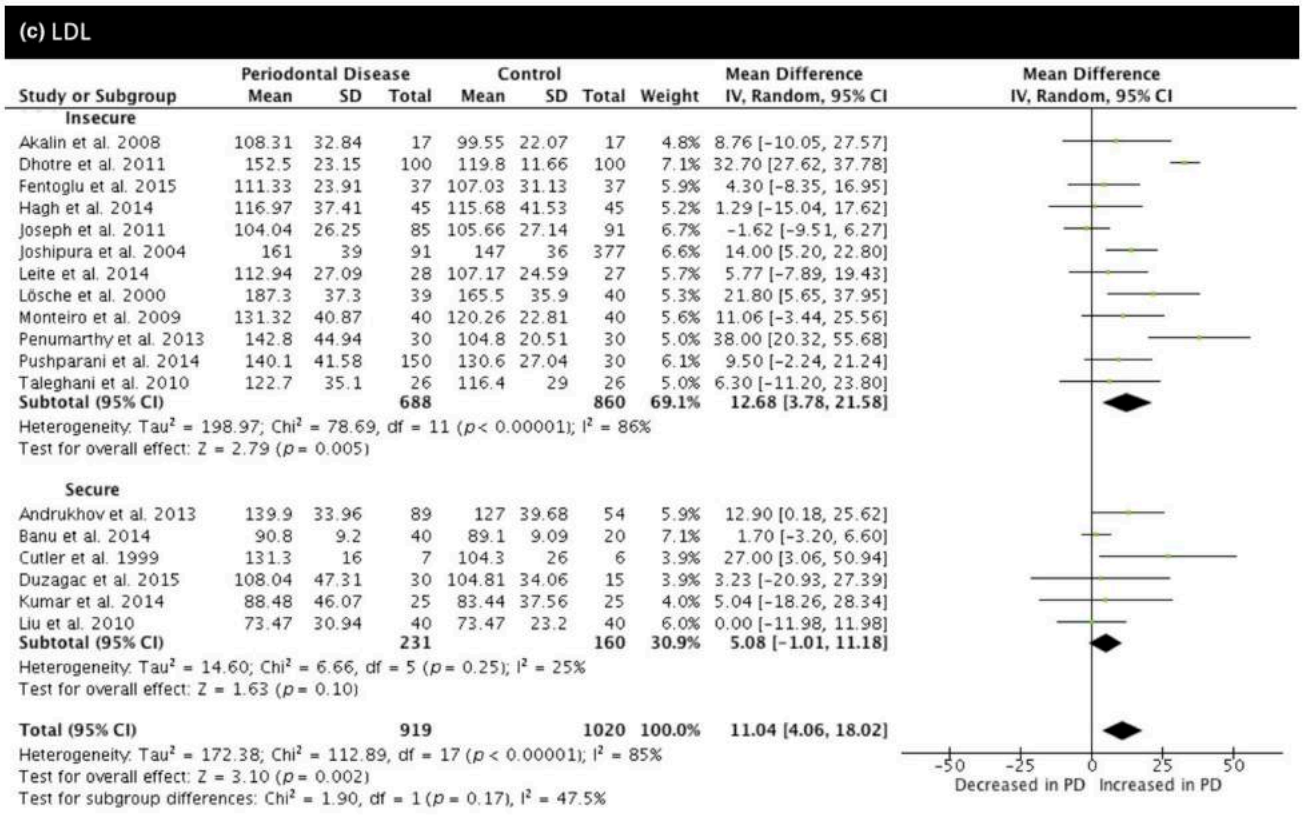


FIGURE 2 Forest plot of mean difference (MD) for comparison: periodontitis *versus* non-periodontitis in studies with Insecure and Secure diagnoses for PD before meta-regression. Outcomes: (a) Total Cholesterol; (b) HDL; (c) LDL; (d) Triglycerides



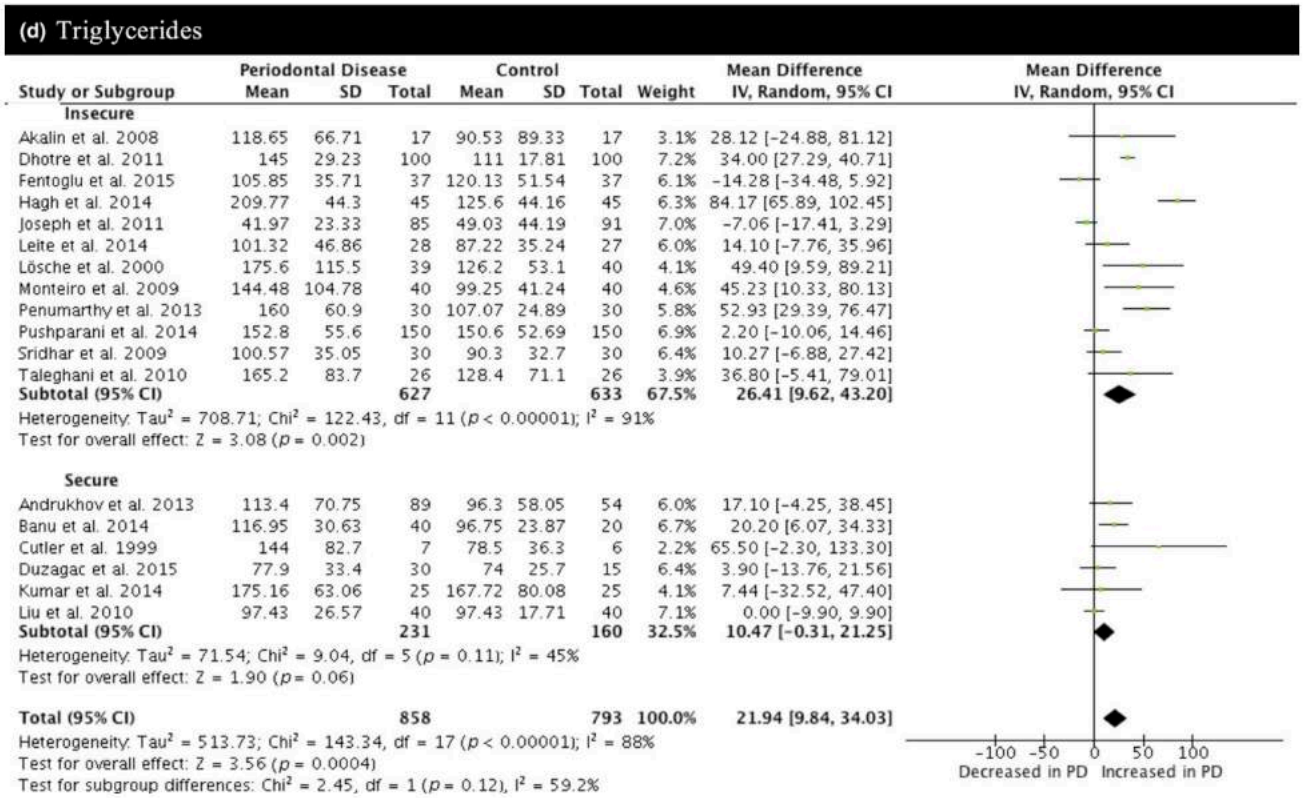


FIGURE 2 (Continued)

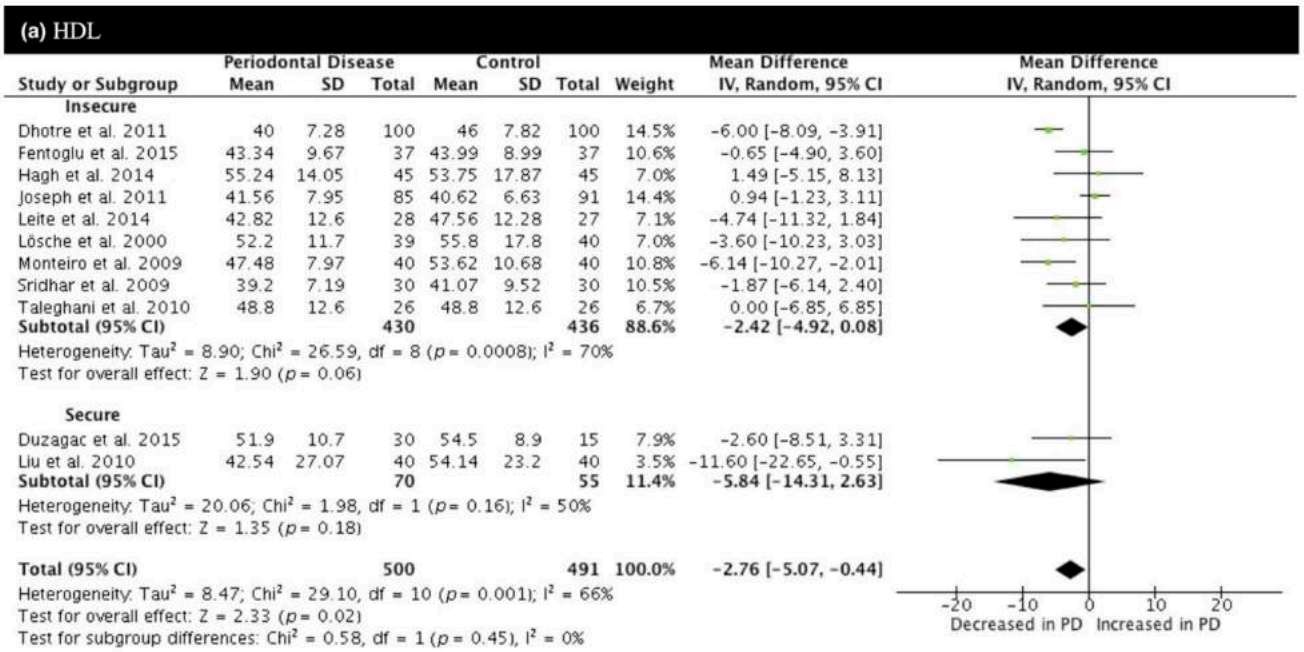


FIGURE 3 Forest plot of mean difference (MD) for comparison: periodontitis *versus* non-periodontitis in studies with Insecure and Secure diagnoses for PD after meta-regression. Outcomes: (a) HDL; (b) LDL; (c) Triglycerides

that introduced significantly increased risk of bias were excluded. All meta-regression analyses were carried out using OpenMeta[Analyst] statistical software (Wallace, Schmid, Lau, & Trikalinos, 2009).

To test the influence of each study on the overall effect size, sensitivity analysis was conducted using Leave-One-Out method after meta-regression; that is removing one study each time and repeating the analysis (Patsopoulos, Evangelou, & Ioannidis, 2008). Other details regarding Statistical Analysis are shown in Appendix S7: Materials and Methods.

Descriptive subgroup analysis according to the ethnicity of the subjects was detailed in the Appendix S7: Statistical Analysis.

3 | RESULTS

3.1 | Literature search and study characteristics

The electronic search generated 7,960 hits, which represented 957 unique citations. A total of 375 publications were obtained as full-text

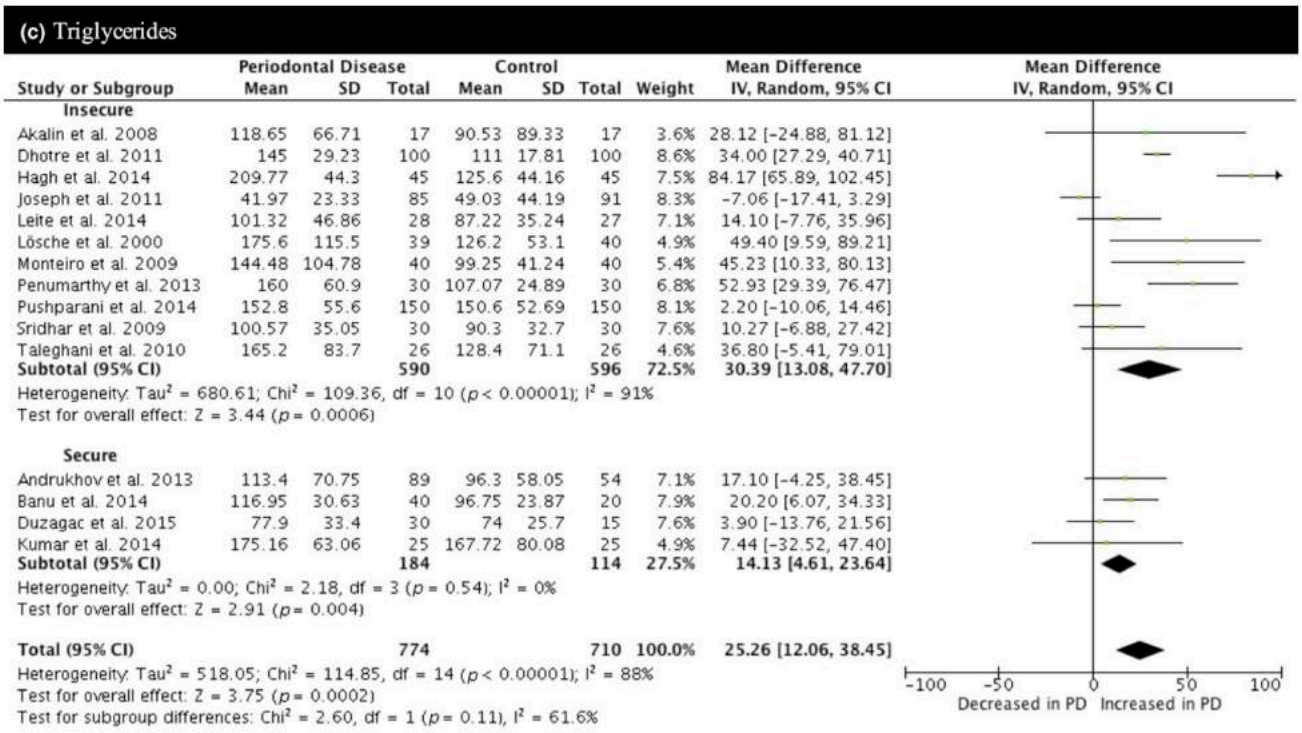


FIGURE 3 (Continued)

copies, and 355 of these publications were later excluded on the basis of a priori criteria. In total, 19 publications conducted in eight different countries on four different continents (North America, South America, Europe and Asia) were included for meta-analysis (Akalin, Işiksal, Baltacioğlu, Renda, & Karabulut, 2008; Andrukhov et al., 2013; Banu et al., 2015; Cutler, Machen, Jotwani, & Iacopino, 1999; Dhotre, Suryakar, & Bhogade, 2011; Duzagac et al., 2016; Fentoglu et al., 2015; Golpasand Hagh, Zakavi, Hajizadeh, & Saleki, 2014; Joseph, Nath, & Joseraj, 2011; Joshipura, Wand, Merchant, & Rimm, 2004; Kumar, Ranganath, Naik, Banu, & Nichani, 2014; Leite, de Araújo Carneiro, & do Carmo Machado Guimarães, 2014; Liu et al., 2010; Lösche, Karapetow, Pohl, Pohl, & Kocher, 2000; Monteiro et al., 2009; Penumarthy, Penmetsa, & Mannem, 2013; Pushparani, Anandan, & Theagarayan, 2014; Sridhar, Byakod, Pudakalkatti, & Patil, 2009; Taleghani, Shamaei, & Shamaei, 2010). The PRISMA flow chart of the complete process of study selection was illustrated in Figure 1, including the reasons for exclusion after full-text screening of the excluded papers. More information about literature search, study characteristics and quality assessment can be found in the Appendix S7: Results.

3.2 | Comparison of blood lipid parameters levels between participants with and without PD

All the outcomes showed χ^2 test = $p < .10$, confirming heterogeneity amongst the included trials and leading to the utilization of a random-effects model analysis (Figures 2a–d and 3a–c). Fixed-effects model analysis was used only for all parameters after Leave-One-Out (Figure 4a–d).

3.2.1 | Total cholesterol

Seventeen studies involving 1,496 participants provided comparative data regarding serum levels of total cholesterol. Considering all studies combined (studies with secure together with insecure diagnosis of PD) we found that participants with chronic periodontitis presented significantly higher serum levels of total cholesterol than healthy participants (Mean Difference, MD = 13.41; $Z = 2.86$; $p = .004$; Figure 2a). The heterogeneity in the serum levels of total cholesterol amongst studies was significant ($\chi^2 = 103.95$; $p < .00001$; $I^2 = 87\%$). The four studies with secure PD diagnosis involved 296 participants and presented increased total cholesterol in the PD group, without significant difference in comparison with healthy participants (MD = 4.86; $Z = 1.04$; $p = .30$). The heterogeneity of serum total cholesterol amongst these studies was relatively lower and insignificant ($\chi^2 = 5.75$; $p = .12$; $I^2 = 48\%$).

In Table 2, we show by meta-regression that for total cholesterol any of the investigated covariates had a significant impact on the high heterogeneity found amongst the studies included in the meta-analysis.

Sensitivity analysis was performed to assess the robustness of the results from the meta-analysis, through the Leave-One-Out analyses that assessed the key studies that had substantial impact on between-study heterogeneity. Three studies (Dhotre et al., 2011; Golpasand Hagh et al., 2014; Penumarthy et al., 2013) were found to contribute to between-study heterogeneity. The heterogeneity decreased from 87% to 32% ($\chi^2 = 16.13$; $p = .14$) with MD of 2.02 ($Z = 1.10$; $p = .27$) in total cholesterol meta-analysis with 12 studies (Figure 4a).

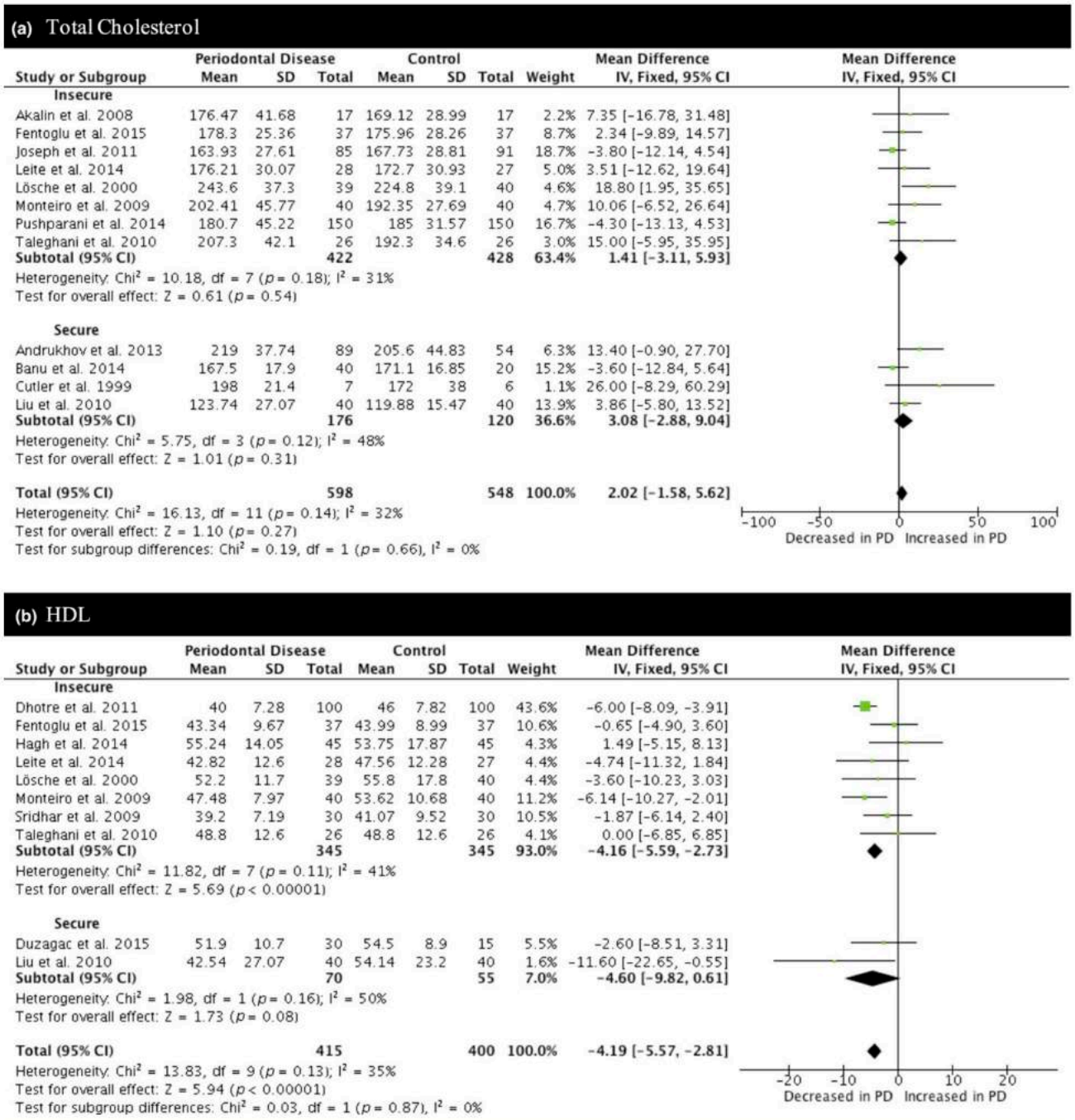


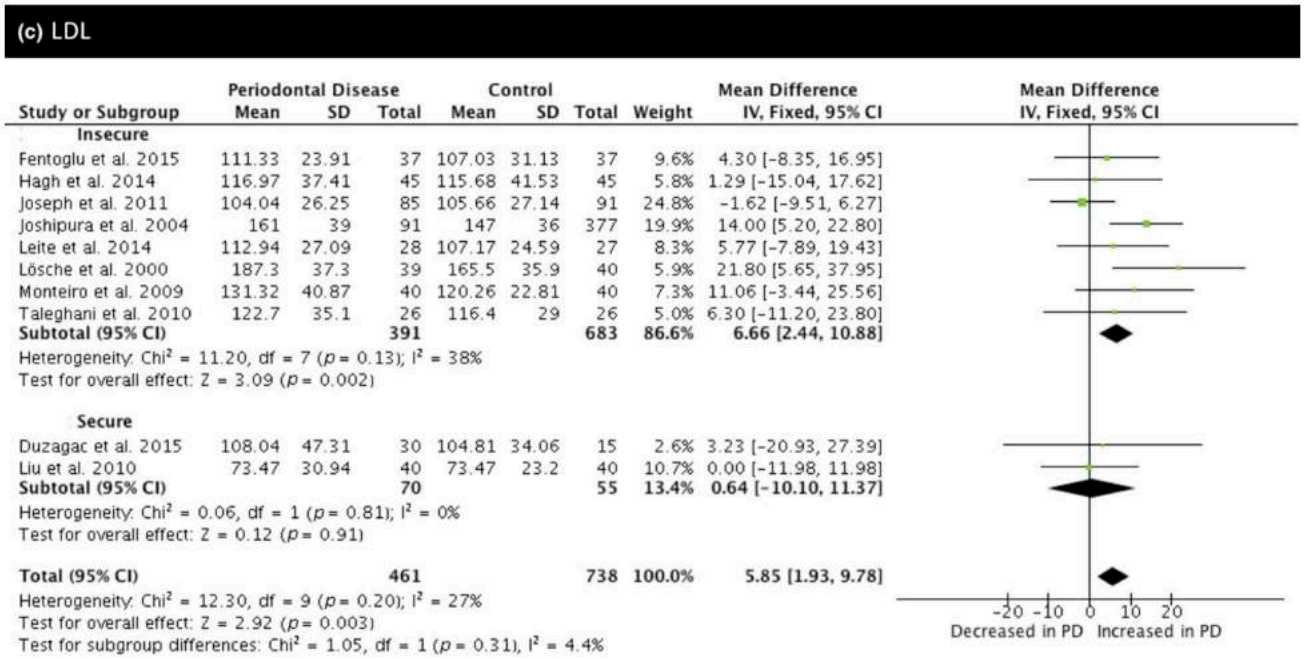
FIGURE 4 Forest plot of mean difference (MD) for comparison: periodontitis *versus* non-periodontitis in studies with Insecure and Secure diagnoses for PD after Leave-One-Out analysis. Outcomes: (a) Total Cholesterol; (b) HDL; (c) LDL; (d) Triglycerides

3.2.2 | HDL

Eighteen studies involving 1,651 participants showed, considering all studies combined, significantly lower HDL serum levels in patients with chronic periodontitis than in healthy subjects (MD = −4.60; $Z = 3.47$; $p = .0005$; Figure 2b). The heterogeneity amongst studies that investigated HDL serum levels was significant ($\chi^2 = 124.82$; $p < .00001$; $I^2 = 86\%$).

Reduced levels of HDL in periodontal disease participants when compared with the levels in periodontally healthy ones were confirmed by the analysis with the six studies with secure PD diagnosis (MD = −3.56; $Z = 3.41$; $p = .0007$), in which the heterogeneity amongst these studies was statistically insignificant ($\chi^2 = 4.28$; $p = .51$; $I^2 = 0\%$).

Meta-regression analysis was performed to assess the association of different covariates with HDL. In these analyses, age ratio (centred on PD Group: Control group ratio) ($\beta = -23.904$; $SE = 6.592$; $p < .001$), per cent of males in study ($\beta = -0.222$; $SE = 0.077$; $p = .004$) and BMI-matched studies ($\beta = 4.577$; $SE = 2.208$; $p = .038$)



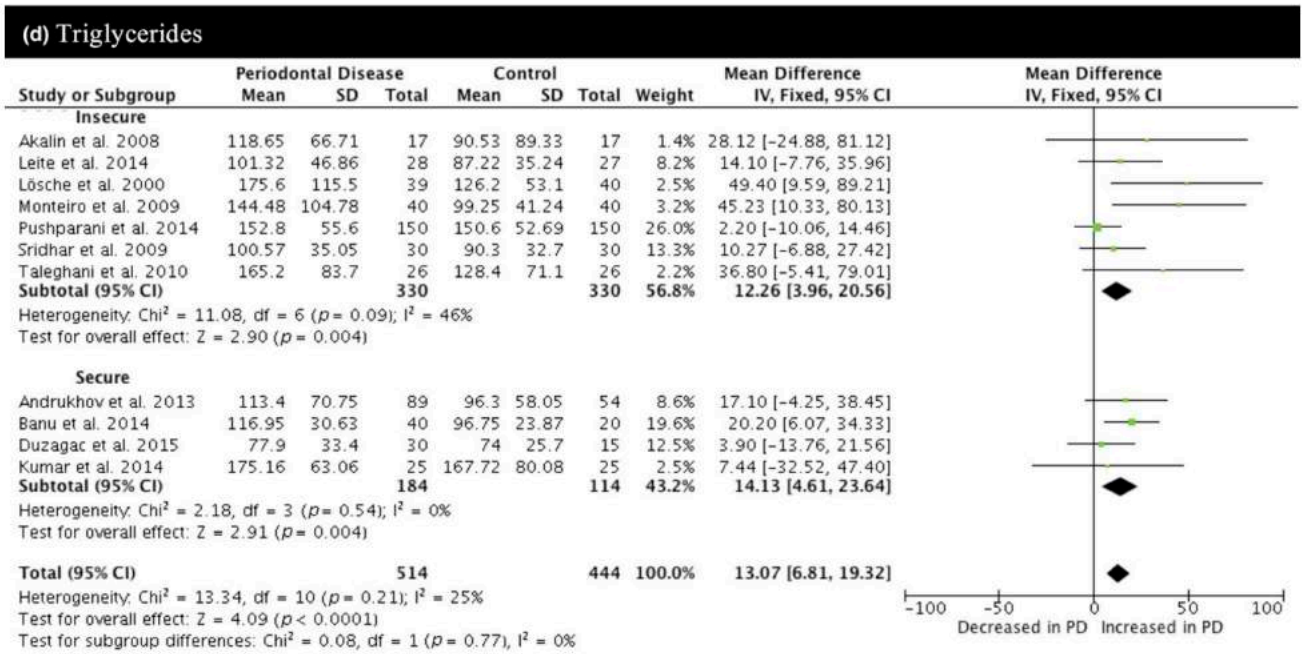


FIGURE 4 (Continued)

demonstrated significant correlation with HDL in the univariate meta-regression. However, only age ratio (β = −19.635; SE = 7.067; p = .005) was identified as potential source of heterogeneity in the multivariate meta-regression (Table 2). These results seemed to indicate that in the PD group, the presence of older patients (in comparison with the control group), the high percentage of men and the inclusion of studies without matching for BMI, influenced the reduction of HDL levels.

To reduce the effect of bias in the analysis, studies without 1.1–0.9 PD Group: Control group Age Ratio were excluded (once age was the only variable confirmed to significantly introduce bias by multivariate meta-regression). Then, a new meta-analysis was conducted. Seven studies with influence on the overall effect size were excluded. Low levels of HDL in periodontal disease participants were confirmed again by the analysis with the eleven studies (MD = −2.76; Z = 2.33; p = .02; Figure 3a). However, the heterogeneity amongst these studies was significant (χ^2 = 29.10; p = .001; I^2 = 66%).

In sensitivity analysis, through the Leave-One-Out analyses, one study (Joseph et al., 2011) was found to contribute to between-study heterogeneity. The heterogeneity decreased from 66% (Figure 3a) to

TABLE 2 Meta-regression analysis of covariates as potential sources of heterogeneity

Dependent variable	Total cholesterol			HDL			LDL			Triglycerides		
Independent variable	**β**	**SE**	***p* value**	**β**	**SE**	***p* value**	**β**	**SE**	***p* value**	**β**	**SE**	***p* value**
Univariate regression												
Year of publication	−0.573	1.019	.574	0.128	0.292	.661	−0.931	0.607	.125	−2.127	1.627	.191
Total sample size (*n*)	−0.040	0.059	.498	−0.015	0.013	.263	0.016	0.025	.532	−0.072	0.084	.395
Quality of studies (NOS score)	1.352	3.637	.710	−0.147	0.765	.848	−1.093	1.589	.492	1.441	4.358	.741
Periodontal diagnosis (secure or insecure)	−8.209	9.869	.406	0.432	2.437	.859	−5.964	5.958	.317	−12.363	13.050	.343
Age (mean of all patients)	−0.044	0.662	.947	0.001	0.188	.996	0.385	0.364	.290	−0.140	1.069	.896
Age (PD Group: control group ratio)	19.034	29.906	.524	**−23.904**	**6.592**	**<.001***	**43.553**	**19.698**	**.027***	35.128	48.208	.466
Gender (% male)	0.557	0.345	.107	**−0.222**	**0.077**	**.004***	**0.252**	**0.148**	**.088***	0.335	0.557	.547
Gender (PD group: control group ratio)	−11.809	22.527	.600	2.513	6.599	.703	−4.878	15.080	.746	−30.664	36.734	.404
BMI-matched studies (yes or no)	6.653	9.964	.504	**4.577**	**2.208**	**.038***	−6.316	5.974	.287	12.193	13.661	.372
Blood collection (serum or plasma)	5.292	9.001	.557	−0.895	2.222	.687	−2.889	5.613	.607	−4.945	13.307	.710
Blood test (fasting or non-fasting)	7.033	11.252	.532	0.830	3.575	.816	2.013	6.697	.764	**29.031**	**12.611**	**.021***
Multivariate regression												
Age (PD Group: Control group ratio)	–	–	–	**19.635**	**7.067**	**.005***	**40.036**	**19.257**	**.038***	–	–	–
Gender (% male)	–	–	–	0.088	0.105	.399	0.187	0.146	.200	–	–	–
BMI-matched studies (yes or no)	–	–	–	2.275	1.837	.216	–	–	–	–	–	–
Blood test (fasting or non-fasting)	–	–	–		–	–	–	–	–	**29.031**	**12.611**	**.021***

β, coefficient; *SE*, standard error; PD group, Periodontal disease group; NOS score, Newcastle-Ottawa Scales score.
*Values in bold indicate statistical significance.

35% (Figure 4b, χ^2 = 13.83; p = .13) with MD of −4.19 (Z = 5.94 and p < .00001).

3.2.3 | LDL

LDL serum levels were reported in 18 studies involving 1,939 participants, and combining all studies, significantly higher LDL levels were found in participants with chronic periodontitis than in healthy ones (MD = 11.04; Z = 3.10; p = .002; Figure 2c). The heterogeneity amongst studies that investigated LDL serum levels was significant (χ^2 = 112.89; p < .00001; I^2 = 85%).

In the six studies with secure PD diagnosis, we observed in the forest plot that LDL was increased in the periodontal disease group, but without significant difference (MD = 5.08; Z = 1.63; p = .10). The heterogeneity amongst the studies was statistically insignificant (χ^2 = 6.66; p = .25; I^2 = 25%).

Univariate meta-regression analysis was performed to assess correlation of different variables with LDL. In these analyses, age ratio (centred on PD Group: Control group ratio; β = 43.553; SE = 19.698; p = .027) and per cent of males in study (β = 0.252; SE = 0.148; p = .088) were found significant (Table 2). However, only age ratio (β = 40.036; SE = 19.257; p = .038) was identified as potential source of heterogeneity in the multivariate meta-regression (Table 2).

To reduce the effect of bias in the analysis, studies not in the range of 1.1–0.9 PD Group:Control group age ratio were excluded. Then, a new meta-analysis was conducted, and seven studies showing influence on the overall effect size were excluded. High levels of LDL in periodontal disease subjects were not confirmed again by the analysis with the 11 remaining studies (MD = 9.40; Z = 1.91; p = .06; Figure 3b). However, the heterogeneity amongst these studies was significant (χ^2 = 79.46; p < .00001; I^2 = 87%).

In the Leave-One-Out analyses, one study (Dhotre et al., 2011) was found to contribute to between-study heterogeneity. The heterogeneity decreased from 87% to 27% (Figure 4c, χ^2 = 12.30; p = .20) with MD of 5.85 (Z = 2.92; p = .003) demonstrating that there was a significant association between high levels of LDL and the presence of PD.

3.2.4 | Triglycerides

Serum levels of triglycerides were reported in 18 studies involving 1,651 participants. Patients with chronic periodontitis were found to have significantly higher serum levels of triglycerides than healthy patients (MD = 21.94; Z = 3.56; p = .0004; Figure 2d) in all studies combined. The heterogeneity among the studies was significant (χ^2 = 143.34; p < .00001; I^2 = 88%).

The analysis of the six studies with secure PD diagnosis showed higher, although insignificant, serum triglycerides in diseased participants (MD = 10.47; Z = 1.90; p = .06).

Univariate meta-regression analysis was performed to assess correlation of different covariates with triglycerides. In these analyses, only blood test (fasting or non-fasting) demonstrated significant correlation with triglycerides (β = 29.031; SE = 12.611; p = .021) (Table 2). These results seemed to indicate that studies, which assessed triglycerides levels by non-fasting blood, could have results influenced by this covariate.

To reduce the effect of bias in the analysis, the three studies with non-fasting blood test were excluded. Considering 15 studies with fasting blood test, high levels of triglycerides in subjects presenting with PD were confirmed (MD = 25.26; Z = 3.75; p = .0002; Figure 3c). However, the heterogeneity amongst these remained high (χ^2 = 114.85; p < .00001; I^2 = 88%). Retracing the meta-analysis, none of the tested covariates had a significant impact on the high heterogeneity, meaning that another factor produced residual heterogeneity.

According to the results of sensitivity analysis after meta-regression, four studies (Dhotre et al., 2011; Golpasand Hagh et al., 2014; Joseph et al., 2011; Penumarthy et al., 2013) were found to contribute to between-study heterogeneity. The heterogeneity decreased from 88% to 25% (χ^2 = 13.34; p = .21) with MD of 13.07 (Z = 4.09; p < .0001) in triglycerides meta-analysis with 11 studies after excluding those four (Figure 4d). It was confirmed that there was a significant association of the higher levels of triglycerides in patients with PD.

Descriptive subgroup analyses according to the ethnicity of the subjects were detailed in the Appendices 5, 6 and S7: Results.

4 | DISCUSSION

In this systematic review, we summarized the current literature on the association between periodontal disease and serum lipid levels. A number of biologically plausible mechanisms have been suggested by the literature for results found in relationship to the four lipid parameters analysed. These mechanisms were detailed in the Appendix S7: Discussion. The main hypothesis of this meta-analysis study was partially accepted because after the meta-regressions and Leave-One-Out analysis LDL and triglycerides proved to be significantly higher (Figure 4c,d) and HDL significantly lower (Figure 4b) in patients with PD. This systematic review and meta-analysis provided evidence that PD may be associated with an imbalance in serum lipid parameter levels, and, consequently, with dyslipidaemia.

Naturally, the quality of a meta-analysis depends on the quality of the included studies, particularly on the study design and the risk of bias. In the present study, the total number of patients included was relatively small (n = 2,104). This could be a limitation of the study; however due to the stringent method applied in our analysis, the results represent solely the effect of periodontal disease on lipid parameters, excluding confounders such as diabetes and smoking, which could interfere with the analysis. Therefore, due to the stringent inclusion/exclusion criteria adopted no large epidemiologic studies nor separate analysis for male and female subjects could be included. The rather restrictive exclusion criteria for the PICO question included major covariates and traditional confounders (e.g., diabetes) to enable a more focused measure of the effects of periodontal disease on blood lipid values, albeit simultaneously limiting the generalizability of these findings.

Between-study heterogeneity is common in meta-analyses (Munafo & Flint, 2004), and exploring its sources is essential. In this present

meta-analysis study, comparison among all the included studies showed a significant heterogeneity, being a limitation of the study. This heterogeneity could be mainly associated with the non-standard criteria for periodontal disease definition (Table 1; Savage, Eaton, Moles, & Needleman, 2009). Heterogeneity in periodontal diagnostic criteria emerged clearly from this review. In particular, a whole variety of diagnoses were used to define "periodontitis." Some authors used well-defined criteria, including those suggested by Page and Eke (Page & Eke, 2007) or Russell (Russell, 1956). However, certain studies used self-defined criteria based on thresholds of clinical data (e.g., average PPD, percentage of sites with a certain CAL threshold) or even self-reported information. This further testifies for the difficulties in having a universally accepted clinical case definition for periodontitis (Nibali et al., 2013).

Based on that possibility and trying to minimize this heterogeneity, studies with more secure diagnosis of periodontitis were analysed as a subgroup in an attempt to obtain a more homogeneous sample (Figure 2a–d). Multivariable meta-regression with several variables was performed due to the lack of homogeneous adjustments in the analyses and to explore the potentially important covariates, which might play a role in this significant between-study heterogeneity. Age (PD Group: Control group ratio) and Gender (% male) were two important factors that contributed to heterogeneity in meta-analysis of HDL and LDL outcomes. In some studies, patients with periodontal disease were older than control patients. In addition, some studies showed higher percentage of men than women. This heterogeneity occurred because male gender and increasing age are factors associated with worsening of lipid profiles, and increased risk of cardiovascular diseases (Anagnostis, Stevenson, Crook, Johnston, & Godsland, 2015; Goh, Tong, Mok, & Said, 2007; Romero-Moraleda et al., 2015). Blood test (fasting or non-fasting) covariate had significant impact on the between-study heterogeneity in triglycerides outcome. Postprandial levels of triglycerides remain elevated for several hours, when compared to fasting results, which may explain this heterogeneity (Campos, Khoo, & Sacks, 2005; Nigam, 2011). It is important to remember that a new meta-analysis was performed excluding the studies that, according to the meta-regression and the presence of covariates, were causing high heterogeneity. These new results after the Leave-One-Out analysis were no longer heterogeneous and the results maintained statistical significance, confirming the worsening of the lipid profile in patients with periodontal disease.

This study has strengths and limitations. Although we were able to perform a homogeneous meta-analysis after meta-regression and Leave-One-Out analysis, the major limitation of our meta-analysis is that most studies did not provide enough clinical information. Consequently, we could not perform a meta-regression to explore the source of heterogeneity for some variables. Moreover, it is interesting to note that the different covariates applied to measure the effect of BMI in meta-regression (BMI-matched studies) influenced HDL levels. Further studies could investigate detailed data related with body mass index (BMI) and other factors such as exercise, diet, socio-economic factors, alcohol use and other related lifestyle behaviours that may affect lipid levels and periodontal disease, leading to potential for residual confounding. Finally, studies set different restrictions or missed information regarding, for example, the number of remaining teeth, previous periodontal treatment or the use of antibiotics. Limitations should be acknowledged when interpreting these results.

Regardless of these limitations, it is worth emphasizing some strengths of this study, where we invested a great deal of effort in limiting possible source of bias. First, important modifying factors for periodontal disease, as smoking and diabetes, were excluded. Second, the meta-analysis was performed separating studies with secure and insecure diagnosis for PD. Third, multiple meta-analytic techniques (meta-regression and Leave-One-Out analysis) were applied taking into consideration many covariates. Fourth, statistical tests were performed for detecting publication bias.

It can be concluded that, despite the limitations of this study, after the meta-regression and Leave-One-Out analyses, a homogeneous meta-analysis indicated that PD was significantly associated with a reduction of HDL and elevation of LDL and triglycerides concentration. Therefore, periodontal disease may have an association with lipid metabolic control. Perhaps, in part, periodontal inflammation may negatively affect serum lipid control, contributing to a higher risk for CVD, and the lipid dysregulation increases the susceptibility to periodontitis, through the increase in systemic inflammation. This suggests a two-way relationship between dyslipidaemia and PD. Even though plausible biological mechanisms exist supporting this possible relationship between periodontal disease and dyslipidaemia, the cause and the effect may be hard to establish based on cross-sectional studies. Therefore, further large-scale longitudinal studies enroling ethnically diverse populations with secure and stringent diagnosis for periodontitis and lipid levels need to be conducted to corroborate our findings and to increase our knowledge about the relationship between periodontal disease and lipid profiles.

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CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

ORCID

Rafael Nepomuceno http://orcid.org/0000-0002-3120-0617
Steven Offenbacher http://orcid.org/0000-0002-0727-9441

REFERENCES

Akalin, F. A., Işiksal, E., Baltacioğlu, E., Renda, N., & Karabulut, E. (2008). Superoxide dismutase activity in gingiva in type-2 diabetes mellitus patients with chronic periodontitis. *Archives of Oral Biology*, *53*, 44–52.

Anagnostis, P., Stevenson, J. C., Crook, D., Johnston, D. G., & Godsland, I. F. (2015). Effects of menopause, gender and age on lipids and

high-density lipoprotein cholesterol subfractions. *Maturitas*, *81*, 62–68. https://doi.org/10.1016/j.maturitas.2015.02.262

Andrukhov, O., Haririan, H., Bertl, K., Rausch, W. D., Bantleon, H. P., Moritz, A., & Rausch-Fan, X. (2013). Nitric oxide production, systemic inflammation and lipid metabolism in periodontitis patients: Possible gender aspect. *Journal of Clinical Periodontology*, *40*, 916–923.

Armitage, G. C., & Cullinan, M. P. (2010). Comparison of the clinical features of chronic and aggressive periodontitis. *Periodontology 2000*, *53*, 12–27.

Awartani, F., & Atassi, F. (2010). Evaluation of periodontal status in subjects with hyperlipidemia. *The Journal of Contemporary Dental Practice*, *11*, 033–040.

Banu, S., Jabir, N. R., Mohan, R., Manjunath, N. C., Kamal, M. A., Kumar, K. R. V., … Tabrez, S. (2015). Correlation of toll-like receptor 4, interleukin-18, transaminases, and uric acid in patients with chronic periodontitis and healthy adults. *Journal of Periodontology*, *86*, 431–439. https://doi.org/10.1902/jop.2014.140414

Brown, L. J., Oliver, R. C., & Loe, H. (1990). Evaluating periodontal status of US employed adults. *Journal of the American Dental Association*, *121*, 226–232.

Bullon, P., Morillo, J. M., Ramirez-Tortosa, M. C., Quiles, J. L., Newman, H. N., & Battino, M. (2009). Metabolic syndrome and periodontitis: Is oxidative stress a common link? *Journal of Dental Research*, *88*, 503–518. https://doi.org/10.1177/0022034509337479

Campos, H., Khoo, C., & Sacks, F. M. (2005). Diurnal and acute patterns of postprandial apolipoprotein B-48 in VLDL, IDL, and LDL from normolipidemic humans. *Atherosclerosis*, *181*, 345–351. https://doi.org/10.1016/j.atherosclerosis.2004.12.045

Cutler, C. W., Machen, R. L., Jotwani, R., & Iacopino, A. M. (1999). Heightened gingival inflammation and attachment loss in type 2 diabetics with hyperlipidemia. *Journal of Periodontology*, *70*, 1313–1321.

Dhotre, P. S., Suryakar, A. N., & Bhogade, R. B. (2011). Oxidative stress in periodontitis: A critical link to cardiovascular disease. *Biomedical Research*, *22*, 178–182.

Duzagac, E., Cifcibasi, E., Erdem, M. G., Karabey, V., Kasali, K., Badur, S., & Cintan, S. (2016). Is obesity associated with healing after non-surgical periodontal therapy? A local vs. systemic evaluation *J Periodontal Res*, *51*, 604–612. doi:10.1111/jre.12340.

Ebersole, J. L., Cappelli, D., Mott, G., Kesavalu, L., Holt, S. C., & Singer, R. E. (1999). Systemic manifestations of periodontitis in the non-human primate. *Journal of Periodontal Research*, *34*, 358–362.

Fentoglu, O., Kirzioglu, F. Y., Bulut, M. T., Kumbul Doguc, D., Kulac, E., Onder, C., & Gunhan, M. (2015). Evaluation of lipid peroxidation and oxidative DNA damage in patients with periodontitis and hyperlipidemia. *Journal of Periodontology*, *86*, 682–688. https://doi.org/10.1902/jop.2015.140561

Gjermo, P. (1998). Epidemiology of periodontal diseases in Europe. *Journal de Parodontologie et d'Implantologie Orale*, *17*, 111–121.

Goh, V. H., Tong, T. Y., Mok, H. P., & Said, B. (2007). Differential impact of aging and gender on lipid and lipoprotein profiles in a cohort of healthy Chinese Singaporeans. *Asian Journal of Andrology*, *9*, 787–794. https://doi.org/10.1111/j.1745-7262.2007.00294.x

Golpasand Hagh, L., Zakavi, F., Hajizadeh, F., & Saleki, M. (2014). The association between hyperlipidemia and periodontal infection. *Iranian Red Crescent Medical Journal*, *16*, e6577. https://doi.org/10.5812/ircmj.6577

Joseph, R., Nath, S. G., & Joseraj, M. G. (2011). Elevated plasma homocysteine levels in chronic periodontitis: A hospital-based case-control study. *Journal of Periodontology*, *82*, 439–444. https://doi.org/10.1902/jop.2010.100271

Joshipura, K. J., Wand, H. C., Merchant, A. T., & Rimm, E. B. (2004). Periodontal disease and biomarkers related to cardiovascular disease. *Journal of Dental Research*, *83*, 151–155.

Kinane, D. F., & Lowe, G. D. (2000). How periodontal disease may contribute to cardiovascular disease. *Periodontology 2000*, *23*, 121–126.

Kumar, K. R., Ranganath, V., Naik, R., Banu, S., & Nichani, A. S. (2014). Assessment of high-sensitivity C-reactive protein and lipid levels in healthy adults and patients with coronary artery disease, with and without periodontitis—A cross-sectional study. *Journal of Periodontal Research*, *49*, 836–844.

Kuo, L. C., Poison, A. M., & Kang, T. (2008). Associations between periodontal diseases and systemic diseases: A review of the inter-relationships and interactions with diabetes, respiratory diseases, cardiovascular diseases and osteoporosis. *Public Health*, *122*, 417–433. https://doi.org/Doi 10.1016/J.Puhe. 2007.07.004

Lee, J. B., Yi, H. Y., & Bae, K. H. (2013). The association between periodontitis and dyslipidemia based on the Fourth Korea National Health and Nutrition Examination Survey. *Journal of Clinical Periodontology*, *40*, 437–442. https://doi.org/10.1111/jcpe.12095

Leite, A. C. E., de Araújo Carneiro, V. M., & do Carmo Machado Guimarães, M. (2014). Effects of periodontal therapy on C-reactive protein and HDL in serum of subjects with periodontitis. *Brazilian Journal of Cardiovascular Surgery*, *29*, 69–77. https://doi.org/10.5935/1678-9741.20140013

Liu, J., Wu, Y., Ding, Y., Meng, S., Ge, S., & Deng, H. (2010). Evaluation of serum levels of C-reactive protein and lipid profiles in patients with chronic periodontitis and/or coronary heart disease in an ethnic Han population. *Quintessence International (Berlin, Germany: 1985)*, *41*, 239–247.

Loe, H., Theilade, E., & Jensen, S. B. (1965). Experimental gingivitis in man. *Journal of Periodontology*, *36*, 177–187. https://doi.org/10.1902/jop.1965.36.3.177

Lösche, W., Karapetow, F., Pohl, A., Pohl, C., & Kocher, T. (2000). Plasma lipid and blood glucose levels in patients with destructive periodontal disease. *Journal of Clinical Periodontology*, *27*, 537–541.

Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G., & Prisma Group. (2009). Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *BMJ*, *339*, b2535. https://doi.org/10.1136/bmj.b2535

Monteiro, A. M., Jardini, M. A. N., Alves, S., Giampaoli, V., Aubin, E. C. Q., Figueiredo Neto, A. M., & Gidlund, M. (2009). Cardiovascular disease parameters in periodontitis. *Journal of Periodontology*, *80*, 378–388.

Munafo, M. R., & Flint, J. (2004). Meta-analysis of genetic association studies. *Trends in Genetics: TIG*, *20*, 439–444. https://doi.org/10.1016/j.tig.2004.06.014

Nibali, L., Tatarakis, N., Needleman, I., Tu, Y. K., D'Aiuto, F., Rizzo, M., & Donos, N. (2013). Clinical review: Association between metabolic syndrome and periodontitis: A systematic review and meta-analysis. *The Journal of Clinical Endocrinology and Metabolism*, *98*, 913–920. https://doi.org/10.1210/jc.2012-3552

Nigam, P. K. (2011). Serum lipid profile: Fasting or non-fasting? *Indian Journal of Clinical Biochemistry: IJCB*, *26*, 96–97. https://doi.org/10.1007/s12291-010-0095-x

Page, R. C., & Eke, P. I. (2007). Case definitions for use in population-based surveillance of periodontitis. *Journal of Periodontology*, *78*, 1387–1399. https://doi.org/10.1902/jop.2007.060264

Patsopoulos, N. A., Evangelou, E., & Ioannidis, J. P. (2008). Sensitivity of between-study heterogeneity in meta-analysis: Proposed metrics and empirical evaluation. *International Journal of Epidemiology*, *37*, 1148–1157. https://doi.org/10.1093/ije/dyn065

Penumarthy, S., Penmetsa, G. S., & Mannem, S. (2013). Assessment of serum levels of triglycerides, total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol in periodontitis patients. *Journal of Indian Society of Periodontology*, *17*, 30–35. https://doi.org/10.4103/0972-124X.107471

Persson, G. R. (2006). What has ageing to do with periodontal health and disease? *International Dental Journal*, *56*, 240–249.

Pushparani, D. S., Anandan, S. N., & Theagarayan, P. (2014). Serum zinc and magnesium concentrations in type 2 diabetes mellitus with periodontitis. *Journal of Indian Society of Periodontology*, *18*, 187–193. https://doi.org/10.4103/0972-124X.131322

Romero-Moraleda, B., Peinado Lozano, A. B., Morencos Martinez, E., Lopez-Plaza, B., Gomez Candela, C., Calderon Montero, F. J., & PRONAF Study group. (2015). Lipid profile response to weight loss program in overweight and obese patient is related with gender and age. *Nutricion Hospitalaria*, *31*, 2455–2464. https://doi.org/10.3305/nh.2015.31.6.8926

Ross, R. (1999). Atherosclerosis—An inflammatory disease. *The New England Journal of Medicine*, *340*, 115–126. https://doi.org/10.1056/NEJM199901143400207

Russell, A. L. (1956). A system of classification and scoring for prevalence surveys of periodontal disease. *Journal of Dental Research*, *35*, 350–359.

Savage, A., Eaton, K. A., Moles, D. R., & Needleman, I. (2009). A systematic review of definitions of periodontitis and methods that have been used to identify this disease. *Journal of Clinical Periodontology*, *36*, 458–467. https://doi.org/10.1111/j.1600-051X.2009.01408.x

Saxlin, T., Suominen-Taipale, L., Kattainen, A., Marniemi, J., Knuuttila, M., & Ylostalo, P. (2008). Association between serum lipid levels and periodontal infection. *Journal of Clinical Periodontology*, *35*, 1040–1047. https://doi.org/10.1111/j.1600-051X.2008.01331.x

Sridhar, R., Byakod, G., Pudakalkatti, P., & Patil, R. (2009). A study to evaluate the relationship between periodontitis, cardiovascular disease and serum lipid levels. *International Journal of Dental Hygiene*, *7*, 144–150.

Stroup, D. F., Berlin, J. A., Morton, S. C., Olkin, I., Williamson, G. D., Rennie, D., … Thacker, S. B. (2000). Meta-analysis of observational studies in epidemiology: A proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA*, *283*, 2008–2012.

Taleghani, F., Shamaei, M., & Shamaei, M. (2010). Association between chronic periodontitis and serum lipid levels. *Acta Medica Iranica*, *48*, 47–50.

Tonetti, M. S., Van Dyke, T. E., & Working group 1 of the joint, E. F. P. A. A. P. w. (2013). Periodontitis and atherosclerotic cardiovascular disease: Consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. *Journal of Clinical Periodontology*, *40*(Suppl 14), S24–S29. https://doi.org/10.1111/jcpe.12089

Wallace, B. C., Schmid, C. H., Lau, J., & Trikalinos, T. A. (2009). Meta-analyst: Software for meta-analysis of binary, continuous and diagnostic data. *BMC Medical Research Methodology*, *9*, 80. https://doi.org/10.1186/1471-2288-9-80

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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SUPPLEEMENTAL MATERIAL

Serum lipid levels in patients with periodontal disease. A meta-analysis and meta-regression

INTRODUCTION

It was suggested that dyslipidemia and PD are involved in a two-way relationship, in which dyslipidemia increases the risk of PD, and periodontal inflammation negatively affects serum lipid control (Awartani and Atassi, 2010). The biological rationale for this association that PD, as a chronic infectious disease, produces high levels of pro-inflammatory cytokines; which in turn, lead to alteration of the serum lipid parameters, thereby promoting hyperlipidemia (Heasman et al., 1993, Page, 1991). High levels of pro-inflammatory cytokines occur as a consequence of infection by periodontal microorganisms whose surface components (often described as endotoxins), particularly lipopolysaccharides (LPS), elicit an immune system reaction (Cutler et al., 1999b, Lakio et al., 2006). For the other way, when serum lipid levels are elevated toward the upper limit of the normal physiologic range, it alters the immune cell function. Lipids may alter macrophage gene expression for essential polypeptide growth factors and therefore increase the production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha) and interleukin 1 beta (IL-1B) (Cutler et al., 1999b, Iacopino, 1995). The release of pro-inflammatory cytokines and interleukin is believed to compromise tissue response and affect wound healing, thereby increasing the susceptibility to periodontitis (Stashenko et al., 1991).

MATERIALS AND METHODS

Quality Assessment

Risk of bias was assessed separately for each study. The assessment was performed by two investigators (RN, SCP) independently, and the inter-examiner agreement was analyzed. Any discrepancy between the two examiners in quality assessment was resolved via discussion. For cross-sectional (case-control and cohort) and longitudinal studies, the Newcastle-Ottawa Scale (NOS) was used to assess study quality. NOSs were developed to assess the quality of studies being easy and convenient for systematic reviews/meta-analyses. Briefly, it consists of nine items, categorized in three main groups: (i) selection of the study groups, (ii) comparability of the groups and (iii) the determination of the exposure of interest (Wells et al., 2013). A study can be awarded a minimum of zero and maximum of nine point score. Agreement rate between investigators was 83.3%.

Data Extraction

The initial search for assessment of titles and abstracts was carefully independently performed by two investigators (RN and SCP), and checked for agreement. Then, the full texts of the articles judged by title and abstract to be relevant were read independently and assessed in relation to the selection criteria for inclusion and exclusion. The agreement rate between investigators was 92.1%. Disagreements were adjudicated by a third investigator (RMSC), after referring back to the original articles.

The following parameters were extracted from each selected study using a standardized form: name of first author and year of publication, country, total sample sizes, percent male, male ratio (centred on 1:1 – PD Group : Control group ratio), mean age of participants, age ratio (centred on 1:1 – PD Group : Control group ratio), diagnostic criteria for periodontal disease, blood collection (serum or plasma) and blood test (fasting or non-fasting) and studies matched for BMI between groups (yes or no). Moreover, serum lipid parameter levels (HDL, LDL, triglycerides and total cholesterol) were extracted from periodontally healthy and diseased

groups in each study for statistical analyses (sample size, mean and standard deviation from each group).

In the case of more than one article by the same author utilizing the same sample and showing the same results for the levels of lipid profile, only one of those articles was selected. In the case of two or more groups of patients with chronic periodontitis based on both the extent and severity of the disease, only the data collected from the more severe group were included in the analysis to minimize any heterogeneity between included studies.

Statistical Analysis

Among the studies, we found two different units of measure used to quantify serum lipid parameter levels: mg/dL or mmol/L (the same as mM). To standardize units, here we converted the lipid parameter levels of all studies to mg/dL. To convert TC, HDL and LDL data from SI units (mmol/L) to mg/dL we multiplied the SI value by 38.67. In addition, to convert TG from SI units (mmol/L) to mg/dL we multiplied by 88.57 (Rugge et al., 2011).

In case of standard errors (SE) were used in an eligible study, to be considered in the present meta-analysis they were converted to standard deviations (SD) depending on both the standard errors and the sample size (N), using a simple relation $SD = SE \times \sqrt{N}$ (Altman and Bland, 2005).

Forest plots for each meta-analysis present the raw data (i.e. means, SDs and sample sizes), point estimates (displayed as blocks) and CIs (displayed as lines) for the chosen effect, as well as heterogeneity statistics (Chi^2 and I^2), total number of participants per group, overall average effect (MD and Z-statistics) and percent weight given to each study.

Chi-square (Chi^2) and inconsistency index (I^2) tests were used to assess the heterogeneity of the studies included in this meta-analysis. The heterogeneity of the trials was significantly indicated by $p<0.1$, instead of $p<0.05$, since that test had low power. Fixed-effects

model (Mantel-Haenszel method) analysis was used when no evidence of heterogeneity was detected in outcomes; otherwise, a random-effects model (DerSimonian-Laird method) was applied.

Publication bias was tested using a funnel plot test confirmed by Begg's rank correlation and Egger's regression method. Begg's method is a correlation test between the effect estimate and standard error (Begg and Mazumdar, 1994). The Egger test uses linear regression logistics, based on the standard deviation and the accuracy of studies (Egger et al., 1997). Two-tailed $p < 0.10$ was used as an indication of the presence of potential publication bias for both tests. If publication bias was indicated (by Begg's or Egger's tests) in meta-analysis after leave-one-out, Duval and Tweedie's trim-and-fill method was used for adjusting mean difference (Duval and Tweedie, 2000). All publication bias analyses were carried out using Comprehensive Meta-Analysis version 3.3.070 (2014) statistical software.

In order to evaluate whether the ethnicity of patients could be a potential variable causing heterogeneity among the studies included in the meta-analysis, we conducted additional meta-analyses subgrouping the studies according to the continent where the research was developed, in other words, the continent in which the investigated subjects from that ethnicity reside.

RESULTS

Literature search and study characteristics

Table 1 reports the main characteristics of all the studies selected for this meta-analysis. The subject samples ranged from 13 patients (Cutler et al., 1999a) to 468 (Joshipura et al., 2004) (median number of subjects: 74; interquartile range: 53.5 to 116.5). The percentage of male in the studies ranged from 23.08%(Cutler et al., 1999a) to 100% (Joshipura et al., 2004). The mean age of participants ranged from 33.73 (Joseph et al., 2011) to 57.58 (Joshipura et al., 2004). The

age ratio (centered on 1:1 – PD Group : Control group ratio) was higher in PD group in 13 studies, lower in 4 studies and not informed in other 2 studies. 12 studies evaluated the lipid profile in blood serum and seven in plasma. Most studies (15 studies) conducted the blood collection fasting. Only two studies reported the lipid parameter levels separately for women and men(Andrukhov et al., 2013, Monteiro et al., 2009). 16 papers were case-control studies, 1 cohort and 2 longitudinal studies.

Significant variation in the criteria for chronic periodontitis diagnosis was observed among the studies. Only 6 studies were evaluated as **secure PD diagnosis** criteria (Andrukhov et al., 2013, Banu et al., 2015, Cutler et al., 1999a, Duzagac et al., 2015, Kumar et al., 2014, Liu et al., 2010). Two studies assessed only probing pocket depth (PPD) and one assessed only clinical attachment level (CAL). Six studies utilized both PPD and CAL and two, in addition to clinical criteria (PPD and CAL) evaluated bone loss by radiography. One study did not report the criteria used (Leite et al., 2014) and one study the criterion was through anamnesis (Joshipura et al., 2004).

Quality assessment

The summary result of quality assessment for each study was described in Table 1. Study quality as assessed by the Newcastle Ottawa scale varied considerably across the studies (minimum=0 and maximum=9). The mean NOS score was 6 (range: 1–8). The main lack issues were: adequate periodontal diagnosis, definition of controls, and adjustment for potential confounders.

Publication Bias

Visual inspection of the funnel plot for all investigated outcomes suggested the presence of a publication bias (Appendix 1, 2 and 3). However, Begg's method of rank correlation and

Egger's regression method revealed that the differences between the original estimate and the adjusted effect for those outcomes were non-significant. An exception was found for Begg's and Egger's tests in the total cholesterol analysis before and after meta-regression and leave-one-out analysis (Appendix 4). In Duval and Tweedie's trim-and-fill method, using the random-effects model, six potentially missing studies was imputed, leading to a corrected effect size that was insignificant (MD = -1.34; 95 % CI: -4.59 – 1.92) (Table 3). Other exception was found for Begg's and Egger's tests in the triglycerides just after meta-regression and leave-one-out analysis (P = 0.06 and P=0.01, respectively) (Appendix 4). In trim-and-fill method, four potentially missing studies were imputed, leading to a corrected effect size. Serum levels of triglycerides in chronic periodontitis than healthy patients kept significantly higher likewise meta-analysis after meta-regression and leave-one-out analysis (MD = 9.86; 95 % CI: 3.84 - 15.87) (Table 3).

Subgroup analysis and meta-regression according to the ethnicity of patients

We carried out subgroup analyses according to the ethnicity of patients and we presented the results according the continent where the research was developed (North/South America, Europe and Asia - Appendix 5-6). Total Cholesterol was significantly higher in periodontal diseased patients than in healthy ones in Europe and Asia subgroups, even though the high heterogeneity amongst the studies in Asia subgroup (Appendix 5A). Regarding HDL, this lipid level was significantly lower in periodontal diseased patients than in healthy ones in South America and Asia subgroups (Appendix 5B), while LDL patients from North America and Europe this result were significantly higher in PD patients and without significant heterogeneity (Appendix 5C). Triglycerides were significantly higher only in Asia subgroup, but with high heterogeneity amongst the studies (Appendix 5D). The heterogeneity was higher in some analyses, mainly in the Asians analyses. Interestingly, meta-regression made according

to the continent that resides the subjects did not confirm that ethnicity might be a main source of heterogeneity for any outcome (total cholesterol, HDL, LDL and triglycerides) (Appendix 6).

DISCUSSION

Abnormal serum lipids is a common medical condition associated with high risk of developing cardiovascular disease and, consequently, with unquestionable public health impact (Ross, 1999). PD is a chronic infection that produces local and systemic inflammatory responses (Kuo et al., 2008). The high incidence of PD in the population has generated growing interest from researchers regarding its effects on systemic health and possible associations between PD and systemic diseases. Therefore, periodontitis may have direct quality-of-life relevance (Nibali et al., 2013).

The wide age range (28 to 65 years old) of patients enrolled in the six studies with secure PD diagnosis included in the meta-analysis can be pointed to as the possible reason for the insignificant association of high levels of total cholesterol with PD observed by that separated analysis (Figure 2A). Total cholesterol usually increases with age and normally less alteration in the levels occurs in patients who are less than 60 years old (Kannel, 2002). Therefore, the ratio between total cholesterol and HDL (atherogenic index of plasma) is considered a better and easier index for measuring the risk of developing cardiovascular disease than the total cholesterol level in those patients (Kinosian et al., 1994, Genest et al., 2003). Moreover, periodontal disease was also related to changes in the ratio of total cholesterol to HDL (Pradnya Shree and Abdul Kayyum, 2014).

HDL can resist atherosclerosis and is the protective factor for CVD because it participates in reverse cholesterol transport and eliminates the lipid component of atheromatous plaques (Toth, 2005). Our present study showed significantly lower serum levels of HDL in

periodontitis participants (Figures 2B, 3A and 4B). This significant result is important, since it suggests that periodontal disease might influence blood lipid concentrations and thereby the risk of cardiovascular disease (Buhlin et al., 2003). One biological explanation for the relationship between periodontitis and low HDL levels might be that chronic inflammation in the periodontal tissue leads to the release of pro-inflammatory cytokines, such as IL-1β and TNF-α, which affect other inflammatory systemic mediators (Iacopino and Cutler, 2000). These mediators have the capacity to influence lipid metabolism, promoting the reduction of serum HDL levels (Iacopino and Cutler, 2000). Furthermore, gram-negative bacteria and free LPS in the plasma from PD patients induce the release of inflammatory mediators. In addition, plasma lipoproteins, such as HDL, interact with LPS and prevent LPS-mediated cellular activation, resulting in a reduction of free HDL levels in the serum (Monteiro et al., 2009). This effect is particularly interesting because it also explains why the patients with chronic periodontitis had significantly lower levels of HDL than the healthy group.

High levels of free fatty acids such as LDL and triglycerides were previously associated with the action of the pro-inflammatory cytokines TNF and IL-1 induced by microorganisms/endotoxins (Fentoglu and Bozkurt, 2008, Griffiths and Barbour, 2010). In the present study, association was observed between the high levels of LDL and PD in total patients before and after leave-one-out analysis (Figure 2C and 4C).

In the present meta-analysis, elevated triglycerides level was also associated with periodontitis, including secure diagnosis subgroup after meta-regression and leave-one-out analysis (Figures 2D, 3C and 4D). The periodontal tissue has been described as a potential reservoir of endotoxins and cytokines (Cutler et al., 1999a). The administration of low dosages of endotoxin in rodents rapidly induces changes in lipid metabolism resulting in hypertriglyceridemia (Feingold et al., 1992). Infection with Gram-negative periodontal pathogens could trigger the release of IL-1β and TNF-α into the circulation, inhibiting the

lipoprotein lipase (LPL) activity. LPL is a key enzyme in triglycerides catabolism and the formation of adipose tissue, resulting in chronic hypertriglyceridemia (Cutler et al., 1999b, Assmann et al., 1991, Cutler et al., 1999a). Although the systemic exposure to LPS in animal models is likely much greater than in human periodontitis, this mechanism may help to explain our present findings of significantly higher levels of triglycerides in PD patients.

There are many studies that investigate race-ethnic differences in the relationship between lipid profile components (Willey et al., 2011, Sliwa et al., 2012, Zhang et al., 2010, Davis et al., 2001). Overall, there are important differences in lipid profile according to ethnicity. The causes of ethnic differences are complex. Possible contributors include genetic, environmental, psychosocial, cultural, and unmeasured factors, and many are not well clarified (Zaninotto et al., 2007). Our subgroup meta-analysis according to the ethnicity of patients showed small variations between continents, but no statistically significant differences were found even after meta-regression (Appendix 6), meaning that the ethnicity did not influence the outcomes. However, some meta-analyses even after meta-regressions remained highly heterogeneous. This might be occurred due to the presence of co-factors (such as age and gender) that could influence the results (Table 2). Subgroup meta-analysis according to gender was not possible to execute because only two study separating women from men was found according to the adopted inclusion/exclusion criteria (Monteiro et al., 2009, Andrukhov et al., 2013).

References

Altman, D. G. & Bland, J. M. (2005) Standard deviations and standard errors. *BMJ* **331,** 903. doi:10.1136/bmj.331.7521.903.

Andrukhov, O., Haririan, H., Bertl, K., Rausch, W. D., Bantleon, H. P., Moritz, A. & Rausch-Fan, X. (2013) Nitric oxide production, systemic inflammation and lipid metabolism in periodontitis patients: Possible gender aspect. *Journal of Clinical Periodontology* **40,** 916-923.

Assmann, G., Betteridge, D. J., Gotto, A. M., Jr. & Steiner, G. (1991) Management of hypertriglyceridemic patients. A. Treatment classifications and goals. *Am J Cardiol* **68,** 30A-34A.

Awartani, F. & Atassi, F. (2010) Evaluation of periodontal status in subjects with hyperlipidemia. *J Contemp Dent Pract* **11,** 033-040.

Banu, S., Jabir, N. R., Mohan, R., Manjunath, N. C., Kamal, M. A., Kumar, K. R. V., Zaidi, S. K., Khan, M. S. & Tabrez, S. (2015) Correlation of toll-like receptor 4, interleukin-18, transaminases, and uric acid in patients with chronic periodontitis and healthy adults. *Journal of Periodontology* **86,** 431-439. doi:10.1902/jop.2014.140414.

Begg, C. B. & Mazumdar, M. (1994) Operating characteristics of a rank correlation test for publication bias. *Biometrics* **50,** 1088-1101.

Buhlin, K., Gustafsson, A., Pockley, A. G., Frostegard, J. & Klinge, B. (2003) Risk factors for cardiovascular disease in patients with periodontitis. *Eur Heart J* **24,** 2099-2107.

Cutler, C. W., Machen, R. L., Jotwani, R. & Iacopino, A. M. (1999a) Heightened gingival inflammation and attachment loss in type 2 diabetics with hyperlipidemia. *Journal of Periodontology* **70,** 1313-1321.

Cutler, C. W., Shinedling, E. A., Nunn, M., Jotwani, R., Kim, B. O., Nares, S. & Iacopino, A. M. (1999b) Association between periodontitis and hyperlipidemia: cause or effect? *J Periodontol* **70,** 1429-1434. doi:10.1902/jop.1999.70.12.1429.

Davis, T. M., Cull, C. A., Holman, R. R. & Group, U. K. P. D. S. (2001) Relationship between ethnicity and glycemic control, lipid profiles, and blood pressure during the first 9 years of type 2 diabetes: U.K. Prospective Diabetes Study (UKPDS 55). *Diabetes Care* **24,** 1167-1174.

Duval, S. & Tweedie, R. (2000) Trim and fill: A simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics* **56,** 455-463.

Duzagac, E., Cifcibasi, E., Erdem, M. G., Karabey, V., Kasali, K., Badur, S. & Cintan, S. (2015) Is obesity associated with healing after non-surgical periodontal therapy? A local vs. systemic evaluation. *J Periodontal Res*. doi:10.1111/jre.12340.

Egger, M., Davey Smith, G., Schneider, M. & Minder, C. (1997) Bias in meta-analysis detected by a simple, graphical test. *BMJ* **315,** 629-634.

Feingold, K. R., Staprans, I., Memon, R. A., Moser, A. H., Shigenaga, J. K., Doerrler, W., Dinarello, C. A. & Grunfeld, C. (1992) Endotoxin rapidly induces changes in lipid metabolism that produce hypertriglyceridemia: low doses stimulate hepatic triglyceride

production while high doses inhibit clearance. *Journal of Lipid Research* **33,** 1765-1776.

Fentoglu, O. & Bozkurt, F. Y. (2008) The Bi-Directional Relationship between Periodontal Disease and Hyperlipidemia. *Eur J Dent* **2,** 142-146.

Genest, J., Frohlich, J., Fodor, G., McPherson, R., Working Group on, H. & Other, D. (2003) Recommendations for the management of dyslipidemia and the prevention of cardiovascular disease: summary of the 2003 update. *CMAJ* **169,** 921-924.

Griffiths, R. & Barbour, S. (2010) Lipoproteins and lipoprotein metabolism in periodontal disease. *Clinical Lipidology* **5,** 397-411. doi:10.2217/clp.10.27.

Heasman, P. A., Collins, J. G. & Offenbacher, S. (1993) Changes in Crevicular Fluid Levels of Interleukin-1-Beta, Leukotriene-B4, Prostaglandin-E2, Thromboxane-B2 and Tumor-Necrosis-Factor-Alpha in Experimental Gingivitis in Humans. *Journal of Periodontal Research* **28,** 241-247. doi:Doi 10.1111/J.1600-0765.1993.Tb02090.X.

Iacopino, A. M. (1995) Diabetic periodontitis: possible lipid-induced defect in tissue repair through alteration of macrophage phenotype and function. *Oral Dis* **1,** 214-229.

Iacopino, A. M. & Cutler, C. W. (2000) Pathophysiological relationships between periodontitis and systemic disease: recent concepts involving serum lipids. *Journal of Periodontology* **71,** 1375-1384. doi:10.1902/jop.2000.71.8.1375.

Joseph, R., Nath, S. G. & Joseraj, M. G. (2011) Elevated plasma homocysteine levels in chronic periodontitis: a hospital-based case-control study. *J Periodontol* **82,** 439-444. doi:10.1902/jop.2010.100271.

Joshipura, K. J., Wand, H. C., Merchant, A. T. & Rimm, E. B. (2004) Periodontal disease and biomarkers related to cardiovascular disease. *Journal of Dental Research* **83,** 151-155.

Kannel WB. (2002) Coronary heart disease risk factors in the elderly. *Am J Geriatr Cardiol*. **11[1]**,101-7.

Kinosian, B., Glick, H. & Garland, G. (1994) Cholesterol and coronary heart disease: predicting risks by levels and ratios. *Anals of Internal Medicine* **121,** 641-647.

Kumar, K. R., Ranganath, V., Naik, R., Banu, S. & Nichani, A. S. (2014) Assessment of high-sensitivity C-reactive protein and lipid levels in healthy adults and patients with coronary artery disease, with and without periodontitis--a cross-sectional study. *Journal of Periodontal Research* **49,** 836-844.

Kuo, L. C., Poison, A. M. & Kang, T. (2008) Associations between periodontal diseases and systemic diseases: A review of the inter-relationships and interactions with diabetes,

respiratory diseases, cardiovascular diseases and osteoporosis. *Public Health* **122,** 417-433. doi:Doi 10.1016/J.Puhe.2007.07.004.

Lakio, L., Lehto, M., Tuomainen, A. M., Jauhiainen, M., Malle, E., Asikainen, S. & Pussinen, P. J. (2006) Pro-atherogenic properties of lipopolysaccharide from the periodontal pathogen Actinobacillus actinomycetemcomitans. *J Endotoxin Res* **12,** 57-64. doi:10.1179/096805106X89099.

Leite, A. C. E., de Araújo Carneiro, V. M. & do Carmo Machado Guimarães, M. (2014) Effects of periodontal therapy on C-reactive protein and HDL in serum of subjects with periodontitis. *Brazilian Journal of Cardiovascular Surgery* **29,** 69-77. doi:10.5935/1678-9741.20140013.

Liu, J., Wu, Y., Ding, Y., Meng, S., Ge, S. & Deng, H. (2010) Evaluation of serum levels of C-reactive protein and lipid profiles in patients with chronic periodontitis and/or coronary heart disease in an ethnic Han population. *Quintessence international (Berlin, Germany : 1985)* **41,** 239-247.

Monteiro, A. M., Jardini, M. A. N., Alves, S., Giampaoli, V., Aubin, E. C. Q., Figueiredo Neto, A. M. & Gidlund, M. (2009) Cardiovascular disease parameters in periodontitis. *Journal of Periodontology* **80,** 378-388.

Nibali, L., Tatarakis, N., Needleman, I., Tu, Y. K., D'Aiuto, F., Rizzo, M. & Donos, N. (2013) Clinical review: Association between metabolic syndrome and periodontitis: a systematic review and meta-analysis. *J Clin Endocrinol Metab* **98,** 913-920. doi:10.1210/jc.2012-3552.

Page, R. C. (1991) The Role of Inflammatory Mediators in the Pathogenesis of Periodontal-Disease. *Journal of Periodontal Research* **26,** 230-242. doi:Doi 10.1111/J.1600-0765.1991.Tb01649.X.

Pradnya Shree, D. & Abdul Kayyum, S. (2014) Assessment of Atherogenic Index of Plasma in Periodontitis as a Risk Factor for Cardiovascular Disease. *Ijhsr* **4,** 117-122.

Ross, R. (1999) Atherosclerosis--an inflammatory disease. *N Engl J Med* **340,** 115-126. doi:10.1056/NEJM199901143400207.

Rugge, B., Balshem, H., Sehgal, R., Relevo, R., Gorman, P. & Helfand, M. (2011) In: *Screening and Treatment of Subclinical Hypothyroidism or Hyperthyroidism*. Rockville (MD).

Sliwa, K., Lyons, J. G., Carrington, M. J., Lecour, S., Marais, A. D., Raal, F. J. & Stewart, S. (2012) Different lipid profiles according to ethnicity in the Heart of Soweto study cohort of de novo presentations of heart disease. *Cardiovasc J Afr* **23,** 389-395. doi:10.5830/CVJA-2012-036.

Stashenko, P., Fujiyoshi, P., Obernesser, M. S., Prostak, L., Haffajee, A. D. & Socransky, S. S. (1991) Levels of interleukin 1 beta in tissue from sites of active periodontal disease. *J Clin Periodontol* **18,** 548-554.

Toth, P. P. (2005) Cardiology patient page. The "good cholesterol": high-density lipoprotein. *Circulation* **111,** e89-91. doi:10.1161/01.CIR.0000154555.07002.CA.

Wells, G., Shea, B., O'Connell, D., Peterson, J., Welch, V., Losos, M. & Tugwell, P. (2013) The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses [WWW document]. URL http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp

Willey, J. Z., Rodriguez, C. J., Carlino, R. F., Moon, Y. P., Paik, M. C., Boden-Albala, B., Sacco, R. L., DiTullio, M. R., Homma, S. & Elkind, M. S. (2011) Race-ethnic differences in the association between lipid profile components and risk of myocardial infarction: The Northern Manhattan Study. *Am Heart J* **161,** 886-892. doi:10.1016/j.ahj.2011.01.018.

Zaninotto, P., Mindell, J. & Hirani, V. (2007) Prevalence of cardiovascular risk factors among ethnic groups: results from the Health Surveys for England. *Atherosclerosis* **195,** e48-57. doi:10.1016/j.atherosclerosis.2007.02.017.

Zhang, L., Qiao, Q., Tuomilehto, J., Janus, E. D., Lam, T. H., Ramachandran, A., Mohan, V., Stehouwer, C. D., Dong, Y., Nakagami, T., Onat, A., Soderberg, S. & Group, D. D. S. (2010) Distinct ethnic differences in lipid profiles across glucose categories. *J Clin Endocrinol Metab* **95,** 1793-1801. doi:10.1210/jc.2009-2348.

Appendix 4. Quantitative Analysis for Publication Bias Assessments

	Original Meta-Analysis		Meta-Analysis after Meta-regression and leave-one-out analysis		Trim-and-Fill Analysis[&]	
Outcome	P of Begg's test	P of Egger's test	P of Begg's test	P of Egger's test	MD (95% CI)	Studies Trimmed / Total Studies
Total Cholesterol	**0.05***	0,30	**0.01***	**0.00***	-1.34 (-4.59 - 1.92)	6/12
HDL	0.45	0.36	0.88	0.23	-	-
LDL	0.32	0.62	0.47	0.69	-	-
Triglycerides	0.11	0.66	**0.06***	**0.01***	**9.86 (3.84 - 15.87)**[#]	4/11

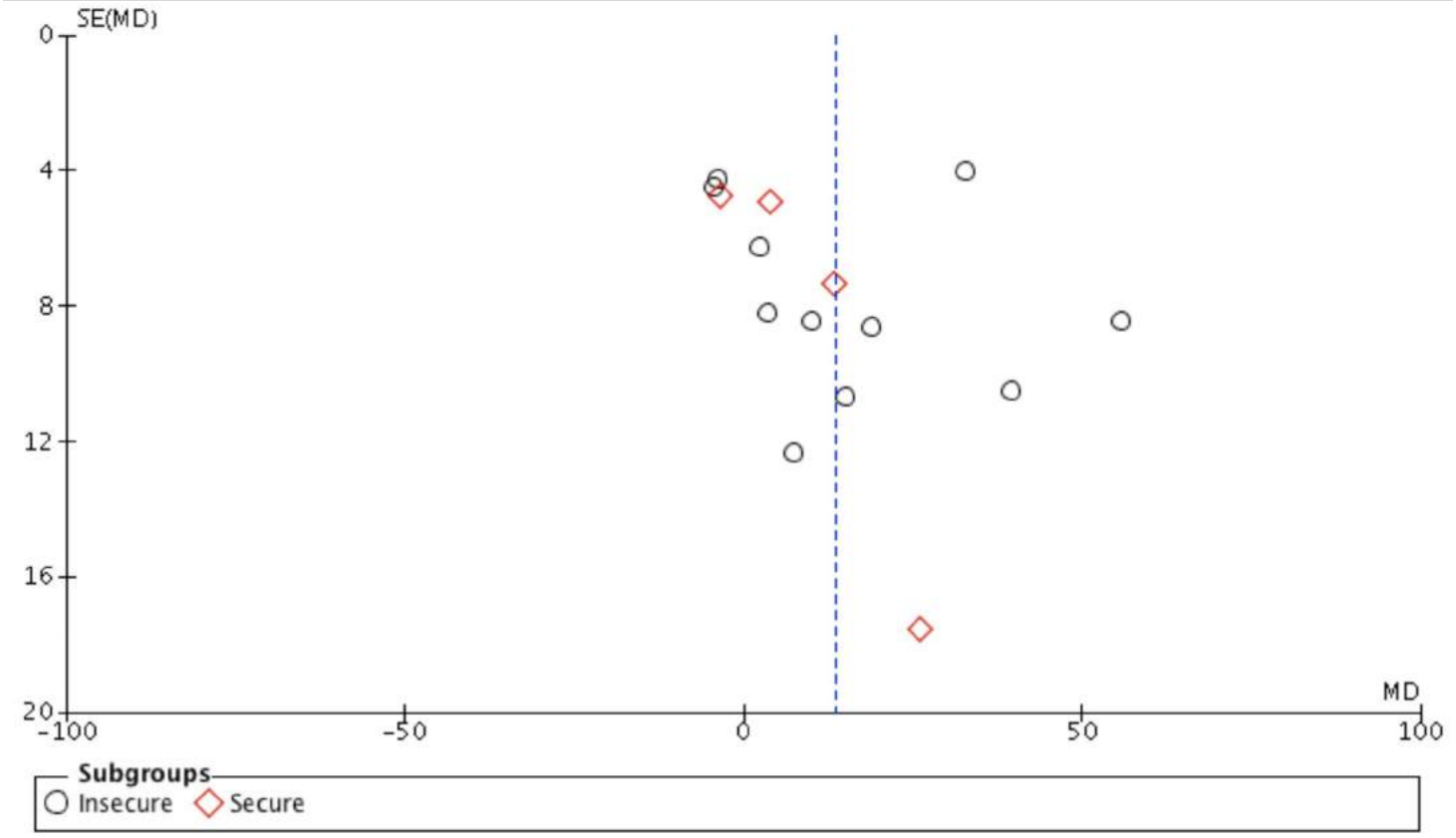
*Variable present P <0.10 indicate statistical significance for publication bias through linear regression logistics. [&]Bias was indicated (by Begg and Egger's tests) in meta-analysis after meta-regression and leave-one-out analysis for total cholesterol and triglycerides; Duval and Tweedie's trim-and-fill method was used for adjusting mean difference. [#]Variable present P <0.05 indicate significantly higher serum levels of triglycerides in chronic periodontitis than healthy patients.

Appendix 6. Meta-regression analysis according to the continent where the research was developed.

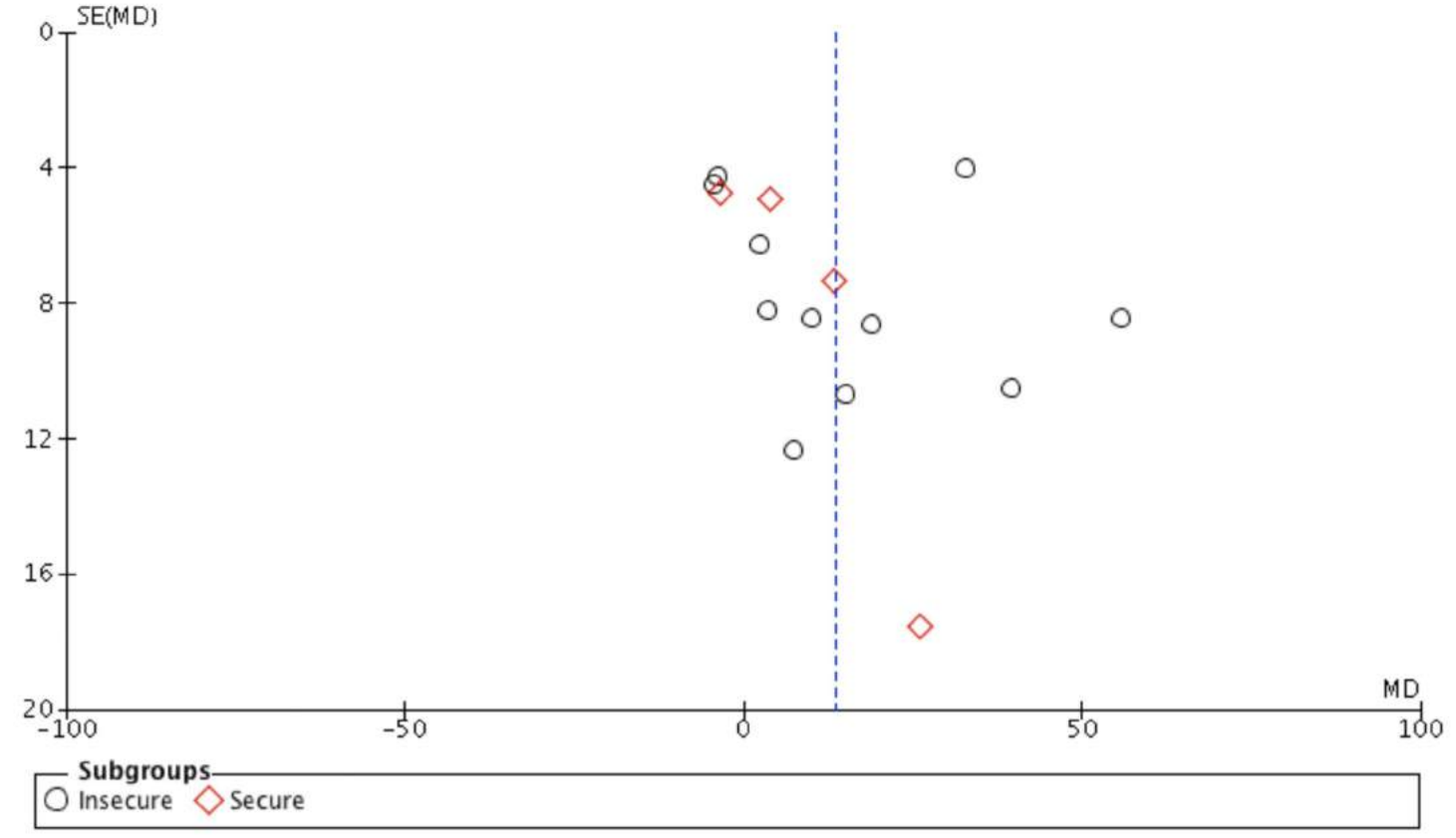
Dependent variable	Total Cholesterol			HDL			LDL			Triglycerides		
Independent variable	β	SE	p-value	β	SE	p-value	β	SE	p-value	β	SE	p-value
North America (reference)	-	-	-	-	-	-	-	-	-	-	-	-
South America	-19.236	26.054	0.460	8.444	9.259	0.362	-9.706	11.979	0.418	-37.924	45.229	0.402
Europe	-14.821	24.993	0.553	11.724	9.073	0.196	-5.589	10.937	0.609	-52.169	43.835	0.234
Asia	-11.387	23.713	0.631	9.176	8.791	0.297	-8.135	9.312	0.382	-43.754	41.783	0.295
Omnibus p-value			0.874			0.551			0.822			0.674

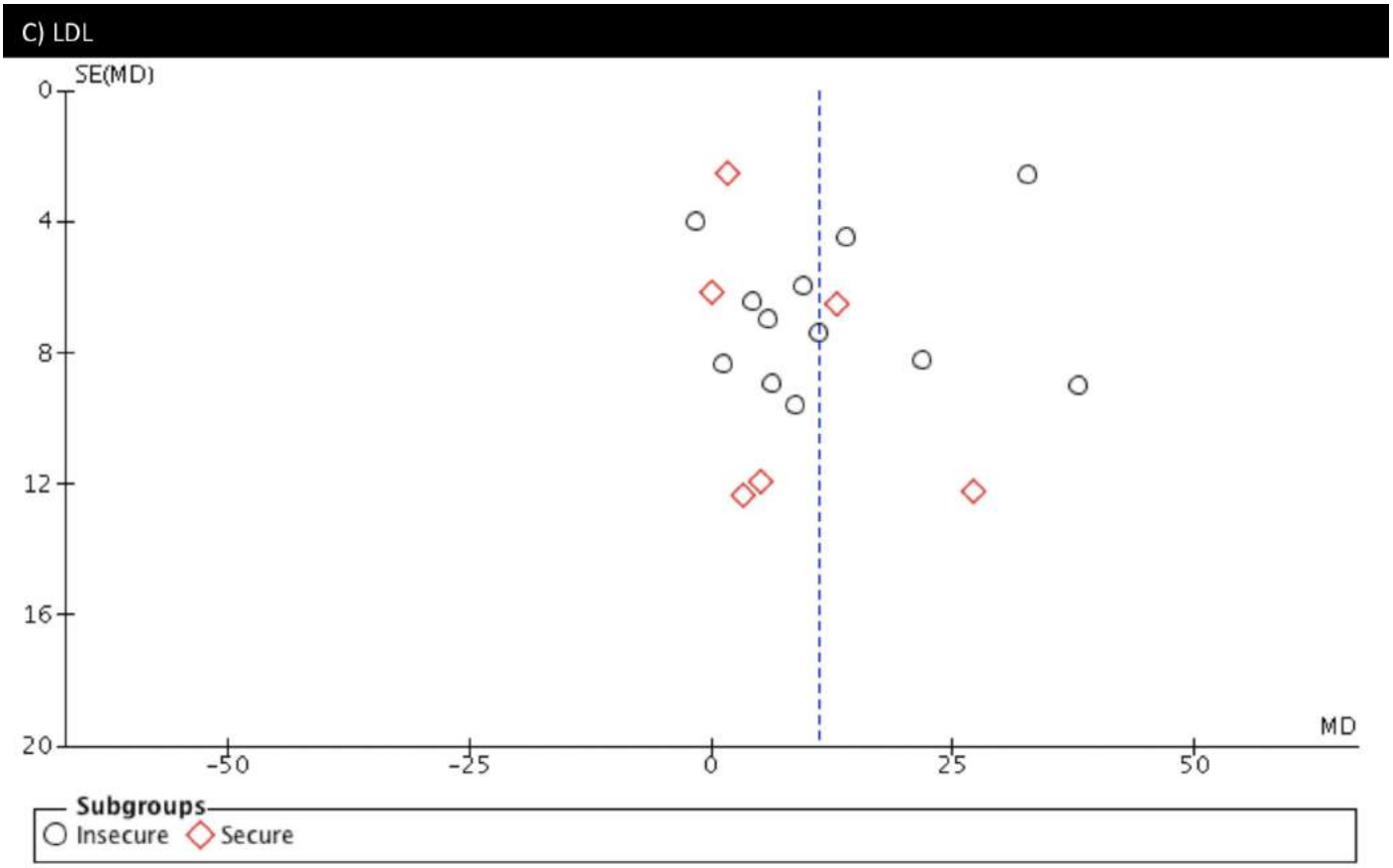
Abbreviations: β, coefficient; SE, standard error. *No statistical significance was found.

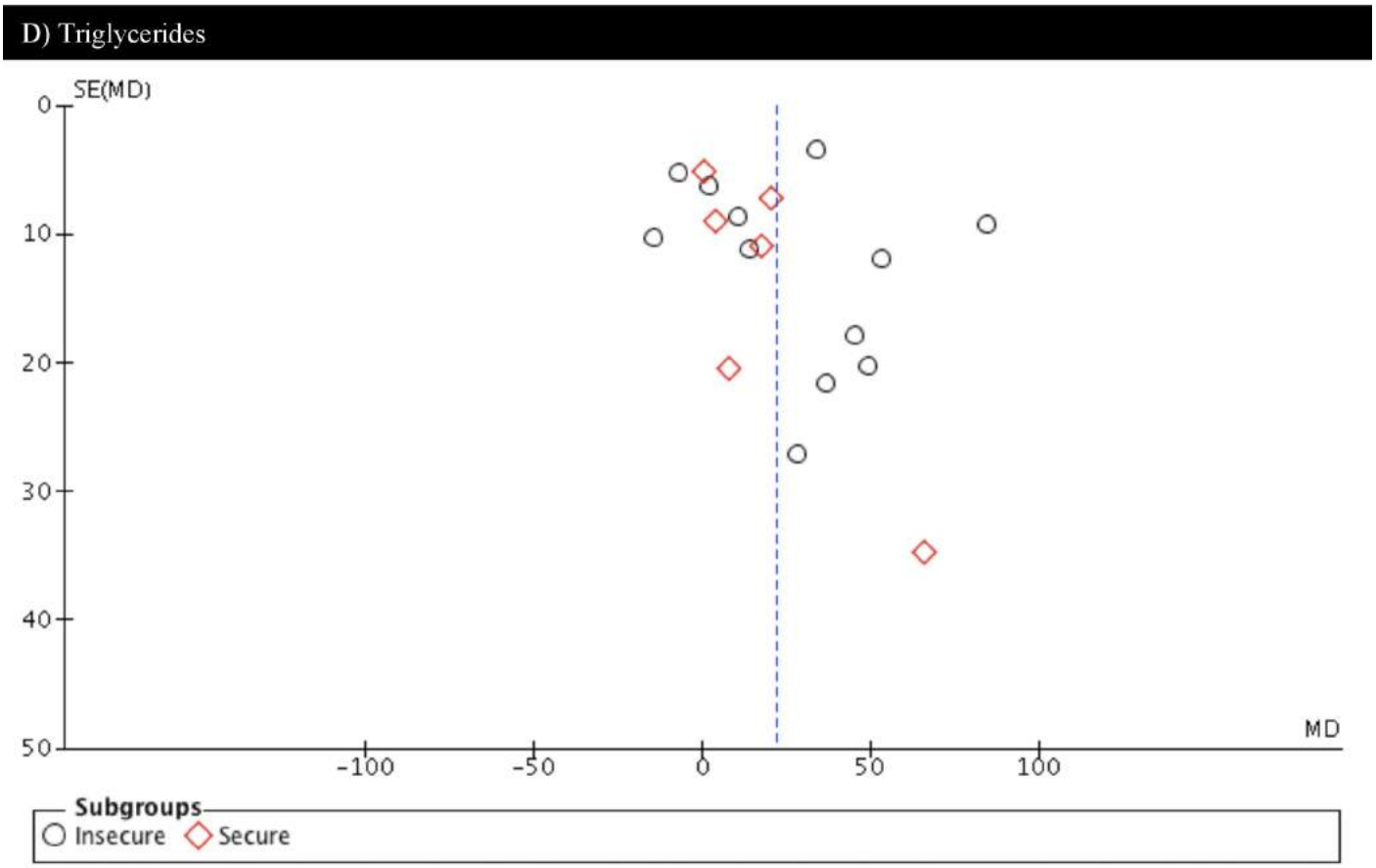
A) Total Cholesterol



B) HDL

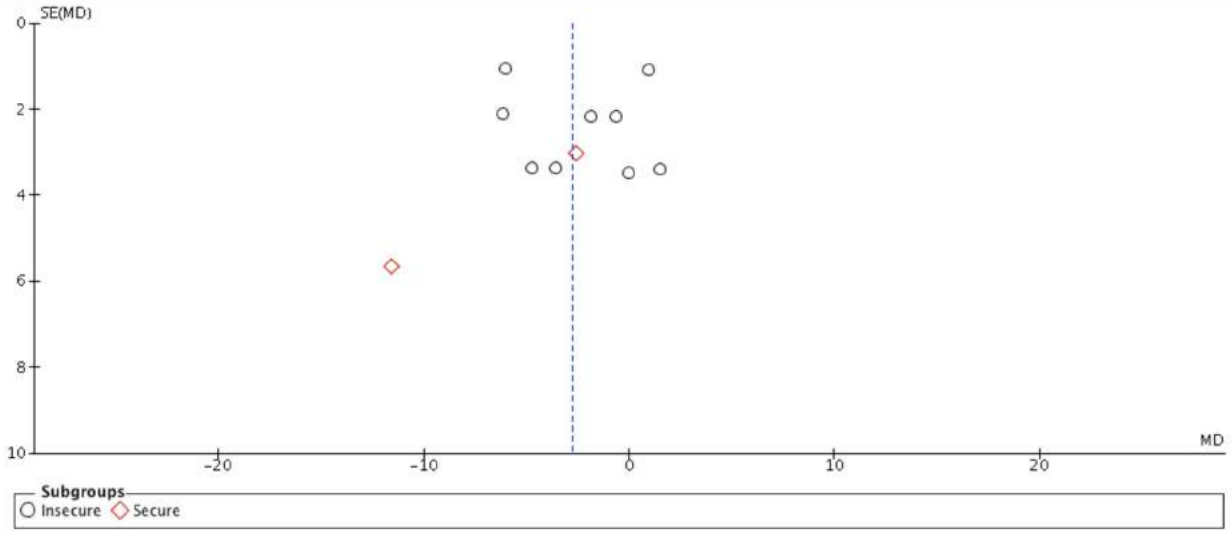




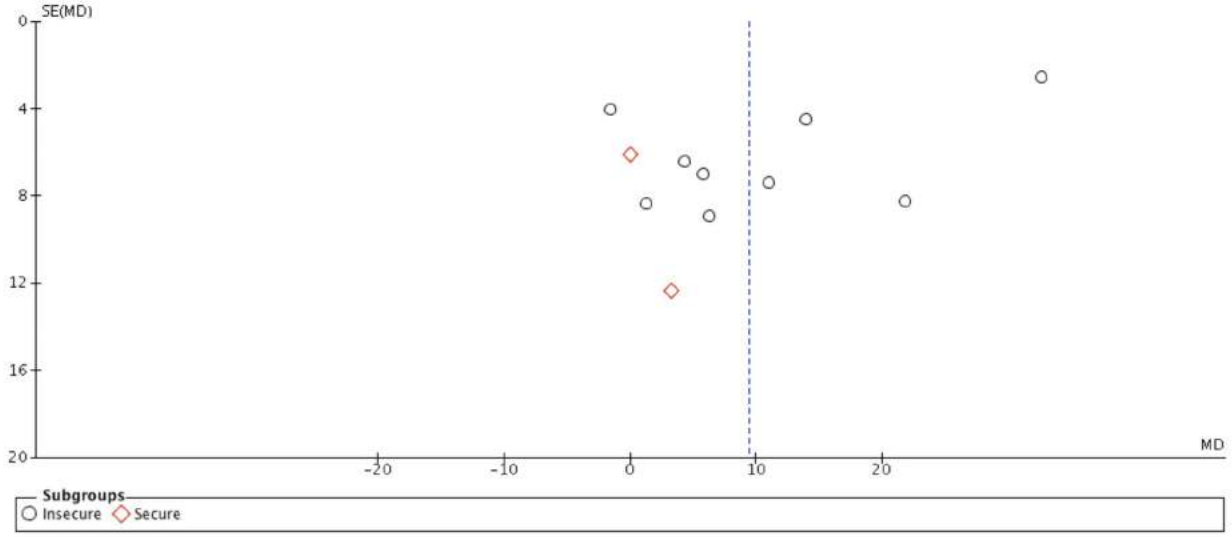


Appendix 1. Funnel Plot for comparison: periodontitis vs. non-periodontitis in studies with Insecure and Secure diagnoses for PD before meta-regression. Outcomes: (**A**) Total Cholesterol; (**B**) HDL; (**C**) LDL; (**D**) Triglycerides.

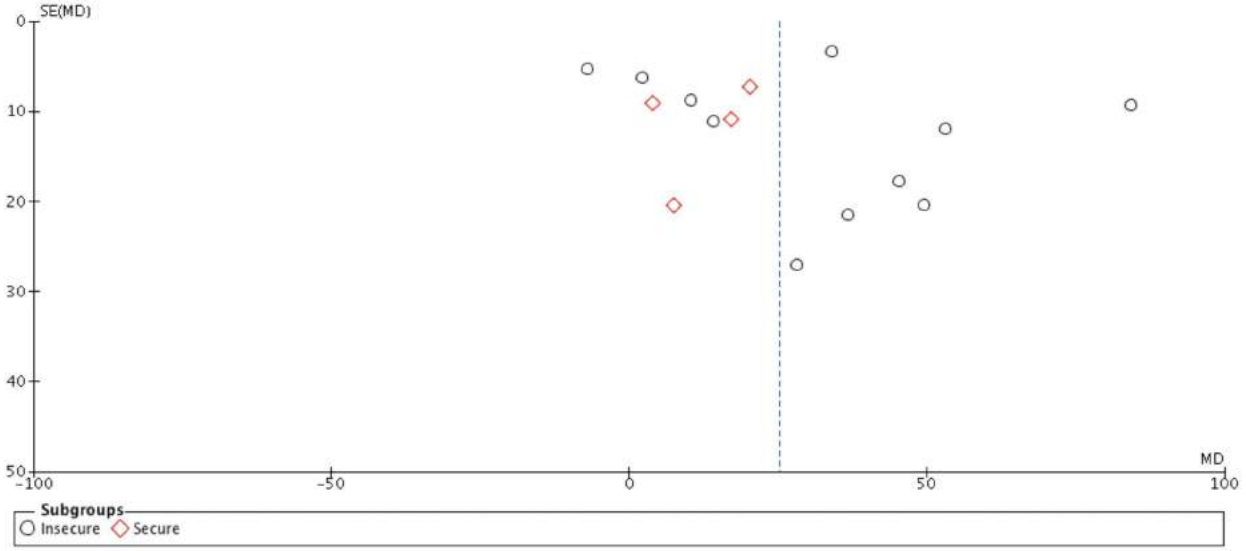
A) HDL



B) LDL

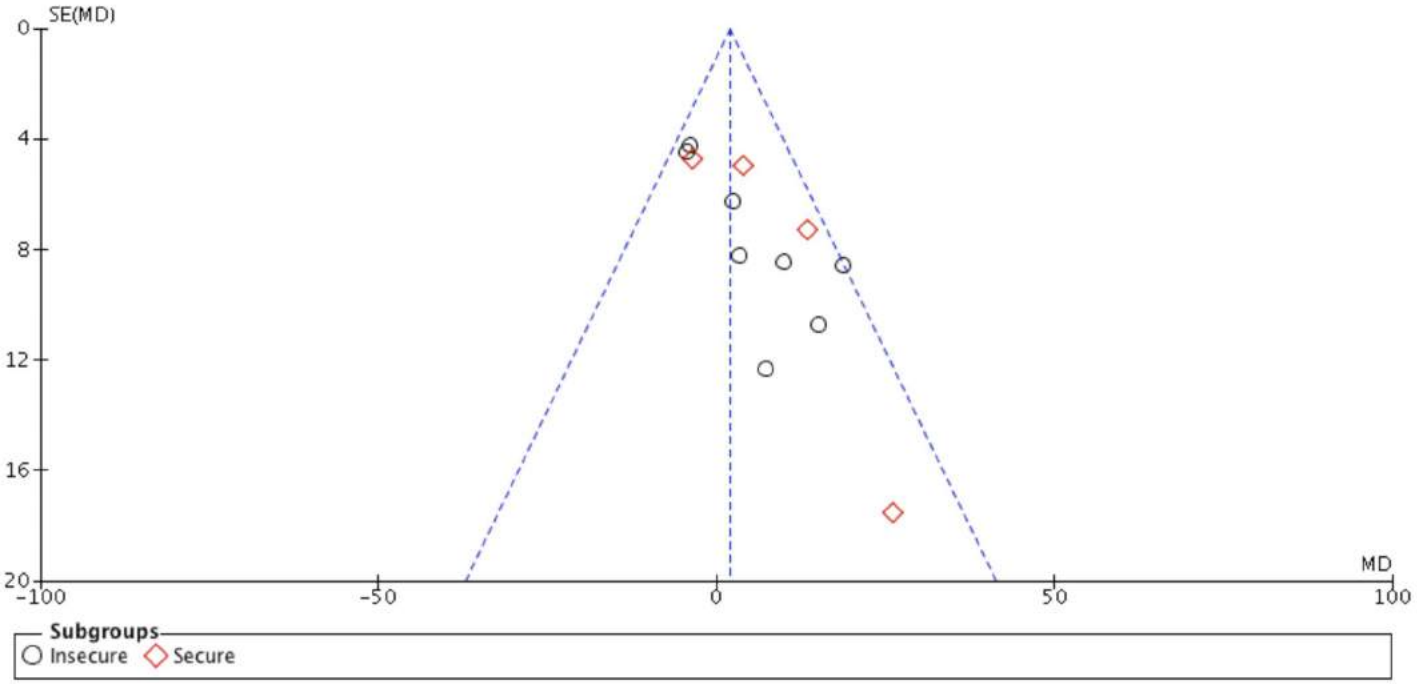


C) Triglycerides

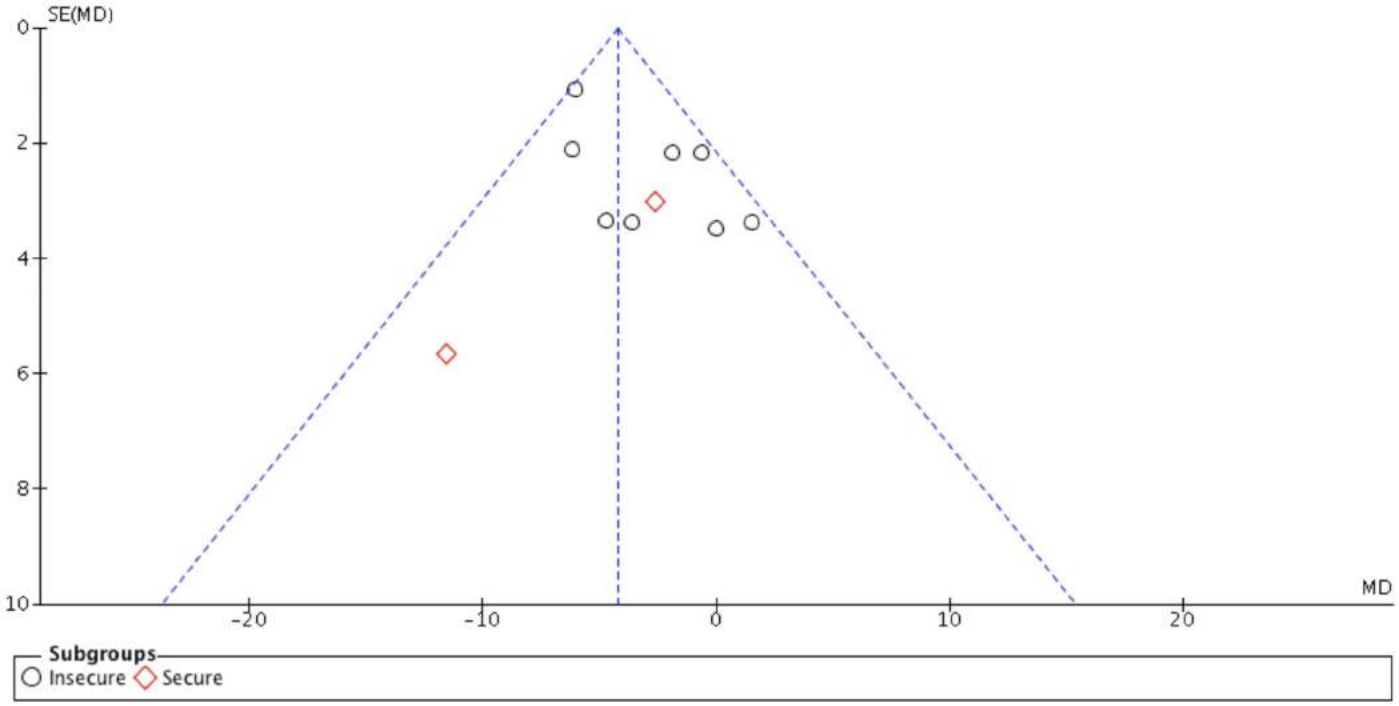


Appendix 2. Funnel Plot for comparison: periodontitis vs. non-periodontitis in studies with Insecure and Secure diagnoses for PD after meta-regression. Outcomes: (**A**) HDL; (**B**) LDL; (**C**) Triglycerides.

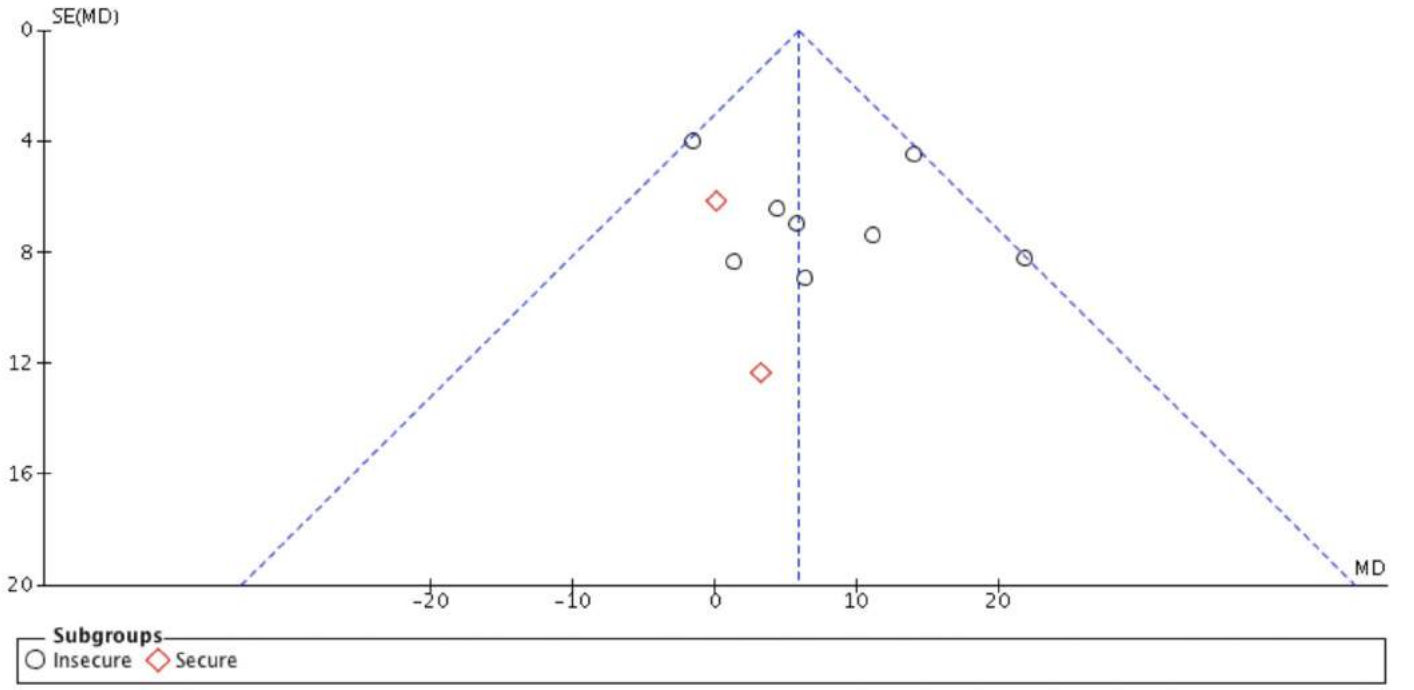
A) Total Cholesterol



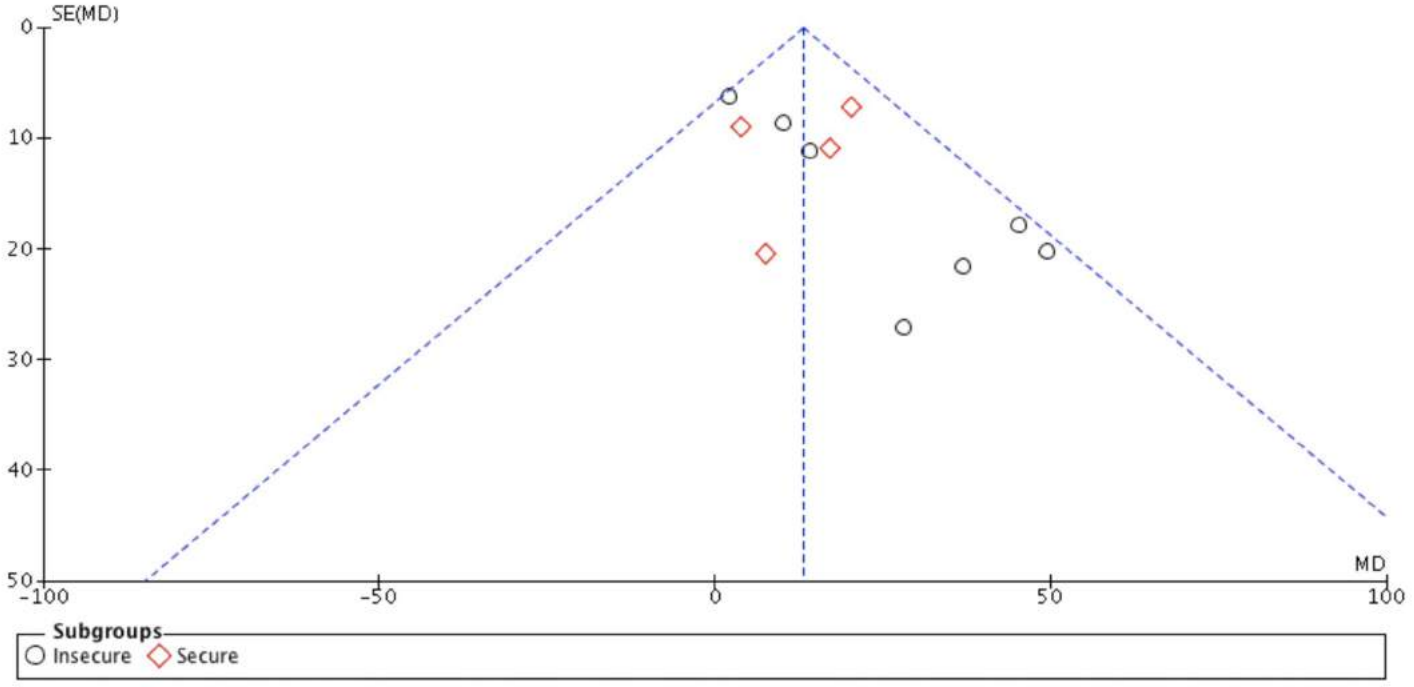
B) HDL



C) LDL



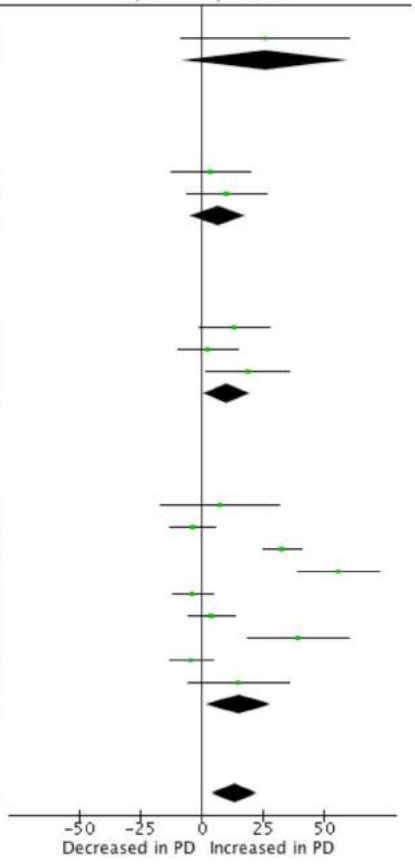
D) Triglycerides



Appendix 3. Funnel Plot for comparison: periodontitis vs. non-periodontitis in studies with Insecure and Secure diagnoses for PD after leave-one-out analysis. Outcomes: (**A**) Total Cholesterol; (**B**) HDL; (**C**) LDL; (**D**) Triglycerides.

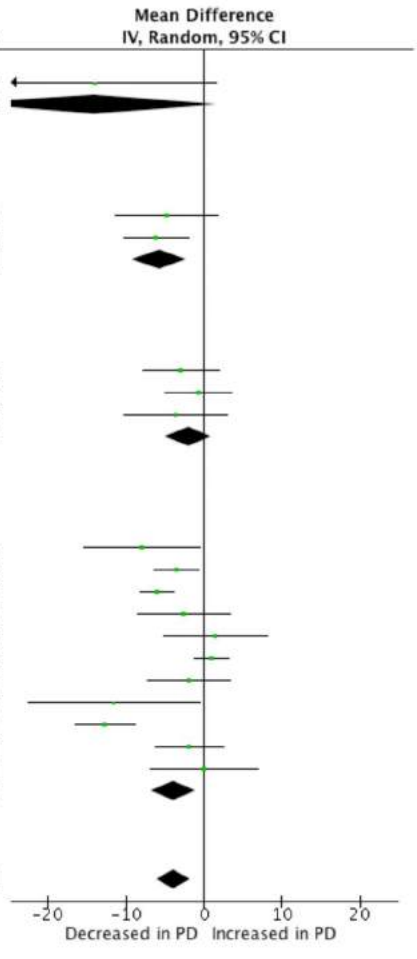
A) Total Cholesterol

Study or Subgroup	Periodontal Disease Mean	SD	Total	Control Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
North America								
Cutler et al. 1999	198	21.4	7	172	38	6	3.9%	26.00 [-8.29, 60.29]
Subtotal (95% CI)			**7**			**6**	**3.9%**	**26.00 [-8.29, 60.29]**
Heterogeneity: Not applicable								
Test for overall effect: Z = 1.49 (P = 0.14)								
South America								
Leite et al. 2014	176.21	30.07	28	172.7	30.93	27	6.6%	3.51 [-12.62, 19.64]
Monteiro et al. 2009	202.41	45.77	40	192.35	27.69	40	6.6%	10.06 [-6.52, 26.64]
Subtotal (95% CI)			**68**			**67**	**13.2%**	**6.70 [-4.87, 18.26]**
Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 0.31$, df = 1 (P = 0.58); $I^2 = 0\%$								
Test for overall effect: Z = 1.14 (P = 0.26)								
Europe								
Andrukhov et al. 2013	219	37.74	89	205.6	44.83	54	6.9%	13.40 [-0.90, 27.70]
Fentoglu et al. 2015	178.3	25.36	37	175.96	28.26	37	7.3%	2.34 [-9.89, 14.57]
Lösche et al. 2000	243.6	37.3	39	224.8	39.1	40	6.5%	18.80 [1.95, 35.65]
Subtotal (95% CI)			**165**			**131**	**20.7%**	**10.26 [0.57, 19.94]**
Heterogeneity: $Tau^2 = 20.54$; $Chi^2 = 2.77$, df = 2 (P = 0.25); $I^2 = 28\%$								
Test for overall effect: Z = 2.08 (P = 0.04)								
Asia								
Akalin et al. 2008	176.47	41.68	17	169.12	28.99	17	5.3%	7.35 [-16.78, 31.48]
Banu et al. 2014	167.5	17.9	40	171.1	16.85	20	7.7%	-3.60 [-12.84, 5.64]
Dhotre et al. 2011	222.7	35.46	100	190	19.1	100	7.9%	32.70 [24.81, 40.59]
Hagh et al. 2014	218.11	29.77	45	162.31	48.24	45	6.6%	55.80 [39.24, 72.36]
Joseph et al. 2011	163.93	27.61	85	167.73	28.81	91	7.8%	-3.80 [-12.14, 4.54]
Liu et al. 2010	123.74	27.07	40	119.88	15.47	40	7.6%	3.86 [-5.80, 13.52]
Penumarthy et al. 2013	208.03	52.82	30	168.7	22.46	30	5.9%	39.33 [18.79, 59.87]
Pushparani et al. 2014	180.7	45.22	150	185	31.57	150	7.7%	-4.30 [-13.13, 4.53]
Taleghani et al. 2010	207.3	42.1	26	192.3	34.6	26	5.8%	15.00 [-5.95, 35.95]
Subtotal (95% CI)			**533**			**519**	**62.3%**	**15.04 [1.49, 28.60]**
Heterogeneity: $Tau^2 = 374.74$; $Chi^2 = 99.73$, df = 8 (P < 0.00001); $I^2 = 92\%$								
Test for overall effect: Z = 2.18 (P = 0.03)								
Total (95% CI)			**773**			**723**	**100.0%**	**13.41 [4.22, 22.59]**
Heterogeneity: $Tau^2 = 263.46$; $Chi^2 = 103.95$, df = 14 (P < 0.00001); $I^2 = 87\%$								
Test for overall effect: Z = 2.86 (P = 0.004)								
Test for subgroup differences: $Chi^2 = 1.63$, df = 3 (P = 0.65), $I^2 = 0\%$								

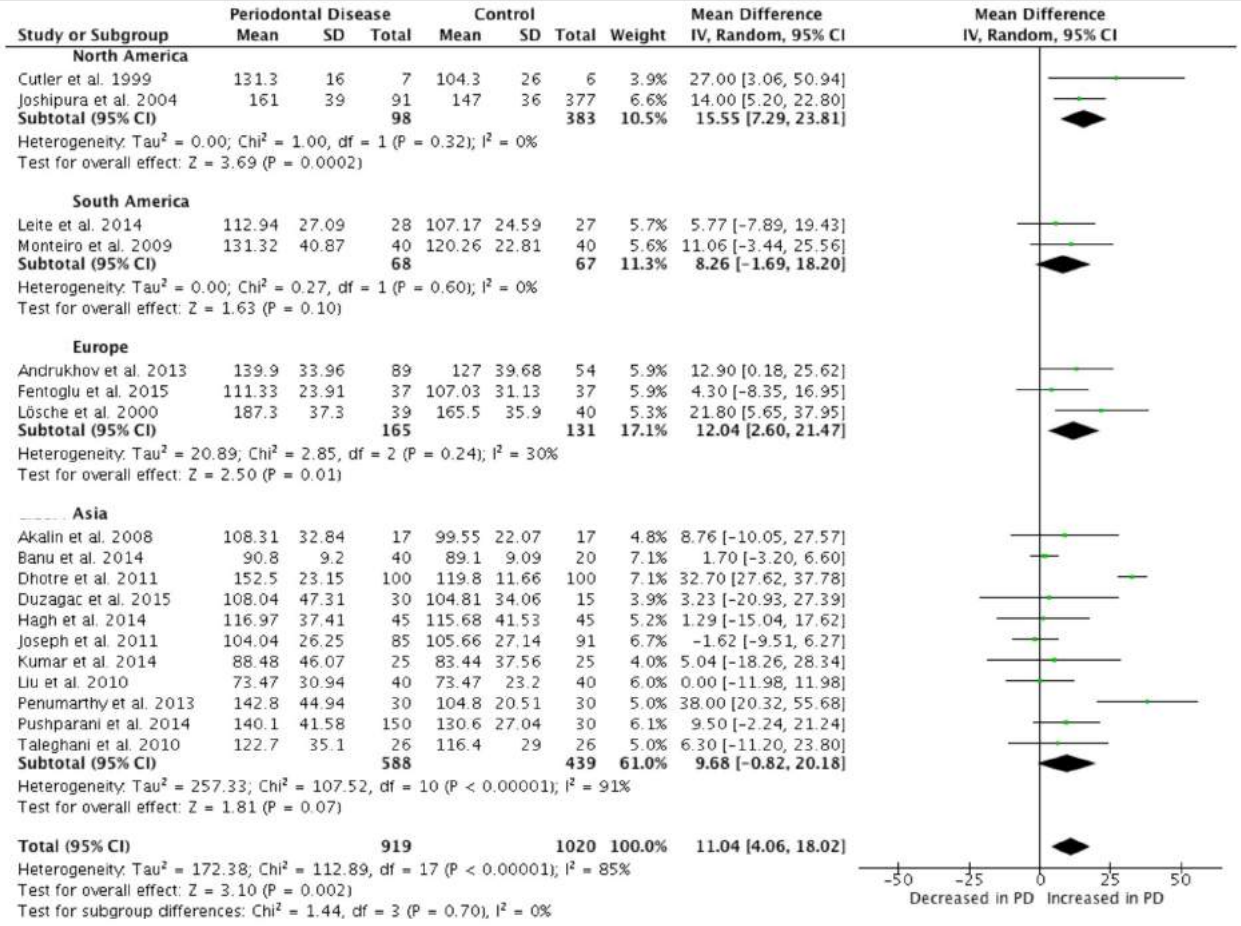


B) HDL

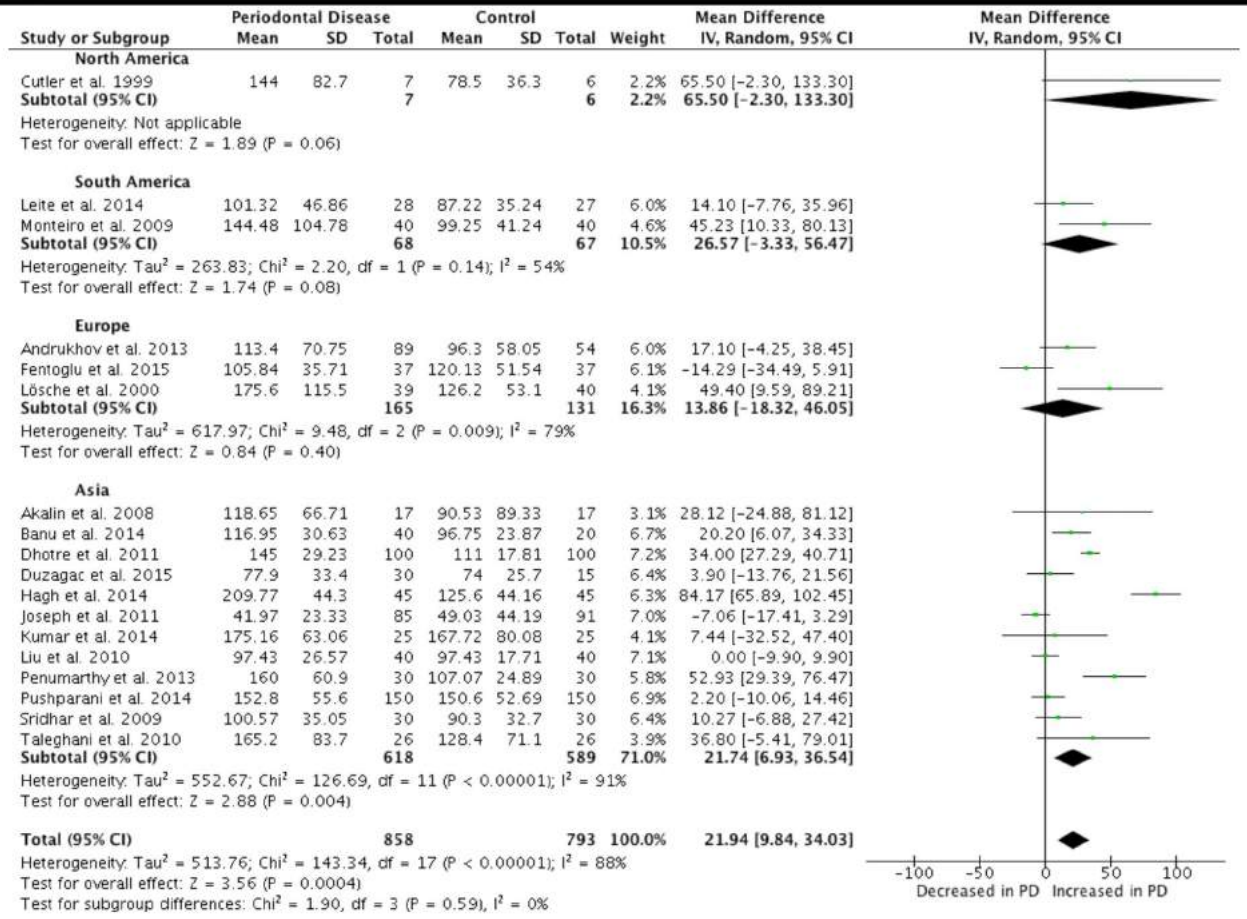
Study or Subgroup	Periodontal Disease Mean	SD	Total	Control Mean	SD	Total	Weight	Mean Difference IV, Random, 95% CI
North America								
Cutler et al. 1999	38	7.9	7	52	18	6	1.5%	-14.00 [-29.55, 1.55]
Subtotal (95% CI)			**7**			**6**	**1.5%**	**-14.00 [-29.55, 1.55]**
Heterogeneity: Not applicable								
Test for overall effect: Z = 1.77 (P = 0.08)								
South America								
Leite et al. 2014	42.82	12.6	28	47.56	12.28	27	5.0%	-4.74 [-11.32, 1.84]
Monteiro et al. 2009	47.48	7.97	40	53.62	10.68	40	7.1%	-6.14 [-10.27, -2.01]
Subtotal (95% CI)			**68**			**67**	**12.0%**	**-5.74 [-9.24, -2.25]**
Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 0.12$, df = 1 (P = 0.72); $I^2 = 0\%$								
Test for overall effect: Z = 3.22 (P = 0.001)								
Europe								
Andrukhov et al. 2013	56.3	15.09	89	59.3	13.96	54	6.4%	-3.00 [-7.87, 1.87]
Fentoglu et al. 2015	43.34	9.67	37	43.99	8.99	37	6.9%	-0.65 [-4.90, 3.60]
Lösche et al. 2000	52.2	11.7	39	55.8	17.8	40	4.9%	-3.60 [-10.23, 3.03]
Subtotal (95% CI)			**165**			**131**	**18.2%**	**-2.03 [-4.92, 0.85]**
Heterogeneity: $Tau^2 = 0.00$; $Chi^2 = 0.77$, df = 2 (P = 0.68); $I^2 = 0\%$								
Test for overall effect: Z = 1.38 (P = 0.17)								
Asia								
Akalin et al. 2008	45.18	11.84	17	53.18	10.32	17	4.4%	-8.00 [-15.47, -0.53]
Banu et al. 2014	40.1	4.48	40	43.65	5.74	20	8.2%	-3.55 [-6.42, -0.68]
Dhotre et al. 2011	40	7.28	100	46	7.82	100	8.8%	-6.00 [-8.09, -3.91]
Duzagac et al. 2015	51.9	10.7	30	54.5	8.9	15	5.5%	-2.60 [-8.51, 3.31]
Hagh et al. 2014	55.24	14.05	45	53.75	17.87	45	4.9%	1.49 [-5.15, 8.13]
Joseph et al. 2011	41.56	7.95	85	40.62	6.63	91	8.8%	0.94 [-1.23, 3.11]
Kumar et al. 2014	36.75	9.93	25	38.66	9.35	25	6.0%	-1.91 [-7.26, 3.44]
Liu et al. 2010	42.54	27.07	40	54.14	23.2	40	2.6%	-11.60 [-22.65, -0.55]
Penumarthy et al. 2013	33.27	5.82	30	46.03	8.99	30	7.3%	-12.76 [-16.59, -8.93]
Sridhar et al. 2009	39.2	7.19	30	41.07	9.52	30	6.9%	-1.87 [-6.14, 2.40]
Taleghani et al. 2010	48.8	12.6	26	48.8	12.6	26	4.8%	0.00 [-6.85, 6.85]
Subtotal (95% CI)			**468**			**439**	**68.2%**	**-3.89 [-6.74, -1.04]**
Heterogeneity: $Tau^2 = 16.20$; $Chi^2 = 51.58$, df = 10 (P < 0.00001); $I^2 = 81\%$								
Test for overall effect: Z = 2.68 (P = 0.007)								
Total (95% CI)			**708**			**643**	**100.0%**	**-3.94 [-6.04, -1.84]**
Heterogeneity: $Tau^2 = 11.86$; $Chi^2 = 56.78$, df = 16 (P < 0.00001); $I^2 = 72\%$								
Test for overall effect: Z = 3.67 (P = 0.0002)								
Test for subgroup differences: $Chi^2 = 4.29$, df = 3 (P = 0.23), $I^2 = 30.0\%$								



C) LDL



D) Triglycerides



Appendix 5. Forest plot of Mean Difference (MD) for comparison: periodontitis vs. non-periodontitis in studies according to the continent where the research was conducted. Outcomes: (**A**) Total Cholesterol; (**B**) HDL; (**C**) LDL; (**D**) Triglycerides.

3.2 Capítulo 2*

Lack of association *of Low-Density Lipoprotein Receptor and Apolipoprotein B* genes polymorphisms with chronic periodontitis in the Brazilian Population

Running title: *LDLR* and *APOB* polymorphisms in periodontitis

Keywords: Periodontal Disease, Genetic risk, Single Nucleotide Polymorphism, Low density lipoprotein receptor, Apolipoprotein B

Rafael Nepomuceno[1], Thamiris Cirelli[1], Silvana R P Orrico[1], Joni A Cirelli[1], Carlos Rossa Jr[1], Kari E. North[2,3], Mariaelisa Graff[2,4], Silvana P Barros[5], Steven Offenbacher[5], Raquel M Scarel-Caminaga[6]

1. Department of Diagnosis and Surgery, São Paulo State University – UNESP, School of Dentistry at Araraquara, Araraquara, SP, Brazil
2. Department of Epidemiology, University of North Carolina at Chapel Hill - UNC, School of Public Health, Chapel Hill, NC, USA
3. Carolina Population Center, University of North Carolina at Chapel Hill - UNC, Chapel Hill, NC, USA
4. Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill - UNC, School of Public Health, Chapel Hill, NC, USA
5. Department of Periodontology, University of North Carolina at Chapel Hill – UNC, School of Dentistry, Chapel Hill, NC, USA
6. Department of Morphology, São Paulo State University – UNESP, School of Dentistry at Araraquara, Araraquara, SP, Brazil

Correspondence:

Raquel Mantuaneli Scarel-Caminaga, PhD

Department of Morphology, School of Dentistry at Araraquara, UNESP – São Paulo State University, CP 331, CEP 14801-903, Araraquara, SP, Brazil, E-mail: raquel@foar.unesp.br

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ABSTRACT

Aim: Since chronic periodontitis (CP) is associated with higher LDL plasma levels, and polymorphisms in the *Apolipoprotein B* (*APOB*) and *Low-Density Lipoprotein Receptor* (*LDLR*) genes may increase plasma LDL concentrations, the present study aimed to assess whether polymorphisms in those genes (rs5925, rs688, rs676210, rs693) contribute to the susceptibility for periodontal disease.

Materials and Methods: Genomic DNA was obtained from No/mild CP group (n=356) and moderate/severe CP group (n=358). Genotyping of polymorphisms was performed using real-time polymerase chain reaction by OpenArray® platform. Multinomial logistic regression was used to determine the degree of association between polymorphisms and periodontal status adjusted for known confounders. Multiplicative and additive interactions between the evaluated single nucleotide polymorphisms (SNPs) under study and smoking were developed with regard to the risk of CP. The linkage disequilibrium was calculated for the multi-locus polymorphisms studied and the associations between the haplotypes and CP were tested by calculating the multiple logistic regression.

Results: No significant association was verified between the clinical periodontal parameters and genotype frequencies of any of the evaluated SNPs. In the multivariate model, there were no significant differences in the genotype frequencies for all SNPs between the control and CP groups. The multiplicative and additive interactions between each SNPs and smoking were not statistically significant. Moreover, none of the haplotypes in *APOB* and *LDLR* genes were significantly related to the risk of periodontal disease.

Conclusions: None of the individual SNPs in the *LDLR* and *APOB* genes, or their haplotypes, was found to be statistically significantly associated with CP in the investigated Brazilian population.

1. Introduction

In recent years, the chronic periodontitis (CP) has been receiving increased attention, since it not only impairs the general state of oral health, but also appears to have a relationship with a variety of systemic diseases, including the metabolic syndrome (D'Aiuto et al., 2008, Bullon et al., 2009) and diabetes mellitus type 2 (T2DM) (Graves et al., 2004, Mealey et al., 2006, Susanto et al., 2011). Motivated by this bidirectional relationship between CP and T2DM, some researchers have investigated the influence of polymorphisms in genes associated with T2DM and glycemic metabolism in patients with periodontitis (Wu et al., 2015, Folwaczny et al., 2011, Holla et al., 2001, Wang et al., 2013).

During the past decade, an enormous amount of literature has emerged linking CP with atherosclerosis. The correlation between periodontitis and atherosclerosis is substantiated by meta-analyzes (Mustapha et al., 2007, Paraskevas et al., 2008), and 80% of cross-sectional, longitudinal and case-control studies reported associations between CP and measures of atherosclerosis / vascular disease (Offenbacher and Beck, 2005).

Some evidences demonstrated that associations between CP and atherosclerosis are strongest with subclinical measures of atherosclerosis (Mustapha et al., 2007). Dyslipidemia, the abnormality in levels of triglycerides, total cholesterol, or lipoproteins in the plasma, is a hallmark of atherosclerosis, leading to cardiovascular disease (Griffiths and Barbour, 2010). However, polymorphisms in genes associated with dyslipidemia have not been studied in patients with CP.

Recently, Nepomuceno et al. (Nepomuceno et al., 2017) concluded by meta-analysis comprising 19 publications, that patients with CP present significantly higher serum levels of low-density lipoprotein (LDL) and triglycerides ($p = 0.003$ and $p < .0001$, respectively), and lower high-density lipoprotein (HDL) serum levels ($p = 0.0005$) than healthy subjects. These results support the rationale that periodontal disease is associated with lipid metabolic control. Moreover, high levels of free fatty acids such as LDL were previously associated with the action of the pro-inflammatory cytokines TNF and IL-1 induced by microorganisms/endotoxins (Fentoglu and Bozkurt, 2008, Griffiths and Barbour, 2010). The release of pro-inflammatory cytokines and interleukin is believed to compromise tissue response and affect wound healing, thereby increasing the susceptibility to periodontitis (Stashenko et al., 1991).

Apolipoprotein B (ApoB) is scaffold of LDL particles that are the major natural transporter of cholesterol and phospholipids, acting as a constant supply of cholesterol for peripheral tissues and cells (Oram and Heinecke, 2005). ApoB contains multiple lipid-

associating regions to adopt the required structure for binding to the LDL receptor (LDLR) (Chen et al., 1988, Fernandez-Higuero et al., 2015). Several polymorphisms in human *APOB* and *LDLR* loci have been described (Wojczynski et al., 2010, Ye et al., 2014, Kathiresan et al., 2009, Lu et al., 2010). It has been suggested that some of these variants (rs688, rs5925, rs693, rs676210) might effectively change the total and LDL cholesterol levels and frequently develop early coronary heart disease (CHD) (Lee et al., 2012, Xiao et al., 2017).

Since CP is associated with higher LDL plasma levels (Nepomuceno et al., 2017), and it has been reported that polymorphisms in the *APOB* and *LDLR* genes may increase plasma LDL concentrations, the present study aimed to assess whether these polymorphisms contribute to the susceptibility for CP.

2. Material and Methods

2.1. Study subjects, sample collection and DNA extraction

Three hundred and fifty-eight patients diagnosed with moderate or severe CP and 356 (no/mild = control) participants were recruited through random selection from the Department of Periodontology, School of Dentistry at Araraquara, São Paulo State University (UNESP), Brazil. This study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1983, and was approved by the Ethics in Human Research Committee of the Araraquara School of Dentistry (UNESP; Protocol number 50/06). All the patients gave written informed consent before any study related procedure.

The study participants were at least 30 years old and genetically unrelated. Participants were excluded from the study if they had chronic or systemic diseases (i.e. T2DM, HIV infection, or immunosuppressive chemotherapy), if they had received systemic antibiotic treatments in the previous 3 months, if they had received periodontal treatment in the previous 6 months, if they have less then 10 teeth (excluding third molars) or if they were pregnant or lactating. For both cases and controls, smoking status were confirmed by a questionnaire. A history of smoking was defined as having smoked at least once per day for at least 1 year (Miyake et al., 2014).

The patients were diagnosed based on the criteria defined by the CDC / AAP (Centers for Disease Control and Prevention / American Academy of Periodontology) (Page and Eke, 2007) and they were divided into two groups: Group A (n=356), patients with mild periodontitis and healthy patients; Group B (n=358), patient with moderate or severe periodontitis.

The clinical periodontal parameters were assessed by a University of North Carolina-15

periodontal probe at four sites (interproximal sites) around each tooth of the participants: interproximal probing pocket depth (PPDi) and interproximal clinical attachment level (CALi). Moreover, bleeding on probing (BOP), visible plaque and marginal bleeding were registered. All fully erupted teeth, except third molars and retained roots were examined, and buccal epithelial samples were collected for laboratory analysis.

The genomic DNA from the buccal epithelial cells was extracted using standard protocols involving proteinase K digestion, 8M ammonium acetate and subsequent ethanol precipitation. The genomic DNA was quantified by NanoDrop® 2000 spectrophotometer (Thermo Scientific) and a Qubit® 2.0 fluorometer (Invitrogen). The samples showing $A_{260/280}$ ratio between 1.7 and 2.0 and a concentration of 50 ng/μL were selected.

2.2. Polymerase Chain Reaction (PCR) Amplification and SNP Genotyping

A total of 4 polymorphisms (rs688, rs5925, rs693, rs676210) were selected in two key genes associated with dyslipidemia: *APOB* and *LDLR*. We included all single-nucleotide polymorphisms (SNPs) with Hardy–Weinberg equilibrium for controls > 0.05 and minor allele frequency (MAF) > 0.05 according to the manufacturer's standard protocols.

Genotyping was conducted utilizing 2.5 μL of each individual sample of DNA (total of 125 ng of genomic DNA) to be analyzed in a well of a 384-well plate, together with 2.5 μL of 2X TaqMan®OpenArray® Genotyping Master Mix, according to the manufacturer's standard protocols, using the QuantStudio 12K Flex Real-Time PCR System. The genotyping analysis was made with Applied Biosystems™ Analysis Software, Genotyping Analysis Module, version 3.2. (available at: Thermo Fisher Cloud) using autocalling as the call method. The quality value of the data points genotype was determined by a threshold above 95%. To ensure quality control, genotyping was performed without the knowledge of individual case–control status.

2.3. Statistical analysis

Statistical analyses were performed using the PLINK software (Purcell et al., 2007) to detect differences in distributions for each SNP between groups. All other analyses were performed by STATA software, version 12.0 for Mac (Statistics/Data Analysis, Stata Corporation, College Station, Tex., USA).

Demographic data were expressed as the mean ± SD for numerical data and as frequency

distributions for categorical data. Differences in the frequencies of demographic factors were tested using the chi-squared or Student's t analysis. Kruskal-Wallis test was used to assess the association between periodontal parameters (number of teeth, visible plaque, marginal bleeding, bleeding on probing, interproximal periodontal pockets depth and interproximal clinical attachment loss) and individual genotype of each SNP independently.

We tested for Hardy–Weinberg equilibrium with the chi-squared goodness-of-fit for biallelic markers test. The association between genotypes and periodontal disease was computed using logistic regression. A multivariate logistic regression analysis was used to adjust for potential confounding factors, such as age, sex and smoking status. The effect size was expressed as an odds ratio (OR) with a 95% confidence interval (CI). Experiment-wise significance (*p-value* = 0.0125) was determined by Bonferroni correction based on the total number of markers genotyped of the study.

We examined multiplicative and additive interactions between the SNPs under study and smoking with regard to the risk of CP. A more-than-multiplicative gene–environment interaction was evaluated using logistic regression analysis (i.e. (Case/Control * Covariates + SNP1 + Smoking + SNP1×Smoking). For the additive interaction, three measures and their 95% CIs were calculated: 1) Relative Excess Risk Due to Interaction (RERI); 2) Attributable Proportion due to interaction (AP); and 3) Synergy index (S). RERI is the excess risk due to an interaction relative to the risk without exposure. AP refers to the attributable proportion of disease among individuals exposed to both factors that is due to the factors' interaction. S is the excess risk from both exposures when there is an additive interaction, relative to the risk from both exposures without an interaction (Andersson et al., 2005). RERI =0, AP =0, and S =1 indicate no interaction or strict additivity, whereas RERI >0, AP >0, or S >1 indicates synergistic interaction or more than additivity; and RERI < 0, AP < 0 or S < 1 means negative interaction or less than additivity (Knol et al., 2011). If 95% CI of any of these measures did not include the null values (0 for RERI/AP or 1 for S), the additive interaction was considered statistically significant (Andersson et al., 2005).

The linkage disequilibrium (LD) coefficients r^2 and D' were then calculated for the multi-locus polymorphisms studied in *LDLR* and *APOB* to determine any co-segregation. The associations between the haplotypes and CP were tested by calculating the logistic regression (adjustments) statistic and the corresponding *p-values* and odds ratios (ORs) with 95% confidence intervals (CIs).

3. Results

The characteristics of cases and controls are shown in Table 1. The investigated population was composed mainly of female and non-smoker subjects. There were no differences between cases and control subjects regarding sex.

Among our control subjects, the genetic distributions of SNPs rs5925, rs688, rs676210 and rs693 did not deviate from the Hardy–Weinberg equilibrium (p = 0.74, 0.42, 0.05 and 0.14, respectively) (Table 2). Kruskal-Wallis test revealed no significant association between the clinical periodontal parameters and genotype frequencies of any of the evaluated SNPs (Table 3). In the multivariate model, compared with the control group, there were no significant differences in the genotype frequencies between all four investigated SNPs and periodontal disease after adjusting for age, sex and smoking (Table 4).

Compared with subjects with the TT genotype of the rs5925 SNP who had never smoked, those with the TC or CC genotype who had ever smoked had a significantly increased risk of periodontal disease: the adjusted OR was 1.61 (95% CI: 1.01 – 2.58); nevertheless, neither multiplicative nor additive interaction was significant (Table 5). Likewise, subjects carrying the CT or TT genotype of the rs688 SNP who had ever smoked had a significantly increased risk of periodontal disease: the adjusted OR was 1.61 (95% CI: 1.01 – 2.59). However, the multiplicative and additive interactions between the rs688 SNP and smoking were not statistically significant. Subjects who had ever smoked, independently from the genotype of rs676210 and rs693 SNPs had a significantly increased risk of periodontal disease (rs676210 GG genotype, OR: 1.67, CI: 1.09 - 2.56; rs676210 AG or AA genotype, OR: 2.24, CI: 1.24 - 4.05; rs693 CC genotype, OR: 2.16, CI: 1.20 - 3.88; rs693 CT or TT genotype, OR: 1.66, CI: 1.04 - 2.64). No multiplicative or additive interactions were observed between the two SNPs and smoking.

Of the two SNP pairs, both were in strong linkage disequilibrium: D’ between rs676210 and rs693 (*APOB* gene), D’ between rs688 and rs5925 (*LDLR* gene) were 1.00 and 0.997, respectively (Table 6). Given that haplotype order for the *LDLR* gene was rs688 and rs5925, the CT, TC and CC haplotypes were identified after haplotypes with a frequency of <1% in either cases or control subjects were excluded. Regarding the *APOB* gene, the haplotypes with frequency > 1% were GC, GT and AC, considering that the SNPs order was rs676210 and rs693 (Table 7). None of these were significantly related to the risk of periodontal disease.

4. Discussion

To our knowledge, the present study is the first to investigate the lipid metabolic important rs688, rs5925 SNPs in the *LDLR* gene and rs693 and rs676210 SNPs in the *APOB* gene in relation to CP. Indeed, these SNPs were not ever investigated in the Brazilian population with CP. Contradicting our hypothesis, we demonstrated here that none of the four SNPs associated to dyslipidemia were statistically significantly associated with the Brazilian patients affected by CP.

There is a large body of literature in candidate-gene studies investigating the genetics of host inflammatory response, inflammatory mediators, and cytokines in periodontitis (Zhang et al., 2011). The LDLR plays a key role in the regulation of cholesterol metabolism by removing excess of LDL in blood (Dammerman and Breslow, 1995). This receptor is particularly abundant in the liver, the organ responsible for removing most excess of cholesterol from the body (Li et al., 2001). *LDLR* gene mutations can lead to deficiency or abnormality of LDLR in the cell membrane surface and thus disrupt lipid metabolism (Zhang et al., 2016). *LDLR* gene mutations are known to cause familial hypercholesterolemia (FH) (Brown and Goldstein, 1974, Alonso et al., 2008), which is a common genetic disorder characterized by elevated circulating LDL (Hobbs et al., 1992). Moreover, recent genome-wide association studies (GWASs) showed that *LDLR* gene mutations were significantly associated with the abnormal blood lipid levels (Kathiresan et al., 2009, Sandhu et al., 2008, Waterworth et al., 2010, Sanna et al., 2011).

The rs5925 and rs688 SNPs in the *LDLR* gene were predicted to be silent mutations due to the preservation of the codon sense. In a study with 3575 subjects from the Netherlands, 23 SNPs (including the rs688 and rs5925) were found to be significantly associated with total cholesterol levels after adjustment for age, sex, current smoking status, alcohol consumption and body mass index (BMI) (Lu et al., 2010).

The rs688 SNP, located in the coding region of the *LDLR* gene, is suggested to produce a functional effect on the plasma LDL level in 5414 European, 7616 Asian and 1936 Canadian study subjects (Park et al., 2011, Kathiresan et al., 2008a, Boright et al., 1998). The SNP rs688 was also identified associated with triglycerides and glycated hemoglobin (HbA1C) (Park et al., 2011), representing an additional risk factor for diabetes and atherosclerosis. Additionally, the rs688 was associated with increased plasmatic LDL in pre-menopausal women (Zhu et al., 2007), increased Alzheimer's disease risk in men (Zou et al., 2008) and coronary artery disease risk (Martinelli et al., 2010). Regarding the rs5925 SNP in the *LDLR* gene, it was associated with blood total cholesterol and LDL levels in Hispanic women (Ahn et al., 1994). In a Mexican

population, the rs5925 showed association with low triglycerides levels in hypertensive patients and hyperlipidemia subgroup, in a multiple linear regression model (Rios-Gonzalez et al., 2014). Furthermore, the same *LDLR* SNP may be strongly involved in the pathogenesis of cerebral infarction (Yan et al., 2014). A specific *LDLR* haplotype consisting of the rs5925 and other two SNPs was overrepresented in women affected with Alzheimer's disease when compared to matched normal controls (Lamsa et al., 2008).

Besides the analyses of individual SNPs, we carried out the haplotype analysis of the tested two SNPs of each gene between CP patients and controls. The haplotype analysis is likely to continue to play an important role in genetic epidemiology studies because it effectively captures both the joint marker correlations and the evolutionary history (Schaid, 2004). However, the present haplotype analysis showed no significant association with CP. In a Taiwanese population, the haplotype TC formed by the rs688 and rs5925 was associated with 65% increased risk of overall ischemic stroke, 72% increased risk of atherothrombotic stroke, and 70% increased risk of lacunar infarction; indicating a synergistic effect between these 2 SNPs (Lee et al., 2012). In spite of the previous association of the rs688 and/or the rs5925 SNPs with diabetes, dyslipidemia and cardiovascular disease, that increased their potential as candidate for CP, we did not find any significant association of those SNPs, or their haplotypes, with the CP.

Apolipoproteins are the structural components of lipoprotein particles, such as LDL, and serve as cofactors for lipid-metabolizing enzymes and ligands for lipoprotein receptors, such as LDLR (Zaman et al., 1997). ApoB is necessary for the cellular uptake and catabolism of LDL by the LDL receptor. Polymorphisms in the *APOB* gene have been associated with the variability of serum cholesterol levels and coronary atherosclerosis in some populations (Rajput-Williams et al., 1988). The rs676210 SNP is a missense mutation involving A to G substitution that results in the conversion of proline to leucine, also named as Pro2739Leu. The rare allele at the rs676210 SNP was significantly associated with the increased triglyceride-lowering effects of fenofibrate, commonly drug used to treat hypertriglyceridemia (Wojczynski et al., 2010), in reduction of LDL and triglycerides levels in Europeans (Barbosa et al., 2012), and associated by GWAS with circulating oxidized LDL (oxLDL) (Makela et al., 2013). The haplotype "TGAG" in *APOB* constructed by the two SNPs in this present study and other two (rs1042034 and rs673548) was associated with increased ischemic stroke risk (OR = 1.583; 95 % CI = 1.045 - 2.397; p = 0.031) (Xiao et al., 2017). The rs693 SNP lies in the coding region of the *APOB* gene. *APOB* rs693 consists of a silent cytosine to thymine substitution at

nucleotide 7673 (Au et al., 2017). The rs693 SNP was associated with decreased LDL and triglycerides levels and increased HDL in Asian and European subjects (Park et al., 2011, Kathiresan et al., 2008a, Barbosa et al., 2012). Obesity and coronary disease were associated with haplotypes involving the rs693 in men (Rajput-Williams et al., 1988). The same SNP was also significantly associated with the CVD risk factors waist circumference, waist-to-hip ratio and Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) (Park et al., 2011); and ischemic stroke (Xiao et al., 2017). The more important results about this SNP were three different GWAS. In two GWAS studies, the SNP rs693 was associated with reduction of LDL in Northern Finnish and European ancestry (Sabatti et al., 2009, Kathiresan et al., 2008b); and one GWAS, the SNP was associated with reduction total cholesterol in 16 European population cohorts (Aulchenko et al., 2009).

Incubation of whole blood with the oral pathogen *Porphyromonas gingivalis* results in proteolysis and degradation of ApoB in LDL (Bengtsson et al., 2008, Lonn et al., 2018). Other investigators have also demonstrated degradation of ApoB by intact *Porphyromonas gingivalis*, its lipopolysaccharides (LPS), or outer membrane vesicles from the organism (Miyakawa et al., 2004). Interestingly, the LDLR (which binds ApoB) in the liver does not recognize ApoB-modified LDL (e.g., produced by gingipains of *Porphyromonas gingivalis*), thereby resulting in the accumulation of LDL in the circulation (Lonn et al., 2018). LDL modification induced by selective degradation of the ApoB protein by *Porphyromonas gingivalis* results in aggregated lipid particles that can be taken up by macrophages to form foam cells and consequently accelerates the progression of atherosclerosis in mice fed with a high-fat diet (Hashimoto et al., 2006).

ApoB, the major protein component in plasma LDL, is present in gingival crevicular fluid (GCF) (Sakiyama et al., 2010). Besides that, the levels of ApoB and oxLDL in GCF are higher at the sites with periodontitis than at the healthy sites (Ishizuka et al., 2017); as well as in patients with T2DM than normoglycemic subjects (Noguchi et al., 2014). The levels of ApoB and oxLDL at periodontal sites decreased after scaling and root planning (Ishizuka et al., 2017).

In the present study, no statistically significant association between the polymorphisms in the candidate genes *LDLR* or *APOB* and CP susceptibility was observed. As a complex disease, the genetic susceptibility of CP may be determined by a number of genes that may interact with each other, with none of them demonstrating a dominant effect on CP susceptibility (Ioannidis et al., 2001). In addition, the gene–systemic diseases interactions may play an important role in CP, although the present study did not have data regarding the lipid

profile of patients. Altogether, although we investigated four SNPs in two genes highest related with altered lipid metabolism, other SNPs located in other regions of the *LDLR* and *APOB* genes could be related with CP. A better option is to verify more SNPs in the genes using tag SNPs, since it reduces the expense and time of mapping genome areas associated with disease, since it eliminates the need to study every individual SNP (Mahdevar et al., 2010).

The study of the interactions between host genetic factors and oral microbial colonization, termed "periodontal infectogenomics" (Nibali et al., 2011), is an important emerging area of research. Two GWAS for host genetic markers showed that there are SNPs in different populations associated with specific colonization with periodontal bacteria (Rhodin et al., 2014, Divaris et al., 2012). As observed in several studies, the *LDLR* and *APOB* genes are more associated with the presence of periodontopathogenic microorganisms, such as *Porphyromonas gingivalis* than the presence or absence of CP. Perhaps, if we had information on the levels of bacteria in health and CP sites of subjects, we could find association of the 4 studied SNPs, contributing with the periodontal infectogenomics.

Alternatively, the periodontal disease can be actually a group of distinct conditions with similar and overlapping clinical presentations. A new investigation defined the disease phenotype as complex traits using principal component analyses (PCA) to identify genetic determinants (Offenbacher et al., 2016). This new approach used clinical data combined with the levels of eight classic periodontal pathogens and gingival crevicular fluid (GCF)–IL-1b to derive six periodontal complex traits (PCTs). The intent is to identify loci related to the biological underpinning and pathogenesis of CP and not strictly to serve as population markers for clinical CP. This new classification has identified more genes related to CP in GWAS studies when compared to the traditional American Academy of Periodontology (AAP) / American Dental Association (ADA) periodontal disease classification, as used in the present study (Page and Eke, 2007). This present study is limited by presenting only clinical information beyond the genotyping of patients.

Study of gene–environment interaction is important for improving accuracy and precision in the assessment of both genetic and environmental influences. An understanding of gene–environment interaction also has important implications for public health. It aids in predicting disease rates and provides a basis for well-informed recommendations for disease prevention (Ottman, 1996). The joint effect between two factors can be measured on either an additive or multiplicative scale. Although there is a debate as to which scale should be used, there has been general consensus that the additive scale is more appropriate for assessing the

public health importance of interactions (Chantarangsu et al., 2016). Despite gene-environment interactions seem to be an obvious topic for consideration, the analysis of such interactions is not yet routine in genetic studies (Lanktree and Hegele, 2009). There are few studies investigating the gene-environment interaction associated with chronic periodontitis, particularly with smoking (Tanaka et al., 2017, Chantarangsu et al., 2016, Tanaka et al., 2014, Ribeiro et al., 2016). Risk genotypes on SNP in vitamin D receptor gene (VDR) synergistically interacted with smoking in increasing CP susceptibility (Chantarangsu et al., 2016). To our knowledge, there is only one study of gene-environment interaction with SNPs in the *APOB* and *LDLR* genes; unfortunately, they were not the same SNPs studied in this present study (Andreotti et al., 2008). Accounting for gene-environment interactions will probably be important for future strategies of diagnosis, prognosis and management of CP. Combinations of specific genetic variants in the context of specific environmental variables - reflecting gene-environment interactions - could help to re-stratify an individual between risk strata (Lanktree and Hegele, 2009).

Further studies are suggested focusing information about the microbiome, proteome and systemic metabolic profile and fractions that could be combined to clinical data to enrich the investigation on the pathogenesis of CP. Moreover, extensive analysis of gene–gene, gene–environment and gene-systemic disease interactions using different and larger populations would be valuable to obtain a better insight into the influence of genetics in the occurrence and progression of CP.

We concluded that none of the individual SNPs rs5925 and rs688 in the *LDLR* gene, and rs676210 and rs693 in the *APOB* gene, their haplotypes or the gene interactions analyses were found to be statistically significantly associated with CP in the Brazilian population.

5. References

Ahn, Y. I., Kamboh, M. I., Aston, C. E., Ferrell, R. E. & Hamman, R. F. (1994) Role of common genetic polymorphisms in the LDL receptor gene in affecting plasma cholesterol levels in the general population. *Arterioscler Thromb* **14,** 663-670.

Alonso, R., Mata, N., Castillo, S., Fuentes, F., Saenz, P., Muniz, O., Galiana, J., Figueras, R., Diaz, J. L., Gomez-Enterria, P., Mauri, M., Piedecausa, M., Irigoyen, L., Aguado, R., Mata, P. & Spanish Familial Hypercholesterolaemia, G. (2008) Cardiovascular disease in familial hypercholesterolaemia: influence of low-density lipoprotein receptor mutation type and classic risk factors. *Atherosclerosis* **200,** 315-321. doi:10.1016/j.atherosclerosis.2007.12.024.

Andersson, T., Alfredsson, L., Kallberg, H., Zdravkovic, S. & Ahlbom, A. (2005) Calculating measures of biological interaction. *Eur J Epidemiol* **20,** 575-579.

Andreotti, G., Chen, J., Gao, Y. T., Rashid, A., Chen, B. E., Rosenberg, P., Sakoda, L. C., Deng, J., Shen, M. C., Wang, B. S., Han, T. Q., Zhang, B. H., Yeager, M., Welch, R., Chanock, S., Fraumeni, J. F., Jr. & Hsing, A. W. (2008) Polymorphisms of genes in the lipid metabolism pathway and risk of biliary tract cancers and stones: a population-based case-control study in Shanghai, China. *Cancer Epidemiol Biomarkers Prev* **17,** 525-534. doi:10.1158/1055-9965.EPI-07-2704.

Au, A., Griffiths, L. R., Irene, L., Kooi, C. W. & Wei, L. K. (2017) The impact of APOA5, APOB, APOC3 and ABCA1 gene polymorphisms on ischemic stroke: Evidence from a meta-analysis. *Atherosclerosis* **265,** 60-70. doi:10.1016/j.atherosclerosis.2017.08.003.

Aulchenko, Y. S., Ripatti, S., Lindqvist, I., Boomsma, D., Heid, I. M., Pramstaller, P. P., Penninx, B. W., Janssens, A. C., Wilson, J. F., Spector, T., Martin, N. G., Pedersen, N. L., Kyvik, K. O., Kaprio, J., Hofman, A., Freimer, N. B., Jarvelin, M. R., Gyllensten, U., Campbell, H., Rudan, I., Johansson, A., Marroni, F., Hayward, C., Vitart, V., Jonasson, I., Pattaro, C., Wright, A., Hastie, N., Pichler, I., Hicks, A. A., Falchi, M., Willemsen, G., Hottenga, J. J., de Geus, E. J., Montgomery, G. W., Whitfield, J., Magnusson, P., Saharinen, J., Perola, M., Silander, K., Isaacs, A., Sijbrands, E. J., Uitterlinden, A. G., Witteman, J. C., Oostra, B. A., Elliott, P., Ruokonen, A., Sabatti, C., Gieger, C., Meitinger, T., Kronenberg, F., Doring, A., Wichmann, H. E., Smit, J. H., McCarthy, M. I., van Duijn, C. M., Peltonen, L. & Consortium, E. (2009) Loci influencing lipid levels and coronary heart disease risk in 16 European population cohorts. *Nat Genet* **41,** 47-55. doi:10.1038/ng.269.

Barbosa, E. J., Glad, C. A., Nilsson, A. G., Filipsson Nystrom, H., Gotherstrom, G., Svensson, P. A., Vinotti, I., Bengtsson, B. A., Nilsson, S., Boguszewski, C. L. & Johannsson, G. (2012) Genotypes associated with lipid metabolism contribute to differences in serum lipid profile of GH-deficient adults before and after GH replacement therapy. *Eur J Endocrinol* **167,** 353-362. doi:10.1530/EJE-12-0263.

Bengtsson, T., Karlsson, H., Gunnarsson, P., Skoglund, C., Elison, C., Leanderson, P. & Lindahl, M. (2008) The periodontal pathogen Porphyromonas gingivalis cleaves apoB-100 and increases the expression of apoM in LDL in whole blood leading to cell proliferation. *J Intern Med* **263,** 558-571. doi:10.1111/j.1365-2796.2007.01917.x.

Boright, A. P., Connelly, P. W., Brunt, J. H., Morgan, K. & Hegele, R. A. (1998) Association and linkage of LDLR gene variation with variation in plasma low density lipoprotein cholesterol. *J Hum Genet* **43,** 153-159. doi:10.1007/s100380050060.

Brown, M. S. & Goldstein, J. L. (1974) Expression of the familial hypercholesterolemia gene in heterozygotes: mechanism for a dominant disorder in man. *Science* **185,** 61-63.

Bullon, P., Morillo, J. M., Ramirez-Tortosa, M. C., Quiles, J. L., Newman, H. N. & Battino, M. (2009) Metabolic syndrome and periodontitis: is oxidative stress a common link? *J Dent Res* **88,** 503-518. doi:10.1177/0022034509337479.

Chantarangsu, S., Sura, T., Mongkornkarn, S., Donsakul, K. & Torrungruang, K. (2016) Vitamin D Receptor Gene Polymorphism and Smoking in the Risk of Chronic Periodontitis. *J Periodontol* **87,** 1343-1351. doi:10.1902/jop.2016.160222.

Chen, P. F., Marcel, Y. L., Yang, C. Y., Gotto, A. M., Jr., Milne, R. W., Sparrow, J. T. & Chan, L. (1988) Primary sequence mapping of human apolipoprotein B-100 epitopes. Comparisons of trypsin accessibility and immunoreactivity and implication for apoB conformation. *Eur J Biochem* **175,** 111-118.

D'Aiuto, F., Sabbah, W., Netuveli, G., Donos, N., Hingorani, A. D., Deanfield, J. & Tsakos, G. (2008) Association of the metabolic syndrome with severe periodontitis in a large U.S. population-based survey. *J Clin Endocrinol Metab* **93,** 3989-3994. doi:10.1210/jc.2007-2522.

Dammerman, M. & Breslow, J. L. (1995) Genetic basis of lipoprotein disorders. *Circulation* **91,** 505-512.

Divaris, K., Monda, K. L., North, K. E., Olshan, A. F., Lange, E. M., Moss, K., Barros, S. P., Beck, J. D. & Offenbacher, S. (2012) Genome-wide association study of periodontal pathogen colonization. *J Dent Res* **91,** 21S-28S. doi:10.1177/0022034512447951.

Fentoglu, O. & Bozkurt, F. Y. (2008) The Bi-Directional Relationship between Periodontal Disease and Hyperlipidemia. *Eur J Dent* **2,** 142-146.

Fernandez-Higuero, J. A., Etxebarria, A., Benito-Vicente, A., Alves, A. C., Arrondo, J. L., Ostolaza, H., Bourbon, M. & Martin, C. (2015) Structural analysis of APOB variants, p.(Arg3527Gln), p.(Arg1164Thr) and p.(Gln4494del), causing Familial Hypercholesterolaemia provides novel insights into variant pathogenicity. *Sci Rep* **5,** 18184. doi:10.1038/srep18184.

Folwaczny, M., Manolis, V., Markus, C. & Glas, J. (2011) Variants of the human PPARG locus and the susceptibility to chronic periodontitis. *Innate Immun* **17,** 541-547. doi:10.1177/1753425910392089.

Graves, D. T., Al-Mashat, H. & Liu, R. (2004) Evidence that diabetes mellitus aggravates periodontal diseases and modifies the response to an oral pathogen in animal models. *Compend Contin Educ Dent* **25,** 38-45.

Griffiths, R. & Barbour, S. (2010) Lipoproteins and lipoprotein metabolism in periodontal disease. *Clin Lipidol* **5,** 397-411. doi:10.2217/clp.10.27.

Hashimoto, M., Kadowaki, T., Tsukuba, T. & Yamamoto, K. (2006) Selective proteolysis of apolipoprotein B-100 by Arg-gingipain mediates atherosclerosis progression accelerated by bacterial exposure. *J Biochem* **140,** 713-723. doi:10.1093/jb/mvj202.

Hobbs, H. H., Brown, M. S. & Goldstein, J. L. (1992) Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum Mutat* **1,** 445-466. doi:10.1002/humu.1380010602.

Holla, L. I., Kankova, K., Fassmann, A., Buckova, D., Halabala, T., Znojil, V. & Vanek, J. (2001) Distribution of the receptor for advanced glycation end products gene polymorphisms in patients with chronic periodontitis: a preliminary study. *J Periodontol* **72,** 1742-1746. doi:10.1902/jop.2001.72.12.1742.

Ioannidis, J. P., Ntzani, E. E., Trikalinos, T. A. & Contopoulos-Ioannidis, D. G. (2001) Replication validity of genetic association studies. *Nat Genet* **29,** 306-309. doi:10.1038/ng749.

Ishizuka, M., Kato, R., Moriya, Y., Noguchi, E., Koide, Y., Inoue, S., Itabe, H. & Yamamoto, M. (2017) Changes in apolipoprotein B and oxidized low-density lipoprotein levels in gingival crevicular fluids as a result of periodontal tissue conditions. *J Periodontal Res* **52,** 594-602. doi:10.1111/jre.12427.

Kathiresan, S., Melander, O., Anevski, D., Guiducci, C., Burtt, N. P., Roos, C., Hirschhorn, J. N., Berglund, G., Hedblad, B., Groop, L., Altshuler, D. M., Newton-Cheh, C. & Orho-

Melander, M. (2008a) Polymorphisms associated with cholesterol and risk of cardiovascular events. *N Engl J Med* **358,** 1240-1249. doi:10.1056/NEJMoa0706728.

Kathiresan, S., Melander, O., Guiducci, C., Surti, A., Burtt, N. P., Rieder, M. J., Cooper, G. M., Roos, C., Voight, B. F., Havulinna, A. S., Wahlstrand, B., Hedner, T., Corella, D., Tai, E. S., Ordovas, J. M., Berglund, G., Vartiainen, E., Jousilahti, P., Hedblad, B., Taskinen, M. R., Newton-Cheh, C., Salomaa, V., Peltonen, L., Groop, L., Altshuler, D. M. & Orho-Melander, M. (2008b) Six new loci associated with blood low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides in humans. *Nat Genet* **40,** 189-197. doi:10.1038/ng.75.

Kathiresan, S., Willer, C. J., Peloso, G. M., Demissie, S., Musunuru, K., Schadt, E. E., Kaplan, L., Bennett, D., Li, Y., Tanaka, T., Voight, B. F., Bonnycastle, L. L., Jackson, A. U., Crawford, G., Surti, A., Guiducci, C., Burtt, N. P., Parish, S., Clarke, R., Zelenika, D., Kubalanza, K. A., Morken, M. A., Scott, L. J., Stringham, H. M., Galan, P., Swift, A. J., Kuusisto, J., Bergman, R. N., Sundvall, J., Laakso, M., Ferrucci, L., Scheet, P., Sanna, S., Uda, M., Yang, Q., Lunetta, K. L., Dupuis, J., de Bakker, P. I., O'Donnell, C. J., Chambers, J. C., Kooner, J. S., Hercberg, S., Meneton, P., Lakatta, E. G., Scuteri, A., Schlessinger, D., Tuomilehto, J., Collins, F. S., Groop, L., Altshuler, D., Collins, R., Lathrop, G. M., Melander, O., Salomaa, V., Peltonen, L., Orho-Melander, M., Ordovas, J. M., Boehnke, M., Abecasis, G. R., Mohlke, K. L. & Cupples, L. A. (2009) Common variants at 30 loci contribute to polygenic dyslipidemia. *Nat Genet* **41,** 56-65. doi:10.1038/ng.291.

Knol, M. J., VanderWeele, T. J., Groenwold, R. H., Klungel, O. H., Rovers, M. M. & Grobbee, D. E. (2011) Estimating measures of interaction on an additive scale for preventive exposures. *Eur J Epidemiol* **26,** 433-438. doi:10.1007/s10654-011-9554-9.

Lamsa, R., Helisalmi, S., Herukka, S. K., Tapiola, T., Pirttila, T., Vepsalainen, S., Hiltunen, M. & Soininen, H. (2008) Genetic study evaluating LDLR polymorphisms and Alzheimer's disease. *Neurobiol Aging* **29,** 848-855. doi:10.1016/j.neurobiolaging.2006.12.009.

Lanktree, M. B. & Hegele, R. A. (2009) Gene-gene and gene-environment interactions: new insights into the prevention, detection and management of coronary artery disease. *Genome Med* **1,** 28. doi:10.1186/gm28.

Lee, J. D., Lee, T. H., Kuo, Y. W., Huang, Y. C., Hsu, H. L., Lin, Y. H., Wu, C. Y., Huang, Y. C., Lee, M. & Hsiao, K. M. (2012) Polymorphisms at the LDLR locus may be associated with ischemic cerebrovascular disease independent of lipid profile. *Curr Neurovasc Res* **9,** 200-206.

Li, Y., Cam, J. & Bu, G. (2001) Low-density lipoprotein receptor family: endocytosis and signal transduction. *Mol Neurobiol* **23,** 53-67. doi:10.1385/MN:23:1:53.

Lonn, J., Ljunggren, S., Klarstrom-Engstrom, K., Demirel, I., Bengtsson, T. & Karlsson, H. (2018) Lipoprotein modifications by gingipains of Porphyromonas gingivalis. *J Periodontal Res*. doi:10.1111/jre.12527.

Lu, Y., Feskens, E. J., Boer, J. M., Imholz, S., Verschuren, W. M., Wijmenga, C., Vaarhorst, A., Slagboom, E., Muller, M. & Dolle, M. E. (2010) Exploring genetic determinants of plasma total cholesterol levels and their predictive value in a longitudinal study. *Atherosclerosis* **213,** 200-205. doi:10.1016/j.atherosclerosis.2010.08.053.

Mahdevar, G., Zahiri, J., Sadeghi, M., Nowzari-Dalini, A. & Ahrabian, H. (2010) Tag SNP selection via a genetic algorithm. *J Biomed Inform* **43,** 800-804. doi:10.1016/j.jbi.2010.05.011.

Makela, K. M., Seppala, I., Hernesniemi, J. A., Lyytikainen, L. P., Oksala, N., Kleber, M. E., Scharnagl, H., Grammer, T. B., Baumert, J., Thorand, B., Jula, A., Hutri-Kahonen, N., Juonala, M., Laitinen, T., Laaksonen, R., Karhunen, P. J., Nikus, K. C., Nieminen, T., Laurikka, J., Kuukasjarvi, P., Tarkka, M., Viik, J., Klopp, N., Illig, T., Kettunen, J., Ahotupa, M., Viikari, J. S., Kahonen, M., Raitakari, O. T., Karakas, M., Koenig, W., Boehm, B. O., Winkelmann, B. R., Marz, W. & Lehtimaki, T. (2013) Genome-wide association study pinpoints a new functional apolipoprotein B variant influencing oxidized low-density lipoprotein levels but not cardiovascular events: AtheroRemo Consortium. *Circ Cardiovasc Genet* **6,** 73-81. doi:10.1161/CIRCGENETICS.112.964965.

Martinelli, N., Girelli, D., Lunghi, B., Pinotti, M., Marchetti, G., Malerba, G., Pignatti, P. F., Corrocher, R., Olivieri, O. & Bernardi, F. (2010) Polymorphisms at LDLR locus may be associated with coronary artery disease through modulation of coagulation factor VIII activity and independently from lipid profile. *Blood* **116,** 5688-5697. doi:10.1182/blood-2010-03-277079.

Mealey, B. L., Oates, T. W. & American Academy of, P. (2006) Diabetes mellitus and periodontal diseases. *J Periodontol* **77,** 1289-1303. doi:10.1902/jop.2006.050459.

Miyakawa, H., Honma, K., Qi, M. & Kuramitsu, H. K. (2004) Interaction of Porphyromonas gingivalis with low-density lipoproteins: implications for a role for periodontitis in atherosclerosis. *J Periodontal Res* **39,** 1-9.

Miyake, Y., Tanaka, K. & Arakawa, M. (2014) IL3 rs40401 polymorphism and interaction with smoking in risk of asthma in Japanese women: the Kyushu Okinawa Maternal and Child Health study. *Scand J Immunol* **79,** 410-414. doi:10.1111/sji.12171.

Mustapha, I. Z., Debrey, S., Oladubu, M. & Ugarte, R. (2007) Markers of systemic bacterial exposure in periodontal disease and cardiovascular disease risk: a systematic review and meta-analysis. *J Periodontol* **78,** 2289-2302. doi:10.1902/jop.2007.070140.

Nepomuceno, R., Pigossi, S. C., Finoti, L. S., Orrico, S. R. P., Cirelli, J. A., Barros, S. P., Offenbacher, S. & Scarel-Caminaga, R. M. (2017) Serum lipid levels in patients with periodontal disease: A meta-analysis and meta-regression. *J Clin Periodontol* **44,** 1192-1207. doi:10.1111/jcpe.12792.

Nibali, L., Madden, I., Franch Chillida, F., Heitz-Mayfield, L., Brett, P. & Donos, N. (2011) IL6 -174 genotype associated with Aggregatibacter actinomycetemcomitans in Indians. *Oral Dis* **17,** 232-237. doi:10.1111/j.1601-0825.2010.01731.x.

Noguchi, E., Kato, R., Ohno, K., Mitsui, A., Obama, T., Hirano, T., Itabe, H. & Yamamoto, M. (2014) The apolipoprotein B concentration in gingival crevicular fluid increases in patients with diabetes mellitus. *Clin Biochem* **47,** 67-71. doi:10.1016/j.clinbiochem.2013.09.016.

Offenbacher, S. & Beck, J. D. (2005) A perspective on the potential cardioprotective benefits of periodontal therapy. *Am Heart J* **149,** 950-954. doi:10.1016/j.ahj.2005.01.046.

Offenbacher, S., Divaris, K., Barros, S. P., Moss, K. L., Marchesan, J. T., Morelli, T., Zhang, S., Kim, S., Sun, L., Beck, J. D., Laudes, M., Munz, M., Schaefer, A. S. & North, K. E. (2016) Genome-wide association study of biologically informed periodontal complex traits offers novel insights into the genetic basis of periodontal disease. *Hum Mol Genet* **25,** 2113-2129. doi:10.1093/hmg/ddw069.

Oram, J. F. & Heinecke, J. W. (2005) ATP-binding cassette transporter A1: a cell cholesterol exporter that protects against cardiovascular disease. *Physiol Rev* **85,** 1343-1372. doi:10.1152/physrev.00005.2005.

Ottman, R. (1996) Gene-environment interaction: definitions and study designs. *Prev Med* **25,** 764-770.

Page, R. C. & Eke, P. I. (2007) Case definitions for use in population-based surveillance of periodontitis. *J Periodontol* **78,** 1387-1399. doi:10.1902/jop.2007.060264.

Paraskevas, S., Huizinga, J. D. & Loos, B. G. (2008) A systematic review and meta-analyses on C-reactive protein in relation to periodontitis. *J Clin Periodontol* **35,** 277-290. doi:10.1111/j.1600-051X.2007.01173.x.

Park, M. H., Kim, N., Lee, J. Y. & Park, H. Y. (2011) Genetic loci associated with lipid concentrations and cardiovascular risk factors in the Korean population. *J Med Genet* **48,** 10-15. doi:10.1136/jmg.2010.081000.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A., Bender, D., Maller, J., Sklar, P., de Bakker, P. I., Daly, M. J. & Sham, P. C. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* **81,** 559-575. doi:10.1086/519795.

Rajput-Williams, J., Knott, T. J., Wallis, S. C., Sweetnam, P., Yarnell, J., Cox, N., Bell, G. I., Miller, N. E. & Scott, J. (1988) Variation of apolipoprotein-B gene is associated with obesity, high blood cholesterol levels, and increased risk of coronary heart disease. *Lancet* **2,** 1442-1446.

Rhodin, K., Divaris, K., North, K. E., Barros, S. P., Moss, K., Beck, J. D. & Offenbacher, S. (2014) Chronic periodontitis genome-wide association studies: gene-centric and gene set enrichment analyses. *J Dent Res* **93,** 882-890. doi:10.1177/0022034514544506.

Ribeiro, M. S., Pacheco, R. B., Fischer, R. G. & Macedo, J. M. (2016) Interaction of IL1B and IL1RN polymorphisms, smoking habit, gender, and ethnicity with aggressive and chronic periodontitis susceptibility. *Contemp Clin Dent* **7,** 349-356. doi:10.4103/0976-237X.188560.

Rios-Gonzalez, B. E., Ibarra-Cortes, B., Ramirez-Lopez, G., Sanchez-Corona, J. & Magana-Torres, M. T. (2014) Association of polymorphisms of genes involved in lipid metabolism with blood pressure and lipid values in mexican hypertensive individuals. *Dis Markers* **2014,** 150358. doi:10.1155/2014/150358.

Sabatti, C., Service, S. K., Hartikainen, A. L., Pouta, A., Ripatti, S., Brodsky, J., Jones, C. G., Zaitlen, N. A., Varilo, T., Kaakinen, M., Sovio, U., Ruokonen, A., Laitinen, J., Jakkula, E., Coin, L., Hoggart, C., Collins, A., Turunen, H., Gabriel, S., Elliot, P., McCarthy, M. I., Daly, M. J., Jarvelin, M. R., Freimer, N. B. & Peltonen, L. (2009) Genome-wide association analysis of metabolic traits in a birth cohort from a founder population. *Nat Genet* **41,** 35-46. doi:10.1038/ng.271.

Sakiyama, Y., Kato, R., Inoue, S., Suzuki, K., Itabe, H. & Yamamoto, M. (2010) Detection of oxidized low-density lipoproteins in gingival crevicular fluid from dental patients. *J Periodontal Res* **45,** 216-222. doi:10.1111/j.1600-0765.2009.01226.x.

Sandhu, M. S., Waterworth, D. M., Debenham, S. L., Wheeler, E., Papadakis, K., Zhao, J. H., Song, K., Yuan, X., Johnson, T., Ashford, S., Inouye, M., Luben, R., Sims, M., Hadley, D., McArdle, W., Barter, P., Kesaniemi, Y. A., Mahley, R. W., McPherson, R., Grundy,

S. M., Wellcome Trust Case Control, C., Bingham, S. A., Khaw, K. T., Loos, R. J., Waeber, G., Barroso, I., Strachan, D. P., Deloukas, P., Vollenweider, P., Wareham, N. J. & Mooser, V. (2008) LDL-cholesterol concentrations: a genome-wide association study. *Lancet* **371,** 483-491. doi:10.1016/S0140-6736(08)60208-1.

Sanna, S., Li, B., Mulas, A., Sidore, C., Kang, H. M., Jackson, A. U., Piras, M. G., Usala, G., Maninchedda, G., Sassu, A., Serra, F., Palmas, M. A., Wood, W. H., 3rd, Njolstad, I., Laakso, M., Hveem, K., Tuomilehto, J., Lakka, T. A., Rauramaa, R., Boehnke, M., Cucca, F., Uda, M., Schlessinger, D., Nagaraja, R. & Abecasis, G. R. (2011) Fine mapping of five loci associated with low-density lipoprotein cholesterol detects variants that double the explained heritability. *PLoS Genet* **7,** e1002198. doi:10.1371/journal.pgen.1002198.

Schaid, D. J. (2004) Evaluating associations of haplotypes with traits. *Genet Epidemiol* **27,** 348-364. doi:10.1002/gepi.20037.

Stashenko, P., Fujiyoshi, P., Obernesser, M. S., Prostak, L., Haffajee, A. D. & Socransky, S. S. (1991) Levels of interleukin 1 beta in tissue from sites of active periodontal disease. *J Clin Periodontol* **18,** 548-554.

Susanto, H., Nesse, W., Dijkstra, P. U., Agustina, D., Vissink, A. & Abbas, F. (2011) Periodontitis prevalence and severity in Indonesians with type 2 diabetes. *J Periodontol* **82,** 550-557. doi:10.1902/jop.2010.100285.

Tanaka, K., Miyake, Y., Hanioka, T. & Arakawa, M. (2014) Relationship between IL1 gene polymorphisms and periodontal disease in Japanese women. *DNA Cell Biol* **33,** 227-233. doi:10.1089/dna.2013.2202.

Tanaka, K., Miyake, Y., Hanioka, T., Furukawa, S., Miyatake, N. & Arakawa, M. (2017) The IL18 Promoter Polymorphism, rs1946518, Is Associated with the Risk of Periodontitis in Japanese Women: The Kyushu Okinawa Maternal and Child Health Study. *Tohoku J Exp Med* **243,** 159-164. doi:10.1620/tjem.243.159.

Wang, Y., Sugita, N., Yoshihara, A., Iwasaki, M., Miyazaki, H., Nakamura, K. & Yoshie, H. (2013) Peroxisome proliferator-activated receptor (PPAR) gamma polymorphism, vitamin D, bone mineral density and periodontitis in postmenopausal women. *Oral Dis* **19,** 501-506. doi:10.1111/odi.12032.

Waterworth, D. M., Ricketts, S. L., Song, K., Chen, L., Zhao, J. H., Ripatti, S., Aulchenko, Y. S., Zhang, W., Yuan, X., Lim, N., Luan, J., Ashford, S., Wheeler, E., Young, E. H., Hadley, D., Thompson, J. R., Braund, P. S., Johnson, T., Struchalin, M., Surakka, I., Luben, R., Khaw, K. T., Rodwell, S. A., Loos, R. J., Boekholdt, S. M., Inouye, M.,

Deloukas, P., Elliott, P., Schlessinger, D., Sanna, S., Scuteri, A., Jackson, A., Mohlke, K. L., Tuomilehto, J., Roberts, R., Stewart, A., Kesaniemi, Y. A., Mahley, R. W., Grundy, S. M., Wellcome Trust Case Control, C., McArdle, W., Cardon, L., Waeber, G., Vollenweider, P., Chambers, J. C., Boehnke, M., Abecasis, G. R., Salomaa, V., Jarvelin, M. R., Ruokonen, A., Barroso, I., Epstein, S. E., Hakonarson, H. H., Rader, D. J., Reilly, M. P., Witteman, J. C., Hall, A. S., Samani, N. J., Strachan, D. P., Barter, P., van Duijn, C. M., Kooner, J. S., Peltonen, L., Wareham, N. J., McPherson, R., Mooser, V. & Sandhu, M. S. (2010) Genetic variants influencing circulating lipid levels and risk of coronary artery disease. *Arterioscler Thromb Vasc Biol* **30,** 2264-2276. doi:10.1161/ATVBAHA.109.201020.

Wojczynski, M. K., Gao, G., Borecki, I., Hopkins, P. N., Parnell, L., Lai, C. Q., Ordovas, J. M., Chung, B. H. & Arnett, D. K. (2010) Apolipoprotein B genetic variants modify the response to fenofibrate: a GOLDN study. *J Lipid Res* **51,** 3316-3323. doi:10.1194/jlr.P001834.

Wu, T. L., Tsai, C. C., Wang, Y. Y., Ho, K. Y., Wu, Y. M., Hung, H. C. & Lin, Y. C. (2015) The association between the RAGE G82S polymorphism, sRAGE and chronic periodontitis in Taiwanese individuals with and without diabetes. *J Periodontal Res* **50,** 881-889. doi:10.1111/jre.12282.

Xiao, R., Sun, S., Zhang, J., Ouyang, Y., Zhang, N., Yang, M., Jin, T. & Xia, Y. (2017) Association analysis of APO gene polymorphisms with ischemic stroke risk: a case-control study in a Chinese Han population. *Oncotarget* **8,** 60496-60503. doi:10.18632/oncotarget.15549.

Yan, H. C., Wang, W., Dou, C. W., Tian, F. M. & Qi, S. T. (2014) Relationships of LDLR genetic polymorphisms with cerebral infarction: a meta-analysis. *Mol Biol Rep* **41,** 4425-4434. doi:10.1007/s11033-014-3313-4.

Ye, H., Zhao, Q., Huang, Y., Wang, L., Liu, H., Wang, C., Dai, D., Xu, L., Ye, M. & Duan, S. (2014) Meta-analysis of low density lipoprotein receptor (LDLR) rs2228671 polymorphism and coronary heart disease. *Biomed Res Int* **2014,** 564940. doi:10.1155/2014/564940.

Zaman, M. M., Ikemoto, S., Yoshiike, N., Date, C., Yokoyama, T. & Tanaka, H. (1997) Association of apolipoprotein genetic polymorphisms with plasma cholesterol in a Japanese rural population. The Shibata Study. *Arterioscler Thromb Vasc Biol* **17,** 3495-3504.

Zhang, J., Sun, X., Xiao, L., Xie, C., Xuan, D. & Luo, G. (2011) Gene polymorphisms and periodontitis. *Periodontol 2000* **56,** 102-124. doi:10.1111/j.1600-0757.2010.00371.x.

Zhang, Y., Ma, K. L., Ruan, X. Z. & Liu, B. C. (2016) Dysregulation of the Low-Density Lipoprotein Receptor Pathway Is Involved in Lipid Disorder-Mediated Organ Injury. *Int J Biol Sci* **12,** 569-579. doi:10.7150/ijbs.14027.

Zhu, H., Tucker, H. M., Grear, K. E., Simpson, J. F., Manning, A. K., Cupples, L. A. & Estus, S. (2007) A common polymorphism decreases low-density lipoprotein receptor exon 12 splicing efficiency and associates with increased cholesterol. *Hum Mol Genet* **16,** 1765-1772. doi:10.1093/hmg/ddm124.

Zou, F., Gopalraj, R. K., Lok, J., Zhu, H., Ling, I. F., Simpson, J. F., Tucker, H. M., Kelly, J. F., Younkin, S. G., Dickson, D. W., Petersen, R. C., Graff-Radford, N. R., Bennett, D. A., Crook, J. E., Younkin, S. G. & Estus, S. (2008) Sex-dependent association of a common low-density lipoprotein receptor polymorphism with RNA splicing efficiency in the brain and Alzheimer's disease. *Hum Mol Genet* **17,** 929-935. doi:10.1093/hmg/ddm365.

Table 1. Comparison of demographic characteristics

	Control **n=356**	Case **n=358**
Demographic Characteristics		
Age (mean ± SD)*	43.6 (± 11.0)	49.1 (± 9.3)
Sex, n (%)		
Males	115 (32.3)	146 (40.8)
Females	241 (67.7)	212 (59.2)
Smoking, n (%)*		
Never	275 (77.3)	229 (64.0)
Ever	81 (22.7)	129 (36.00)

**p-value* < 0.05 (comparisons performed by Student's t or chi-square test).

Table 2. Data regarding the assays of the genotyped polymorphisms and alleles frequencies

SNP	Assay ID	Cytogenetic Band	Gene and classification of mutation	Allele	MAF		HWE	Call rate (%)*
rs5925	C___2804279_10	19p13.2b	*LDLR* (silent mutation)	**T|C**	**C|C**	0.16	0.74	96.92
rs688	C___2804264_20	19p13.2b	*LDLR* (silent mutation)	**C|T**	**T|T**	0.14	0.42	96.36
rs676210	C___3216558_10	2p24.1c	*APOB* (missense mutation)	**G|A**	**A|A**	0.06	0.05	95.66
rs693	C___7615420_20	2p24.1c	*APOB* (silent mutation)	**C|T**	**T|T**	0.16	0.14	96.92

MAF, minor allele frequency (in this study); HWE, Hardy–Weinberg equilibrium.
*Represents the amount of genotyping performed with success, in percentage, for OpenArray™ plate.

Table 3. Clinical parameters (mean ± SD) distributed by the genotypes

	Genotypes			
	1.1	**1.2**	**2.2**	*p-value**
(T>C) rs5925				
Number of teeth	23.88 (4.12)	24.48 (4.27)	24.05 (4.48)	0.58
Visible plaque (% of sites)	37.11 (26.96)	33.74 (26.46)	34.80 (26.03)	0.30
Marginal bleeding (% of sites)	15.82 (20.39)	13.68 (18.68)	13.53 (19.48)	0.34
BOP (% of sites)	23.40 (25.96)	19.98 (23.43)	22.64 (26.03)	0.58
PPDi ≤ 4mm (% of sites)	92.98 (12.57)	93.78 (11.64)	92.92 (10.94)	0.42
PPDi ≥ 5mm (% of sites)	7.02 (12.57)	6.22 (11.64)	7.08 (10.94)	0.42
CALi ≤ 3mm (% of sites)	83.07 (21.94)	85.58 (20.12)	81.36 (24.38)	0.52
CALi ≥ 6mm (% of sites)	5.77 (10.76)	5.19 (10.88)	7.36 (12.80)	0.51
(C>T) rs688				
Number of teeth	23.94 (4.10)	24.37 (4.38)	24.17 (4.28)	0.55
Visible plaque (% of sites)	37.18 (27.41)	33.62 (26.16)	34.41 (25.77)	0.30
Marginal bleeding (% of sites)	16.08 (21.16)	13.44 (18.11)	13.27 (18.69)	0.34
BOP (% of sites)	23.60 (26.28)	20.17 (23.56)	21.47 (24.64)	0.55
PPDi ≤ 4mm (% of sites)	92.56 (13.41)	94.14 (10.64)	92.92 (11.04)	0.36
PPDi ≥ 5mm (% of sites)	7.44 (13.41)	5.86 (10.64)	7.08 (11.04)	0.36
CALi ≤ 3mm (% of sites)	82.99 (22.41)	85.23 (20.19)	82.60 (23.44)	0.68
CALi ≥ 6mm (% of sites)	5.96 (11.19)	5.14 (10.74)	7.18 (12.55)	0.63
(G>A) rs676210				
Number of teeth	24.11 (4.36)	24.37 (3.95)	23.55 (4.64)	0.84
Visible plaque (% of sites)	34.60 (26.16)	35.42 (26.30)	38.80 (29.48)	0.68
Marginal bleeding (% of sites)	14.80 (20.21)	14.41 (17.77)	12.06 (21.04)	0.18
BOP (% of sites)	21.70 (25.49)	22.14 (24.06)	21.45 (23.16)	0.84
PPDi ≤ 4mm (% of sites)	93.41 (11.69)	92.89 (12.65)	94.02 (11.03)	0.53
PPDi ≥ 5mm (% of sites)	6.59 (11.69)	7.11 (12.65)	5.98 (11.03)	0.53
CALi ≤ 3mm (% of sites)	83.86 (22.51)	83.89 (20.06)	84.01 (20.41)	0.68
CALi ≥ 6mm (% of sites)	6.08 (11.74)	5.29 (10.37)	5.40 (10.12)	0.88
(C>T) rs693				
Number of teeth	24.20 (4.40)	24.06 (4.10)	24.33 (4.29)	0.96
Visible plaque (% of sites)	33.15 (26.50)	37.53 (26.84)	33.10 (25.41)	0.07
Marginal bleeding (% of sites)	13.98 (18.56)	14.97 (19.71)	14.37 (21.12)	0.44
BOP (% of sites)	21.22 (24.00)	21.55 (24.40)	23.16 (28.04)	0.96
PPDi ≤ 4mm (% of sites)	93.28 (12.50)	93.22 (11.25)	93.85 (12.20)	0.61

PPDi ≥ 5mm (% of sites)	6.72 (12.50)	6.78 (11.25)	6.15 (12.20)	0.61
CALi ≤ 3mm (% of sites)	84.48 (20.25)	84.34 (21.15)	81.81 (25.65)	0.97
CALi ≥ 6mm (% of sites)	4.97 (9.48)	5.74 (11.21)	7.72 (14.44)	0.86

*The *p-value* was estimated by Kruskal-Wallis test.
Abbreviations: BOP, bleeding on probing; PPDi, interproximal probing pocket depth; CALi, interproximal clinical attachment level. 1 and 2 refer to common and rare alleles. respectively. 1.1 (homozygous for the major allele), 1.2 (heterozygous for the major and minor alleles), and 2.2 (homozygous for the minor allele).

Table 4. Adjusted OR and 95% CI for periodontal disease associated with polymorphisms

	n (%)			
SNP / Genotype	Control	Cases	Adjusted OR* (95% CI)	*p-value*
(T>C) rs5925				
TT	126 (36.52)	130 (37.46)	1.00	
TC	162 (46.96)	159 (45.82)	0.98 (0.69 - 1.38)	0.90
CC	57 (16.52)	58 (16.71)	0.96 (0.60 - 1.52)	0.85
TC+CC	219 (63.48)	217 (62.54)	0.97 (0.70 - 1.35)	0.86
(C>T) rs688				
CC	137 (39.94)	142 (41.16)	1.00	
CT	154 (44.90)	152 (44.06)	0.97 (0.69 - 1.37)	0.91
TT	54 (15.16)	51 (14.78)	0.93 (0.58 - 1.51)	0.78
CT+TT	206 (60.06)	203 (58.84)	0.96 (0.69 - 1.33)	0.81
(G>A) rs676210				
GG	228 (67.06)	213 (62.10)	1.00	
AG	94 (27.65)	108 (31.49)	1.32 (0.93 - 1.88)	0.12
AA	18 (5.29)	22 (6.41)	1.09 (0.55 - 2.17)	0.80
AG+AA	112 (32.94)	130 (37.90)	1.28 (0.92 - 1.78)	0.14
(C>T) rs693				
CC	137 (39.60)	137 (39.60)	1.00	
CT	151 (43.64)	157 (45.38)	1.09 (0.77 - 1.54)	0.64
TT	58 (16.76)	52 (15.03)	0.87 (0.54 - 1.39)	0.57
CT+TT	209 (60.40)	209 (60.40)	1.02 (0.74 - 1.42)	0.88

*The OR with 95% CI were estimated by multiple logistic regression model after controlling for age, sex and smoking.
Abbreviations: OR, odds ratio; CI, confidence interval.

Table 5. Risk of periodontal disease associated with genotypes by smoking status

					Multiplicative interaction	Additive interaction (95% CI)		
SNP / Genotype	Smoking	Controls, n(%)	Cases, n(%)	Adjusted OR (95% CI)*	*p* -value	RERI	AP	S
(T>C) rs5925								
TT	Never	100 (28.99)	91 (26.22)	1.00				
TC+CC	Never	168 (48.70)	133 (38.33)	0.95 (0.65 - 1.39)	0.80	0.29	0.17	1.75
TT	Ever	26 (7.54)	39 (11.24)	1.54 (0.86 - 2.80)		(-0.79 - 1.37)	(-0.44 - 0.78)	(0.15 - 20.96)
TC+CC	Ever	51 (14.78)	84 (24.21)	**1.61 (1.01 - 2.58)**				
(C>T) rs688								
CC	Never	110 (32.07)	98 (28.41)	1.00		0.03	0.02	1.05
CT+TT	Never	157 (45.77)	124 (35.94)	0.97 (0.66 - 1.42)	0.92	(-1.10 - 1.17)	(-0.67 - 0.71)	(0.17 - 6.61)
CC	Ever	27 (7.87)	44 (12.75)	1.72 (0.97 - 3.05)				
CT+TT	Ever	49 (14.29)	79 (22.90)	**1.61 (1.01 - 2.59)**				
(G>A) rs676210								
GG	Never	174 (51.18)	134 (39.07)	1.00		-0.10	-0.06	0.89
AG+AA	Never	91 (26.76)	87 (25.36)	1.26 (0.86 - 1.85)	0.87	(-1.44 - 1.23)	(-0.80 - 0.69)	(0.20 - 3.95)
GG	Ever	54 (15.88)	79 (23.03)	**1.67 (1.09 - 2.56)**				
AG+AA	Ever	21 (6.18)	43 (12.54)	**2.24 (1.24 - 4.05)**				
(C>T) rs693								
CC	Never	113 (32.66)	91 (26.30)	1.00		-0.85	-0.57	0.36
CT+TT	Never	156 (45.09)	131 (37.86)	1.14 (0.78 - 1.67)	0.29	(-2.20 - 0.50)	(-1.58 - 0.44)	(0.08 - 1.69)
CC	Ever	24 (6.94)	46 (13.29)	**2.16 (1.20 - 3.88)**				
CT+TT	Ever	53 (15.32)	78 (22.54)	**1.66 (1.04 - 2.64)**				

*The OR with 95% CI and *p-value* were estimated by multiple logistic regression model after controlling for age and sex.

Abbreviations: OR, odds ratio; CI, confidence interval; RERI, Relative excess risk due to interaction; AP, Attributable proportion due to interaction; S, Synergy index.

Table 6. Pairwise linkage disequilibrium of polymorphisms (r^2 and D' values)

			Linkage disequilibrium	
Chromosome	**SNP1**	**SNP2**	***r*²**	**D'**
2	rs676210	rs693	0.161	1.000
19	rs688	rs5925	0.886	0.997

D’ = the linkage disequilibrium coefficient D standardized by the maximum value it can take (Dmax). given the allele frequencies; r^2 = the square of the correlation coefficient between allele frequencies.

Table 7. Distribution of *LDLR* and *APOB* haplotypes in the studied groups

		Estimated frequencies*			
SNP markers	Haplotype	Controls	Cases	Adjusted OR (95% CI)[a]	*p-value*
(C>T) rs688	CT	0.5983	0.6023	1.00	
(T>C) rs5925	TC	0.3755	0.3672	0.97 (0.77 - 1.22)	0.8
	CC	0.0262	0.0291	1.01 (0.51 - 2.01)	0.98
(G>A) rs676210	GC	0.4227	0.4011	1.00	
(C>T) rs693	GT	0.3858	0.3764	1.03 (0.80 - 1.32)	0.84
	AC	0.1915	0.2225	1.20 (0.90 - 1.61)	0.22

*Frequency threshold for rare haplotypes = 0.01.
[a]The OR with 95% CI and *p-value* were estimated by multiple logistic regression model after controlling for age. sex and smoking.
Abbreviations: OR, odds ratio; CI, confidence interval.

3.3 Capítulo 3*

Validation of the *Neuropeptide Y* and *Interleukin 37* polymorphisms as associated with moderate or severe chronic periodontitis in a Brazilian population

Running title: *NPY* and *IL37* polymorphisms in periodontitis

Keywords: Periodontal Disease, Genetic Risk, Single Nucleotide Polymorphism, Neuropeptide Y, Interleukin 37

Rafael Nepomuceno[1], Thamiris Cirelli[1], Silvana R P Orrico[1], Carlos Rossa Jr[1], Joni A Cirelli[1], Kari E. North[2,3], Mariaelisa Graff[2,4], Silvana P Barros[5], Steven Offenbacher[5], Raquel M Scarel-Caminaga[6]

1. Department of Diagnosis and Surgery, São Paulo State University – UNESP, School of Dentistry at Araraquara, Araraquara, SP, Brazil
2. Department of Epidemiology, University of North Carolina at Chapel Hill - UNC, School of Public Health, Chapel Hill, NC, USA
3. Carolina Population Center, University of North Carolina at Chapel Hill - UNC, Chapel Hill, NC, USA
4. Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill - UNC, School of Public Health, Chapel Hill, NC, USA
5. Department of Periodontology, University of North Carolina at Chapel Hill – UNC, School of Dentistry, Chapel Hill, NC, USA
6. Department of Morphology, São Paulo State University – UNESP, School of Dentistry at Araraquara, Araraquara, SP, Brazil

Correspondence:
Raquel Mantuaneli Scarel-Caminaga, PhD
Department of Morphology, School of Dentistry at Araraquara, UNESP – São Paulo State University, CP 331, CEP 14801-903, Araraquara, SP, Brazil, E-mail: raquel@foar.unesp.br

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ABSTRACT

Aim: The number of publications using bioinformatics methods to identify candidate genes and genome-wide association studies (GWAS) has expanded to identify genes variants associated with chronic periodontitis (CP). Results from some of these studies enrolling different populations have not yet been validated for Brazilian population. The purpose of the present study is to investigate the association of 7 candidate polymorphisms in the *IL18*, *TLR9*, *FZD8*, *NPY*, *IL37*, *EMR1* and *NCR2* genes (rs187238, rs352140, rs1360573, rs2521634, rs3811046, rs3826782, rs7762544) with CP in a Brazilian population.

Materials and Methods: Genomic DNA was obtained from No/mild CP group (n=356) and moderate or severe CP group (n=358). Genotyping of polymorphisms was performed using real-time polymerase chain reaction by OpenArray® platform. Multinomial logistic regression was used to determine degree of association between polymorphisms and periodontal status adjusted for known confounders. The same analysis was conducted by sex to identify the sex-specific effects of gene polymorphisms on periodontal disease. A multiple linear regression was used to assess the relationship of periodontal parameters with each SNP independently.

Results: The AA genotype of the rs2521634 SNP was associated with increased risk to severe CP (OR = 2.34; 95% CI=1.19 – 4.59; $p = 0.01$). Considering the rs3811046 SNP, TG and GG genotypes were shown to increase the susceptibility to moderate CP. However, these results were not significant after the Bonferroni correction. Women carrying the homozygous genotype for the minor allele on rs3811046 SNP were more susceptible to develop moderate CP (OR = 4.02, CI: 1.65 – 9.80, $p = 0.002$), even after the Bonferroni correction. For the rs2521634, the homozygotes for rare allele (AA) were associated with higher percentage of PPDi ≥ 5mm ($\beta = 4.91$, $p = 0.004$).

Conclusions: Here we validated in a Brazilian population the association of the rs2521634 SNP, close to the *NPY* (*Neuropeptide Y*) gene, and the rs3811046 SNP, in the intronic region of the *IL37* (*Interleukin 37*) gene, with increased chance to moderate and severe CP, and this risk was sex-specific. These results reinforce the evidence that the *NPY* and *IL37* polymorphisms, previously found by GWAS, might play a role on the genetic profile predisposing to CP.

1. Introduction

The chronic periodontitis (CP) is among the most common inflammatory diseases, affecting human populations at worldwide with prevalence rates of 11% for the severe forms (Marcenes et al., 2013). CP is a multifactorial pathology that is primarily provoked by specific pathogenic bacterial consortia in subgingival biofilm (Darveau, 2010), and the destruction of periodontium, the tooth supporting tissues, is immunologically mediated (Yoshie et al., 2007). It is well established that inherent genetic factors and environmental factors, such as smoking, affect the occurrence, extent and severity of the periodontal destructive process (Kornman, 2008). The evidence for a genetic susceptibility to periodontitis comes from twins (Michalowicz et al., 1991, Corey et al., 1993), which suggested that the susceptibility to CP is related to heritability in approximately 50% of cases (Michalowicz et al., 2000).

In the last decades, a variety of association studies focusing on candidate genes related to host immunity and inflammatory response (such as cytokines, cell-surface receptors, chemokines and enzymes) have demonstrated association with CP in different populations (Kornman et al., 1997, Laine et al., 2012, Zhang et al., 2011, Scarel-Caminaga et al., 2011, Ding et al., 2014). The majority of positive association of a genetic polymorphism was found with CP, and since this disease is multifactorial and polygenic, it is common to find weak association of such candidate gene polymorphism individually. Moreover, most of these studies have employed non-standardized clinical parameters and cohorts of fairly small size, resulting in high variability across studies and limited statistical power and a large potential for false-positive/ false-negative results (Laine et al., 2012). Therefore, the genes involved in the pathogenesis of CP should be better investigated.

The number of publications using genetic databases and bioinformatics methods to identify disease candidate genes has been expanded. A combination of computational tools will be useful in mining candidate genes for periodontitis. These theoretical results provide new clues for experimental biologists to plan targeted experiments. Just few studies have been published in the literature using bioinformatics to identify the most promising candidate genes for CP. Just one study used the multiple data sources and different computational methods to prioritize periodontitis genes within the whole human genome (Zhan et al., 2014). Through this integrative method of gene ranking, *TLR9* and *IL18* (*Toll Like Receptor 9* and *Interleukin 18*) are some that stand out.

The limitation of the candidate gene approach is that hundreds of genes will not be selected because their functions are unknown; therefore, hypothesis-free strategy studies of the

entire genome might be conducted (Vaithilingam et al., 2014). Genome-wide association studies (GWAS) are an agnostic approach to scan the entire human genome to identify genes or genetic loci having even modest effects on complex human diseases or traits (Teumer et al., 2013). Evidences are supporting that the GWAS approach, set of approximately 650,000 genotyped SNPs with a less frequent random frequency (MAF) ≥ 5%, shows approximately 22% of the phenotypic variance observed in severe chronic periodontitis (Divaris et al., 2013). A number of GWAS have reported novel findings for complex diseases, and interestingly, most of these genes have not been thought of as likely candidates before. This was also observed regarding CP, since GWAS evidenced genetic variants associated with susceptibility to periodontitis. It can be highlighted polymorphisms in the *IL37* (*Interleukin 37*) gene, *EMR1* (*Adhesion G Protein-Coupled Receptor E1*) and close to *FZD8* (*Frizzled Class Receptor 8*), *NPY* (*Neuropeptide Y*), and *NCR2* (*Natural Cytotoxicity Triggering Receptor 2*) genes (Divaris et al., 2013, Freitag-Wolf et al., 2014, Shaffer et al., 2014).

It has been acknowledged that results from GWAS need to be reproduced in further independent samples. A previous GWAS result is 'replicated' when it is reproduced in a similar population from the original study; but when the GWAS result is reproduced in a different population (e.g. ethnically) this result was 'validated' (Igl et al., 2009). Considering that the mentioned polymorphisms prospected from the bioinformatics and GWAS studies have not yet been validated for the Brazilian population, the purpose of the present study is to investigate the association of 7 candidate single nucleotide polymorphisms (SNPs) (rs187238, rs352140, rs1360573, rs2521634, rs3811046, rs3826782, rs7762544) in different genes or close to them (*IL18*, *TLR9, FZD8, NPY*, *IL37, EMR1* and *NCR2,* respectively) in a Brazilian population with moderate and severe chronic periodontitis.

2. Material and Methods

2.1. Study subjects

The study was a case–control trial. According to the sample size calculation (described in Results), after detailed and complete periodontal examination, a total of 714 unrelated subjects, with at least 30 years old, were recruited from the School of Dentistry at Araraquara, São Paulo State University (UNESP). The patients were allocated to each of the 2 different groups according to the criteria defined by the CDC / AAP (Centers for Disease Control and Prevention / American Academy of Periodontology) (Page and Eke, 2007): Group A (n = 356): patients with mild periodontitis and healthy patients; Group B (n=358) patients with moderate

(B1; n = 89) or severe periodontitis (B2; n = 269) (Figure 1). This study was performed in accordance with the Helsinki Declaration of 1975, as revised in 1983, and was approved by the Ethics in Human Research Committee of the Araraquara School of Dentistry (UNESP; Protocol number 50/06). All the patients gave written informed consent before any study related procedure.

The exclusion criteria for enrolling patients in the study were periodontal treatment during the last 6 months, antibiotic therapy during the last 3 months, have less then 10 teeth (excluding third molars), chronic usage of anti-inflammatory drugs, current pregnant or lactating and self-declared history of diseases that influence the immune system (i.e. diabetes mellitus, HIV infection, or immunosuppressive chemotherapy). For both cases and controls, smoking status was confirmed by a questionnaire.

The following clinical signs and parameters were assessed at four sites (interproximal sites) around each tooth: interproximal probing pocket depth (PPDi) and interproximal clinical attachment level (CALi), measured to the nearest millimeter by a periodontal probe with University of North Carolina-15 periodontal probe; visible plaque, marginal bleeding and bleeding on probing (BOP), registered, as a percentage of the total number of sites. All fully erupted teeth, except third molars and retained roots, were examined.

2.2. DNA Isolation and Quantification

Each participants' buccal epithelial cells was collected by a mouthwash of 3% glucose for 2 minutes and stored with buffer solution (10mM Tris HCl, 0.1M EDTA, 0.5% SDS - pH 8.0) at −80°C until used for subsequent genomic DNA extraction by proteinase K digestion and 8M ammonium acetate protocol extraction adapted (Aidar and Line, 2007). The purity and concentration of the samples were checked using a NanoDrop® 2000 spectrophotometer (Thermo Scientific) and a Qubit® 2.0 fluorometer (Invitrogen), respectively. All the samples showed $A_{260/230}$ and $A_{260/280}$ ratios between 1.7 and 2.0, and a concentration of 50 ng/μL were standardized.

2.3. Genotyping

Genotyping was performed using the OpenArray® platform (Thermo Fisher Scientific, Waltham, MA, USA), which employs a chip-based TaqMan genotyping technology. Custom Open Array Chips (Thermo Fisher Scientific, Waltham, MA, USA) were designed with the

selected polymorphisms of interest which were previously associated with CP: *FZD8, IL37, IL18, TLR9, NPY*, *EMR1* and *NCR2* (Table 1). Genotyping was conducted utilizing 2.5 µL of each individual sample of DNA (total of 125 ng of genomic DNA) to be analyzed in a well of a 384-well plate, together with 2.5 µL of 2X TaqMan®OpenArray® Genotyping Master Mix, according to the manufacturer's standard protocols, using the QuantStudio 12K Flex Real-Time PCR System. The genotyping analysis was made with Applied Biosystems™ Analysis Software, Genotyping Analysis Module, version 3.2. (available at: Thermo Fisher Cloud) using autocalling as the call method. The quality value of the data points genotype was determined by a threshold above 95%. To ensure quality control, genotyping was performed without the knowledge of individual case–control status.

2.4. Statistical analysis

Demographic variables between cases and controls were compared using Student's *t* test for continuous variables (age and periodontal parameters) and the χ^2 test for categorical variables (gender and smoking). The Hardy–Weinberg equilibrium (HWE) formula was applied to the genotype frequencies among the control subjects to test, using chi-square goodness-of-fit, for any deviations from the expected genotype equilibrium.

Three different binary independent variables (Group A *vs*. Group B; Group A *vs*. GroupB1; Group A *vs*. Group B2) were used to ascertain the association between each SNP and the diagnosis of chronic periodontitis. All case-control association analyses were carried out, which included allelic and genotypic associations. Genotypes were categorized into three groups (major allele homozygous - 11, heterozygous - 12, and homozygous variant - 22). Different degrees of disease severity (moderate or severe) were assessed by the Chi-square test in four models: i) allele 1 versus 2; ii) genotype 11 versus 12 versus 22 (additive model); iii) genotype 11+12 versus 22 (recessive model); and iv) genotype 11 versus 12+22 (dominant model). Significance threshold was set to 0.007 (0.05/7), according to Bonferroni correction based on the total number of polymorphisms of the study (n = 7), similar to other studies (Nibali et al., 2008, Wu et al., 2015, Su et al., 2015b).

The association between each SNP with moderate and severe periodontal disease was estimated as an odds ratio (OR) and 95% confidence intervals (CI) by multiple logistic regression adjusted by age, sex and smoking. Furthermore, the gene polymorphisms were evaluated, through the logistic regression model, separately by the sex of patients to investigate the susceptibility to CP, adjusted by age and smoking. The analysis was performed for SNPs

that presented at least a weak association ($p < 0.05$) with CP after multiple logistic regression. A multiple linear regression was used to assess the relationship of periodontal parameters (BOP, PPDi and CALi) with each SNP independently. Models were adjusted for age, sex and smoking status, which were used as independent variables.

The PLINK software (Purcell et al., 2007) was used to detect differences in distributions for each SNP between groups. All other analyses were performed by STATA software, version 12.0 for Mac (Statistics/Data Analysis, Stata Corporation, College Station, Tex., USA).

3. Results

3.1. Characteristics of study population

To calculate the sample size necessary for the study to have power to attest the association between the alleles with CP, the G * Power Calculator, version 3.1 (Faul et al., 2007) program was used, considering the parameters: logistic regression; two tail, OR: 1.5; Pr (Y = 1 / X = 1) H0 = 0.2; alpha of 0.005 and 90% power, R2 = 0. It is important to note that alpha = 0.005 was obtained from p = 0.05 divided by 10 (7 SNPs +3 adjustment variables [age, gender and smoking]). This calculation resulted in a total sample of 654 patients, 327 patients per group. This number is smaller than the total of subjects enrolled in this present study (n = 716).

The demographic and periodontal characteristics of the 2 study groups (356 controls and 358 patients with moderate and severe CP) were analyzed (Table 2). No difference in sex distribution was found between the groups. Significantly different distributions ($p < 0.05$) of age and smoking status were detected between controls and CP patients. Table 2 also showed, as expected, that periodontal tissue destruction (including bone loss) and local inflammation were significantly higher in CP patients, particularly in the severe CP group, which presented higher percentage of periodontal sites with bleeding on probing, interproximal probing pocket ≥ 5 mm and interproximal clinical attachment loss ≥ 6 mm.

3.2. SNP analysis

For all SNPs, the distribution of the genotypes in controls was in Hardy–Weinberg equilibrium ($p > 0.05$). The position and the minor allele frequency (MAF) of the 7 SNPs was estimated and presented in Table 1. Just one SNP had a MAF < 5% (rs3826782). The call rate of all the investigated SNPs was > 95%.

The genotype and allele distributions were compared between the no/mild CP with moderate, severe and moderate + severe CP patients by association analysis (chi-square test, Table 3). Although some results presented *p-value* < 0.5, the majority of these were not significant after the Bonferroni correction. In regard to the rs2521634 SNP close to the *NPY* gene, the control group (no/mild CP) had significantly higher proportions of G allele than severe CP and severe + moderate CP ($p = 0.005$ and $p = 0.006$, respectively). Furthermore, there were different proportion of rs2521634 genotypes between control group and severe CP group, but not significant after Bonferroni correction in additive, dominant and recessive models ($p = 0.02$, $p = 0.02$ and $p = 0.03$, respectively). Concerning the intronic rs3811046 SNP in the *IL37* gene, the control group had significantly higher proportion of T allele ($p = 0.004$) in comparison with the moderate CP. Moreover, there were different proportion of genotypes between control group and moderate CP group in the same SNP ($p < 0.05$) in additive and dominant models ($p = 0.02$ and $p = 0.01$, respectively), which lack significance after Bonferroni correction.

To more accurately evaluate the strength of any association and to eliminate the distortion caused by confounding effects (sex, age and smoking), multiple logistic regressions were performed (Table 4). When we compared the genotype distributions between the Control and Moderate or Severe CP groups, we found that the following genotypes showed associations with: (i) Moderate CP: in rs3811046, it was observed that TG and GG genotypes were shown to increase the susceptibility to moderate periodontal disease (TG, OR = 1.81; 95% CI=1.05 - 3.12; $p = 0.03$ / GG, OR=2.58; 95% CI=1.28 - 5.18; $p = 0.008$ / TG + GG, OR = 1.99; 95% CI=1.18 - 3.32; $p = 0.009$); (ii) Severe CP: in rs2521634, it was observed that AA genotype was shown to increase the risk to severe CP (OR = 2.34; 95% CI=1.19 – 4.59; $p = 0.01$). However, considering the Bonferroni correction, those differences lack the significance ($p < 0.007$).

3.3. Sex-specific effects of gene polymorphisms on periodontal disease

We further analyzed the association between gene polymorphisms and CP by sex (Table 5). Women carrying the rs3811046 SNP (heterozygous or homozygous for the minor allele) are more susceptible to develop moderate CP, especially women carrying the homozygous genotype, since the results were statistically significant after Bonferroni correction (GG, OR = 4.02; CI: 1.65 – 9.80; $p = 0.002$ / TG, OR = 2.16; CI: 1.02 – 4.58; $p = 0.04$). The sex-specific OR to GG genotype were 1.19 for men, but this result was not significant.

The opposite sex-specific effect was observed for the rs2521634 SNP. Men who carried the rs2521634 (homozygote for the minor allele) may are more prone to develop severe CP (OR = 3.61; CI: 1.11 – 11.76; $p = 0.03$). The same effect wan not verified for women.

3.4. Association of periodontal parameters with gene polymorphisms

Instead of just performing only the binary analysis (case / control), we also analyzed the effects of these 7 genetic variants on periodontal parameters (Table 6). The direction of the regression coefficient (β) represents the effect of each genotype increasing (+) or decreasing (−) the percentage of sites with bleeding on probing, in interproximal periodontal pockets depth with 5mm (PPDi ≥ 5 mm) or more and interproximal clinical attachment loss with 6 mm (CALi ≥ 6 mm) or more, that means more severity of CP. We observed that regarding the rs2521634 SNP the homozygous genotype for the rare allele (AA) was positively associated with higher percentage of PPDi ≥ 5 mm (β = 4.91, $p = 0.004$). In addition, we also found that patients carrying the homozygous variant for the rs2521634 SNP and the rs3811046 SNP were positively, associated with higher percentage of bleeding on probing (AA, β = 8.18; $p = 0.02$ / GG, β = 5.95; $p = 0.03$, respectively); and higher percentage of sites with CALi ≥ 6mm (AA, β = 3.27; $p = 0.04$ / GG, β = 2.93; $p = 0.02$, respectively), but not statistically significant after the Bonferroni correction.

4. Discussion

Genetic association studies based on the candidate-gene or genome-wide approach to search for the statistical correlation between genetic variants and a pathophysiologic trait have made important strides in developing numerous diagnostic and therapeutic strategies for various kinds of human diseases (Su et al., 2015a). In this present study, we selected SNPs previously associated with CP in other populations by GWAS or bioinformatic approaches in order to validate those results in a Brazilian population. For the first time, we demonstrated the validation of the association between the rs2521634 SNP, close to the *NPY* gene, and the rs3811046 SNP in the intronic region of the *IL37* gene and increased risk to moderate and severe chronic CP in a Brazilian population.

The validated rs2521634 SNP is an intergenic SNP, located on 47 Kb downstream from the *NPY* gene at the 7p15 locus (Divaris et al., 2013). This SNP was firstly associated with severe CP (OR = 1.47; 95% CI = 1.25–1.73; p-value = 1.6×10^{-6}) in a cohort of 4504 European Americans (EA) participating in the Atherosclerosis Risk in Communities (ARIC) study

through GWAS (Divaris et al., 2013). Subsequently, the *NPY* locus (rs2521634 SNP) was highlighted by a gene-centered reanalysis of ARIC, and the results obtained by Rhodin et al. (2014) showed association with chronic severe periodontitis (Rhodin et al., 2014), in agreement with Divaris et al. (2013). Interestingly, in a Japanese population investigated by GWAS, it was not found significant association between the rs2521634 SNP close to the *NPY* gene and periodontitis (Shimizu et al., 2015).

In the present study, even after the multiple logistic regression and the Bonferroni`s correction, the rs2521634 SNP was positively associated with higher percentage of sites with PPDi ≥ 5 mm. This SNP was also associated with higher percentage of sites with bleeding on probing and CALi ≥ 6 mm, although not statistically significant after the Bonferroni correction. The NPY protein levels, recently investigated by Haririan et al. (2017) on the saliva of patients with CP, were significantly correlated with the clinical parameters of the mean CAL ($r = 0.29$, $p < 0.05$), bleeding on probing ($r = 0.29$, $p < 0.05$) and the number of teeth with PPD ≥ 5 mm ($r = 0.34$, $p < 0.005$) (Haririan et al., 2017).

Neuropeptide Y (NPY) is a potent vasoconstrictor which is co-localized with noradrenaline in the sympathetic nervous system (Lundberg et al., 1985). NPY has been shown to have several roles including modulation of the immune response, angiogenesis, suggesting a role in tissue development and repair (Bedoui et al., 2003, Zukowska et al., 2003, Pedrazzini et al., 2003, Lundy and Linden, 2004). Evidence for NPY-mediated effects on immune cells has arisen from studies showing the presence of NPY-positive nerves fibers in direct contact with immune cells (Wheway et al., 2007).

NPY is the most abundant neuropeptide in bone and has recently been shown to have a role in maintaining the balance between hard tissue formation and resorption, processes that are relevant to the periodontal metabolism (Ahmed et al., 1994, Haug and Heyeraas, 2006). There is a significantly lower production of NPY in bone after sympathectomy, and it is follow by increased osteoclast number and activity (Haug and Heyeraas, 2003). NPY is therefore potentially important in the coordination of inflammation and bone metabolism, both of which are central to the pathogenesis of periodontal disease (Lundy et al., 2009). Altogether, NPY have been shown to be associated with blood vessels in the periodontal ligament, suggesting a role for this molecule in regulating blood flow in this region (Kato et al., 1996). Besides that, NPY is present in gingival crevicular fluid (GCF), an immunologically relevant exudate of the gingival crevice. The NPY level is significantly higher from healthy periodontal sites compared with periodontitis sites (Lundy et al., 2009). This indicates a possible role for NPY in

periodontal health and disease, since it is known that NPY is bound and recognized by receptors on immune cells. Multiple signaling systems are likely to be involved in the pathogenesis of periodontitis which is a complex disease, and NPY, which is predominantly anti-inflammatory, may have an important role in the maintenance of periodontal health. In contrast, higher levels of NPY were found in the saliva of patients with aggressive and chronic periodontitis compared to healthy patients (Haririan et al., 2017). The authors suggest that substances with anti-inflammatory activities are released in abundance in periodontal disease to reestablish a healthy state, and this would also apply to NPY.

After a paper published of GWAS in a cohort of 4910 Caucasians as the dental part of ARIC study (Divaris et al., 2013), the GWAS data from this population was re-analyzed. Instead of using clinical measurements (bleeding on probing, probing depth, or attachment loss) used to diagnose chronic periodontitis as the phenotype, that new analysis focused on biomarkers of inflammation to define pro-inflammatory phenotype. The upper quartile of IL-1ß concentration in GCF was used to define the pro-inflammatory phenotype. the study was recently conducted (Kim, 2017) and it is in preparation to submission to be published. Those authors looked into 2.5 million SNPs in 22 somatic chromosomes and the re-analysis identified a SNP (rs3811046) within the coding region of the anti-inflammatory gene *Interleukin-37* (*IL37*), with significant association with high levels of GCF-IL-1ß (*p*-value of less than $1x10^{-21}$). This locus is not only strongly associated with high local IL-1ß levels, but it is also associated with more severe periodontal disease (Kim, 2017).

Noteworthy, in the investigated Brazilian population the rs3811046 SNP was statistically associated to moderate CP before correction for covariates (p=0.004, Table 3). Considering the multiple logistic regression, the same SNP was associated with moderate CP (Table 4) and with higher percentage of sites with bleeding on probing and CALi ≥ 6mm (Table 6).

In an alternative analysis, the association of polymorphisms regarding chronic periodontitis was explored by stratification for gender. Although there is no well-established evidence of a differential susceptibility of CP between males and females, in several different populations males had presented worse periodontal health compared with females (Albandar, 2002). There are some studies showing a sex-specific CP susceptibility, since the dimorphic role of some polymorphisms in humans was demonstrated as associated with complex diseases, including CP (Chen et al., 2014, Anovazzi et al., 2010, Meisel et al., 2002, Ericson et al., 2013, Gbadoe et al., 2016). A gender differential in gene transcription is necessary as males and

females use a similar genome to achieve distinct phenotypes (Rinn and Snyder, 2005). It has been suggested that gender-biased differences in gene transcription 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 (Rinn et al., 2004). Clinical experience confirms the influence of sex hormones on the pathogenesis and expression of many diseases, and the observed sex-specific differences suggest that of sexual hormones may be affect, at least in part, the association between polymorphisms and periodontal disease (Meisel et al., 2002).

The present study showed a weak sex-specific effect between the rs2521634 SNP close to the *NPY* gene and severe CP, after the Bonferroni correction, since men who carry the homozygote for the minor allele genotype (AA) may be more prone to develop severe CP ($p = 0.03$, Table 5). Interestingly, sex-dependent effects of NPY were previously described in human and mice (Freitag-Wolf et al., 2014, Forbes et al., 2012, Painsipp et al., 2011). Ten SNPs in strong linkage disequilibrium, upstream to the *NPY* gene, suggested gene–sex interaction with respect to the risk for aggressive periodontitis on a genome-wide scale in German/Austrian population (Freitag-Wolf et al., 2014). A different SNP (rs198712) than the studied in this present study showed strongest association in interaction ($p = 5.4 \times 10^{-6}$) with odds ratios in males and females of 1.63 and 0.69 respectively. This study enrolling Brazilian individuals demonstrated a sexually dimorphic role of alleles at the rs2521634 SNP close to the *NPY* gene in humans and support previous genome-wide findings of a role of the *NPY* in the severe CP. Concerning the SNP investigated in the *IL37* gene, to our knowledge, this study, even after de Bonferroni`s correction, is the first to observe a sexually dimorphic potential role of *IL37* in humans in regard to CP. Yan et al. (2015) investigated the same SNP, and after gender stratification the authors observed that this polymorphism was significantly associated with a decreased risk of Graves' disease in female patients (G allele, *p-value* = 0.03, OR = 0.777) (Yan et al., 2015). Intriguingly, the same SNP has opposite sex-specific effect in the two diseases. While an increased risk for CP was observed for women in this present study, females presented a reduction in risk for Graves' disease.

The IL-37, also known as IL-1F7, is the most recently described member of the IL-1 cytokine family (Kumar et al., 2000). It is a potent anti-inflammatory cytokine, produced by peripheral blood mononuclear cells, monocytes, dendritic cells, plasma cells and epithelial cells in different tissues that inhibits innate inflammatory responses (Nold et al., 2010, Boraschi et al., 2011). Under normal conditions IL-37 attenuates the innate immune response to TLR

agonists to include suppression of pro-inflammatory cytokines (IL-1ß, TNF-α, IL-6, IL-17 and IFN-γ) (Boraschi et al., 2011). In addition to its inhibitory effect on innate immunity; IL-37 plays a pivotal role in regulating adaptive immunity by inducing regulatory T cells and impairing effector T-cell functions (Luo et al., 2014). In bacterial infections, IL-37 inhibits infection-induced inflammation. These findings indicate that IL-37 plays an essential role in a negative-feedback system to control excessive inflammation, but its role in host susceptibility/resistance to infectious diseases is not yet completely understood (Nold et al., 2010).

There is only one study that investigated the gingival crevicular fluid (GCF), salivary and plasma levels of IL-37 in individuals with chronic periodontitis and in systemically periodontally healthy control individuals (Saglam et al., 2015). The total amounts of IL-37 in GCF and the salivary and plasma were similar in the control and periodontal disease sites. In the light of these findings, it seems 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.

This study has some potential limitations. The genes investigated in this study present more than one SNP and it might be interesting to evaluate whether other genetic SNPs and possible haplotypes play a role in subjects with CP. The true association may be missed, or the true causal SNPs may be located in other regions of the seven studied genes, including SNPs in the vicinity. The individuals in the two groups were not matched for important risk factors, i.e. age and smoking status. Another limitation of this study was that, confounders (i.e. sex, age and smoking status) were controlled for this study using multinomial logistic regression. However, it is possible that results may be confounded by other factors not included in the model, such as lipid profile, alcohol, body mass index, nutritional status and education of each participant. There is a well-known limitation of the logistic regression model in general, as it can describe only multiplicative effects of odds ratios (Witte and Greenland, 1997). The ethnically admixed Brazilian population is other limitation. Therefore, the individuals have not been stratified into ethnic groups or skin color due to the remarkable miscegenation of the Brazilian population. Parra et al. (2003) does not recommend grouping Brazilians into ethnic groups based on skin color and other physical characteristics associated with racial divisions (Parra et al., 2003). Brazilian individuals classified as ‘white’ or ‘black’ have significantly overlapping genotypes considered race-associated loci for Caucasians, Africans and Indigenous people. Brazilians form one of the most heterogeneous populations in the world, resulting of the 5 centuries of

interethnic crosses between Europeans (mainly Portuguese colonizers and Italian immigrants), Africans (brought to Brazil as slaves) and the autochthonous Amerindians (Alves-Silva et al., 2000). In the present study, all the patients and controls were selected from the same geographical area (Araraquara region, São Paulo State, Southeast of Brazil) and were of the same socio-economic status.

In this study, it was observed that a specific factor, i.e. sex, masked the genetic effect in a way that the genetic influence could be observed only in one of the sexes. This showed that adding an interaction term towards a more comprehensive model attempting to describe more precisely the interplay of environmental and genetic factors. In spite of these limitations, the present study shows consistent and relevant evidence on the validation in a Brazilian population of GWAS loci for CP. It is expected that a GWAS loci could be validated in ethnic diverse populations, since this give useful genetic information that would be invaluable in identifying CP high-risk individuals intending the development of periodontal disease.

Further studies are necessary to investigate the functional role of the validated SNPs in the periodontal disease context, and whether there is influence of gender in their transcriptional or translational levels. Moreover, although the number of Brazilian subjects enrolled in this study permitted the statistical power, it is suggested to investigate the same genetic variants in larger sample sizes of both Brazilians from different regions of the country, as well of multiple ethnic populations of the world, in agreement with Laine et al (2014). Also, it is important to use mathematical modeling of the various causative factors to further explore the complexity of periodontitis (Laine et al., 2014).

In conclusion, here we validated for a Brazilian population the association of the rs2521634 and the rs3811046 SNPs, previously found by GWAS, with increased risk to moderate and severe chronic CP. These results also support the hypothesis that *NPY* and *IL37* polymorphisms might play a role on the genetic profile predisposing to periodontal disease, and the risk is sex-specific. Functional analyses of the role of these polymorphisms and possible existence of confident haplotypes useful in the diagnosis of susceptible CP individuals might contribute with the better understanding of the pathogenic process.

5. References

Ahmed, M., Srinivasan, G. R., Theodorsson, E., Bjurholm, A. & Kreicbergs, A. (1994) Extraction and quantitation of neuropeptides in bone by radioimmunoassay. *Regul Pept* **51,** 179-188.

Aidar, M. & Line, S. R. (2007) A simple and cost-effective protocol for DNA isolation from buccal epithelial cells. *Braz Dent J* **18,** 148-152.

Albandar, J. M. (2002) Global risk factors and risk indicators for periodontal diseases. *Periodontol 2000* **29,** 177-206.

Alves-Silva, J., da Silva Santos, M., Guimaraes, P. E., Ferreira, A. C., Bandelt, H. J., Pena, S. D. & Prado, V. F. (2000) The ancestry of Brazilian mtDNA lineages. *Am J Hum Genet* **67,** 444-461. doi:10.1086/303004.

Anovazzi, G., Kim, Y. J., Viana, A. C., Curtis, K. M., Orrico, S. R., Cirelli, J. A. & Scarel-Caminaga, R. M. (2010) Polymorphisms and haplotypes in the interleukin-4 gene are associated with chronic periodontitis in a Brazilian population. *J Periodontol* **81,** 392-402. doi:10.1902/jop.2009.090392.

Bedoui, S., Miyake, S., Lin, Y., Miyamoto, K., Oki, S., Kawamura, N., Beck-Sickinger, A., von Horsten, S. & Yamamura, T. (2003) Neuropeptide Y (NPY) suppresses experimental autoimmune encephalomyelitis: NPY1 receptor-specific inhibition of autoreactive Th1 responses in vivo. *J Immunol* **171,** 3451-3458.

Boraschi, D., Lucchesi, D., Hainzl, S., Leitner, M., Maier, E., Mangelberger, D., Oostingh, G. J., Pfaller, T., Pixner, C., Posselt, G., Italiani, P., Nold, M. F., Nold-Petry, C. A., Bufler, P. & Dinarello, C. A. (2011) IL-37: a new anti-inflammatory cytokine of the IL-1 family. *Eur Cytokine Netw* **22,** 127-147. doi:10.1684/ecn.2011.0288.

Chen, D., Zhang, T. L. & Wang, L. M. (2014) The association of CSF-1 gene polymorphism with chronic periodontitis in the Han Chinese population. *J Periodontol* **85,** e304-312. doi:10.1902/jop.2014.130688.

Corey, L. A., Nance, W. E., Hofstede, P. & Schenkein, H. A. (1993) Self-reported periodontal disease in a Virginia twin population. *J Periodontol* **64,** 1205-1208. doi:10.1902/jop.1993.64.12.1205.

Darveau, R. P. (2010) Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol* **8,** 481-490. doi:10.1038/nrmicro2337.

Ding, C., Ji, X., Chen, X., Xu, Y. & Zhong, L. (2014) TNF-alpha gene promoter polymorphisms contribute to periodontitis susceptibility: evidence from 46 studies. *J Clin Periodontol* **41,** 748-759. doi:10.1111/jcpe.12279.

Divaris, K., Monda, K. L., North, K. E., Olshan, A. F., Reynolds, L. M., Hsueh, W. C., Lange, E. M., Moss, K., Barros, S. P., Weyant, R. J., Liu, Y., Newman, A. B., Beck, J. D. & Offenbacher, S. (2013) Exploring the genetic basis of chronic periodontitis: a genome-wide association study. *Hum Mol Genet* **22,** 2312-2324. doi:10.1093/hmg/ddt065.

Ericson, U., Rukh, G., Stojkovic, I., Sonestedt, E., Gullberg, B., Wirfalt, E., Wallstrom, P. & Orho-Melander, M. (2013) Sex-specific interactions between the IRS1 polymorphism and intakes of carbohydrates and fat on incident type 2 diabetes. *Am J Clin Nutr* **97,** 208-216. doi:10.3945/ajcn.112.046474.

Faul, F., Erdfelder, E., Lang, A. G. & Buchner, A. (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* **39,** 175-191.

Forbes, S., Herzog, H. & Cox, H. M. (2012) A role for neuropeptide Y in the gender-specific gastrointestinal, corticosterone and feeding responses to stress. *Br J Pharmacol* **166,** 2307-2316. doi:10.1111/j.1476-5381.2012.01939.x.

Freitag-Wolf, S., Dommisch, H., Graetz, C., Jockel-Schneider, Y., Harks, I., Staufenbiel, I., Meyle, J., Eickholz, P., Noack, B., Bruckmann, C., Gieger, C., Jepsen, S., Lieb, W., Schreiber, S., Konig, I. R. & Schaefer, A. S. (2014) Genome-wide exploration identifies sex-specific genetic effects of alleles upstream NPY to increase the risk of severe periodontitis in men. *J Clin Periodontol* **41,** 1115-1121. doi:10.1111/jcpe.12317.

Gbadoe, K. M., Berdouzi, N., Aguinano, A. A., Ndiaye, N. C. & Visvikis-Siest, S. (2016) Cardiovascular diseases-related GNB3 C825T polymorphism has a significant sex-specific effect on serum soluble E-selectin levels. *J Inflamm (Lond)* **13,** 39. doi:10.1186/s12950-016-0146-z.

Haririan, H., Andrukhov, O., Bottcher, M., Pablik, E., Wimmer, G., Moritz, A. & Rausch-Fan, X. (2017) Salivary Neuropeptides, Stress and Periodontitis. *J Periodontol***,** 1-15. doi:10.1902/jop.2017.170249.

Haug, S. R. & Heyeraas, K. J. (2003) Effects of sympathectomy on experimentally induced pulpal inflammation and periapical lesions in rats. *Neuroscience* **120,** 827-836.

Haug, S. R. & Heyeraas, K. J. (2006) Modulation of dental inflammation by the sympathetic nervous system. *J Dent Res* **85,** 488-495. doi:10.1177/154405910608500602.

Igl, B. W., Konig, I. R. & Ziegler, A. (2009) What do we mean by 'replication' and 'validation' in genome-wide association studies? *Hum Hered* **67,** 66-68. doi:10.1159/000164400.

Kato, J., Wakisaka, S. & Kurisu, K. (1996) Immunohistochemical changes in the distribution of nerve fibers in the periodontal ligament during an experimental tooth movement of the rat molar. *Acta Anat (Basel)* **157,** 53-62.

Kim, S. J. D. (2017) Functional analysis of rs3811046 and rs3811047 variants in Caucasians. In: *Oral and Craniofacial Biomedicine, School of Dentistry,* . Chapel Hill - NC: University of North Carolina at Chapel Hill.

Kornman, K. S. (2008) Mapping the pathogenesis of periodontitis: a new look. *J Periodontol* **79,** 1560-1568. doi:10.1902/jop.2008.080213.

Kornman, K. S., Crane, A., Wang, H. Y., di Giovine, F. S., Newman, M. G., Pirk, F. W., Wilson, T. G., Jr., Higginbottom, F. L. & Duff, G. W. (1997) The interleukin-1 genotype as a severity factor in adult periodontal disease. *J Clin Periodontol* **24,** 72-77.

Kumar, S., McDonnell, P. C., Lehr, R., Tierney, L., Tzimas, M. N., Griswold, D. E., Capper, E. A., Tal-Singer, R., Wells, G. I., Doyle, M. L. & Young, P. R. (2000) Identification and initial characterization of four novel members of the interleukin-1 family. *J Biol Chem* **275,** 10308-10314.

Laine, M. L., Crielaard, W. & Loos, B. G. (2012) Genetic susceptibility to periodontitis. *Periodontol 2000* **58,** 37-68. doi:10.1111/j.1600-0757.2011.00415.x.

Laine, M. L., Jepsen, S. & Loos, B. G. (2014) Progress in the Identification of Genetic Factors in Periodontitis. *Current Oral Health Reports* **1,** 272-278. doi:10.1007/s40496-014-0037-4.

Lundberg, J. M., Pernow, J., Tatemoto, K. & Dahlof, C. (1985) Pre- and postjunctional effects of NPY on sympathetic control of rat femoral artery. *Acta Physiol Scand* **123,** 511-513. doi:10.1111/j.1748-1716.1985.tb07619.x.

Lundy, F. T., El Karim, I. A. & Linden, G. J. (2009) Neuropeptide Y (NPY) and NPY Y1 receptor in periodontal health and disease. *Arch Oral Biol* **54,** 258-262. doi:10.1016/j.archoralbio.2008.10.002.

Lundy, F. T. & Linden, G. J. (2004) Neuropeptides and Neurogenic Mechanisms in Oral and Periodontal Inflammation. *Crit Rev Oral Biol Med* **15,** 82-98.

Luo, Y., Cai, X., Liu, S., Wang, S., Nold-Petry, C. A., Nold, M. F., Bufler, P., Norris, D., Dinarello, C. A. & Fujita, M. (2014) Suppression of antigen-specific adaptive immunity by IL-37 via induction of tolerogenic dendritic cells. *Proc Natl Acad Sci U S A* **111,** 15178-15183. doi:10.1073/pnas.1416714111.

Marcenes, W., Kassebaum, N. J., Bernabe, E., Flaxman, A., Naghavi, M., Lopez, A. & Murray, C. J. (2013) Global burden of oral conditions in 1990-2010: a systematic analysis. *J Dent Res* **92,** 592-597. doi:10.1177/0022034513490168.

Meisel, P., Krause, T., Cascorbi, I., Schroeder, W., Herrmann, F., John, U. & Kocher, T. (2002) Gender and smoking-related risk reduction of periodontal disease with variant myeloperoxidase alleles. *Genes Immun* **3,** 102-106. doi:10.1038/sj.gene.6363840.

Michalowicz, B. S., Aeppli, D., Virag, J. G., Klump, D. G., Hinrichs, J. E., Segal, N. L., Bouchard, T. J., Jr. & Pihlstrom, B. L. (1991) Periodontal findings in adult twins. *J Periodontol* **62,** 293-299. doi:10.1902/jop.1991.62.5.293.

Michalowicz, B. S., Diehl, S. R., Gunsolley, J. C., Sparks, B. S., Brooks, C. N., Koertge, T. E., Califano, J. V., Burmeister, J. A. & Schenkein, H. A. (2000) Evidence of a substantial genetic basis for risk of adult periodontitis. *J Periodontol* **71,** 1699-1707. doi:10.1902/jop.2000.71.11.1699.

Nibali, L., Griffiths, G. S., Donos, N., Parkar, M., D'Aiuto, F., Tonetti, M. S. & Brett, P. M. (2008) Association between interleukin-6 promoter haplotypes and aggressive periodontitis. *J Clin Periodontol* **35,** 193-198. doi:10.1111/j.1600-051X.2007.01188.x.

Nold, M. F., Nold-Petry, C. A., Zepp, J. A., Palmer, B. E., Bufler, P. & Dinarello, C. A. (2010) IL-37 is a fundamental inhibitor of innate immunity. *Nat Immunol* **11,** 1014-1022. doi:10.1038/ni.1944.

Page, R. C. & Eke, P. I. (2007) Case definitions for use in population-based surveillance of periodontitis. *J Periodontol* **78,** 1387-1399. doi:10.1902/jop.2007.060264.

Painsipp, E., Herzog, H., Sperk, G. & Holzer, P. (2011) Sex-dependent control of murine emotional-affective behaviour in health and colitis by peptide YY and neuropeptide Y. *Br J Pharmacol* **163,** 1302-1314. doi:10.1111/j.1476-5381.2011.01326.x.

Parra, F. C., Amado, R. C., Lambertucci, J. R., Rocha, J., Antunes, C. M. & Pena, S. D. (2003) Color and genomic ancestry in Brazilians. *Proc Natl Acad Sci U S A* **100,** 177-182. doi:10.1073/pnas.0126614100.

Pedrazzini, T., Pralong, F. & Grouzmann, E. (2003) Neuropeptide Y: the universal soldier. *Cell Mol Life Sci* **60,** 350-377.

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A., Bender, D., Maller, J., Sklar, P., de Bakker, P. I., Daly, M. J. & Sham, P. C. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* **81,** 559-575. doi:10.1086/519795.

Rhodin, K., Divaris, K., North, K. E., Barros, S. P., Moss, K., Beck, J. D. & Offenbacher, S. (2014) Chronic periodontitis genome-wide association studies: gene-centric and gene set enrichment analyses. *J Dent Res* **93,** 882-890. doi:10.1177/0022034514544506.

Rinn, J. L., Rozowsky, J. S., Laurenzi, I. J., Petersen, P. H., Zou, K., Zhong, W., Gerstein, M. & Snyder, M. (2004) Major molecular differences between mammalian sexes are involved in drug metabolism and renal function. *Dev Cell* **6,** 791-800. doi:10.1016/j.devcel.2004.05.005.

Rinn, J. L. & Snyder, M. (2005) Sexual dimorphism in mammalian gene expression. *Trends Genet* **21,** 298-305. doi:10.1016/j.tig.2005.03.005.

Saglam, M., Koseoglu, S., Savran, L., Pekbagriyanik, T., Saglam, G. & Sutcu, R. (2015) Levels of interleukin-37 in gingival crevicular fluid, saliva, or plasma in periodontal disease. *J Periodontal Res* **50,** 614-621. doi:10.1111/jre.12241.

Scarel-Caminaga, R. M., Kim, Y. J., Viana, A. C., Curtis, K. M., Corbi, S. C., Sogumo, P. M., Orrico, S. R. & Cirelli, J. A. (2011) Haplotypes in the interleukin 8 gene and their association with chronic periodontitis susceptibility. *Biochem Genet* **49,** 292-302. doi:10.1007/s10528-010-9407-3.

Shaffer, J. R., Polk, D. E., Wang, X., Feingold, E., Weeks, D. E., Lee, M. K., Cuenco, K. T., Weyant, R. J., Crout, R. J., McNeil, D. W. & Marazita, M. L. (2014) Genome-wide association study of periodontal health measured by probing depth in adults ages 18-49 years. *G3 (Bethesda)* **4,** 307-314. doi:10.1534/g3.113.008755.

Shimizu, S., Momozawa, Y., Takahashi, A., Nagasawa, T., Ashikawa, K., Terada, Y., Izumi, Y., Kobayashi, H., Tsuji, M., Kubo, M. & Furuichi, Y. (2015) A genome-wide association study of periodontitis in a Japanese population. *J Dent Res* **94,** 555-561. doi:10.1177/0022034515570315.

Su, S., Chien, M., Lin, C., Chen, M. & Yang, S. (2015a) RAGE gene polymorphism and environmental factor in the risk of oral cancer. *J Dent Res* **94,** 403-411. doi:10.1177/0022034514566215.

Su, S. L., Yang, H. Y., Lee, H. S., Huang, G. S., Lee, C. H., Liu, W. S., Wang, C. C., Peng, Y. J., Lai, C. H., Chen, C. Y., Lin, C., Pan, Y. T., Salter, D. M. & Chen, H. C. (2015b) Gene-gene interactions between TGF-beta/Smad3 signalling pathway polymorphisms affect susceptibility to knee osteoarthritis. *BMJ Open* **5,** e007931. doi:10.1136/bmjopen-2015-007931.

Teumer, A., Holtfreter, B., Volker, U., Petersmann, A., Nauck, M., Biffar, R., Volzke, H., Kroemer, H. K., Meisel, P., Homuth, G. & Kocher, T. (2013) Genome-wide association

study of chronic periodontitis in a general German population. *J Clin Periodontol* **40,** 977-985. doi:10.1111/jcpe.12154.

Vaithilingam, R. D., Safii, S. H., Baharuddin, N. A., Ng, C. C., Cheong, S. C., Bartold, P. M., Schaefer, A. S. & Loos, B. G. (2014) Moving into a new era of periodontal genetic studies: relevance of large case-control samples using severe phenotypes for genome-wide association studies. *J Periodontal Res* **49,** 683-695. doi:10.1111/jre.12167.

Wheway, J., Herzog, H. & Mackay, F. (2007) NPY and receptors in immune and inflammatory diseases. *Curr Top Med Chem* **7,** 1743-1752.

Witte, J. S. & Greenland, S. (1997) A nested approach to evaluating dose-response and trend. *Ann Epidemiol* **7,** 188-193.

Wu, L., Hu, Y., Li, D., Jiang, W. & Xu, B. (2015) Screening toll-like receptor markers to predict latent tuberculosis infection and subsequent tuberculosis disease in a Chinese population. *BMC Med Genet* **16,** 19. doi:10.1186/s12881-015-0166-1.

Yan, N., Meng, S., Song, R. H., Qin, Q., Wang, X., Yao, Q., Jiang, Y., Jiang, W., Shi, L., Xu, J. & Zhang, J. (2015) Polymorphism of IL37 gene as a protective factor for autoimmune thyroid disease. *J Mol Endocrinol* **55,** 209-218. doi:10.1530/JME-15-0144.

Yoshie, H., Kobayashi, T., Tai, H. & Galicia, J. C. (2007) The role of genetic polymorphisms in periodontitis. *Periodontol 2000* **43,** 102-132. doi:10.1111/j.1600-0757.2006.00164.x.

Zhan, Y., Zhang, R., Lv, H., Song, X., Xu, X., Chai, L., Lv, W., Shang, Z., Jiang, Y. & Zhang, R. (2014) Prioritization of candidate genes for periodontitis using multiple computational tools. *J Periodontol* **85,** 1059-1069. doi:10.1902/jop.2014.130523.

Zhang, J., Sun, X., Xiao, L., Xie, C., Xuan, D. & Luo, G. (2011) Gene polymorphisms and periodontitis. *Periodontol 2000* **56,** 102-124. doi:10.1111/j.1600-0757.2010.00371.x.

Zukowska, Z., Grant, D. S. & Lee, E. W. (2003) Neuropeptide Y: a novel mechanism for ischemic angiogenesis. *Trends Cardiovasc Med* **13,** 86-92.

Table 1. Information for 7 genotyped polymorphisms and assays

SNP	Assay ID	Cytogenetic Band	Closest gene(s) and position or distance	Allele	MAF		HWE	Call rate (%)*
rs187238	C___2408543_10	11q23.1d	*IL18* (intronic)	**C|G**	**G|G**	0.06	0.69	97.62
rs352140	C___2301954_20	3p21.2a	*TLR9* (exonic)	**C|T**	**T|T**	0.23	0.16	96.49
rs1360573	C___8858561_20	10p11.21a	*ANKRD30A (529 Kb); FZD8 (956 Kb)*	**A|G**	**G|G**	0.05	0.55	95.79
rs2521634	C__15891260_10	7p15.3b	*NPY* (47Kb)	**A|G**	**A|A**	0.07	0.66	95.65
rs3811046	C__27487174_10	2q13d	*IL37* (intronic)	**G|T**	**G|G**	0.14	0.55	97.76
rs3826782	C__27506722_10	19p13.3a	*EMR1* (intronic)	**A|G**	**A|A**	0.01	0.26	96.77
rs7762544	C__29802770_10	6p21.1g	*NCR2* (61Kb)	**A|G**	**G|G**	0.05	0.99	96.21

MAF, minor allele frequency (in this study); HWE, Hardy–Weinberg equilibrium.
*Represents the amount of genotyping performed with success, in percentage, for OpenArray™ plate.

Table 2. Comparison of demographic characteristics and periodontal parameters (comparisons performed by Student's t or chi-square test)

	Chronic periodontitis			
	No/Mild n=356	**Moderate + Severe n=358**	**Moderate n=89**	**Severe n=269**
Demographic Characteristics				
Age (mean ± SD)*	43.6 (±11.0)	49.1 (±9.3)*	42.9 (±6.6)	51.2 (±9.2)*
Sex, n (%)				
Males	115 (32.3)	146 (40.8)	39 (43.8)	107 (39.8)
Females	241 (67.7)	212 (59.2)	50 (56.2)	162 (60.2)
Smoking, n (%)				
Never	275 (77.3)	229 (64.0)*	66 (74.2)*	163 (60.6)*
Ever	81 (26.7)	129 (36.0)	23 (25.8)	106 (39.4)
Periodontal Parameters**				
Number of teeth	25.5 (±3.5)	22.8 (±4.5)	23.8 (±4.3)	22.5 (±4.6)
Visible plaque (% of sites)	24.7 (±20.0)	45.7 (±28.2)	41.0 (±28.7)	47.2 (±27.9)
Marginal bleeding (% of sites)	8.6 (±11.6)	20.0 (±23.3)	19.8 (±23.9)	20.0 (±23.2)
BOP (% of sites)	3.2 (±4.9)	40.2 (±23.2)	23.2 (±16.8)	45.8 (±22.3)
PPDi ≤ 4mm (% of sites)	99.3 (±0.3)	86.8 (±14.2)	96.0 (±6.6)	83.8 (±14.7)
PPDi ≥ 5mm (% of sites)	0.1 (±0.3)	13.2 (±14.2)	4.0 (±6.6)	16.2 (±14.7)
CALi ≤ 3mm (% of sites)	99.5 (±0.8)	68.3 (±21.5)	87.2 (±9.0)	62.1 (±20.8)
CALi = 4-5mm (% of sites)	0.5 (±0.8)	20.1 (±11.9)	11.6 (±8.6)	22.9 (±11.5)
CALi ≥ 6mm (% of sites)	0.0 (±0.2)	11.6 (±14.1)	1.3 (±2.1)	15.0 (±14.7)

*$p < 0.05$ comparing to No/Mild CP. **$p < 0.001$ for all periodontal parameters, comparing No/Mild *versus* Moderate, Severe and Moderate + Severe CP.

Abbreviations: CP, chronic periodontitis; BOP, bleeding on probing; PPDi, interproximal probing pocket depth; CALi, interproximal clinical attachment level.

Table 3. Alleles and genotype distribution of the 7 SNPs in the studied individuals (performed by chi-square test)

	Periodontal Disease, n (%)				χ2 (*p* -value)[a]		
Allele / Genotype	No/Mild	Moderate + Severe	Moderate	Severe	a	b	c
(C>G) rs187238							
C/G	504 (72.83) / 188 (27.17)	533 (76.14) / 167 (23.86)	133 (77.33) / 39 (22.67)	400 (75.76) / 128 (24.24)	0.16	0.23	0.25
CC	185 (53.47)	197 (56.29)	50 (58.14)	147 (55.68)			
CG	134 (38.73)	139 (39.71)	33 (38.37)	106 (40.15)	0.10	0.35	0.18
GG	27 (7.80)	14 (4.00)	3 (3.49)	11 (4.17)			
CC/CG+GG	185 (53.47) / 161 (46.53)	197 (56.29) / 153 (43.71)	50 (58.14) / 36 (41.86)	147 (55.68) / 117 (44.32)	0.46	0.44	0.59
CC+CG/GG	319 (92.20) / 27 (7.80)	336 (96.00) / 14 (4.00)	83 (96,51) / 3 (3.49)	253 (95.83) / 11 (4.17)	**0.03**	0.16	0.07
(C>T) rs352140							
C/T	336 (49.12) /348 (50.88)	378 (54.62) / 314 (45.38)	93 (53.45) / 81 (46.55)	285 (55.02) / 233 (44.98)	**0.04**	0.31	**0.04**
CC	76 (22.22)	106 (30.64)	25 (28.74)	81 (31.27)			
CT	184 (53.80)	166 (47.98)	43 (49.43)	123 (47.49)	**0.04**	0.44	**0.04**
TT	82 (23.98)	74 (21.39)	19 (21.84)	55 (21.24)			
CC/CT+TT	76 (22.22) / 266 (77.78)	106 (30.64) / 240 (69,36)	25 (28.74) / 62 (71.26)	81 (31.27) / 178 (68,73)	**0.01**	0.20	**0.01**
CC+CT/TT	260 (76.02) / 82 (23.98)	272 (78,62) / 74 (21.39)	68 (78.16) / 19 (21.84)	204 (78.76) / 55 (21.24)	0.42	0.67	0.43
(A>G) rs1360573							
A/G	532 (78.24) / 148 (21.76)	514 (74.93) / 172 (25.07)	139 (79.89) / 35 (20.11)	375 (73.24) / 137 (26.76)	0.15	0.64	**0.04**
AA	204 (60.00)	192 (55.98)	57 (65.52)	135 (52.73)			
AG	124 (36.47)	130 (37.90)	25 (28.74)	105 (41.02)	0.23	0.30	0.11
GG	12 (3.53)	21 (6.12)	5 (5.75)	16 (6.25)			
AA/AG+GG	204 (60.00) / 136 (40.00)	192 (55.98) / 151 (44.02)	57 (65.52) / 30 (34.48)	135 (52.73) / 121 (47.27)	0.29	0.35	0.08
AA+AG/GG	328 (96.47) / 12 (3.53)	322 (93.88) / 21 (6.12)	82 (94.25) / 5 (5.75)	240 (93.75) / 16 (6.25)	0.11	0.35	0.12
(G>A) rs2521634							
G/A	529 (77.34) / 155 (22.66)	482 (70.88) / 198 (29.12)	126 (73.26) / 46 (26.74)	356 (70.08) / 152 (29.92)	**0.006***	0.26	**0.005***
GG	206 (60.23)	173 (50.88)	45 (52.33)	128 (50.39)			
AG	117 (34.21)	136 (40.00)	36 (41.86)	100 (39.37)	**0.03**	0.39	**0.02**
AA	19 (5.56)	31 (9.12)	5 (5.81)	26 (10.24)			
GG/GA+AA	206 (60.23) / 136 (39.77)	173 (50.88) / 167 (49.12)	45 (52.33) / 41 (47.67)	128 (50.39) / 126 (49.61)	**0.01**	0.18	**0.02**
GG+GA/AA	323 (94.44) / 19 (5.56)	309 (90.88) / 31 (9.12)	81 (94.19) / 5 (5.81)	228 (89.76) / 26 (10.24)	0.07	0.92	**0.03**
(T>G) rs3811046							
T/G	457 (65.86) / 237 (34.15)	428 (61.14) / 272 (38.86)	94 (54.02) / 80 (45.98)	334 (63.50) / 192 (36.50)	0.07	**0.004***	0.39
TT	153 (44.09)	136 (38.86)	25 (28.74)	111 (42.21)			
TG	151 (43.52)	156 (44.57)	44 (50.57)	112 (42.59)	0.19	**0.02**	0.60
GG	43 (12.39)	58 (16.57)	18 (20.69)	40 (15.21)			
TT/TG+GG	153 (44.09) / 194 (55.91)	136 (38.86) / 214 (61.64)	25 (28.74) / 62 (71.26)	111 (42.21) / 152 (57.79)	0.16	**0.01**	0.64
TT+TG/GG	304 (87.61) / 43 (12.39)	292 (83.43) / 58 (16.57)	69 (79.31) / 18 (20.69)	223 (84.79) / 40 (15.21)	0.12	0.05	0.32
(G>A) rs3826782							
G/A	620 (90.38) / 66 (9.62)	620 (89.34) / 74 (10.66)	156 (89.66) / 18 (10.34)	464 (89.23) / 56 (10.77)	0.52	0.77	0.51
GG	282 (82.22)	278 (80.12)	71 (81.61)	207 (79.62)			
AG	56 (16.33)	64 (18.44)	14 (16.09)	50 (19.23)	0.76	0.86	0.94
AA	5 (1.46)	5 (1.44)	2 (2.30)	3 (1.15)			
GG/GA+AA	282 (82.22) / 61 (17.78)	278 (80.12) / 69 (19.88)	71 (81.61) / 16 (18.39)	207 (79.62) / 53 (20.38)	0.48	0.90	0.42
GG+GA/AA	338 (98.54) / 5 (1.46)	342 (98.56) / 5 (1.44)	85 (97.70) / 2 (2.30)	257 (98.85) / 3 (1.15)	0.99	0.58	0.75
(A>G) rs7762544							
A/G	529 (77.11) / 157 (22.89)	521 (75.95) / 165 (24.05)	130 (75.58) / 42 (24.42)	391 (76.07) / 123 (23.93)	0.61	0.67	0.67
AA	204 (59.48)	196 (57.14)	48 (55.81)	148 (57.59)			
AG	121 (35.28)	129 (37.61)	34 (39.53)	95 (36.96)	0.81	0.76	0.89
GG	18 (5.25)	18 (5.25)	4 (4.65)	14 (5.45)			
AA/AG+GG	204 (59.48) / 139 (40.52)	196 (57.14) / 147 (42.86)	48 (55.81) / 38 (44.19)	148 (57.59) / 109 (42.41)	0.54	0.54	0.64
AA+AG/GG	325 (94.75) / 18 (5.25)	325 (94.74) / 18 (5.25)	82 (95.35) / 4 (4.65)	243 (94.55) / 14 (5.45)	1.00	0.82	0.91

[a]The *p-value* was estimated by chi-square test, comparing No/Mild *versus* Moderate + Severe PD (a); Moderate PD (b); Severe PD (c).

Bold font indicates $p < 0.05$; *indicates statistical significance after Bonferroni correction ($p < 0.007$).

Abbreviations: PD, periodontal disease.

Table 4. Adjusted OR and 95% CI for association among SNPs and moderate / severe CP (performed by multiple logistic regression analysis)

Allele / Genotype	No/Mild *verus* Moderate + Severe CP		No/Mild *versus* Moderate CP		No/Mild *versus* Severe CP	
	Adjusted OR[a] (95% CI)	*p-value*	Adjusted OR[a] (95% CI)	*p-value*	Adjusted OR[a] (95% CI)	*p-value*
(C>G) rs187238						
CC	1.00		1.00		1.00	
CG	0.89 (0.65 - 1.24)	0.51	0.94 (0.57 - 1.54)	0.80	0.87 (0.60 - 1.25)	0.44
GG	0.55 (0.28 - 1.11)	0.10	0.41 (0.12 - 1.42)	0.16	0.65 (0.30 - 1.41)	0.27
CC/CG+GG	0.84 (0.61 - 1.15)	0.28	0.85 (0.52 - 1.37)	0.50	0.83 (0.59 - 1.18)	0.31
(C>T) rs352140						
CC	1.00		1.00		1.00	
CT	0.74 (0.51 - 1.08)	0.12	0.71 (0.41 - 1.26)	0.24	0.76 (0.50 - 1.16)	0.20
TT	0.68 (0.43 - 1.07)	0.09	0.69 (0.35 - 1.36)	0.28	0.66 (0.40 - 1.09)	0.11
CC/CT+TT	0.72 (0.50 - 1.03)	0.07	0.71 (0.41 - 1.20)	0.20	0.73 (0.49 - 1.08)	0.12
(A>G) rs1360573						
AA	1.00		1.00		1.00	
AG	1.09 (0.78 - 1.51)	0.61	0.73 (0.43 - 1.24)	0.25	1.26 (0.87 - 1.81)	0.22
GG	1.65 (0.78 - 3.52)	0.19	1.42 (0.47 - 4.24)	0.53	1.76 (0.77 - 3.99)	0.18
AA/AG+GG	1.14 (0.83 - 1.60)	0.41	0.80 (0.49 - 1.31)	0.37	1.31 (0.92 - 1.86)	0.14
(G>A) rs2521634						
GG	1.00		1.00		1.00	
AG	1.25 (0.90 - 1.75)	0.19	1.45 (0.88 - 2.39)	0.14	1.18 (0.81 - 1.72)	0.38
AA	1.95 (1.04 - 3.66)	**0.04**	1.20 (0.42 - 3.41)	0.73	2.34 (1.19 - 4.59)	**0.01**
GG/GA+AA	1.35 (0.98 - 1.85)	0.07	1.41 (0.88 - 2.29)	0.16	1.33 (0.94 - 1.90)	0.11
(T>G) rs3811046						
TT	1.00		1.00		1.00	
TG	1.15 (0.82 - 1.62)	0.40	1.81 (1.05 - 3.12)	**0.03**	0.95 (0.66 - 1.39)	0.81
GG	1.59 (0.99 - 2.56)	0.06	2.58 (1.28 - 5.18)	**0.008**	1.25 (0.73 - 2.12)	0.42
TT/TG+GG	1.25 (0.91 - 1.72)	0.17	1.99 (1.18 - 3.32)	**0.009**	1.02 (0.72 - 1.45)	0.91
(G>A) rs3826782						
GG	1.00		1.00		1.00	
AG	1.13 (0.74 - 1.70)	0.57	0.99 (0.52 - 1.88)	0.97	1.15 (0.73 - 1.81)	0.55
AA	0.93 (0.25 - 3.41)	0.91	1.58 (0.30 - 8.38)	0.59	0.63 (0.14 - 2.87)	0.55
GG/GA+AA	1.11 (0.74 - 1.66)	0.61	1.04 (0.56 - 1.91)	0.91	1.10 (0.71 - 1.71)	0.68
(A>G) rs7762544						
AA	1.00		1.00		1.00	
AG	1.11 (0.80 - 1.54)	0.55	1.23 (0.74 - 2.01)	0.43	1.13 (0.78 - 1.64)	0.52
GG	1.07 (0.53 - 2.19)	0.84	1.02 (0.33 - 3.17)	0.98	1.06 (0.49 - 2.31)	0.88
AA/AG+GG	1.10 (0.80 - 1.52)	0.54	1.19 (0.74 - 1.93)	0.47	1.12 (0.79 - 1.60)	0.53

[a]The OR with 95% CI were estimated by multiple logistic regression models after controlling for age, sex and smoking.
Bold font indicates $p < 0.05$. There is no statistical significance after Bonferroni correction ($p < 0.007$).
Abbreviations: CP, chronic periodontitis; OR, odds ratio; CI, confidence interval.

Table 5. Adjusted OR and 95% CI for association among SNPs and moderate / severe CP by sex (performed by multiple logistic regression analysis)

Allele / Genotype	No/Mild *versus* Moderate + Severe CP		No/Mild *versus* Moderate CP		No/Mild *versus* Severe CP	
	Adjusted OR[a] (95% CI)	*p*-value	Adjusted OR[a] (95% CI)	*p*-value	Adjusted OR[a] (95% CI)	*p*-value
(G>A) rs2521634						
Male						
GG	1.00		1.00		1.00	
AG	1.27 (0.72 - 2.24)	0.40	1.50 (0.68 - 3.31)	0.31	1.20 (0.63 - 2.27)	0.58
AA	3.12 (1.04 - 9.40)	**0.04**	2.34 (0.50 - 10.87)	0.28	3.61 (1.11 - 11.76)	**0.03**
Female						
GG	1.00		1.00		1.00	
AG	1.28 (0.85 - 1.95)	0.24	1.42 (0.75 - 2.70)	0.29	1.24 (0.78 - 1.97)	0.37
AA	1.51 (0.69 - 3.34)	0.31	0.73 (0.16 - 3.41)	0.69	1.88 (0.81 - 4.37)	0.14
(T>G) rs3811046						
Male						
TT	1.00		1.00		1.00	
TG	1.04 (0.59 - 1.83)	0.89	1.41 (0.63 - 3.16)	0.41	0.87 (0.46 - 1.65)	0.68
GG	0.94 (0.42 - 2.13)	0.88	1.19 (0.36 - 3.90)	0.77	0.76 (0.31 - 1.88)	0.56
Female						
TT	1.00		1.00		1.00	
TG	1.22 (0.80 - 1.87)	0.36	2.16 (1.02 - 4.58)	**0.04**	1.00 (0.63 - 1.60)	0.98
GG	2.04 (1.13 - 3.68)	**0.02**	4.02 (1.65 - 9.80)	**0.002***	1.58 (0.81 - 3.06)	0.18

[a]The OR with 95% CI were estimated by multiple logistic regression models after controlling for age and smoking. **Bold** font indicates $p < 0.05$. *indicates statistical significance after Bonferroni correction ($p < 0.007$).
Abbreviations: CP, chronic periodontitis; OR, odds ratio; CI, confidence interval.

Table 6. Regression coefficient and 95% CI for effects of SNPs in periodontal parameters (performed by multiple linear regression analysis)

Allele / Genotype	Bleeding on probing (% of sites)		PPDi ≥ 5mm (% of sites)		CALi ≥ 6mm (% of sites)	
	adjusted β[a] (95% CI)	*p*-value	adjusted β[a] (95% CI)	*p*-value	adjusted β[a] (95% CI)	*p*-value
(C>G) rs187238						
CC	1.00		1.00		1.00	
CG	-0.85 (-4.59 - 2.88)	0.65	0.04 (-1.78 - 1.87)	0.96	-0.38 (-2.10 - 1.34)	0.67
GG	-2.63 (-10.37 - 5.11)	0.51	0.01 (-3.78 - 3.80)	1.00	-1.25 (-4.82 - 2.33)	0.49
(C>T) rs352140						
CC	1.00		1.00		1.00	
CT	-3.85 (-8.15 - 0.45)	0.08	-1.17 (-3.29 - 0.95)	0.28	-1.18 (-3.17 - 0.82)	0.25
TT	-4.74 (-9.85 - 0.37)	0.07	-1.89 (-4.41 - 0.63)	0.14	-2.14 (-4.51 - 0.23)	0.08
(A>G) rs1360573						
AA	1.00		1.00		1.00	
AG	3.01 (-0.75 - 6.76)	0.12	1.52 (-0.29 - 3.34)	0.10	0.77 (-0.91 - 2.46)	0.37
GG	-1.40 (-9.89 - 7.08)	0.75	2.11 (-2.00 - 6.22)	0.31	1.21 (-2.60 - 5.01)	0.53
(G>A) rs2521634						
GG	1.00		1.00		1.00	
AG	0.46 (-3.33 - 4.25)	0.81	1.42 (-0.41 - 3.25)	0.13	1.25 (-0.45 - 2.95)	0.15
AA	8.18 (1.19 - 15.17)	**0.02**	4.91 (1.54 - 8.28)	**0.004***	3.27 (0.14 - 6.40)	**0.04**
(T>G) rs3811046						
TT	1.00		1.00		1.00	
TG	-0.72 (-4.56 - 3.12)	0.72	-0.38 (-2.26 - 1.51)	0.70	-0.87 (-2.64 - 0.90)	0.33
GG	5.95 (0.55 - 11.35)	**0.03**	2.08 (-0.57 - 4.73)	0.12	2.93 (0.44 - 5.42)	**0.02**
(G>A) rs3826782						
GG	1.00		1.00		1.00	
AG	0.89 (-3.80 - 5.59)	0.71	0.12 (-2.15 - 2.40)	0.92	-0.47 (-2.57 - 1.64)	0.66
AA	-4.90 (-19.79 - 9.98)	0.52	2.41 (-4.80 - 9.63)	0.51	0.79 (-5.88 - 7.45)	0.82
(A>G) rs7762544						
AA	1.00		1.00		1.00	
AG	-0.54 (-4.31 - 3.23)	0.78	-0.38 (-2.20 - 1.45)	0.68	-0.25 (-1.94 - 1.44)	0.77
GG	-1.15 (-9.28 - 6.98)	0.78	-0.60 (-4.54 - 3.34)	0.77	-0.71 (-4.36 - 2.93)	0.70

[a]The regression coefficient with 95% CI were estimated by multiple linear regression models after controlling for age. sex and smoking.

Bold font indicates $p < 0.05$. *indicates statistical significance after Bonferroni correction ($p < 0.007$).

Abbreviations: β, regression coefficient; CI, confidence interval; PPDi, interproximal probing pocket depth; CALi, interproximal clinical attachment level.

4 DISCUSSÃO GERAL

O presente estudo realizou pela primeira vez uma metanálise investigando a relação entre dislipidemia e periodontite crônica (PC), examinando os níveis séricos de parâmetros lipídicos. Embora muitos estudos tenham investigado esta relação, nenhuma metanálise foi realizada até o momento. As questões importantes voltadas para saúde geralmente são estudadas por diferentes grupos de pesquisa em diferentes populações. Em muitos casos, os resultados desses múltiplos estudos são diferentes e conflitantes, o que dificulta a tomada de decisões clínicas. A necessidade de chegar a decisões que afetam a prática clínica promoveu o impulso para a "medicina baseada em evidências" [117,118]. Portanto, a metanálise, um procedimento estatístico que integra os resultados de vários estudos independentes, desempenha um papel central na medicina baseada em evidências [115]. Na verdade, na hierarquia da evidência clínica, as metanálises estão no topo.

Embora a intenção de uma metanálise seja encontrar e avaliar todos os estudos que atendam aos critérios de inclusão, nem sempre é possível obtê-los. Uma preocupação crítica nas metanálises voltadas para Periodontia são os diferentes critérios clínicos utilizados para classificação da doença periodontal [119]. Estudos epidemiológicos da PC são complicados pela diversidade de definições e medidas utilizadas para descrever e quantificar essa doença. A heterogeneidade nos critérios de diagnóstico periodontal apresentou-se claramente nesta revisão sistemática. Alguns autores utilizaram critérios bem definidos, incluindo principalmente por Page e Eke [120] ou Russell [121]. No entanto, certos estudos utilizaram critérios próprios, com base em dados clínicos (por exemplo, profundida de sondagem média, porcentagem de sítios com um certo limiar de perda inserção clinica ou perda óssea radiográfica) ou mesmo auto-relatos de pacientes. Isso demostra as dificuldades de se ter um critério universalmente aceito para diagnóstico da periodontite [122,123]. Na tentativa de reduzir esse viés, na metanálise aqui apresentada, separamos os estudos em dois grupos (que utilizaram critérios seguros e inseguros para diagnóstico da PC) de acordo como descrito previamente por Nibali et al. [119] (APÊNDICE A).

Existe uma forte necessidade de futura uniformidade nos estudos epidemiológicos em Periodontia. Tais estudos deveriam adotar critérios clínicos semelhantes para permitir que os dados resultantes sejam comparados com aqueles obtidos de outros estudos [123]. Para refletir verdadeiramente a atividade da doença, o uso combinado de profundidade de sondagem (PS), nível de inserção clínico (NIC) e sangramento à sondagem (SS) devem ser considerados como

variáveis a serem avaliadas em futuros estudos epidemiológicos [123].

Em nossos dois estudos caso-controle aqui presentes (Capítulos 2 e 3) os pacientes foram classificados em dois diferentes grupos, de acordo com os critérios definidos pela CDC/AAP (*Centers for Disease Control and Prevention/ American Academy of Periodontology*) [120], um dos critérios definidos por Nibali et al. [119] como diagnóstico seguro para doença periodontal (APÊNDICE A). Neste critério, duas definições são fornecidas para periodontite: uma para periodontite severa e outra para periodontite moderada. A definição do caso para a periodontite severa é rigorosa para garantir que os pacientes identificados pela definição tenham a doença. A definição provavelmente exclui alguns casos genuínos de periodontite. Para resolver este problema, as definições de casos para periodontite moderada destinam-se a detectar pacientes com periodontite menos severa. As medidas de NIC e PS são necessárias por vários motivos. Embora o NIC seja considerado uma medida mais precisa do que a PS, o uso do NIC sozinho poderia incluir por engano alguns sítios periodontalmente saudáveis porque a perda de inserção pode ocorrer por uma recessão gengival não inflamatória [120]. Além disso, um paciente com periodontite pode ser tratado com sucesso sem retorno do NIC para o normal [124].

Recentemente, Offenbacher et al. [125] definiram o fenótipo da doença periodontal como traços complexos usando análise de componentes principais (PCA). Neste modelo de PCA foram criados traços complexos periodontais (*periodontal complex traits* - PCTs). Para esta investigação, o PCA inclui a análise de microrganismos periodontopatogênicos (oito patógenos), níveis do mediador inflamatório IL-1β no fluido crevicular gengival (GCF) e classificação clínica da PC de acordo com os mesmos critérios utilizados por nós, da CDC / AAP [120], e foram identificados seis PCTs. Neste modelo, a doença periodontal é um grupo de condições biológicas distintas, que podem ter apresentações clínicas sobrepostas. O objetivo desta análise é favorecer, principalmente para estudos genéticos, a identificação de loci relacionados às características biológicas e à patogênese da doença periodontal, e não somente a característica clínica da doença [126]. Essa análise, apesar de interessante, não foi possível ser realizada em nosso estudo pois não foi realizada coleta de GCF e placa bacteriana dos pacientes aqui avaliados.

Um outro importante fator a ser considerado em nossa metanálise que pode provocar viés dos resultados é a grande variedade de grupos étnicos-raciais envolvidos no estudo. Existem muitos estudos que investigaram as diferenças raciais-étnicas na relação entre os componentes do perfil lipídico [127-130]. No geral, existem diferenças importantes no perfil

lipídico de acordo com a etnia, cujas causas são complexas. Dentre os possíveis contribuintes, incluem-se fatores genéticos [131]. A metanálise em subgrupos de acordo com a etnia dos pacientes mostrou pequenas variações entre os continentes, mas não foram encontradas diferenças estatisticamente significativas mesmo após a meta-regressão. No entanto, estes resultados foram altamente heterogêneos. Da mesma forma que ocorre em relação ao perfil lipídico, os diferentes grupos étnicos, ou diferentes cores de pele, carregam diferentes combinações de variantes genéticas. Para alguns pesquisadores a utilização de "rótulos" étnicos, raciais ou cores de pele são marcadores biológicos pobres associados a diferenças genotípicas [132], porém a classificação étnica ainda pode explicar interações complexas entre genética, meio ambiente, sociedade e outros fatores [133].

Entretanto, esta análise é muito difícil de ser realizada no Brasil. Muitos estudos genéticos em populações brasileiras não tem estratificado os pacientes em relação a cor da pele [134-137], incluindo nossos 2 estudos caso-controle. A explicação para esta observação é que a miscigenação característica da população brasileira resultou de cinco séculos de cruzamentos interétnicos entre europeus (principalmente colonizadores portugueses e imigrantes italianos), africanos (trazidos para o Brasil como escravos) e indígenas [138]. Como todos os grupos étnicos brasileiros podem apresentar algum grau de mistura racial [138], e há uma dissociação da cor da pele individual e uma ascendência genômica [139,140], os indivíduos brasileiros incluídos no presente estudo não foram distinguidos por características étnicas ou cor da pele . Parra et al. [140] não recomenda o agrupamento de brasileiros em grupos étnicos com base em cores e outras características físicas associadas a divisões raciais. Os indivíduos brasileiros classificados como "brancos" ou "negros" têm genótipos significativamente sobrepostos considerando os loci associados à raça para caucasianos, africanos e indígenas, provavelmente devido a miscigenação [140]. Uma outra possibilidade mais sofisticada seria determinar a ascendência genômica de cada indivíduo, genotipando um conjunto de polimorfismos previamente validados como marcadores informativos de ancestralidade (ou AIMs, do inglês *ancestry informative marker*), polimorfismos de frequências alélicas substancialmente diferentes entre as populações. Entretanto, a quantidade de marcadores necessários para caracterizar ancestralmente uma população é alvo de debate. Está técnica vem sendo utilizada em pesquisas odontológicas no Brasil [141-143].

Apesar de não realizarmos a estratificação por cor de pele, é muito importante que os resultados encontrados em nossos estudos possam ser validados em outras regiões do Brasil. Estudos mostram que existem, apesar de pequenas, diferenças de ancestralidade em relação à

cor da pele nas diferentes regiões do Brasil [144]. Em todas as regiões estudadas, a ascendência europeia era predominante, porém com proporções variando de 60,6% no Nordeste a 77,7% no Sul [144]. Nos presentes estudos (Capítulos 2 e 3), todos os indivíduos (casos e controles) foram selecionados da mesma área geográfica e tinham o mesmo status socioeconômico. Assim, compreende-se que os indivíduos analisados da região de Araraquara, que é central do Estado de São Paulo, no Sudeste do Brasil, são representativos da população brasileira, uma vez que possui proporções intermediárias em relação as ascendências, quando comparado a outras regiões do Brasil [136,144]. Acredita-se que a tendência a longo prazo é que pessoas de cor de pele branca ou negra, tenham uma proporção similar de ascendência africana [140]. Ou seja, comprovando que a cor de pele não é representativa para os estudos genéticos enfocando a população brasileira.

Em nossa metanálise, após a meta-regressão, uma metanálise homogênea indicou que a PC foi significativamente associada a uma redução da HDL e elevação da concentração de LDL e triglicerídeos. O único parâmetro estudado que não apresentou associação com a PC foi o colesterol total. Isso pode ser justificável pelo fato do colesterol total geralmente aumentar com a idade e, normalmente, poucas alterações nos níveis ocorrem em pacientes com menos de 60 anos [1]. Entretanto a média de idade dos pacientes neste estudo era menor que 60 anos. Os níveis elevados de LDL e triglicerídeos foram previamente associados à ação das citocinas pró-inflamatórias, como TNF e IL-1β, induzidas por microrganismos e suas endotoxinas [145,146]. No presente estudo, observou-se associação entre os níveis elevados de LDL e triglicérides nos pacientes com PC.

Foram investigados no Capítulo 2 deste estudo, dois genes importantes associados à LDL: a *Apolipoprotein B* (*APOB*) e o *Receptor de LDL* (*LDLR*). ApoB é uma apolipoproteina primária da LDL atuando no transporte constante de colesterol para tecidos e células periféricas [82]. ApoB possui estruturas para ligar-se ao LDLR, que são receptores presentes em células. O LDLR desempenha um papel fundamental na regulação do metabolismo do colesterol, removendo o excesso de LDL no sangue [85]. Existem estudos relacionando as duas proteínas e/ou seus respectivos genes à PC e bactérias periodontopatogênicas. Por meio de imunohistoquímica foi mostrado que o estímulo de LPS de *Porphyromonas gingivalis* (*P.g.*) durante 16 horas diminuiu os níveis de expressão de LDLR nas células mesenquimais da medula óssea e no tecido periodontal de ratos, em comparação com o grupo controle, sem a estimulação com LPS bacteriano [147]. Estudos com *P.g.* também foram realizados em relação à ApoB. Incubação de sangue total com *P.g.* resultou em proteólise e degradação de ApoB em

LDL [148-150]. Curiosamente, o LDLR (que se liga a ApoB) no fígado não reconhece LDL modificado pela degradação de ApoB, resultando assim no acúmulo de LDL na circulação [149]. A modificação de LDL induzida pela degradação seletiva da proteína ApoB por *P.g.* resulta em partículas lipídicas agregadas que podem ser absorvidas por macrófagos e consequentemente acelera a progressão da aterosclerose em camundongos [151]. Além disso, ApoB, está presente no fluido crevicular gengival [152]. Além disso, os níveis de ApoB no GCF são maiores em sítios com periodontite do que nos sítios saudáveis [87]; e os níveis de ApoB em sítios com doença diminuem após tratamento periodontal com raspagem e alisamento radicular [87].

Uma vez que a PC está associada a níveis plasmáticos de LDL mais elevados [153], e foi relatado que os polimorfismos nos genes *APOB* e *LDLR* podem aumentar as concentrações plasmáticas de LDL, o artigo 2 teve como objetivo avaliar se esses polimorfismos contribuem para a suscetibilidade para PC. Entretanto, demonstramos pela primeira vez que nenhum dos quatro SNPs associados à dislipidemia nos dois genes estavam significativamente associados aos pacientes brasileiros afetados pela PC. A publicação de resultados negativos, como os encontrados em relação aos SNPs nos genes *APOB* e *LDLR* são muito importantes para a literatura científica, inclusive para futuras metanálises. Um dos grandes problemas em metanálises, incluindo a publicada nesta Tese (Capítulo 1) é o viés de publicação relacionado ao fato de existirem diversos resultados de pesquisa não publicados (como resultados negativos). Há uma boa razão para se preocupar com essa perda potencial, porque estudos com resultados significativamente positivos são mais prováveis de serem publicados em comparação a estudos com resultados "negativos" ou sem associação com a doença [115].

O estudo da interação gene-fator ambiental é importante para melhorar a precisão na avaliação de influências de ambos genética e fatores ambientais nas doenças. Uma compreensão da interação gene-ambiente também tem implicações importantes para a saúde pública. Ela ajuda a prever as taxas de doenças e fornece uma base para recomendações de prevenção de doenças [154]. O efeito conjunto entre dois fatores pode ser medido em uma escala aditiva ou multiplicativa. Embora exista um debate sobre qual escala deve ser utilizada, tem havido consenso geral de que a escala aditiva é mais adequada para avaliar as interações [155]. Apesar das interações entre genes e meio ambiente parecerem ser um análise óbvia a ser considerada, testes de tais interações ainda não são rotina em estudos genéticos [156]. Há poucos estudos que investigam estas interações associadas à periodontite crônica, particularmente com o tabagismo [155,157-159]. Os genótipos de risco em SNP do gene receptor de vitamina D (*VDR*) interagiram sinergicamente com o tabagismo na susceptibilidade à PC [155]. Para o nosso conhecimento,

existe apenas um estudo sobre a interação gene-ambiente com SNPs nos genes *APOB* e *LDLR*; infelizmente, eles não eram os mesmos SNPs estudados neste presente estudo [160]. As interações gene-ambiente provavelmente serão importantes para futuras estratégias de diagnóstico, prognóstico e manejo da PC [156].

Em relação ao Capítulo 3, dois genes previamente estudados em *GWAS* para PC foram validados na população brasileira. O genótipo AA do SNP rs2521634 no gene *NPY* foi associado com risco aumentado para PC severa (OR = 2,34; IC 95% = 1,19 - 4,59; p = 0,01). Além disso, o mesmo genótipo foi associado à maior porcentagem de profundidade de sondagem interproximal (PSi) ≥ 5mm (β = 4,91, p = 0,004). Este SNP foi primeiramente associado a DP severa (OR = 1,47; IC 95% = 1,25-1,73; p-valor = 1,6 × 10-6) em um estudo com 4504 americanos em um *GWAS* [54]. Posteriormente, o resultado foi confirmado na mesma população, mostrando associação com PC severa [161]. Os níveis de proteína NPY, recentemente investigados por Haririan et al. [162] na saliva de pacientes com DP, correlacionaram-se significativamente com os parâmetros clínicos periodontais: média do nível clínico de inserção (r = 0,29, p <0,05), sangramento à sondagem (r = 0,29, p <0,05) e o número de dentes com profundidade de sondagem ≥ 5 mm (r = 0,34, p <0,005).

Considerando o SNP rs3811046, os genótipos TG e GG mostraram um aumento à suscetibilidade à PC moderada. Além disso, as mulheres portadoras do genótipo homozigótico para o alelo raro no mesmo SNP foram mais suscetíveis a desenvolver DP moderada (OR = 4,02, IC: 1,65 - 9,80, p = 0,002), mesmo após a correção de Bonferroni. Como descrito previamente, Offenbacher et al. [125] definiu o fenótipo da doença periodontal como traços complexos usando análise de componentes principais (PCA). Nos resultados de *GWAS* utilizando PCA, os autores analisaram 2,5 milhões de SNPs e identificaram um SNP (rs3811046) dentro da região codificante do gene anti-inflamatório *IL37*, com associação significativa com altos níveis de FCG-IL-1ß (p-valor inferior a 1x10-21) e também fortemente associado à PC severa [163].

Existem alguns estudos que mostram suscetibilidade sexo-específica à PC, uma vez que o papel dimórfico de alguns polimorfismos em seres humanos foi associado a doenças complexas, incluindo PC [135,164-167]. Um diferencial na transcrição de genes entre os gêneros é necessário, pois homens e mulheres usam um genoma similar para alcançar fenótipos distintos [168]. Yan[169] (2015) investigou o SNP rs3811046, o mesmo por nós investigado para PC e, após a estratificação por gênero, os autores observaram que esse polimorfismo estava significativamente associado a um menor risco de doença de Graves em pacientes do sexo

feminino (alelo G, p-valor = 0,03, OR = 0,777). Intrigantemente, o mesmo SNP tem efeito sexo-específico contrário nas duas doenças. Embora o risco de PC, neste presente estudo, tenha sido associado a mulheres, na doença de Graves, as mulheres apresentaram redução de risco.

Foi reconhecido que os resultados do GWAS precisam ser reproduzidos em outras amostras independentes. Um resultado de GWAS anterior é "replicado" quando é reproduzido em uma população similar do estudo original; mas quando o resultado de GWAS é reproduzido em uma população diferente (por exemplo, etnicamente), esse resultado foi "validado" [170]. No Capítulo 3 do presente estudo, selecionamos SNPs anteriormente associados à CP em outras populações por GWAS ou abordagens de bioinformática para validar esses resultados em uma população brasileira. Pela primeira vez, demonstramos a validação da associação entre o SNP rs2521634, perto do gene *NPY* e o SNP rs3811046 na região intronic do gene *IL37* e aumento do risco para PC moderado e severa em uma população brasileira. Estudos adicionais são necessários para investigar o papel funcional dos SNPs validados no contexto da doença periodontal, interação genética com fatores ambientais e se há influência do gênero, em seus níveis de transcrição ou translacional.

5 CONCLUSÃO

- Capítulo 1: Pode-se concluir que, apesar das limitações deste estudo, após meta-regressão, uma metanálise homogênea indicou que a periodontite crônica foi significativamente associada a uma redução da HDL e elevação da concentração de LDL e triglicerídeos. Portanto, a periodontite crônica pode ter associação com o controle metabólico lipídico.

- Capítulo 2: Não foi verificada associação significativa entre os parâmetros clínicos periodontais e as frequências de genótipos de qualquer dos SNPs avaliados (rs5925, rs688, rs676210, rs693) nos genes *LDLR* e *APOB*. No modelo multivariável, não houve diferenças significativas nas frequências de genótipos para todos os SNPs que compararam o controle e os grupos de pacientes com periodontite crônica. As interações multiplicativas e aditivas entre cada SNP e tabagismo não foram estatisticamente significantes. Além disso, nenhum dos haplótipos nos genes *APOB* e *LDLR* estava significativamente relacionado ao risco de doença periodontal.

- Capítulo 3: Foi possível validar em uma população brasileira, a associação do SNP rs2521634 próximo do gene *NPY*, e do SNP rs3811046 na região intrônica do gene *IL37*, com risco aumentado para periodontite crônica moderada e severa, sendo esse risco sexo-específico. Esses resultados reforçam a evidência de que os polimorfismos nos genes *NPY* e *IL37*, previamente encontrados por *GWAS*, podem desempenhar um papel no perfil genético que predispõe a periodontite crônica.

REFERÊNCIAS*

1 Kannel WB. Coronary heart disease risk factors in the elderly. Am J Geriatr Cardiol. 2002; 11(2):101-7.

2 Armitage GC. Development of a classification system for periodontal diseases and conditions. Ann Periodontol. 1999; 4(1):1-6.

3 Nath SG, Raveendran R. "What is there in a name?": A literature review on chronic and aggressive periodontitis. J Indian Soc Periodontol . 2011; 15(4):318-22.

4 Highfield J. Diagnosis and classification of periodontal disease. Aust Dent J. 2009; 54 Suppl:S11-26.

5 Hunter DJ. Gene-environment interactions in human diseases. Nat Rev Genet. 2005; 6(4):287-98.

6 Dempfle A, Scherag A, Hein R, Beckmann L, Chang-Claude J, Schafer H. Gene-environment interactions for complex traits: definitions, methodological requirements and challenges. Eur J Hum Genet. 2008; 16(10):1164-72.

7 Covani U, Marconcini S, Giacomelli L, Sivozhelevov V, Barone A, Nicolini C. Bioinformatic prediction of leader genes in human periodontitis. J Periodontol. 2008; 79(10):1974-83.

8 Grigoriadou ME, Koutayas SO, Madianos PN, Strub JR. Interleukin-1 as a genetic marker for periodontitis: review of the literature. Quintessence Int. 2010; 41(6):517-25.

9 Greenstein G, Hart TC. A critical assessment of interleukin-1 (IL-1) genotyping when used in a genetic susceptibility test for severe chronic periodontitis. J Periodontol. 2002; 73(2):231-47.

10 Corey LA, Nance WE, Hofstede P, Schenkein HA. Self-reported periodontal disease in a Virginia twin population. J Periodontol. 1993; 64(12):1205-8.

11 Michalowicz BS, Aeppli D, Virag JG, Klump DG, Hinrichs JE, Segal NL, et al. Periodontal findings in adult twins. J Periodontol. 1991; 62(5):293-9.

12 Michalowicz BS, Diehl SR, Gunsolley JC, Sparks BS, Brooks CN, Koertge TE, et al. Evidence of a substantial genetic basis for risk of adult periodontitis. J Periodontol. 2000; 71(11):1699-707.

13 Mealey BL, Oates TW. Diabetes mellitus anal periodontal diseases. J Periodontol. 2006; 77(8):1289-303.

14 Page RC, Kornman KS. The pathogenesis of human periodontitis: an introduction. Periodontol 2000. 1997; 14:9-11.

* De acordo com o Guia de Trabalhos Acadêmicos da FOAr, adaptado das Normas Vancouver. Disponível no site da Biblioteca: http://www.foar.unesp.br/Home/Biblioteca/guia-de-normalizacao-atualizado.pdf

15 Williams RC, Offenbacher S. Periodontal medicine: the emergence of a new branch of periodontology. Periodontol 2000. 2000; 23:9-12.

16 Andrukhov O, Haririan H, Bertl K, Rausch WD, Bantleon HP, Moritz A, et al. Nitric oxide production, systemic inflammation and lipid metabolism in periodontitis patients: possible gender aspect. J Clin Periodontol. 2013; 40(10):916-23.

17 Duzagac E, Cifcibasi E, Erdem MG, Karabey V, Kasali K, Badur S, et al. Is obesity associated with healing after non-surgical periodontal therapy? A local vs. systemic evaluation. Journal of periodontal research. 2016; 51(5):604-12.

18 Fentoglu O, Kirzioglu FY, Bulut MT, Kumbul Doguc D, Kulac E, Onder C, et al. Evaluation of lipid peroxidation and oxidative DNA damage in patients with periodontitis and hyperlipidemia. Journal of periodontology. 2015; 86(5):682-8.

19 Golpasand Hagh L, Zakavi F, Hajizadeh F, Saleki M. The association between hyperlipidemia and periodontal infection. Iran Red Crescent Med J. 2014; 16(12):e6577.

20 Penumarthy S, Penmetsa GS, Mannem S. Assessment of serum levels of triglycerides, total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol in periodontitis patients. J Indian Soc Periodontol. 2013; 17(1):30-5.

21 Susin C, Dalla Vecchia CF, Oppermann RV, Haugejorden O, Albandar JM. Periodontal attachment loss in an urban population of Brazilian adults: Effect of demographic, behavioral, and environmental risk indicators. J Periodontol. 2004; 75(7):1033-41.

22 Taylor JJ, Preshaw PM, Donaldson PT. Cytokine gene polymorphism and immunoregulation in periodontal disease. Periodontology 2000. 2004; 35:158-82.

23 Schafer AS, Jepsen S, Loos BG. Periodontal genetics: a decade of genetic association studies mandates better study designs. J Clin Periodontol. 2011; 38(2):103-7.

24 Vijayalakshmi R, Geetha A, Ramakrishnan T, Emmadi P. Genetic polymorphisms in periodontal diseases: an overview. Indian J Dent Res. 2010; 21(4):568-74.

25 Kornman KS, di Giovine FS. Genetic variations in cytokine expression: a risk factor for severity of adult periodontitis. Ann Periodontol. 1998; 3(1):327-38.

26 Stern DL. Evolutionary biology. The problem of variation. Nature. 2000; 408(6812):529, 31.

27 Hodge PJ, Riggio MP, Kinane DF. Failure to detect an association with IL1 genotypes in European Caucasians with generalised early onset periodontitis. J Clin Periodontol. 2001; 28(5):430-6.

28 Lopez NJ, Jara L,Valenzuela CY. Association of interleukin-1 polymorphisms with periodontal disease. J Periodontol. 2005; 76(2):234-43.

29 Franch-Chillida F, Nibali L, Madden I, Donos N, Brett P. Association between interleukin-6 polymorphisms and periodontitis in Indian non-smokers. J Clin Periodontol. 2010; 37(2):137-44.

30 Kim YJ, Viana AC, Curtis KM, Orrico SR, Cirelli JA, Scarel-Caminaga RM. Lack of association of a functional polymorphism in the interleukin 8 gene with susceptibility to periodontitis. DNA Cell Biol. 2009; 28(4):185-90.

31 Tervonen T, Raunio T, Knuuttila M, Karttunen R. Polymorphisms in the CD14 and IL-6 genes associated with periodontal disease. J Clin Periodontol. 2007; 34(5):377-83.

32 Kornman KS, Crane A, Wang HY, diGiovine FS, Newman MG, Pirk FW, et al. The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol. 1997; 24(1):72-7.

33 Kornman KS, Crane A, Wang HY, di Giovine FS, Newman MG, Pirk FW, et al. The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol. 1997; 24(1):72-7.

34 Laine ML, Crielaard W, Loos BG. Genetic susceptibility to periodontitis. Periodontol 2000. 2012; 58(1):37-68.

35 Zhang J, Sun X, Xiao L, Xie C, Xuan D, Luo G. Gene polymorphisms and periodontitis. Periodontol 2000. 2011; 56(1):102-24.

36 Scarel-Caminaga RM, Kim YJ, Viana AC, Curtis KM, Corbi SC, Sogumo PM, et al. Haplotypes in the interleukin 8 gene and their association with chronic periodontitis susceptibility. Biochem Genet. 2011; 49(5-6):292-302.

37 Ding C, Ji X, Chen X, Xu Y, Zhong L. TNF-alpha gene promoter polymorphisms contribute to periodontitis susceptibility: evidence from 46 studies. J Clin Periodontol. 2014; 41(8):748-59.

38 Sachidanandam R, Weissman D, Schmidt SC, Kakol JM, Stein LD, Marth G, et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature. 2001; 409(6822):928-33.

39 Scarel-Caminaga RM, Trevilatto PC, Souza AP, Brito RB, Line SR. Frequencies of the -330 (T --> G) IL-2 and -590 (T --> C) IL-4 gene polymorphisms in a population from south-eastern Brazil. Eur J Immunogenet. 2002; 29(4):293-6.

40 Astolfi CM, Shinohara AL, da Silva RA, Santos MCLG, Line SRP, de Souza AP. Genetic polymorphisms in the MMP-1 and MMP-3 gene may contribute to chronic periodontitis in a Brazilian population. J Clin Periodontol. 2006; 33(10):699-703.

41 Moreira PR, Costa JE, Gomez RS, Gollob KJ, Dutra WO. The IL1A (-889) gene polymorphism is associated with chronic periodontal disease in a sample of Brazilian individuals. J Periodontal Res. 2007; 42(1):23-30.

42 Oliveira RN, Corbi SCT, Bastos AS, Orrico SRP, Scarel-Caminaga RM. Doença periodontal em pacientes com Diabetes Mellitus: influência de polimorfismos genéticos? Rev Odontol UNESP. 2011; 40(4):187-94.

43 Pontes CC, Gonzales JR, Novaes AB, Jr., Taba Junior M, Grisi MF, Michel J, et al. Interleukin-4 gene polymorphism and its relation to periodontal disease in a Brazilian population of African heritage. J Dent. 2004; 32(3):241-6.

44 Reid ME. Transfusion in the age of molecular diagnostics. Hematology Am Soc Hematol Educ Program. 2009; 171-7.

45 Martins FT, Ramos PZ, Svidnicki MC, Castilho AM, Sartorato EL. Optimization of simultaneous screening of the main mutations involved in non-syndromic deafness using the TaqMan(R) OpenArray Genotyping platform. BMC Med Genet. 2013; 14:112.

46 Martins FTA, Ramos PZ, Svidnicki MCCM, Castilho AM, Sartorato EL. Optimization of simultaneous screening of the main mutations involved in non-syndromic deafness using the TaqMan (R) OpenArray (TM) Genotyping Platform. BMC Med Genet. 2013; 14:112.

47 Vaithilingam RD, Safii SH, Baharuddin NA, Ng CC, Cheong SC, Bartold PM, et al. Moving into a new era of periodontal genetic studies: relevance of large case-control samples using severe phenotypes for genome-wide association studies. J Periodontal Res. 2014; 49(6):683-95.

48 Zhan Y, Zhang R, Lv H, Song X, Xu X, Chai L, et al. Prioritization of candidate genes for periodontitis using multiple computational tools. J Periodontol. 2014; 85(8):1059-69.

49 Okamura H, Tsutsui H, Kashiwamura S, Yoshimoto T, Nakanishi K. Interleukin-18: a novel cytokine that augments both innate and acquired immunity. Adv Immunol. 1998; 70:281-312.

50 Ozcaka O, Nalbantsoy A, Buduneli N. Interleukin-17 and interleukin-18 levels in saliva and plasma of patients with chronic periodontitis. J Periodontal Res. 2011; 46(5):592-8.

51 Kajita K, Honda T, Amanuma R, Domon H, Okui T, Ito H, et al. Quantitative messenger RNA expression of Toll-like receptors and interferon-alpha1 in gingivitis and periodontitis. Oral Microbiol Immunol. 2007; 22(6):398-402.

52 Teumer A, Holtfreter B, Volker U, Petersmann A, Nauck M, Biffar R, et al. Genome-wide association study of chronic periodontitis in a general German population. J Clin Periodontol. 2013; 40(11):977-85.

53 Vaithilingam RD, Safii SH, Baharuddin NA, Ng CC, Cheong SC, Bartold PM et al. Moving into a new era of periodontal genetic studies: relevance of large case-control samples using severe phenotypes for genome-wide association studies. J Periodontal Res. 2014; 49(6):683-95.

54 Divaris K, Monda KL, North KE, Olshan AF, Reynolds LM, Hsueh WC, et al. Exploring the genetic basis of chronic periodontitis: a genome-wide association study. Hum Mol Genet. 2013; 22(11):2312-24.

55 Freitag-Wolf S, Dommisch H, Graetz C, Jockel-Schneider Y, Harks I, Staufenbiel I, et al. Genome-wide exploration identifies sex-specific genetic effects of alleles upstream NPY to increase the risk of severe periodontitis in men. J Clin Periodontol. 2014; 41(12):1115-21.

56 Shaffer JR, Polk DE, Wang X, Feingold E, Weeks DE, Lee MK, et al. Genome-wide association study of periodontal health measured by probing depth in adults ages 18-49 years. G3 (Bethesda). 2014; 4(2):307-14.

57 Lundberg JM, Pernow J, Tatemoto K, Dahlof C. Pre- and postjunctional effects of NPY on sympathetic control of rat femoral artery. Acta Physiol Scand. 1985; 123(4):511-3.

58 Bedoui S, Miyake S, Lin Y, Miyamoto K, Oki S, Kawamura N, et al. Neuropeptide Y (NPY) suppresses experimental autoimmune encephalomyelitis: NPY1 receptor-specific inhibition of autoreactive Th1 responses in vivo. J Immunol. 2003; 171(7):3451-8.

59 Zukowska Z, Grant DS, Lee EW. Neuropeptide Y: a novel mechanism for ischemic angiogenesis. Trends Cardiovasc Med. 2003; 13(2):86-92.

60 Pedrazzini T, Pralong F, Grouzmann E. Neuropeptide Y: the universal soldier. Cell Mol Life Sci. 2003; 60(2):350-77.

61 Lundy FT, Linden GJ. Neuropeptides and neurogenic mechanisms in oral and periodontal inflammation. Crit Rev Oral Biol Med. 2004; 15(2):82-98.

62 Ahmed M, Srinivasan GR, Theodorsson E, Bjurholm A, Kreicbergs A. Extraction and quantitation of neuropeptides in bone by radioimmunoassay. Regul Pept. 1994; 51(3):179-88.

63 Haug SR, Heyeraas KJ. Modulation of dental inflammation by the sympathetic nervous system. J Dent Res. 2006; 85(6):488-95.

64 Lundy FT, El Karim IA, Linden GJ. Neuropeptide Y (NPY) and NPY Y1 receptor in periodontal health and disease. Arch Oral Biol. 2009; 54(3):258-62.

65 Kumar S, McDonnell PC, Lehr R, Tierney L, Tzimas MN, Griswold DE, et al. Identification and initial characterization of four novel members of the interleukin-1 family. J Biol Chem. 2000; 275(14):10308-14.

66 Nold MF, Nold-Petry CA, Zepp JA, Palmer BE, Bufler P, Dinarello CA. IL-37 is a fundamental inhibitor of innate immunity. Nat Immunol. 2010; 11(11):1014-22.

67 Boraschi D, Lucchesi D, Hainzl S, Leitner M, Maier E, Mangelberger D, et al. IL-37: a new anti-inflammatory cytokine of the IL-1 family. Eur Cytokine Netw. 2011; 22(3):127-47.

68 Saglam M, Koseoglu S, Savran L, Pekbagriyanik T, Saglam G, Sutcu R. Levels of interleukin-37 in gingival crevicular fluid, saliva, or plasma in periodontal disease. J Periodontal Res. 2015; 50(5):614-21.

69 Kuo LC, Poison AM, Kang T. Associations between periodontal diseases and systemic diseases: A review of the inter-relationships and interactions with diabetes, respiratory diseases, cardiovascular diseases and osteoporosis. Public Health. 2008; 122(4):417-33.

70 Tonetti MS, Van Dyke TE, Working group 1 of the joint EFP/AAP workshop. Periodontitis and atherosclerotic cardiovascular disease: consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. J Clin Periodontol. 2013; 40 Suppl 14:S24-9.

71 Kinane DF, Lowe GD. How periodontal disease may contribute to cardiovascular disease. Periodontol 2000. 2000; 23:121-6.

72 Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999; 340(2):115-26.

73 National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation. 2002; 106(25):3143-421.

74 Nelson RH. Hyperlipidemia as a risk factor for cardiovascular disease. Prim Care. 2013; 40(1):195-211.

75 Chahil TJ, Ginsberg HN. Diabetic dyslipidemia. Endocrinol Metab Clin North Am. 2006; 35(3):491-510.

76 Almeida Abdo J, Cirano FR, Casati MZ, Ribeiro FV, Giampaoli V, Viana Casarin RC, et al. Influence of dyslipidemia and diabetes mellitus on chronic periodontal disease. J Periodontol. 2013; 84(10):1401-8.

77 Howard BV. Lipoprotein metabolism in diabetes mellitus. J Lipid Res. 1987; 28(6):613-28.

78 Rajput-Williams J, Knott TJ, Wallis SC, Sweetnam P, Yarnell J, Cox N, et al. Variation of apolipoprotein-B gene is associated with obesity, high blood cholesterol levels, and increased risk of coronary heart disease. Lancet. 1988; 2(8626-8627):1442-6.

79 Xiao R, Sun S, Zhang J, Ouyang Y, Zhang N, Yang M, et al. Association analysis of APO gene polymorphisms with ischemic stroke risk: a case-control study in a Chinese Han population. Oncotarget. 2017; 8(36):60496-503.

80 Brown MS, Goldstein JL. Expression of the familial hypercholesterolemia gene in heterozygotes: mechanism for a dominant disorder in man. Science. 1974; 185(4145):61-3.

81 Alonso R, Mata N, Castillo S, Fuentes F, Saenz P, Muniz O, et al. Cardiovascular disease in familial hypercholesterolaemia: influence of low-density lipoprotein receptor mutation type and classic risk factors. Atherosclerosis. 2008; 200(2):315-21.

82 Oram JF, Heinecke JW. ATP-binding cassette transporter A1: a cell cholesterol exporter that protects against cardiovascular disease. Physiol Rev. 2005; 85(4):1343-72.

83 Chen PF, Marcel YL, Yang CY, Gotto AM, Jr., Milne RW, Sparrow JT, et al. Primary sequence mapping of human apolipoprotein B-100 epitopes. Comparisons of trypsin accessibility and immunoreactivity and implication for apoB conformation. Eur J Biochem. 1988; 175(1):111-8.

84 Fernandez-Higuero JA, Etxebarria A, Benito-Vicente A, Alves AC, Arrondo JL, Ostolaza H, et al. Structural analysis of APOB variants, p.(Arg3527Gln), p.(Arg1164Thr) and p.(Gln4494del), causing Familial Hypercholesterolaemia provides novel insights into variant pathogenicity. Sci Rep. 2015; 5:18184.

85 Dammerman M, Breslow JL. Genetic basis of lipoprotein disorders. Circulation. 1995; 91(2):505-12.

86 Ito S, Gojoubori T, Tsunoda K, Yamaguchi Y, Asano M, Goke E, et al. Nicotine-induced expression of low-density lipoprotein receptor in oral epithelial cells. PLoS One. 2013; 8(12):e82563.

87 Ishizuka M, Kato R, Moriya Y, Noguchi E, Koide Y, Inoue S, et al. Changes in apolipoprotein B and oxidized low-density lipoprotein levels in gingival crevicular fluids as a result of periodontal tissue conditions. J Periodontal Res. 2017; 52(3):594-602.

88 American Diabetes Association. Standards of medical care in diabetes--2013. Diabetes Care. 2013; 36 (1):11-66.

89 Mehra R. Global public health problem of sudden cardiac death. J Electrocardiol. 2007; 40(6 Suppl):S118-22.

90 DeStefano F, Anda RF, Kahn HS, Williamson DF, Russell CM. Dental disease and risk of coronary heart disease and mortality. BMJ. 1993; 306(6879):688-91.

91 Morrison HI, Ellison LF, Taylor GW. Periodontal disease and risk of fatal coronary heart and cerebrovascular diseases. J Cardiovasc Risk. 1999; 6(1):7-11.

92 Ebersole JL, Cappelli D, Mott G, Kesavalu L, Holt SC, Singer RE. Systemic manifestations of periodontitis in the non-human primate. J Periodontal Res. 1999; 34(7):358-62.

93 Bullon P, Morillo JM, Ramirez-Tortosa MC, Quiles JL, Newman HN, Battino M. Metabolic syndrome and periodontitis: is oxidative stress a common link? J Dent Res. 2009; 88(6):503-18.

94 Losche W, Karapetow F, Pohl A, Pohl C, Kocher T. Plasma lipid and blood glucose levels in patients with destructive periodontal disease. J Clin Periodontol. 2000; 27(8):537-41.

95 Awartani F, Atassi F. Evaluation of periodontal status in subjects with hyperlipidemia. J Contemp Dent Pract. 2010; 11(2):33-40.

96 Heasman PA, Collins JG, Offenbacher S. Changes in crevicular fluid levels of Interleukin-1-Beta, Leukotriene-B4, Prostaglandin-E2, Thromboxane-B2 and Tumor-Necrosis-Factor-Alpha in experimental gingivitis in humans. J Periodontal Res. 1993; 28(4):241-7.

97 Page RC. The role of inflammatory mediators in the pathogenesis of periodontal-disease. J Periodontal Res. 1991; 26(3):230-42.

98 Cutler CW, Shinedling EA, Nunn M, Jotwani R, Kim BO, Nares S, et al. Association between periodontitis and hyperlipidemia: cause or effect? J Periodontol. 1999; 70(12):1429-34.

99 Lakio L, Lehto M, Tuomainen AM, Jauhiainen M, Malle E, Asikainen S, et al. Pro-atherogenic properties of lipopolysaccharide from the periodontal pathogen Actinobacillus actinomycetemcomitans. J Endotoxin Res. 2006; 12(1):57-64.

100 Chu X, Newman J, Park B, Nares S, Ordonez G, Iacopino AM. In vitro alteration of macrophage phenotype and function by serum lipids. Cell Tissue Res. 1999; 296(2):331-7.

101 Doxey DL, Cutler CW, Iacopino AM. Diabetes prevents periodontitis-induced increases in gingival platelet derived growth factor-B and interleukin 1-beta in a rat model. J Periodontol. 1998; 69(2):113-9.

102 Fentoglu O, Koroglu BK, Hicyilmaz H, Sert T, Ozdem M, Sutcu R, et al. Pro-inflammatory cytokine levels in association between periodontal disease and hyperlipidaemia. J Clin Periodontol. 2011; 38(1):8-16.

103 Krause S, Pohl A, Pohl C, Liebrenz A, Ruhling K, Losche W. Increased generation of reactive oxygen species in mononuclear blood cells from hypercholesterolemic patients. Thromb Res. 1993; 71(3):237-40.

104 Iacopino AM. Diabetic periodontitis: possible lipid-induced defect in tissue repair through alteration of macrophage phenotype and function. Oral Dis. 1995; 1(4):214-29.

105 Stashenko P, Fujiyoshi P, Obernesser MS, Prostak L, Haffajee AD, Socransky SS. Levels of interleukin 1 beta in tissue from sites of active periodontal disease. J Clin Periodontol. 1991; 18(7):548-54.

106 Fentoglu O, Oz G, Tasdelen P, Uskun E, Aykac Y, Bozkurt FY. Periodontal status in subjects with hyperlipidemia. J Periodontol. 2009; 80(2):267-73.

107 Valentaviciene G, Paipaliene P, Nedzelskiene I, Zilinskas J, Anuseviciene OV. The relationship between blood serum lipids and periodontal condition. Stomatologija. 2006; 8(3):96-100.

108 Saxlin T, Suominen-Taipale L, Kattainen A, Marniemi J, Knuuttila M, Ylostalo P. Association between serum lipid levels and periodontal infection. J Clin Periodontol. 2008; 35(12):1040-7.

109 Yoon AJ, Cheng B, Philipone E, Turner R, Lamster IB. Inflammatory biomarkers in saliva: assessing the strength of association of diabetes mellitus and periodontal status with the oral inflammatory burden. J Clinical Periodontol. 2012; 39(5):434-40.

110 Cutler CW, Machen RL, Jotwani R, Iacopino AM. Heightened gingival inflammation and attachment loss in type 2 diabetics with hyperlipidemia. J Periodontol. 1999; 70(11):1313-21.

111 Nepomuceno R, Villela BS, Corbi SC, Bastos AS, Dos Santos RA, Takahashi CS, et al. Dyslipidemia rather than Type 2 Diabetes Mellitus or Chronic Periodontitis affects the systemic expression of pro- and anti-inflammatory genes. Mediators Inflamm. 2017; 2017:1491405.

112 Corbi SCT, Bastos AS, Nepomuceno R, Cirelli T, Dos Santos RA, Takahashi CS, et al. Expression profile of genes potentially associated with adequate glycemic control in patients with type 2 diabetes mellitus. J Diabetes Res. 2017; 2017:2180819.

113 Nepomuceno R. Parâmetros clínicos periodontais e expressão genética em individuos com diabetes mellitus, dislipidemia e doença periodontal [Dissertação de Mestrado]. Araraquara: Faculdade de Odontologia da Unesp; 2014.

114 Glass GV. Primary, secondary, and meta-analysis of research. Educ Res. 1976; 5(10):3-8.

115 Haidich AB. Meta-analysis in medical research. Hippokratia. 2010; 14(Suppl 1):29-37.

116 Gopalakrishnan S, Ganeshkumar P. Systematic reviews and meta-analysis: understanding the best evidence in primary healthcare. J Family Med Prim Care. 2013; 2(1):9-14.

117 Guyatt GH, Sackett DL, Sinclair JC, Hayward R, Cook DJ, Cook RJ. Users' guides to the medical literature. IX. A method for grading health care recommendations. Evidence-Based Medicine Working Group. JAMA. 1995; 274(22):1800-4.

118 Sackett DL, Rosenberg WM, Gray JA, Haynes RB, Richardson WS. Evidence based medicine: what it is and what it isn't. BMJ. 1996; 312(7023):71-2.

119 Nibali L, Tatarakis N, Needleman I, Tu YK, D'Aiuto F, Rizzo M, et al. Clinical review: Association between metabolic syndrome and periodontitis: a systematic review and meta-analysis. J Clin Endocrinol Metab. 2013; 98(3):913-20.

120 Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. J Periodontol. 2007; 78(7 Suppl):1387-99.

121 Russell AL. A system of classification and scoring for prevalence surveys of periodontal disease. J Dent Res. 1956; 35(3):350-9.

122 Preshaw PM. Definitions of periodontal disease in research. J Clin Periodontol. 2009; 36(1):1-2.

123 Savage A, Eaton KA, Moles DR, Needleman I. A systematic review of definitions of periodontitis and methods that have been used to identify this disease. J Clin Periodontol. 2009; 36(6):458-67.

124 Beck JD, Koch GG, Offenbacher S. Attachment loss trends over 3 years in community-dwelling older adults. J Periodontol. 1994; 65(8):737-43.

125 Offenbacher S, Divaris K, Barros SP, Moss KL, Marchesan JT, Morelli T, et al. Genome-wide association study of biologically informed periodontal complex traits offers novel insights into the genetic basis of periodontal disease. Hum Mol Genet. 2016; 25(10):2113-29.

126 Marchesan JT, Jiao Y, Moss K, Divaris K, Seaman W, Webster-Cyriaque J, et al. Common Polymorphisms in IFI16 and AIM2 Genes Are Associated With Periodontal Disease. J Periodontol. 2017; 88(7):663-72.

127 Willey JZ, Rodriguez CJ, Carlino RF, Moon YP, Paik MC, Boden-Albala B, et al. Race-ethnic differences in the association between lipid profile components and risk of myocardial infarction: The Northern Manhattan Study. Am Heart J. 2011; 161(5):886-92.

128 Sliwa K, Lyons JG, Carrington MJ, Lecour S, Marais AD, Raal FJ, et al. Different lipid profiles according to ethnicity in the Heart of Soweto study cohort of de novo presentations of heart disease. Cardiovasc J Africa. 2012; 23(7):389-95.

129 Zhang L, Qiao Q, Tuomilehto J, Janus ED, Lam TH, Ramachandran A, et al. Distinct ethnic differences in lipid profiles across glucose categories. J Clin Endocrinol Metab. 2010; 95(4):1793-801.

130 Davis TM, Cull CA, Holman RR, Prospective Diabetes Study (UKPDS) Group. Relationship between ethnicity and glycemic control, lipid profiles, and blood pressure during the first 9 years of type 2 diabetes: U.K. Prospective Diabetes Study (UKPDS 55). Diabetes Care. 2001; 24(7):1167-74.

131 Zaninotto P, Mindell J, Hirani V. Prevalence of cardiovascular risk factors among ethnic groups: results from the Health Surveys for England. Atherosclerosis. 2007; 195(1):e48-57.

132 Cooper RS, Kaufman JS, Ward R. Race and genomics. N Engl J Med. 2003; 348(12):1166-70.

133 Risch N, Burchard E, Ziv E,Tang H. Categorization of humans in biomedical research: genes, race and disease. Genome Biol. 2002; 3(7):comment2007.

134 Correa JD, Madeira MF, Resende RG, Correia-Silva Jde F, Gomez RS, de Souza Dda G, et al. Association between polymorphisms in interleukin-17A and -17F genes and chronic periodontal disease. Mediators Inflamm. 2012; 2012:846052.

135 Anovazzi G, Kim YJ, Viana AC, Curtis KM, Orrico SR, Cirelli JA, et al. Polymorphisms and haplotypes in the interleukin-4 gene are associated with chronic periodontitis in a Brazilian population. J Periodontol. 2010; 81(3):392-402.

136 Moreira PR, Lima PM, Sathler KO, Imanishi SA, Costa JE, Gomes RS, et al. Interleukin-6 expression and gene polymorphism are associated with severity of periodontal disease in a sample of Brazilian individuals. Clin Exp Immunol. 2007; 148(1):119-26.

137 Plothow A, Benvenutti R, Contieri FL, Bicalho MG. Frequencies at three polymorphic sites of interleukin-10 gene promoter in Brazilian renal recipients. Transplant Proc. 2003; 35(8):2908-10.

138 Alves-Silva J, da Silva Santos M, Guimaraes PE, Ferreira AC, Bandelt HJ, Pena SD, et al. The ancestry of Brazilian mtDNA lineages. Am J Hum Genet. 2000; 67(2):444-61.

139 Pimenta JR, Zuccherato LW, Debes AA, Maselli L, Soares RP, Moura-Neto RS, et al. Color and genomic ancestry in Brazilians: a study with forensic microsatellites. Hum Hered. 2006; 62(4):190-5.

140 Parra FC, Amado RC, Lambertucci JR, Rocha J, Antunes CM, Pena SD. Color and genomic ancestry in Brazilians. Proc Natl Acad Sci U S A. 2003; 100(1):177-82.

141 de Aguiar PK, Coletta RD, de Oliveira AM, Machado RA, Furtado PG, de Oliveira LA, et al. rs1801133C>T polymorphism in MTHFR is a risk factor for nonsyndromic cleft lip with or without cleft palate in the Brazilian population. Birth Defects Res A Clin Mol Teratol. 2015; 103(4):292-8.

142 Messetti AC, Machado RA, de Oliveira CE, Martelli-Junior H, de Almeida Reis SR, Moreira HS, et al. Brazilian multicenter study of association between polymorphisms in CRISPLD2 and JARID2 and non-syndromic oral clefts. J Oral Pathol Med. 2017; 46(3):232-9.

143 Paranaiba LM, Bufalino A, Martelli-Junior H, de Barros LM, Graner E, Coletta RD. Lack of association between IRF6 polymorphisms (rs2235371 and rs642961) and non-syndromic cleft lip and/or palate in a Brazilian population. Oral Dis. 2010; 16(2):193-7.

144 Pena SD, Di Pietro G, Fuchshuber-Moraes M, Genro JP, Hutz MH, Kehdy Fde S, et al. The genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected. PLoS One. 2011; 6(2):e17063.

145 Fentoglu O, Bozkurt FY. The bi-directional relationship between periodontal disease and hyperlipidemia. Eur J Dent. 2008; 2(2):142-6.

146 Griffiths R, Barbour S. Lipoproteins and lipoprotein metabolism in periodontal disease. Clin Lipidol. 2010; 5(3):397-411.

147 Sun HL, Wu YR, Song FF, Gan J, Huang LY, Zhang L, et al. Role of PCSK9 in the development of mouse periodontitis before and after treatment: a double-edged sword. J Infect Dis. 2018; 217(4):667-80.

148 Bengtsson T, Karlsson H, Gunnarsson P, Skoglund C, Elison C, Leanderson P, et al. The periodontal pathogen Porphyromonas gingivalis cleaves apoB-100 and increases the expression of apoM in LDL in whole blood leading to cell proliferation. J Intern Med. 2008; 263(5):558-71.

149 Lonn J, Ljunggren S, Klarstrom-Engstrom K, Demirel I, Bengtsson T, Karlsson H. Lipoprotein modifications by gingipains of Porphyromonas gingivalis. J Periodontal Res. 2018 Jan 17. [Epub ahead of print]

150 Miyakawa H, Honma K, Qi M, Kuramitsu HK. Interaction of Porphyromonas gingivalis with low-density lipoproteins: implications for a role for periodontitis in atherosclerosis. J Periodontal Res. 2004; 39(1):1-9.

151 Hashimoto M, Kadowaki T, Tsukuba T, Yamamoto K. Selective proteolysis of apolipoprotein B-100 by Arg-gingipain mediates atherosclerosis progression accelerated by bacterial exposure. J Biochem. 2006; 140(5):713-23.

152 Sakiyama Y, Kato R, Inoue S, Suzuki K, Itabe H, Yamamoto M. Detection of oxidized low-density lipoproteins in gingival crevicular fluid from dental patients. J Periodontal Res. 2010; 45(2):216-22.

153 Nepomuceno R, Pigossi SC, Finoti LS, Orrico SRP, Cirelli JA, Barros SP, et al. Serum lipid levels in patients with periodontal disease: A meta-analysis and meta-regression. J Clin Periodontol. 2017; 44(12):1192-207.

154 Ottman R. Gene-environment interaction: definitions and study designs. Prev Med. 1996; 25(6):764-70.

155 Chantarangsu S, Sura T, Mongkornkarn S, Donsakul K,Torrungruang K. Vitamin D receptor gene polymorphism and smoking in the risk of chronic periodontitis. J Periodontol. 2016; 87(11):1343-51.

156 Lanktree MB, Hegele RA. Gene-gene and gene-environment interactions: new insights into the prevention, detection and management of coronary artery disease. Genome Med. 2009; 1(2):28.

157 Tanaka K, Miyake Y, Hanioka T, Furukawa S, Miyatake N, Arakawa M. The IL18 promoter polymorphism, rs1946518, is associated with the risk of periodontitis in japanese women: the Kyushu Okinawa Maternal and Child Health Study. Tohoku J Exp Med. 2017; 243(3):159-64.

158 Tanaka K, Miyake Y, Hanioka T, Arakawa M. Relationship between IL1 gene polymorphisms and periodontal disease in Japanese women. DNA Cell Biol. 2014; 33(4):227-33.

159 Ribeiro MS, Pacheco RB, Fischer RG, Macedo JM. Interaction of IL1B and IL1RN polymorphisms, smoking habit, gender, and ethnicity with aggressive and chronic periodontitis susceptibility. Contemp Clin Dent. 2016; 7(3):349-56.

160 Andreotti G, Chen J, Gao YT, Rashid A, Chen BE, Rosenberg P, et al. Polymorphisms of genes in the lipid metabolism pathway and risk of biliary tract cancers and stones: a population-based case-control study in Shanghai, China. Cancer Epidemiol Biomarkers Prev. 2008; 17(3):525-34.

161 Rhodin K, Divaris K, North KE, Barros SP, Moss K, Beck JD, et al. Chronic periodontitis genome-wide association studies: gene-centric and gene set enrichment analyses. J Dent Res. 2014; 93(9):882-90.

162 Haririan H, Andrukhov O, Bottcher M, Pablik E, Wimmer G, Moritz A, et al. Salivary neuropeptides, stress and periodontitis. J Periodontol. 2017 Sep 15:1-15. [Epub ahead of print].

163 Kim SJD. Functional analysis of rs3811046 and rs3811047 variants in caucasians [Doctor of Philosophy thesis]. Chapel Hill: University of North Carolina; 2017.

164 Chen D, Zhang TL, Wang LM. The association of CSF-1 gene polymorphism with chronic periodontitis in the Han Chinese population. J Periodontol. 2014; 85(8):e304-12.

165 Meisel P, Krause T, Cascorbi I, Schroeder W, Herrmann F, John U, et al. Gender and smoking-related risk reduction of periodontal disease with variant myeloperoxidase alleles. Genes Immun. 2002; 3(2):102-6.

166 Ericson U, Rukh G, Stojkovic I, Sonestedt E, Gullberg B, Wirfalt E, et al. Sex-specific interactions between the IRS1 polymorphism and intakes of carbohydrates and fat on incident type 2 diabetes. Am J Clin Nutr. 2013; 97(1):208-16.

167 Gbadoe KM, Berdouzi N, Aguinano AA, Ndiaye NC,Visvikis-Siest S. Cardiovascular diseases-related GNB3 C825T polymorphism has a significant sex-specific effect on serum soluble E-selectin levels. J Inflamm (Lond). 2016; 13:39.

168 Rinn JL, Snyder M. Sexual dimorphism in mammalian gene expression. Trends Genet. 2005; 21(5):298-305.

169 Yan N, Meng S, Song RH, Qin Q, Wang X, Yao Q, et al. Polymorphism of IL37 gene as a protective factor for autoimmune thyroid disease. J Mol Endocrinol. 2015; 55(3):209-18.

170 Igl BW, Konig IR, Ziegler A. What do we mean by 'replication' and 'validation' in genome-wide association studies? Hum Hered. 2009; 67(1):66-8.

APÊNDICE A – Metodologia detalhada

Metodologia detalhada da revisão sistemática com metanálise (Capítulo 1)

Foi realizada uma revisão sistemática de acordo com as diretrizes *Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (PRISMA) e também de acordo com *Meta-analysis of Observational Studies in Epidemiology* (MOOSE) [1,2].

1 Pergunta principal

A questão central foi proposta seguindo os princípios "*PECO*" (P – paciente ou população; E – exposição; C – comparação ou controle; O – *outcome*, resultado, efeito) para descobrir as diferenças nos parâmetros lipídicos séricos de pacientes com e sem periodontite crônica (PC): "Os indivíduos com doença periodontal crônica sem doença sistêmica têm diferentes níveis de parâmetros lipídicos séricos (HDL, DL, triglicerídeos e colesterol total) em comparação à indivíduos periodontalmente saudáveis?"

2 Estratégia de pesquisa

Pesquisas eletrônicas nos bancos de dados *PubMed*, *Web of Science*, *Scopus*, *EMBASE* e *Cochrane Controlled Trials Registry*, complementadas por uma pesquisa através das listas de referência dos estudos incluídos, foram realizadas entre artigos publicados de 1968 até o final de fevereiro de 2016. Os artigos relevantes foram identificados através de uma pesquisa em bancos de dados seguindo a utilização das palavras-chaves:

• As 2 palavras chaves do *MESH* (https://www.ncbi.nlm.nih.gov/mesh) selecionadas e suas derivações foram combinadas em todas as possibilidades: "*Periodontal Diseases*" and "*Lipids*".

• Palavras-chave para *Periodontal Diseases*: "*Periodontal Disease*" ou "*Periodontitis*" ou "*Alveolar bone loss*"; E

• Palavras-chave para *Lipids*: “HDL” ou “High Density Lipoprotein” ou “LDL” ou “Low Density Lipoprotein” ou “Triglycerides” ou “Total Cholesterol” ou “Dyslipidemia” ou “Hyperlipidemia” ou “Hypertriglyceridemia” ou “Hypercholesterolemia” ou “Lipids”.

A pesquisa da literatura em todos os bancos de dados eletrônicos foi exportada para a o *software EndNote Program ™X7* (Thomson Reuters, Nova York, NY, EUA), a fim de eliminar as referências duplicadas.

3 Seleção dos estudos

Selecionamos artigos de pesquisa originais de acordo com os seguintes critérios de inclusão: 1) estudos longitudinais e transversais (estudos coorte e caso-controle); 2) presença dos dados sobre os parâmetros lipídicos séricos (HDL, LDL, triglicerídeos e colesterol total) de adultos com e sem periodontite crônica; 3) indivíduos sem alterações sistêmicas, incluindo histórico de doença cardiovascular (DCV) e diabetes; 4) pacientes não fumantes; 5) estudos que mostram o uso de métodos estatísticos e os valores numéricos de média e desvio padrão, incluindo as unidades de medida para quantificar os níveis lipídicos séricos; 8) artigos publicados na língua inglesa.

Estudos desenhados especificamente para investigar mulheres grávidas e indivíduos com aterosclerose, artrite reumatóide, tratamento de hemodiálise e / ou com tratamento sistêmico para dislipidemia não foram incluídos neste estudo. Foram excluídos também pesquisas que de acordo com seus critérios de inclusão e exclusão, selecionaram apenas pacientes sem hiperlipidemia (relacionada ao desfecho desta metanálise), uma vez que limitou os pacientes a baixos níveis lipídicos. Também foram selecionados artigos sem resumos, mas com títulos que sugeriam relação com os objetivos desta revisão, para análise do texto completo. No entanto, estudos experimentais (modelos animais e celulares), cartas ao editor, revisões de

literatura e artigos não publicados foram excluídos. Os artigos de pesquisa originais que não seguiram a todos os critérios descritos acima também foram excluídos desta metanálise.

Devido à falta de um consenso geral para o diagnóstico de PC, e ao uso de diferentes definições de PC nos artigos científicos, estabelecemos um limiar de diagnóstico como parte de uma avaliação de qualidade metodológica e validação externa. Os critérios de seleção referentes a este **diagnóstico seguro de PC** foram baseados em uma metanálise anterior [3] como segue:

Definições Clínicas de Doença Periodontal:

a. **diagnóstico seguro de PC**:

• Pelo menos 2 sítios em diferentes dentes com nível clínico de inserção (NCI) ≥6 mm e pelo menos 1 sítio com profundidade de sondagem (PS) ≥4 mm, ou

• Pelo menos 2 sítios em dentes não adjacentes com NCI ≥3 mm, ou

• Índice Periodontal Comunitário (CPI) com código 4 em pelo menos 1 quadrante, ou

• Nos casos em que não seja relatada NCI ou PS, apresente perda óssea alveolar radiográfica (POA) ≥ 30% ou de pelo menos 5 mm do comprimento radicular em pelo menos 2 dentes.

b. **diagnóstico inseguro de PC**:

Os parâmetros para a classificação da doença, ou seja, PS, NCI ou POA não foram claramente definidos ou não tão rigorosamente categorizados.

4 Avaliação da qualidade dos estudos

A avaliação do risco de viés foi realizada separadamente para cada estudo. A avaliação foi conduzida por dois investigadores (RN, SCP) independentemente, e a acordância entre os examinadores foi então avaliada. Qualquer discrepância entre os dois examinadores na avaliação da qualidade dos estudos foi resolvida através da discussão entre ambos. Para estudos

transversais (caso-controle e coorte) e longitudinais, a Escala de *Newcastle-Ottawa* (*Newcastle-Ottawa Scale* - NOS) foi utilizada para avaliar a qualidade do estudo. Esta escala foi desenvolvida para avaliar a qualidade dos estudos de forma fácil e conveniente para revisões sistemáticas e metanálises. Em resumo, esta escala consiste em nove itens classificados em três grupos principais: (i) seleção dos grupos de estudo, (ii) comparabilidade dos grupos e (iii) determinação da exposição de interesse [4]. Os estudos foram então ranqueados com um mínimo de zero e máximo de nove pontos. A taxa de concordância entre os investigadores foi de 83,3%.

5 Extração de dados dos estudos

A busca inicial de avaliação de títulos e resumos foi realizada com cuidado independentemente por dois investigadores (RN e SCP), e verificou-se a acordância. Em seguida, os textos completos dos artigos julgados pelo título e resumo por serem relevantes foram lidos de forma independente e avaliados em relação aos critérios de seleção. A taxa de concordância entre os investigadores foi de 92,1%. Um terceiro investigador (RMSC) avaliou os estudos que não foram consenso entre os outros 2 investigadores.

Os seguintes parâmetros foram extraídos de cada estudo selecionado: nome do primeiro autor e ano de publicação, país, tamanho total da amostra, porcentagem de pacientes do sexo masculino, razão de pacientes do sexo masculino (centrado em 1: 1 – razão grupo PC: grupo controle), média de idade dos participantes, razão da média de idade dos pacientes (centrado em 1: 1 - razão grupo PC: grupo controle), critérios diagnósticos para doença periodontal, tipo de coleta de sangue (soro ou plasma) e exame de sangue (em jejum ou não) e estudos pareados para índice de massa corporal (IMC) entre grupos (sim ou não). Além disso, os níveis séricos dos parâmetros lipídicos (HDL, LDL, triglicerídeos e colesterol total) foram extraídos de cada estudo para análises estatísticas (tamanho da amostra, média e desvio padrão de cada grupo).

No caso de mais de um artigo do mesmo autor utilizando a mesma amostra e mostrando os mesmos resultados para os níveis de perfil lipídico, apenas um desses artigos foi selecionado. No caso de dois ou mais grupos de pacientes com periodontite crônica, com base na extensão e gravidade da doença, apenas foram coletados os dados do grupo de pacientes com PC severa, para minimizar qualquer heterogeneidade entre os estudos incluídos.

6 Análise Estatística

Entre os estudos, encontramos duas unidades de medida diferentes usadas para quantificar os níveis séricos de parâmetros lipídicos: mg/dL ou mmol/L (o mesmo que o mM). Para padronizar as unidades, convertemos os níveis de parâmetros lipídicos de todos os estudos para mg/dL. Para converter os dados de colesterol total, HDL e LDL de mmol/L para mg/dL, multiplicamos o valor por 38,67. Além disso, para converter TG de mmol/L em mg/dL, multiplicamos por 88,57 [5].

Nos estudos em que foi utilizado erros padrão (EP), para ser comparável com os outros estudos presentes na metanálise, eles foram convertidos em desvios padrão (DP). O cálculo depende tanto do erro padrão quanto do tamanho da amostra (N), usando uma simples equação $DP = EP \times \sqrt{N}$ [6].

O tamanho do efeito foi calculado e reportado como a diferença média (*mean difference* - MD). O intervalo de confiança (IC) de 95% foi calculado para cada parâmetro lipídico e o efeito combinado foi considerado significativo para os valores de $p < 0,05$. Foi utilizado o software estatístico *(Review Manager [RevMan],* Versão 5.1. Copenhagen: *The Nordic Cochrane Center, The Cochrane Collaboration, 2011*) para agrupar os dados e produzir os *forest plots*. Realizamos também análises de subgrupos para estudos com e sem diagnóstico seguro de PC.

Forest plots para cada metanálise apresentam os dados brutos (ie, desvio padrão e tamanho da amostra), diferença média (exibidas como quadrados) e IC (exibidas como linhas) para o efeito escolhido, bem como estatísticas de heterogeneidade (Chi2 e I^2), número total de participantes por grupo, efeito médio geral (estatísticas de MD e Z) e percentual de peso atribuído a cada estudo.

Os testes do Qui-quadrado e do índice de inconsistência (I^2) foram utilizados para avaliar a heterogeneidade dos estudos incluídos nesta metanálise. A heterogeneidade dos ensaios foi considerada estatisticamente significante para $p < 0,1$, em vez de $p < 0,05$, já que esse teste tem pouco poder. O Modelo de efeito fixo (Método Mantel-Haenszel) foi utilizado quando nenhuma evidência de heterogeneidade foi detectada nos resultados; Caso contrário, um modelo de efeitos aleatórios (Método DerSimonian-Laird) foi aplicado.

Meta-regressões foram realizadas para explorar o impacto do risco de viés dos estudos incluídos usando um modelo de efeitos aleatórios. Análises de regressão univariada e multivariada foram realizadas. Para a análise univariada, adicionamos o ano em que o estudo foi publicado, tamanho total da amostra, qualidade dos estudos (considerando a classificação NOS), diagnóstico periodontal (seguro ou inseguro), idade (centrado em média de todos os pacientes), proporção de idade (centrado em 1: 1 - razão grupo PC: grupo controle), gênero (centrado na porcentagem de homens), proporção de pacientes homens (centrado em 1: 1 - razão grupo PC: grupo controle), estudos pareados para IMC entre grupos (sim ou não), tipo de coleta de sangue (soro ou plasma) e exame de sangue (em jejum ou não) como covariáveis. Se o coeficiente de regressão das covariáveis fosse significativo ($p < 0,1$), então as covariáveis foram inseridas na meta-regressão multivariada. Utilizamos IC 95% e consideramos um valor $p < 0,05$ para ser estatisticamente significante na meta-regressão multivariada.

Se algum dos parâmetros tivesse influenciado o resultado da metanálise, uma nova análise estatística foi realizada para excluir o viés gerado. Os artigos que introduziram aumento

significativo do risco de viés foram excluídos. Todas as análises de meta-regressão foram realizadas utilizando o software estatístico OpenMeta [Analyst] [7].

Para testar a influência de cada estudo sobre o tamanho do efeito geral, a análise de sensibilidade foi realizada usando o método *leave-one-out* após meta-regressão; isto é, removendo um estudo cada vez e repetindo a análise [8].

O viés de publicação foi analisado através de gráfico de funil (*funnel plot*) e confirmado pelo teste de correlação de Begg e pelo método de regressão de Egger. O método de Begg é um teste de correlação entre a estimativa de efeito e o erro padrão [9]. O teste de Egger utiliza regressão logística linear, com base no desvio padrão e na precisão dos estudos [10]. O p-valor < 0,10 foi utilizado como indicação da presença de potencial viés de publicação para ambos os testes. Se o viés de publicação foi indicado (nos testes de Begg ou Egger) nas metanálises após realização dos testes *leave-one-out* e meta-regressão, o método de corte e preenchimento de Duval e Tweedie (*Duval and Tweedie's trim-and-fill*) foi aplicado para ajustar a diferença média [11]. Todas as análises de viés de publicação foram realizadas usando o software Comprehensive Meta-Analysis, versão 3.3.070 (2014).

Para avaliar se a etnia dos pacientes poderia ser uma potencial variável causadora de heterogeneidade entre os estudos incluídos na metanálise, realizamos metanálises adicionais por subgrupo de acordo com o continente onde a pesquisa foi desenvolvida, ou seja, o continente em que residem os sujeitos investigados dessa etnia.

Metodologia detalhada dos estudos caso-controle (Capítulos 2 e 3)

1 Seleção da amostra

Foram convidados para participarem deste estudo pacientes que procuraram atendimento nas diversas clínicas da Faculdade de Odontologia de Araraquara – FOAr - UNESP. Após esclarecer os objetivos e tirar quaisquer possíveis dúvidas da pesquisa, os pacientes que se adequarem aos critérios de inclusão e exclusão e que aceitarem participar voluntariamente, foram convidados a assinar o termo de consentimento livre e esclarecido, conforme já aprovado pelo Comitê de Ética em Seres Humanos da FOAr - UNESP (57/04) (ANEXO

B). Seguem os critérios de inclusão e exclusão:

- CRITÉRIOS DE INCLUSÃO: indivíduos normoglicêmicos acima de 30 anos, de ambos os sexos, com pelo menos 10 dentes na cavidade bucal (excluindo terceiro molar) e de qualquer grupo étnico-racial (pois a tentativa de isolar determinadas características raciais é difícil em um país altamente miscigenado).
- CRITÉRIOS DE EXCLUSÃO: pacientes que realizaram tratamento periodontal nos últimos 6 meses, histórico de antibioticoterapia nos últimos 3 meses, uso crônico de drogas anti-inflamatórias, história autodeclarada de doenças que influenciam o imunológico sistêmico (diabetes mellitus, infecção pelo HIV ou quimioterapia imunossupressora), além de pacientes gravidas ou em lactação.

Após o exame periodontal, os indivíduos foram classificados em dois diferentes grupos, de acordo com os critérios definidos pela CDC/AAP (*Centers for Disease Control and Prevention/ American Academy of Periodontology*) [12]:

- **Grupo B (n = 358):** pacientes com periodontite crônica (PC) severa (2 ou mais sítios com perda de inserção interproximal maior que 5 mm em dentes diferentes e pelo menos um sitio com profundidade de sondagem maior que 4 mm) ou periodontite moderada (2 ou mais sítios com perda de inserção interproximal maior que 3 mm em dentes diferentes ou pelo menos dois sítios com profundidade de sondagem maior que 4 mm em dentes diferentes) (Tabela 1) [12];

Grupo A (n = 356): pacientes sem periodontite crônica (pacientes que não se enquadram nos critérios de periodontite severa ou moderada) (Tabela A1)[12].

Tabela A1 - Classificados da Doença Periodontal, de acordo com os critérios definidos pela CDC/AAP

		Definição Clínica		
	Classificação	NI		PS
Grupo A	Periodontite leve e Saudáveis	Não se enquadram nos critérios de periodontite severa ou moderada		
Grupo B	Periodontite Moderada	≥ 2 sítios com NIi ≥ 4mm (em dentes diferentes)	OU	≥ 2 sítios com PSi ≥ 5mm (em dentes diferentes)
	Periodontite Severa	≥ 2 sítios com NIi ≥ 6mm (em dentes diferentes)	E	≥ 1 sítio com PSi ≥ 5mm

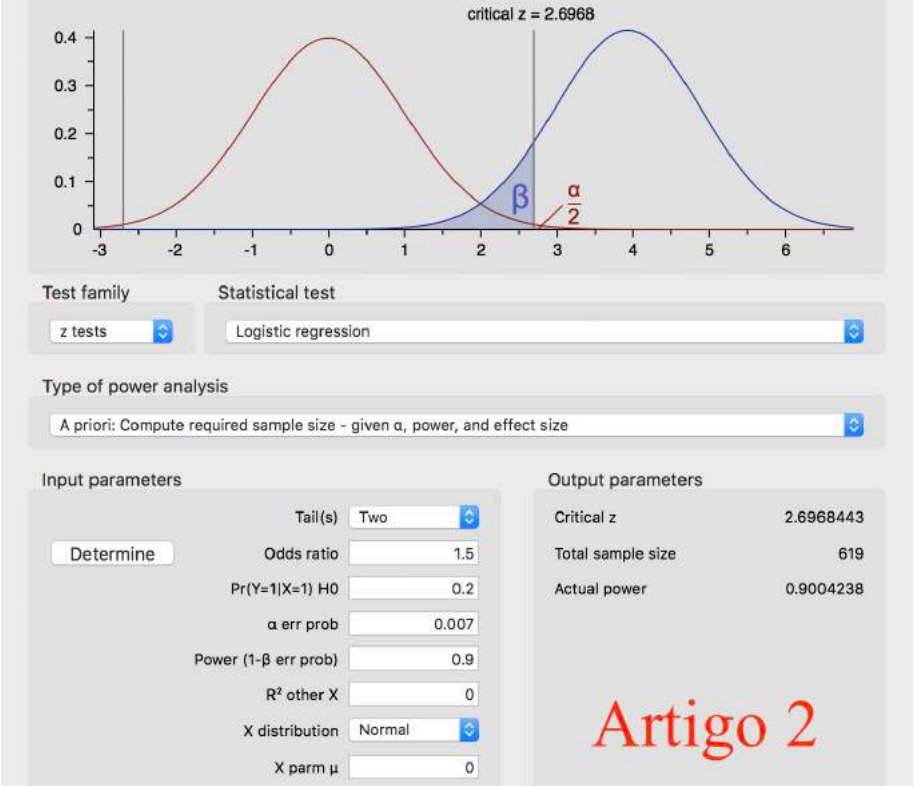
*Exclusão de terceiros molares. PS: profundidade de sondagem interproximal; NIi: nível de inserção interproximal.
Fonte: Elaboração própria.

Os critérios clínicos acima mencionados já foram utilizados em estudos de polimorfismos genéticos, incluindo estudos de varredura ampla do genoma, ou seja, *Genome-Wide Association Study* (*GWAS*) para Periodontite Crônica [13,14]. Pacientes que fumam ou já fumaram não foram excluídos. Foram realizadas análises de regressão logística ou linear múltipla utilizando o status de tabagismo como variável de controle.

2 Cálculo do tamanho amostral

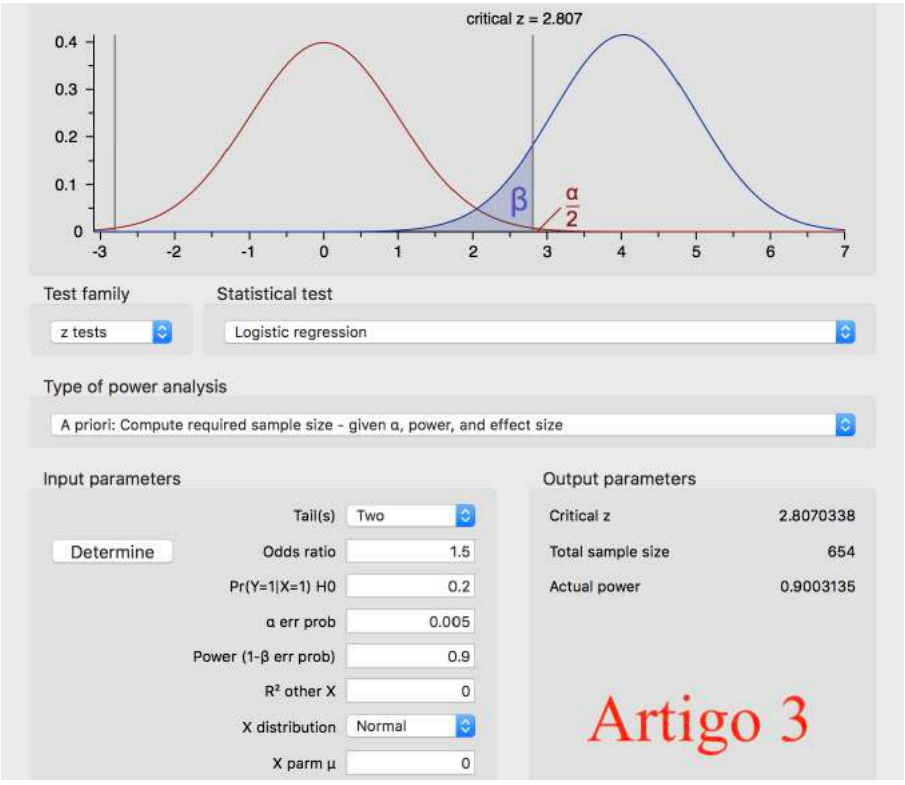
Para calcular o tamanho da população necessária para que o estudo tivesse poder suficiente de atestar a associação do(s) alelo(s) com a PC foi utilizado o programa G*Power Calculator, versão 3.1 [15], considerando os parâmetros: regressão logística; two tail, OR: 1.5; Pr(Y=1/X=1) H0 = 0,2; alfa de 0,007 (para o artigo 2) e 0,005 (para o artigo 3) e poder de 90%, R2 = 0. É importante salientar que o valor alfa=0,007 e 0,005 foram obtidos de p=0,05 dividido por (4 SNPs + 3 variáveis de ajuste [idade, gênero e tabagismo] no Capítulo 2) e (7 SNPs + 3 variáveis de ajuste [idade, gênero e tabagismo] no Capítulo 3). Este cálculo resultou em uma amostra menor que 714 pacientes (n = 619 para o artigo 2; n = 654 para o artigo 3), que foi o tamanho amostral utilizados nos 2 artigos (Figuras A1 e A2).

Figura A1- Resultado do G*Power utilizando os parâmetros descritos no Capítulo 2.



Fonte: Elaboração própria.

Figura A2 - Resultado do G*Power utilizando os parâmetros descritos no Capítulo 3



Fonte: Elaboração própria.

3 Anamnese e exame periodontal

Todos os pacientes foram submetidos à anamnese completa que avaliou o histórico de saúde geral e bucal.

Como mencionado no item 1 (seleção dos pacientes), todos os indivíduos foram submetidos a um exame periodontal completo. Os exames clínicos periodontais foram realizados com a utilização da sonda periodontal milimetrada e padronizada de 15 mm (University of North Carolina – UNC 1-15 Hu-Friedy), e tiveram os parâmetros clínicos de profundidade de sondagem (PS) e nível clínico de inserção (NCI) examinados em seis sítios (mésio-vestibular, vestibular, disto-vestibular, mésio-lingual, lingual e disto-lingual) de cada dente, além de sangramento à sondagem.

Foi considerado nesse estudo:

- 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;
- 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;
- 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;
- Sangramento à sondagem: presença ou ausência de sangramento, decorrido um tempo de 30 segundos depois de mensurada a profundidade de sondagem;
- 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.

Os critérios utilizados para classificar a doença periodontal nos Capítulos 2 e 3, como previamente citado, fora os critérios definidos pela CDC/AAP [12]. Devido a isso, foram considerados nestes dois estudos os parâmetros clínicos de profundidade de sondagem interproximal (PSi) e nível clínico de inserção interproximal (NCIi) examinados em quatro

sítios (mésio-vestibular, disto-vestibular, mésio-lingual e disto-lingual) de cada dente, além de sangramento à sondagem apenas dos sítios interproximais.

Todos os participantes desta pesquisa receberam orientação sobre o que é a doença periodontal e suas implicações na saúde geral. Após o exame periodontal completo, os participantes foram encaminhados para a Clínica de Periodontia desta Faculdade para tratamento periodontal. Todos também receberam instrução de adequada higiene oral.

4 Coleta de material biológico e extração de DNA

Foi solicitado a cada indivíduo que realizasse um bochecho com solução de dextrose a 3% autoclavada 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 foi necessária alta qualidade do DNA, utilizamos o método que se mostrou mais eficiente: o método adaptado do acetato de amônia a 8M [16]. As amostras foram descongeladas e então foi adicionado 5 μL de proteinase K e incubadas 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, centrifugando-se a 14.000 rpm por 5 mim. O pellet foi seco à temperatura ambiente e ressuspendido em 100 μL de TE.

O DNA genômico de cada amostra foi avaliado com relação a concentração (ng/μl) e pureza (260/280nm; 260/230nm) no aparelho NanoVue™ Plus Spectrophotometer (GE Healthcare Life Sciences, EUA), e foi armazenado a -80°C até o momento da genotipagem. O protocolo de genotipagem por OpenArray necessita da quantificação prévia do DNA de cada amostra utilizando o sistema de fluorescência Qubit (Thermo-Fisher).

Para as amostras que não apresentaram concentração igual ou maior que 50 ng/μl e pureza (principalmente a razão 260/280nm) entre 1.7 e 2.1, no aparelho NanoDrop™

2000/2000c Spectrophotometer, foi realizada a precipitação do DNA para purificar e/ou para concentrar amostras, tornando-as ideais para genotipagem utilizando o sistema de genotipagem TaqMan® OpenArray™ (Applied Biosystems).

Para realização desta técnica, à amostra de DNA ressuspendida em TE, adicionou-se 1/20 (do volume da amostra de DNA) de acetato de sódio 3M (pH=5,2) a temperatura ambiente. Estimou-se o volume total e foi adicionado o dobro do volume de etanol absoluto gelado. Homogeneizou-se levemente ate evidenciar a "nuvem" de DNA, e então deixar no freezer *overnight*. No dia seguinte, retirou-se a amostra do freezer para centrifugar a 14000 rpm por 15min a 4ºC. Juntamente ao pellet obtido, foi feita 1 lavagem colocando-se etanol 70%, seguido de centrifugação a 14000rpm por 15min a 4ºC. Ao fim, deixou-se secar o pellet à temperatura ambiente. O pellet foi ressuspendido em 20 μL de TE, objetivando aumentar a concentração do DNA.

5 Seleção dos SNPs

Para a seleção dos SNPs foi utilizado o banco do *International HapMap Project* (www.hapmap.org) e *1000 Genomes Project* (http://www.internationalgenome.org), consórcios internacionais com os padrões 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 e do Projeto 1000 Genomes. Nesse banco de dados estão disponíveis informações sobre os SNPs nos genes de interesse. Foram selecionados genes candidatos, no qual os SNPs deveriam apresentar nas populações CEU ou TSI menor frequência alélica (MAF) ≥ 5% (0.05). Para isso foram selecionados:

- Capítulo 2: variantes genéticas em alguns genes atualmente consideradas por apresentar suscetibilidade para dislipidemia e algumas vezes também a outras doenças sistêmicas. Estes genes e suas proteínas vêm sendo estudadas em relação a doença periodontal, mas ainda não foram estudados SNPs nestes genes em associação a PC (Tabela 2). Pesquisamos minuciosamente de SNPs associados a dislipidemia e frações lipídicas a partir de estudos de *GWAS* utilizando o NHGRI-EBI *Catalog of published genome-wide association studies* (https://www.ebi.ac.uk/GWAS).
- Capítulo 3: variantes genéticas em alguns genes atualmente consideradas por apresentar suscetibilidade para periodontite crônica, porém estes resultados ainda não foram validados para

população brasileira. Estes SNPs apresentaram associação com PC a partir de *GWAS* e de estudos que envolvem análises sofisticadas de bioinformática e bioestatística (Tabela A2). Pesquisamos minuciosamente SNPs associados a PC a partir de estudos de *GWAS* utilizando o mesmo catálogo de *GWAS* (https://www.ebi.ac.uk/GWAS).

Tabela A2 - Informações dos ensaios e SNPs avaliados, incluindo dados de menor frequência alélica (MAF)

Genes relacionados ao metabolismo lipídico						
NCBI SNP ID	Gene	Cytogenetic Band	Alelos	CEU %	TSI %	MAF do estudo
rs5925	*LDLR*	19p13.2b	**T|C**	**C|C**: 0.22	**C|C**: 0.18	**C|C** 0.16
rs688	*LDLR*	19p13.2b	**C|T**	**T|T**: 0.22	**T|T**: 0.17	**T|T** 0.14
rs676210	*Apo B*	2p24.1c	**G|A**	**A|A**: 0.06	**A|A**: 0.06	**A|A** 0.06
rs693	*Apo B*	2p24.1c	**C|T**	**T|T:** 0.20	**T|T:** 0.19	**T|T** 0.14

Genes relacionaods a estudos de *GWAS* ou de Bioinformática para DP						
NCBI SNP ID	Gene	Cytogenetic Band	Alelos	CEU %	TSI %	MAF do estudo
rs1360573	*close FZD8*	10p11.21a	**A|G**	**G|G**: 0.08	**G|G**: 0.08	**G|G** 0.05
rs187238	*IL18*	11q23.1d	**C|G**	**G|G**: 0.10	**G|G**: 0.05	**G|G** 0.06
rs2521634	*close NPY*	7p15.3b	**A|G**	**A|A**: 0.02	**A|A**: 0.05	**A|A** 0.07
rs352140	*TLR9*	3p21.2a	**C|T**	**T|T**: 0.23	**C|C**: 0.11	**T|T** 0.23
rs3811046	*IL37*	2q13d	**G|T**	**G|G**: 0.08	**G|G**: 0.06	**G|G** 0.14
rs3826782	*EMR1*	19p13.3a	**A|G**	**A|G**: 0.16	**A|A**: 0.01	**A|A** 0.01
rs7762544	*close NCR2*	6p21.1g	**A|G**	**G|G**: 0.05	**G|G**: 0.05	**G|G** 0.05

Fonte: Elaboração própria.

6 Genotipagem dos SNPs

Neste estudo, a detecção dos SNPs foi realizada utilizando a plataforma *OpenArray Real Time PCR System*. Trata-se de uma plataforma semi-automatizada de alta demanda e relativamente de baixo custo, pois utiliza quantidades de reagentes extremamente inferiores quando comparado aos outros métodos disponíveis, uma vez que a reação de PCR e a detecção são realizadas em uma lâmina com 3072 orifícios microscópicos compostos por superfícies hidrofílicas e hidrofóbicas.

O ensaio é flexível e permite a disposição de diferentes números de alvos e amostras. O formato escolhido para este ensaio foram chips com 64 ensaios para 48 amostras. 4 SNPs na lâmina de genotipagem foram utilizados para controle interno da técnica, conforme orientação do fabricante. A customização da lâmina é realizada previamente com a seleção de cada SNP a ser investigado por meio de um ensaio específico. Cada ensaio é composto por um par de primers e um par de sondas, marcadas com os fluoróforos VIC e FAM, específicos para a região do SNP, os quais são depositados nos orifícios. Assim, foram escolhidos 60 SNPs para genotipar os pacientes, com enfoque na avaliação da PC e comorbidades associadas, que complementam projetos finalizados que contaram com o apoio da FAPESP. Dentre os 60 SNPs investigados, estão incluídos os 11 SNPs apresentados nos Capítulos 2 e 3 desta Tese.

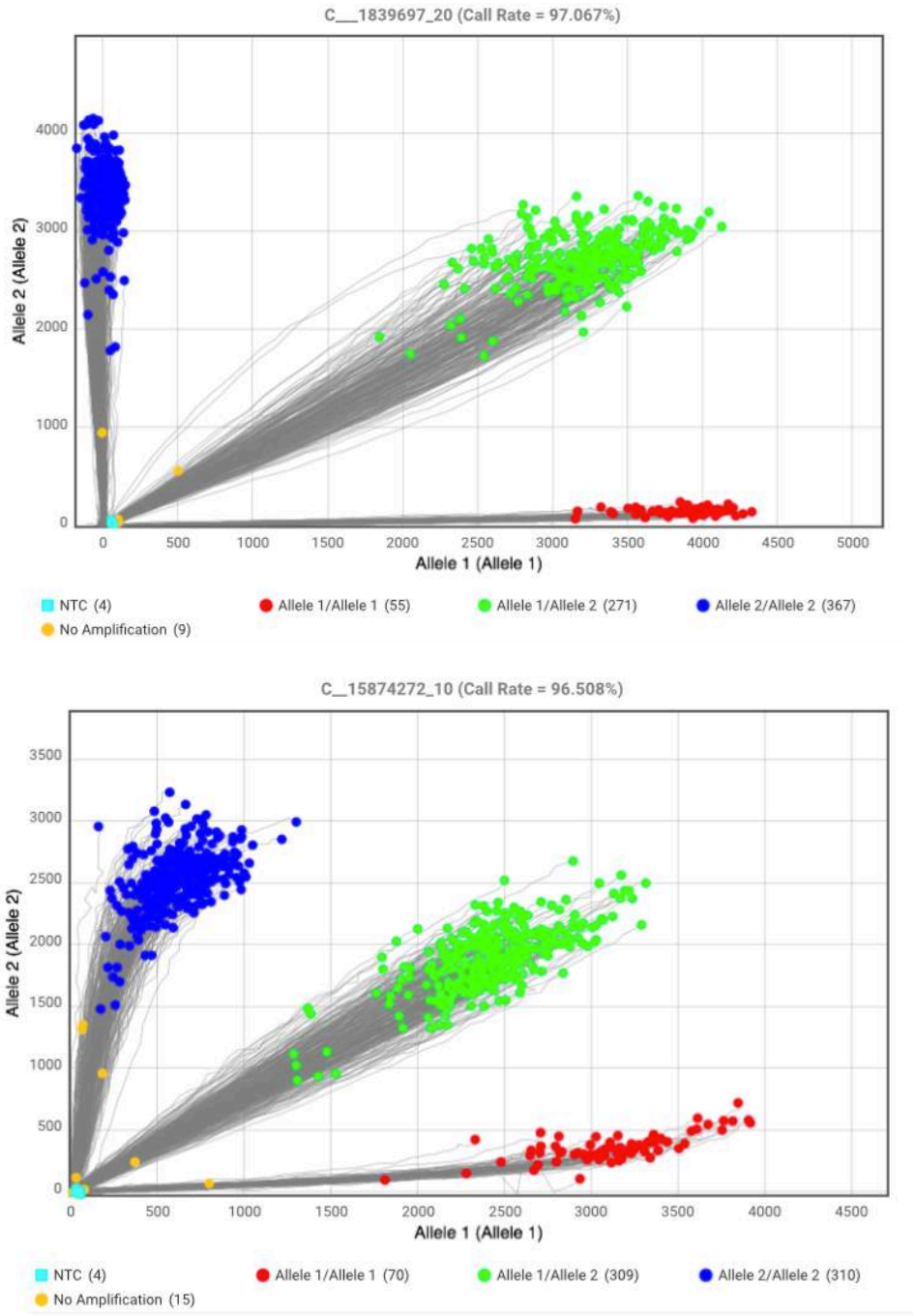
Todas as etapas da genotipagem foram realizadas conforme as instruções do fabricante. A genotipagem foi realizada utilizando 2,5 μL de cada amostra individual de DNA (total de 125 ng de DNA genômico) analisados em um poço de uma placa de 384 poços, juntamente com

2,5 µL de 2X TaqMan®OpenArray® Genotyping Master Misture, de acordo com os protocolos do fabricante. A transferência das amostras de DNA para a placa contendo o mix de reação e subsequentemente a transferência da reação para a lâmina do OpenArray foi realizada pelo instrumento OpenArray AccuFill System (Life Technologies), ocorrendo então a solubilização dos primers e sondas dispostos nos orifícios da lâmina. Pelo menos um controle branco, composto por água DNAse free foi introduzido em cada lâmina para cada ensaio. As lâminas foram seladas e levadas ao sistema de PCR em tempo real QuantStudio 12K Flex (Life Technologies).

A leitura da fluorescência foi realizada em "end point", ou seja, após o final de todos os ciclos de amplificação. A análise dos dados obtidos foi realizada utilizando o software de análises AppliedBiosystems®, através do módulo de análises de genotipagem, versão 3.2, recomendado pelo fabricante do ensaio e equipamentos. Este software é gratuito e online através do Thermo Fisher Cloud (https://apps.thermofisher.com). Importante: a genotipagem foi realizada de modo cego para o fenótipo do paciente, ou seja, as amostras foram codificadas de modo a impedir o conhecimento sobre o grupo ao qual pertencem.

O modo de análise utilizado foi o "*Auto Calling*", em que o próprio software realiza as chamadas dos genótipos de acordo com a intensidade da leitura dos fluoróforos. As amostras foram classificadas como homozigotas para os genótipos 1 e 2 ou heterozigotas. Os resultados foram considerados válidos quando a genotipagem apresentava nível de qualidade (*call rate*) determinada pelo software de 95% ou superior. A Figura A3 apresenta, como exemplo, os gráficos de clusterização dos genótipos de dois SNPs. Pode-se observar que o *call rate* de ambas foi de mais de 95%, o que também ocorreu para a maioria dos ensaios TaqMan. Isso significa que a metodologia produziu resultados confiáveis e que foi possível genotipar quase a totalidade dos pacientes para cada SNP. Eventuais amostras que apresentaram genótipo inconclusivo, não foram consideradas na análise.

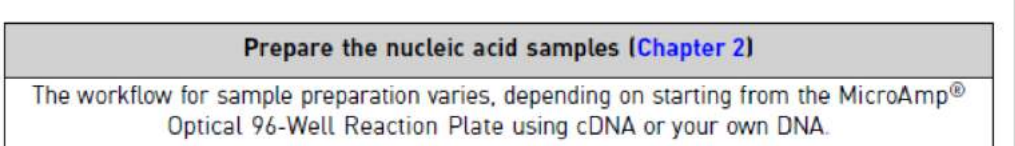
Figura A3 - Gráficos de clusterização de dois SNPs investigados pelo sistema OpenArray



Fonte: Elaboração própria.

Resumidamente, a metodologia consistiu nos procedimentos apresentados no Workflow apresentado na Figura A4 .

Figura A4 - Workflow dos procedimentos que foram realizados para genotipagem das amostras utilizando o sistema OpenArray



Fonte: Applied Biosystems QuantStudio™ 12K Flex Real-Time PCR System: OpenArray® Experiments User Guide, Publication Part Number 4470935 Rev. C

7 Análise estatística dos dados

Os dados demográficos foram expressos como a média ± desvio padrão (DP) para dados numéricos e como frequência para dados categóricos. Diferenças nas frequências e médias de fatores demográficos foram testadas usando a análise de qui-quadrado para variáveis categóricas (gênero e tabagismo) ou t de Student para variáveis contínuas (idade e parâmetros

periodontais). A fórmula de equilíbrio de Hardy-Weinberg (HWE) foi aplicada às frequências de genótipos entre os pacientes do grupo controle para testar qualquer desvio do equilíbrio esperado do genótipo, usando o teste qui-quadrado.

No Capítulo 2, o teste de Kruskal-Wallis foi utilizado para avaliar a diferenças entre os parâmetros periodontais (número de dentes, índice de placa visível, sangramento marginal, sangramento à sondagem, profundidade de sondagem interproximal e perda de inserção clínica interproximal) em cada genótipo para cada SNP de forma independente, para avaliar se existia diferença entre os genótipos. No Capítulo 3, foi utilizada uma regressão linear múltipla para avaliar a relação dos parâmetros periodontais (BOP, PPDi e CALi) com cada SNP de forma independente. Os modelos foram ajustados para idade, sexo e status de tabagismo, que foram utilizados como variáveis independentes.

No Capítulo 2 foi realizada a comparação entre pacientes controle e pacientes com PC (somando-se pacientes com periodontite moderada e severa). Foram utilizadas três variáveis binárias independentes diferentes no Capítulo 3 (Grupo controle vs. PC moderada, Grupo controle vs. PC severa, Grupo controle vs. PC moderada + severa) para determinar a associação entre cada SNP e o diagnóstico de PC. Foram realizadas todas as análises de associação caso-controle, que incluíram associações alélicas e genotípicas. Os genótipos foram categorizados em três grupos (alelo mais frequente homozigoto - 11, heterozigotos - 12 e alelo variante homozigota - 22). As análises de Qui-quadrado foram realizadas em quatro modelos: i) alelo 1 *versus* 2; ii) genótipo 11 *versus* 12 *versus* 22 (modelo aditivo); iii) genótipo 11 + 12 *versus* 22 (modelo recessivo); e iv) genótipo 11 *versus* 12 + 22 (modelo dominante). A associação entre cada SNP com PC foi estimada obtendo-se odds ratio (OR) e intervalos de confiança de 95% (IC) através de regressão logística múltipla ajustada para idade, sexo e tabagismo.

No Capítulo 3, os polimorfismos dos genes foram avaliados, através do modelo de regressão logística, separadamente pelo sexo dos pacientes para investigar a suscetibilidade à PC gênero-especifica, ajustada para idade e tabagismo.

No Capítulo 2, examinamos as interações multiplicativas e aditivas entre os SNPs e tabagismo com relação ao risco de PC. Análise de interação gene-tabagismo foi avaliada através de regressão logística mais do que multiplicativa (por exemplo, Caso / Controle * covariáveis + SNP1 + tabagismo + SNP1 × tabagismo). Para a interação aditiva, foram calculadas três medidas e seus IC de 95%: 1) *Relative Excess Risk Due to Interaction* (RERI); 2) *Attributable Proportion due to interaction* (AP); e 3) *Synergy index* (S). RERI é o excesso de risco devido a uma interação relativa para o risco sem exposição. AP refere-se à proporção atribuível da

doença entre indivíduos expostos a ambos os fatores, devido à interação dos fatores. S é o risco excessivo de ambas as exposições quando existe uma interação aditiva, em relação ao risco de ambas as exposições sem interação [17]. RERI = 0, AP = 0 e S = 1 indicam nenhuma interação, enquanto RERI> 0, AP> 0 ou S> 1 indicam interação sinérgica ou mais do que aditiva; e RERI <0, AP <0 ou S <1 significa interação negativa ou menor que a aditiva [18]. Se IC de 95% de qualquer uma dessas medidas não incluía os valores nulos (0 para RERI / AP ou 1 para S), a interação aditiva foi considerada estatisticamente significante [17].

A significância estatística (p-valor) foi ajustada para 0,0125 (0,05 / 4) no Capítulo 2 e 0,007 (0,05 / 7) no Capítulo 3, de acordo com a correção de Bonferroni com base no número total de polimorfismos estudas nos artigos 2 e 3 (n = 4 e n = 7, respectivamente), semelhante a outros estudos [19-21].

O software PLINK [22] foi utilizado para detectar diferenças nas distribuições de alelos e genótipos de cada SNP entre os grupos. Todas as outras análises foram realizadas pelo software STATA, versão 12.0 para Mac (Statistics / Data Analysis, Stata Corporation, College Station, Tex., EUA).

REFERÊNCIAS DO APÊNDICE A

1 Moher D, Liberati A, Tetzlaff J, Altman DG, PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. BMJ. 2009; 339: b2535.

2 Stroup DF, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. JAMA. 2000; 283(15):2008-12.

3 Nibali L, Tatarakis N, Needleman I, Tu YK, D'Aiuto F, Rizzo M, et al. Clinical review: Association between metabolic syndrome and periodontitis: a systematic review and meta-analysis. J Clin Mol Endocrinol. 2013; 98(3):913-20.

4 Wells G, Shea B, O'Connell D, Peterson J, Welch V, Losos M, et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. 2013. [Epub ahead of print].

5 Rugge B, Balshem H, Sehgal R, Relevo R, Gorman P,Helfand M. Screening and Treatment of Subclinical Hypothyroidism or Hyperthyroidism AHRQ Comparative Effectiveness Reviews. Rockville (MD): Agency for Healthcare Research and Quality (US); 2011 Oct. Report No.: 11(12)-EHC033-EF.

6 Altman DG, Bland JM. Standard deviations and standard errors. BMJ. 2005; 331(7521):903.

7 Wallace BC, Schmid CH, Lau J, Trikalinos TA. Meta-Analyst: software for meta-analysis of binary, continuous and diagnostic data. BMC Med Res Methodol. 2009; 9:80.

8 Patsopoulos NA, Evangelou E, Ioannidis JP. Sensitivity of between-study heterogeneity in meta-analysis: proposed metrics and empirical evaluation. Int J Epidemiol. 2008; 37(5):1148-57.

9 Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. Biometrics. 1994; 50(4):1088-101.

10 Egger M, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. BMJ. 1997; 315(7109):629-34.

11 Duval S, Tweedie R. Trim and fill: A simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. Biometrics. 2000; 56(2):455-63.

12 Page RC, Eke PI. Case definitions for use in population-based surveillance of periodontitis. J Periodontol. 2007; 78(7 Suppl):1387-99.

13 Divaris K, Monda KL, North KE, Olshan AF, Reynolds LM, Hsueh WC, et al. Exploring the genetic basis of chronic periodontitis: a genome-wide association study. Hum Mol Genet. 2013; 22(11):2312-24.

14 Teumer A, Holtfreter B, Volker U, Petersmann A, Nauck M, Biffar R, et al. Genome-wide association study of chronic periodontitis in a general German population. J Clin Periodontol. 2013; 40(11):977-85.

15 Faul F, Erdfelder E, Lang AG, Buchner A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007; 39(2):175-91.

16 Aidar M, Line SR. A simple and cost-effective protocol for DNA isolation from buccal epithelial cells. Braz Dent J. 2007; 18(2):148-52.

17 Andersson T, Alfredsson L, Kallberg H, Zdravkovic S,Ahlbom A. Calculating measures of biological interaction. Eur J Epidemiol. 2005; 20(7):575-9.

18 Knol MJ, VanderWeele TJ, Groenwold RH, Klungel OH, Rovers MM, Grobbee DE. Estimating measures of interaction on an additive scale for preventive exposures. Eur J Epidemiol. 2011; 26(6):433-8.

19 Nibali L, Griffiths GS, Donos N, Parkar M, D'Aiuto F, Tonetti MS, et al. Association between interleukin-6 promoter haplotypes and aggressive periodontitis. J Clin Periodontol. 2008; 35(3):193-8.

20 Wu L, Hu Y, Li D, Jiang W, Xu B. Screening toll-like receptor markers to predict latent tuberculosis infection and subsequent tuberculosis disease in a Chinese population. BMC Med Genet. 2015; 16:19.

21 Su SL, Yang HY, Lee HS, Huang GS, Lee CH, Liu WS, et al. Gene-gene interactions between TGF-beta/Smad3 signalling pathway polymorphisms affect susceptibility to knee osteoarthritis. BMJ Open. 2015; 5(6):e007931.

22 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 2007; 81(3):559-75.

ANEXO A – Autorização da editora (Capítulo 1)

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