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Dissertação de Mestrado

AVALIAÇÃO DA EXPRESSÃO DE PROTEÍNAS REGULADORAS DO METABOLISMO ÓSSEO NA PERIODONTITE CRÔNICA EM FUMANTES E NÃO FUMANTES

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Pelotas, 2014

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Dissertação apresentada ao Programa de Pós Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Mestre em Diagnóstico Bucal.

Orientador: Prof. Dr. Fabio Renato Manzolli Leite
Co-Orientadora: Profª. Drª. Adriana Etges

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ALMEIDA, Aline Ferreira. **Avaliação da expressão de proteínas reguladoras do metabolismo ósseo na periodontite crônica em fumantes e não fumantes**. 2014. XXf. Dissertação (Mestrado em Diagnóstico Bucal) – Programa de Pós Graduação em Odontologia, Faculdade de Odontologia, Universidade Federal de Pelotas.

A periodontite é uma doença crônica que induz resposta imune inata e adaptativa nos tecidos periodontais e pode ser modulada pelo consumo de cigarros. Para avaliar possíveis diferenças na resposta imune foi determinada a expressão dos domínios de oligomerização de ligação de nucleotídeos (Nod1 e Nod2), do ligante do receptor-ativador do fator nuclear kappa B (RANKL), de osteoprotegerina (OPG), de CD20 (marcador de células B), de CD68 (marcador de monócitos/macrófagos), de CD45RO (marcador para células T) e de metaloproteinases de matriz (MMP)-8 e -9 nos tecidos periodontais de fumantes e não fumantes. Os tecidos gengivais foram coletados de pacientes saudáveis (n = 10) e pacientes com periodontite crônica - fumantes (n = 15) e não fumantes (n = 16). As expressões de Nod1, Nod2, RANKL, OPG, CD20, CD68 e CD45RO foram determinadas por reação de imunohistoquímica. A atividade das MMP-2 e MMP-9 foi verificada por zimografia. Sítios com periodontite apresentaram níveis mais elevados de todas as proteínas estudadas, quando comparado aos saudáveis ($P < 0,001$). No entanto, não foram observadas diferenças entre fumantes e não-fumantes ($P > 0,05$). Todas as proteínas foram mais expressas na porção apical da bolsa periodontal em comparação com a porção coronária. Considerando a periodontite como uma doença infecciosa, a sua progressão induz a migração de linfócitos T e B e monócitos/macrófagos para o local inflamado, principalmente regiões mais apicais da bolsa perioodntal. Além disso, os receptores de reconhecimento e reguladores de remodelação óssea mostraram-se com expressão aumentada. Não foram observadas diferenças entre fumantes e não fumantes.

Palavras Chave: doença periodontal; RANKL; osteoprotegerina; metaloproteinase.

ALMEIDA, Aline Ferreira. **Evaluation of the expression of bone metabolism regulating proteins on chronic periodontitis in smoking and nonsmoking individuals.** Dissertation (MSc – Buccal diagnosis) – Graduate Program in Dentistry, Federal University of Pelotas.

Periodontitis is a chronic disease modulated by cigarette consumption inducing innate and adaptive immune responses within the periodontal tissues. To assess these immune responses, we evaluated the expression of nucleotide-binding oligomerization domains (Nod)-1 and -2, receptor activator of nuclear factor kappa-B ligand (*RANKL*), osteoprotegerin (OPG), CD20 (B-cells), CD68 (monocytes/macrophages), CD45RO (T-cells) and matrix metalloproteinases (MMP)-8 and -9 in periodontal tissues of smokers and nonsmokers in order to identify possible differences. Gingival tissues were obtained from periodontally health subjects (n=10), and patients with chronic periodontitis smokers (n=15) and nonsmokers (n=16). Nod1, Nod2, RANKL, OPG, CD20, CD68 and CD45RO expression was determined by immunohistochemistry (IHC). MMP-2 and -9 activity were verified by zymography. Periodontitis-affected patients presented higher levels of all studied proteins when compared with healthy sites ($P < 0.001$). However, no differences were observed between smokers and non-smokers ($P > 0.05$). All proteins were more expressed at the apical portion of periodontal pocket compared with the coronal portion. Considering periodontitis as an infectious disease, its progression induce the migration of B- and T-cells and monocytes/macrophages to inflamed site. In addition, pattern recognition receptors and bone turnover regulators are overexpressed. No differences were observed between smokers and nonsmokers.

Keywords: Periodontal Diseases; NOD; RANKL; Osteoprotegerin; Matrix metalloproteinases

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1. Introdução

As infecções bacterianas são detectadas pelo sistema imune inato através de receptores transmembrânicos e citosólicos (CHAMAILLARD et al., 2003). Sabe-se que o receptor do tipo Toll 4 (TLR-4) reconhece microrganismos gram-negativos e são capazes de desencadear uma cascata de sinalização intracelular resultando na secreção de mediadores, tais como a interleucina (IL-1, IL-6), fator de necrose tumoral alfa, entre outros (Garcia de Aquino et al., 2009). Mais recentemente, dois membros de uma família de proteínas chamados domínios de oligomerização de ligação de nucleotídeos (Nod1 e Nod2) foram encontrados como sítio de reconhecimento de subestruturas de peptidoglicanos bacterianos (Inohara et al., 2003 , Girardin et al., 2001).

Bactérias gram-negativas e algumas espécies gram–positivas são capazes de produzir o ácido mesodiaminopimélico (meso-DAP), responsável pela ativação da Nod-1 (Girardin et al., 2001). Por outro lado, todas as bactérias gram-negativas e positivas apresentam muramildipeptideo (MDP), que é reconhecido por Nod2 (Kim et al., 2008). Mutações no gene NOD2 causam respostas inflamatórias, tais como na doença de Crohn e síndrome de Blau, ambas doenças autoinflamatórias (Hugot et al., 2001). Em casos de reabsorção óssea, osteoblastos estimulados por MDP (agonista de Nod-2) aumentam sinergicamente a formação de osteoclastos induzidos por lipopolissacarídeos (LPS), interleucina 1 alfa (IL-1α), e fator de necrose tumoral alfa (TNF-α), através da expressão do ligante do receptor ativador do fator nuclear kappa B (RANKL) (Yang et al., 2005).

A destruição óssea vista em animais e humanos com periodontite é potencializada pelo hábito de fumar (Cesar- Neto et al., 2006). A nicotina combinada com lipopolissacarídeos (LPS) aumentou a liberação de prostaglandina E2 (PGE2), IL-1β e TNF-α em monócitos circulantes (Payne et al., 1996, Ryder, 2007) e IL-6 em fibroblastos e osteoblastos (Wendell e Stein, 2001). Ratos Wistar com periodontite induzida por exposição à fumaça do cigarro apresentaram níveis mais elevados de perda de massa óssea, MMP-2, IL-6 e interferon gama (IFN- γ) (Cesar-Neto et al., 2006).

Pacientes tabagistas com periodontite tenderam a apresentar níveis mais baixos na corrente sanguínea do ativador do receptor do fator nuclear kappa-B (RANK) e osteoprotegerina (OPG), em comparação com pacientes com periodontite não-fumantes (Lappin et al., 2007). Além disso, a razão RANKL:OPG foi encontrada maior nos pacientes fumantes, possivelmente explicando a maior perda de inserção observada em fumantes.

Assim, este estudo avaliou as diferenças de detecção por meio de imunohistoquímica de alguns tipos celulares, Nod1, Nod2, RANKL e OPG em bolsas periodontais de pacientes fumantes e não fumantes, ambos os grupos com periodontite.

2. Projeto de pesquisa

UNIVERSIDADE FEDERAL DE PELOTAS
FACULDADE DE ODONTOLOGIA

Projeto de Pesquisa

Avaliação da expressão de proteínas reguladoras do metabolismo ósseo na periodontite crônica em fumantes e não fumantes

Equipe de Trabalho

Prof. Dr. Fábio Renato Manzolli Leite
Profª. Drª Adriana Etges

AVALIAÇÃO DA EXPRESSÃO DE PROTEÍNAS REGULADORAS DO METABOLISMO ÓSSEO NA PERIODONTITE CRÔNICA EM FUMANTES E NÃO FUMANTES

Projeto de dissertação apresentado ao Programa de Pós-Graduação em Odontologia da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Mestre em Odontologia, área de concentração Diagnóstico Bucal.

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2012

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Profª. Drª. Ana Paula Neutzling Gomes

Resumo

ALMEIDA, Aline Ferreira de. **Avaliação da expressão de proteínas reguladoras do metabolismo ósseo na periodontite crônica em fumantes e não fumantes.** 2012. 33f. Projeto de Monografia – Qualificação (Mestrado). Programa de Pós-Graduação em Odontologia, Universidade Federal de Pelotas, Pelotas.

O objetivo deste estudo é avaliar a expressão e distribuição tecidual em humanos dos reguladores do metabolismo ósseo de reabsorção/aposição tecidual em pacientes com doença periodontal crônica bem como a influência do tabagismo na expressão e distribuição tecidual em humanos dos reguladores do metabolismo ósseo em pacientes com doença periodontal crônica. Para isso, serão incluídos no estudo 45 pacientes sendo 15 fumantes, 15 não fumantes e 15 pacientes periodontalmente saudáveis (controle). Os voluntários deverão apresentar periodontite crônica, bolsas periodontais >5 mm e pelo menos um dente com necessidade de acesso cirúrgico para raspagem ou indicado para extração por motivo periodontal. Os fumantes deverão consumir ao menos 10 cigarros/dia por mais de cinco anos e os não fumantes nunca tabagistas. As peças cirúrgicas serão fixadas em formol para posterior realização de reação imunohistoquímica onde serão utilizados os anticorpos RANKL, OPG, Nod1 e Nod2 pelo método da estreptavidina-biotina. Será realizada a contagem de células inflamatórias em todo o tecido conjuntivo fibroso da amostra. A contagem de células será executada através da observação em microscópio de luz por um único examinador experiente. Médias representativas de cada grupo serão obtidas, para cada anticorpo e para cada terço das amostras Em seguida um teste de normalidade será aplicado.

Palavras-chave: periodontite, tecido ósseo, inflamação, NOD

Abstract

ALMEIDA, Aline Ferreira de. **Evaluation of the expression of bone metabolism regulating proteins on chronic periodontitis in smoking and nonsmoking individuals.** 2012. 33f. Monograph Project - Qualification (Masters). Postgraduate Program in Dentistry, Federal University of Pelotas, Pelotas.

The aim of this study is to evaluate the expression and tissue distribution in humans of the bone tissue metabolism (resorption/apposition) regulators in patients with periodontal disease as well the influence of smoking in the expression and tissue distribution of these regulators. In this study 45 patients will be included, i.e., 15 nonsmokers, 15 smokers and 15 periodontally healthy (control). The volunteers must have chronic periodontitis, periodontal pockets >5 mm and at least one tooth in need of surgical access for scaling or indicated for extraction due to periodontal disease. The smokers should consume at least 10 cigarrettes/day for more than five years and the nonsmokers should never have been smokers. Surgical pieces will be fixed in formaldehyde for later immunohystochemical reaction where RANKL, OPG, Nod1, and Nod2 antibodies will be employed in the streptavidin-biotin technique. Inflammatory cell counting will be performed throughout the sample of connective tissue. It will be performed by observation under light microscope by the same experienced examiner. Representative averages of each group will be obtained for each antibody, and for each third part of the samples. Following, a normality testing will be performed.

Keywords: periodontitis, bone tissue, inflammation, NOD

Lista de Abreviaturas e Siglas

ERK	*extracellular-regulated kinases*
IFN	*Interferon*
IKK	*Inhibitor of the nuclear factor kappa B*
IL	*Interleukin*
INF	*Interferon*
IRAK	*IL-1 receptor-associated kinase*
JNK	*c-Jun N-terminal kinase*
LPS	*Lipopolisacharide*
LRR	*leucine-rich repeats domain*
MMP	*Metaloproteinase da matriz*
MAPK	*mitogen-activated protein kinases*
M-CSF	*macrophage colony-stimulating factor*
MDP	*muramil-dipeptide*
MyD88	*myeloid differentiation factor 88*
NFkB	*nuclear factor kappa B*
NOD	*Nucleotide-binding Oligomerization Domain*
OPG	*Osteoprotegerin*
PG	*Prostaglandina*
RANK	*receptor activator of nuclear factor-kappaB*
RANKL	*receptor activator of nuclear factor-kappaB ligand*
RIP	*receptor interacting protein*
RNAm	RNA mensageiro
TAK	*TGF-activated kinase*
TIRAP	*TIR domain-containing adaptor protein*
TLR	*Toll-like receptor*
TNF	*Tumor necrosis factor*
TRAM	*TIR domain-containing adaptor-inducing IFN-related adaptor molecule*
TRIF	*TIR domain-containing adaptor-inducing IFN*

1. Introdução Geral

As doenças periodontais destrutivas são infecções nas quais microrganismos Gram-negativos tem um papel fundamental. Um dos principais fatores de virulência destes microrganismos é a endotoxina (LPS), um componente lipossacáride da parede microbiana que é capaz de induzir uma resposta imune/inflamatória (Slots & Genco, 1984; Moore & Moore, 1994). As consequências do estímulo por LPS incluem destruição de tecido conjuntivo e reabsorção óssea, demonstrados em modelos animais de doença periodontal (Ekuni et al., 2003; Garlet et al., 2003; Rossa et al., 2005). Recentemente, uma classe de receptores de membrana foi identificada, denominada Toll-like receptors (TLR) (Wang & Ohura, 2002). TLRs são proteínas transmembrânicas com porções ricas em leucina nos domínios extracelulares, enquanto a porção intracelular é similar à do receptor da interleucina-1, e por isso denominado domínio Toll/IL-1R (TIR). Atualmente, os TLRs são considerados os efetivos receptores de LPS, apesar de alguma disputa em relação ao tipo, TLR-2 ou TLR-4, mais relevante (Kirschning et al., 1998; Poltorak et al., 1998; Chow et al., 1999; Qureshi et al., 1999). Fibroblastos do tecido gengival (GF) (Tabeta et al., 2000) quanto do ligamento periodontal (PDL) (Hatakeyama et al., 2003) expressam TLR2 e TLR4 constitutivamente. Assim, tanto fibroblastos quanto osteoblastos (Kikuchi et al., 2001) são equipados com os receptores necessários para responder ao estímulo por LPS bacteriano causando destruição de colágeno e tecido ósseo.

No balanço entre formação/reabsorção óssea, RANKL foi descoberto em uma busca de um ligante para uma molécula denominada osteoprotegerina (OPG), a qual é capaz de inibir a reabsorção óssea (Aubin & Bonnelye, 2000). RANKL pode ser expresso em duas formas: um peptídeo ancorado na membrana celular, e uma

forma secretada resultante de modificação pós-translacional (Hofbauer & Heufelder, 2001). Segundo o entendimento atual do sistema de citocinas controlando a osteoclastogênese, a ligação de RANKL ao RANK, seu receptor expresso na membrana de células da linhagem hematopoiética precursoras inicia uma cascata de sinalização intracelular e expressão gênica que levará à diferenciação, maturação e/ou ativação de osteoclastos. A osteoprotegerina (OPG) funciona como um receptor falso para RANKL, impedindo sua interação com RANK e, consequentemente, inibindo a osteoclastogênese e reabsorção óssea (figura 1). O papel fundamental e imprescindível do RANKL na osteoclastogênese é demonstrado pelo fato de que, na presença de níveis permissivos de fator estimulador de colônias de macrófagos (M-CSF), RANKL é ao mesmo tempo necessário e suficiente para a formação de osteoclastos (Aubin & Bonnelye, 2000).

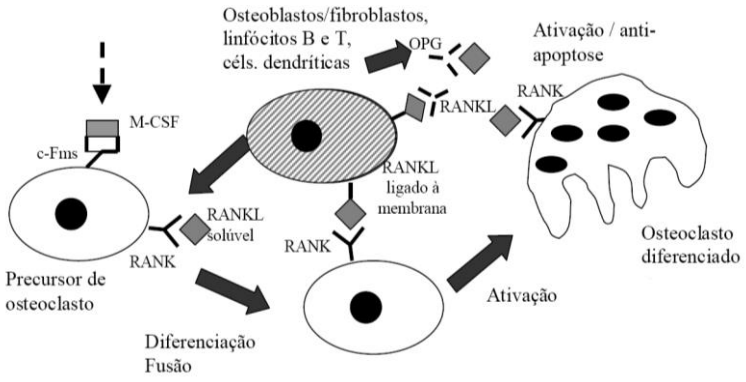

Figura 1 – Modelo de regulação da biologia celular de osteoclastos pelo ligante do receptor ativador do fator nuclear kappa-B (RANKL), receptor ativador do fator nuclear kappa-B (RANK) e osteoprotegerina (OPG). (Adaptado de Hofbauer & Heufelder, 2001 e Khosla, 2001).

A expressão da proteína RANKL é restrita às linhagens de osteoblastos e osteoclastos, células dendríticas, linfócitos e fibroblastos (Hsu et al., 1999). Fibroblastos periodontais também expressam RANKL (Kansaki et al., 2001),

especialmente na presença de outros mediadores inflamatórios (Nagasawa et al., 2002). A expressão destes outros mediadores como IL-1, IL-6 e IL-8 pode ser induzida por bactérias nos tipos celulares considerados (Takada et al., 1991; Tamura et al., 1992; Yamaji et al., 1995; Kent et al., 1999), podendo representar um mecanismo indireto pelo qual o estímulo com LPS induz à expressão de RANKL.

A combinação de nicotina com lipopolissacarídeo (LPS) bacteriano aumentou a secreção de PGE2 pelos monócitos do sangue periférico sugerindo que a nicotina e o LPS bacteriano exercem um efeito sinérgico na produção de mediadores inflamatórios (PAYNE et al., 1996; WENDELL et al., 2001). A nicotina também aumentou a produção de IL-6 em culturas de fibroblastos (WENDELL et al., 2001) e osteoblastos (EL-GHORAB et al., 1997). Uma maior produção de IL-1β foi observada em células mononucleares de fumantes (RYDER et al., 2002) e quando estas células foram expostas à fumaça de cigarro elas aumentavam a produção de TNF-α (RYDER et al., 2002). Em outro estudo investigando a influência da fumaça de cigarro sobre células mononucleares observou-se um aumento na expressão de 20 genes previamente associados com a patogênese das doenças periodontais (RYDER et al., 2004). Histologicamente observou-se que a inalação da fumaça de cigarro potencializa a perda óssea na doença periodontal induzida por ligaduras em ratos. Ao avaliar o tecido gengival adjacente às regiões com doença os autores deste estudo observaram maiores níveis de MMP-2 nos animais que inalaram fumaça (CÉSAR-NETO et al., 2004). Dois estudos recentes avaliaram a expressão de alguns genes em biópsias gengivais de pacientes com saúde periodontal, não fumantes com periodontite e fumantes com periodontite (CÉSAR-NETO et al., 2006; 2007). Observou-se que IL-1β, IL-8, IL-10, TNF-α, MMP-8 e OPG estavam mais baixas nos fumantes, quando comparados aos não fumantes com níveis

semelhantes de periodontite. Por outro lado, IL-6, INF-γ e IL-1ra estavam mais altos. Ao avaliar a proporção de algumas moléculas com seus respectivos antagonistas constatou-se uma maior razão de IL-6:IL-10 nos sítios com periodontite de fumantes (comparados aos de não fumantes). Já para a razão IL1β:IL-1ra o grupo de fumantes apresentou resultados semelhantes ao grupo de pacientes com saúde periodontal. LAPPIN et al. (2007) avaliou as concentrações séricas de RANKL e OPG em fumantes e não fumantes com níveis similares de doença periodontal. Seus resultados foram semelhantes aos de CÉSAR-NETO et al. (2007) mostrando menores valores de OPG e RANKL: OPG nos fumantes. Porém, alguns estudos contestam a tendência de que o fumo aumenta a expressão de genes relacionados à inflamação (MOROZUMI et al., 2004, OUYANG et al., 2000, PETROPOULOS et al., 2004). A maioria dos estudos de patogênese da doença periodontal em fumantes avalia fluido crevicular, cultura de células ou tecidos processados o que impede a observação das diversas áreas da lesão (em toda profundidade da bolsa).

No entanto, as vias de sinalização intracelular envolvidas após o estímulo por LPS são complexas e apenas parcialmente compreendidas. Apesar da similaridade entre os domínios intracelulares dos TLRs e do receptor da IL-1, evidenciada pela ativação de algumas quinases protéicas *upstream* em comum, por exemplo IRAK e TRAF6, assim como a ativação *downstream* dos mesmos fatores de transcrição (como NF-κB), existe uma grande complexidade nas proteínas adaptadoras envolvidas na sinalização intracelular de TLRs. A diferença nas respostas celulares frente a um mesmo estímulo externo podem ser resultantes da ativação de vias celulares alternativas em tipos celulares distintos, incluindo a ativação das proteínas adaptadoras e a participação de possíveis modificadores da transdução de sinais, como as proteínas Nod.

As proteínas Nod, ou Nucleotide-binding Oligomerization Domain, representam uma família de proteínas citoplasmáticas recentemente identificadas caracterizadas pela presença de repetições múltiplas de *motifs* ricos em leucina (*LRR* ou *leucine-rich repeats domain*) no domínio C-terminal, um domain central capaz de interação/ligação com nucleotídeos e um domínio de interação proteína-proteína (*CARD*) na extremidade N-terminal (Chamaillard et al., 2003; Murray, 2005). Duas destas proteínas, denominadas Nod1 e Nod2 vêm sendo mais intensamente estudadas (Ogura et al., 2001; Iwanaga et al., 2003).

Ainda que a função destas proteínas não seja completamente conhecida, sua similaridade estrutural com as proteínas R de plantas sugeriu um papel relacionado ao reconhecimento de antígenos e patógenos intracelulares. As proteínas Nod efetivamente reconhecem antígenos derivados da parede celular microbiana (Inohara et al., 2001), e além disso, mutações no gene da Nod2 em pacientes com doença de Crohn, uma doença inflamatória intestinal associada à alteração na resposta imune aos microrganismos do trato intestinal (Ogura et al., 2001), levou a proposição das proteínas Nod como análogas citoplasmáticas dos TLRs.

No entanto, existe também a hipótese de que Nod1 e Nod2 atuem como modificadores da transdução de sinais iniciada por antígenos microbianos, amplificando e modulando a resposta celular na expressão de citocinas ou indução de apoptose. Esta modulação ocorreria especialmente na presença dos supostos ligantes específicos de Nod1 e Nod2, representados respectivamente por *motifs* de peptideoglicanos derivados de microrganismos Gram-positivos (meso-DAP, ou meso-diaminopimelate) e Gram-negativos (MDP, ou muramil-dipeptídeo) (Marriott et al., 2005).

A interação entre as vias de sinalização induzidas após ativação de TLRs e o papel das proteínas Nod na ativação sinérgica da resposta celular permanecem desconhecidos. Os modelos de sinalização associados a TLR2 e TLR4 estão apresentados resumidamente na figura 2, contudo existem indicações de que diversas vias podem estar implicadas após a ligação de antígenos microbianos a estes receptores. As vias de sinalização envolvidas podem incluir o recrutamento de diferentes proteínas adaptadoras e/ou ativação de diversas cascatas de kinases protéicas ativadas por mitógenos (MAPK) como ERK-1 e -2 (Wright et al., 1992), JNK (kinase protéica c-Jun N-terminal) e p38 (Schumann et al., 1996).

A sinalização intracelular induzida por LPS via TLRs apresenta dois possíveis pontos de bifurcação: o primeiro é *upstream*, próximo ao domínio citoplasmático dos receptores, representado pela proteína adaptadora MyD88. A via MyD88-dependente ativa IRAK, TRAF6 e finalmente NF-κB, essencial na indução da expressão de citocinas. Já a via MyD88-independente não ativa IRAK, mas leva à ativação tardia de NF-κB. Esta via alternativa requer proteínas adaptadoras diferentes, como TIRAP, TRIF e TRAM e se supõe que não seja relevante para a expressão de citocinas. Na verdade, sugere-se que esteja relacionada à secreção de interferon beta e ativação indireta de outros genes IFN-dependentes (Muzio et al., 1998; Swantek et al., 2000, Netea et al., 2004). O segundo ponto de bifurcação é representado pela MAPkinase-kinase-kinase (MAPKKK), também conhecida como TAK-1, e está localizado *downstream* ao primeiro. Pode levar à ativação tanto de AP-1 como de NF-κB, por meio da via do inibidor do fator nuclear kappa B (IKK). Não se sabe a razão da ativação de diversas vias de sinalização pelo mesmo receptor: se é um mecanismo redundante/compensatório, ou uma forma de especificar a resposta em termos de modulação da resposta biológica de diferentes

tipos celulares pelo balanço na produção de citocinas pro e antinflamatórias (Wang & Ohura, 2002; Netea et al., 2004).

A interação e participação das proteínas Nod na sinalização intracelular iniciada por agonistas de TLRs pode resultar em ativação sinérgica de NF-κB (Ogura et al., 2001; Marriott et al., 2005). Esta ativação pode aumentar a expressão de genes regulados por NF-κB, acentuando a resposta inflamatória. A ativação de NF-κB é relevante para diversas condições inflamatórias crônicas, como aterosclerose, artrite reumatóide e as doenças periodontais (Nichols et al., 2001). Tanto Nod1 como Nod2 ativam uma molécula sinalizadora *downstream* comum, Rip2 (também conhecida como RICK ou CARDIAK), a qual leva à ativação de NF-κB pela interação com a subunidade reguladora do IKK (IKKϒ ou NEMO), resultando na fosforilação de IκB-α, subsequente degradação do proteossomo e liberação do NF-κB que é translocado ao núcleo para ativação da transcrição (Inohara et al., 2000). Além disso, outras vias de sinalização podem ser ativadas pelas proteínas Nod, como JNK (Girardin et al., 2001).

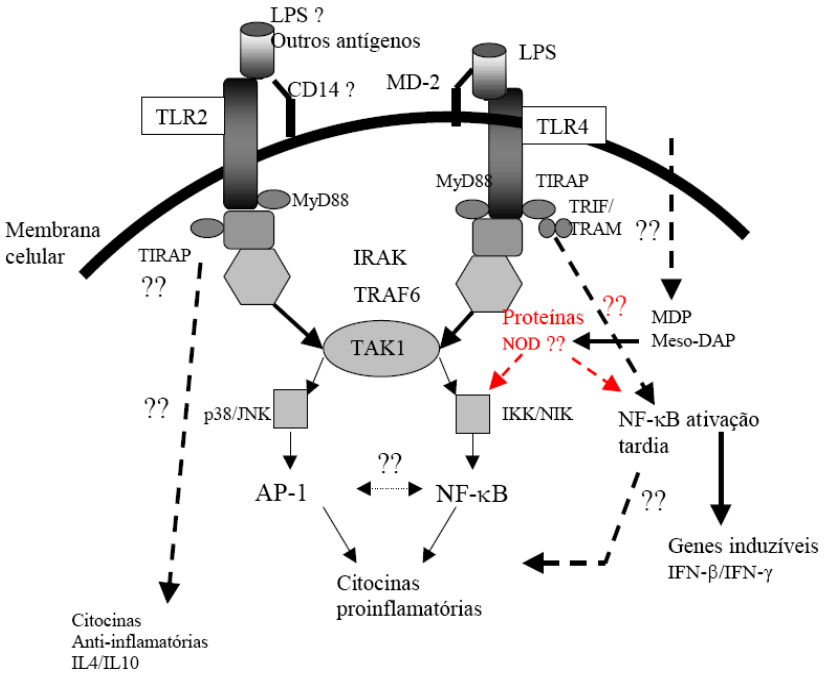

Figura 2 – Vias de sinalização intracelular envolvidas após a estimulação por LPS e outros antígenos bacterianos com possível atuação de proteínas Nod (Adaptado de Netea et al., 2004).

O papel do LPS na osteoclastogênese é complexo devido à evidências recentes indicarem que seu efeito modulador pode ser positivo ou negativo. Assim, LPS pode aumentar a reabsorção óssea pelo favorecimento da osteoclastogênese, por exemplo, por meio da indução de RANKL, contudo em células precursoras de osteoclastos o LPS pode inibir a atividade do RANKL pela redução da expressão tanto de RANK quanto do receptor para o M-CSF (Zou & Bar-Shavit, 2002). É interessante notar que o uso de LPS como único agonista em fibroblastos do tecido conjuntivo gengival não induziu a expressão de mRNA para RANKL. Pelo contrário, o estímulo com LPS nestas células induziu a expressão e secreção de OPG, como evidenciado pela inibição da diferenciação de osteoclastos, quando os precursores de osteoclastos foram cultivados na presença do sobrenadante obtido de culturas de fibroblastos estimulados por LPS (Nagasawa et al., 2002).

Estes fatos ressaltam a importância de avaliar a expressão de RANKL e OPG induzido por bactérias em tecidos gengivais humanos acometidos por doença periodontal crônica nunca antes avaliado *in situ*. Uma melhor compreensão das vias de sinalização ativadas por LPS, incluindo o papel das proteínas Nod na amplificação e/ou ativação sinérgica da expressão gênica, e suas conexões com a expressão das moléculas relacionadas ao remodelamento do tecido ósseo (RANKL, OPG) pode proporcionar alvos potenciais para o desenvolvimento de novos medicamentos ou terapias com objetivo de inibir/reduzir a reabsorção óssea. Uma destas estratégias pode ser baseada no uso de inibidores bioquímicos comercializados (gerando patentes) para o bloqueio ou inibição de vias de sinalização ativadas pela interação das bactérias aos seus potenciais receptores. Além disso, não há relatos dos mecanismos moleculares afetados pelo uso do tabaco em humanos que justifiquem a redução no sangramento gengival, porém maior produção de secreção purulenta.

Objetivos

1.1 Objetivo geral

O objetivo deste estudo será avaliar e comparar a expressão de proteínas reguladoras do metabolismo ósseo no processo inflamatório em sítios com periodontite crônica (bolsas >5 mm) em indivíduos fumantes e não fumantes, através da técnica de imunohistoquímica.

1.2 Objetivos específicos

- Avaliar a expressão e distribuição tecidual em humanos dos reguladores do metabolismo ósseo de reabsorção/aposição tecidual (RANKL e seu antagonista endógeno OPG) em pacientes com doença periodontal crônica.
- Avaliar a influência do tabagismo na expressão e distribuição tecidual em humanos dos reguladores do metabolismo ósseo (RANKL e seu antagonista endógeno OPG) em pacientes com doença periodontal crônica.
- Estudar a expressão das proteínas Nod1 e Nod2, possíveis transdutoras de sinais intracelulares na reabsorção óssea e reconhecedoras de patógenos, e sua distribuição nos tecidos periodontais de pacientes com periodontite.
- Avaliar a possível influência do tabagismo na expressão das proteínas Nod1 e Nod2 nos tecidos periodontais.

3. Materiais e Métodos

Será realizada análise transversal de pacientes fumantes e não fumantes acometidos por periodontite crônica segundo os critérios da Academia Americana de Periodontologia (1999). Todos os voluntários serão submetidos à anamnese e exame periodontal completo. Após estes procedimentos os seguintes critérios de inclusão e exclusão serão levados em consideração para a seleção da amostra.

3.1 Caracterização da amostra

a. Critérios de Inclusão:

Serão incluídos no estudo 45 pacientes sendo 15 fumantes, 15 não fumantes e 15 pacientes periodontalmente saudáveis (controle). De acordo com os critérios de inclusão estabelecidos participarão do estudo, voluntários dentados ou parcialmente edêntulos (com pelo menos 14 dentes naturais) cujo consumo de cigarros seja o único fator de modificação da resposta periodontal frente ao biofilme bacteriano (critério válido para o grupo de fumantes). Os voluntários deverão apresentar periodontite crônica, bolsas periodontais >5 mm e pelo menos um dente com necessidade de acesso cirúrgico para raspagem ou indicado para extração por motivo periodontal. Os fumantes deverão consumir ao menos 10 cigarros/dia por mais de cinco anos e os não fumantes nunca tabagistas.

b. Critérios de Exclusão:

Serão excluídos indivíduos que apresentem sinais de: alcoolismo, imunossupressão adquirida ou induzida, estresse físico/emocional, medicamentos que influenciem os tecidos periodontais, mulheres grávidas ou lactantes, indivíduos que tenham utilizado antibiótico, antinflamatório ou hormônio (anticoncepcional e terapia de reposição hormonal), e/ou que receberam tratamento periodontal nos últimos seis meses anteriores a cirurgia. Tais critérios serão utilizados para evitar a presença de fatores de confundimento que poderiam interferir nos resultados. Todos esses voluntários concordarão em participar do estudo e assinarão um termo de consentimento livre e esclarecido que foi aprovado pelo comitê de ética em pesquisa da Faculdade de Odontologia da UFPel, sob parecer nº. 038/2008.

3.2 Coleta das amostras

Os voluntários selecionados para o estudo serão examinados clinicamente. O exame periodontal incluirá: profundidade de sondagem, índice de placa, índice de sangramento gengival e nível gengival. A coleta do tecido será em uma das faces proximais do dente com necessidade de acesso cirúrgico para raspagem ou indicado para extração por motivo periodontal, a qual deverá apresentar uma bolsa periodontal maior que 5 mm. Durante a cirurgia, o “prisma” de tecido abaixo do ponto de contato será delimitado pelas incisões intrasulculares vestibular e lingual/palatina. Uma incisão na base desse prisma finalizará a remoção do tecido para a análise. O prisma de tecido gengival delimitado por essas incisões, pelo tecido ósseo subjacente e pela parede dental, será removido e utilizado no estudo.

3.3 Processamento Histológico

Imediatamente após sua coleta, o material removido cirurgicamente será fixado em formalina a 10%, tamponado pH 7,4 em temperatura ambiente. Os espécimes será encaminhado ao Laboratório de Histopatologia da FO/UFPel, hemiseccionados e emblocados em parafina. Serão obtidos dois cortes por amostra, na espessura de 3µm, selecionados para a descrição morfológica e corados com hematoxilina e eosina (H & E).

3.4 Imunohistoquímica

De cada bloco de parafina serão obtidos cinco cortes de 3µm estendidos em lâminas de vidro previamente silanizadas. A expressão dos anticorpos RANKL, OPG, Nod1 e Nod2 será avaliada através da técnica de imunohistoquímica, pelo método da estreptavidina-biotina. Os cortes serão desparafinados em dois banhos de xilol, reidratados em séries descendentes de etanóis e imersos em solução de hidróxido de amônio a 10% durante 10 minutos, para retirada de pigmentos. Após lavagem, as lâminas receberão tratamentos de recuperação antigênica para restabelecimento dos sítios antigênicos e rompimento de ligações cruzadas.

Os cortes serão novamente lavados em água corrente seguidos de duas passagens em água destilada e do bloqueio da peroxidase endógena tecidual em solução de peróxido de hidrogênio em metanol (1:1, v/v). Após o bloqueio, os cortes serão lavados em água seguido de dois banhos em solução tampão de TRIS-HCl. Os cortes serão incubados com anticorpo primário de acordo com o fabricante e

lavados em solução tampão de TRIS-HCL. Seguir-se-á a incubação com soro terciário e complexo terciário (ambos por 30 minutos) - Kit LSAB (Dako Corporation, California). Lavagem duas vezes em água destilada e em solução tampão TRIS. Em seguida, serão revelados através da utilização do cromógeno diaminobenzina por 1 minuto. As lâminas serão contracoradas com Hematoxilina de Mayer por 8 minutos e em seguida lavadas.

3.5 Contagem de células

Será realizada a contagem de células com marcação positiva em toda amostra em aumento de 400X (high-power field) (Liu et al., 2012). A contagem de células será executada através da observação em microscópio de luz por um único examinador treinado para o estudo. Será tentado também o teste de dupla marcação para identificar um possível aumento ou redução na produção de Nod1 e Nod2 com produção de RANKL/OPG.

3.6 Análise estatística

Médias representativas de cada grupo serão obtidas, para cada anticorpo e para cada terço das amostras. Será comparada a distribuição das diferentes proteínas entre grupos (controle sem doença gengival, fumante e não fumante com periodontite) e entre os terços apical, médio e cervical do tecido. Em seguida um teste de normalidade será aplicado.

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5. Orçamento

	Item	Descrição	R$
1	Material de escritório	Folhas, gastos com material impresso	200,00
2	Material para realização da imunohistoquimica	Anticorpos, soluções	10.000,00
			***Total: R$ 10.200,00**

***Os custos do projeto serão financiados pelo Auxílio Recém Doutor da Fundação de Amparo a Pesquisa do Estado do Estado do Rio Grande do Sul (FAPERGS) no. 111748-3.**

6. Aprovação no Comitê de Ética em Pesquisa

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE PELOTAS
FACULDADE DE ODONTOLOGIA
COMITÊ DE ÉTICA EM PESQUISA

PELOTAS, 22 de agosto de 2008.

PARECER Nº 038 /2008

O projeto de pesquisa intitulado: **"AVALIAÇÃO IMUNOHISTOQUÍMICA DA PROPORÇÃO DE CÉLULAS MONONUCLEARES E DA EXPRESSÃO DE MMP-2 E 9 NA PERIODONTITE CRÔNICA. COMPARAÇÃO ENTRE FUMANTES E NÃO-FUMANTES,"** está constituído de forma adequada, cumprindo, nas suas plenitudes preceitos éticos estabelecidos por este Comitê e pela legislação vigente, recebendo, portanto, **PARECER FAVORÁVEL** à sua execução.

Prof. Marcos Antonio Torriani
Coordenador do CEP/FO/UFPel

Prof. Marcos A. Torriani
Coordenador
Comitê de Ética e Pesquisa

7. Cronograma

Atividade	Mês (2012/ 2013)																					
	Mar	Abr	Mai	Jun	Jul	Ago	Set	Out	Nov	Dez	Jan	Fev	Mar	Abr	Mai	Jun	Jul	Ago	Set	Out	nov	Dez
Levantamento Bibliográfico																						
Aquisição de materiais																						
Levantamento de casos																						
Realização das reações de Imunohistoquimica																						
Análise e contagem das células																						
Tabulação e Análise de dados																						
Finalização do Artigo e defesa Monografia																						

3. Relatório do trabalho de campo

O presente relatório é parte do estudo realizado em Pelotas, RS com o título “Avaliação da expressão de proteínas reguladoras do metabolismo ósseo na periodontite crônica em fumantes e não-fumantes”, ocorrido de agosto de 2010 à fevereiro de 2014 no Laboratório de Histopatologia da Faculdade de Odontologia da Universidade Federal de Pelotas.

A aquisição dos anticorpos foi realizada previamente e após a qualificação do projeto de dissertação, porém algumas alíquotas só chegaram no laboratório no final de novembro de 2013, e assim realizamos os testes e otimizações até o inicio de janeiro de 2014 onde seguimos com as reações para imunomarcação das amostras.

Para quantificar a expressão dos anticorpos nos tecidos, foi acordado que não poderíamos realizar dupla marcação, conforme proposto inicialmente, em virtude das Nod1 e Nod2 serem marcadores de citoplasma inviabilizando desta forma a distinção de cada um nos tecidos analisados. Devido à necessidade de repetição das reações e o tamanho pequeno dos amostras, a leitura das Nod em terços em todas as lâminas ficou prejudicado. Assim, nas lâminas marcadas para Nod optamos então por fazer contagem das células inflamatórias totais por campo em cada uma das amostras.

Os resultados desta pesquisa serão divulgados das seguintes maneiras: Dissertação de Mestrado, conforme normas do Programa de Pós Graduação em Odontologia da UFPel; artigo científico a ser publicado em revista internacional “Journal of Clinical Periodontology” (normas de publicação no anexo 1).

4. Artigo científico

Title: Increased expression of NOD, RANKL, OPG and MMP in smokers and nonsmokers with periodontitis

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Running title: Nod, RANKL, OPG, MMP in periodontitis

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Abstract

Aim: Periodontitis is a chronic disease modulated by cigarette consumption inducing innate and adaptive immune responses within the periodontal tissues. To assess these immune responses, we evaluated the expression of nucleotide-binding oligomerization domains (Nod)-1 and -2, receptor activator of nuclear factor kappa-B ligand (*RANKL*), osteoprotegerin (OPG), CD20 (B-cells), CD68 (monocytes/macrophages), CD45RO (T-cells) and matrix metalloproteinases (MMP)-8 and -9 in periodontal tissues of smokers and nonsmokers in order to identify possible differences.

Methods: Gingival tissues were obtained from periodontally health subjects (n=10), and patients with chronic periodontitis smokers (n=15) and nonsmokers (n=16). Nod1, Nod2, RANKL, OPG, CD20, CD68 and CD45RO expression was determined by immunohistochemistry (IHC). MMP-8 and -9 activity were verified by zymography.

Results: Periodontitis-affected patients presented higher levels of all studied proteins when compared with healthy sites ($P < 0.001$). However, no differences were observed between smokers and non-smokers ($P > 0.05$). All proteins were more expressed at the apical portion of periodontal pocket compared with the coronal portion.

Conclusions: Considering periodontitis as an infectious disease, its progression induce the migration of B- and T-cells and monocytes/macrophages to inflamed site. In addition, pattern recognition receptors and bone turnover regulators are overexpressed. No differences were observed between smokers and nonsmokers.

Keywords: Periodontal Diseases; Nod; Rankl; Osteoprotegerin; Matrix metalloproteinases

Clinical Relevance

Scientific Relevance for the study: No literature exists considering the expression of Nod-like receptors, bone turnover regulators and immune cells distribution along the periodontal pocket in smokers and nonsmokers.

Principal findings: Differences observed in clinical condition and periodontal disease progression between smokers and nonsmokers are not associated with inflammatory cells and gelatinases expression.

Practical Implications: Since no differences in cell population, bone remodeling and matrix degrading proteins were observed, smoking patients should be advised to reduce or quit the habit and enroll into a more constant supportive periodontal therapy to control disease progression.

Introduction

Bacterial infections are detected by the innate immune system through transmembrane and cytosolic receptors (Chamaillard et al., 2003). It is well known that Toll-like receptor (TLR)-4 recognizes gram-negative microorganisms eliciting a complex intracellular signaling cascade resulting in proinflammatory mediators secretion, such as interleukin (IL)-1, IL-6, tumor necrosis factor alpha, among others (Garcia de Aquino et al., 2009). More recently, two members of a family of proteins called nucleotide-binding oligomerization domain (Nod)-1 and -2 were found to recognize substructures of bacterial peptidoglicans (Inohara et al., 2003, Girardin et al., 2001).

Gram-negative and some species of Gram-positive bacteria produce the amino acid meso-diaminopimelic acid (meso-DAP) responsible for Nod1 activation (Girardin et al., 2001). On the other hand, all Gram-negative and -positive bacteria present muramyl-dipeptide (MDP) which is recognized by Nod2 (Kim et al., 2008). Mutations in NOD2 gene cause hyperinflammatory responses to bacteria components such as in Crohn's disease and Blau´s syndrome, both autoinflammatory diseases (Hugot et al., 2001). In cases of bone resorption, osteoblast stimulated by MDP (Nod2 agonist) synergistically enhances osteoclast formation induced by lipopolysaccharide (LPS), IL-1α, and tumor necrosis factor alpha (TNF-α) through

the receptor activator of nuclear factor kappa-B *ligand (*RANKL) expression (Yang et al., 2005).

Destruction seen in animal and human with periodontitis is potentiated by smoking habits (Cesar-Neto et al., 2006). Nicotine combined with LPS increased the release of prostaglandin E2 (PGE2), IL-1β and TNF-α in circulating monocytes (Payne et al., 1996, Ryder, 2007) and IL-6 in fibroblasts and osteoblasts (Wendell and Stein, 2001). Wistar rats with ligature-induced periodontitis exposed to cigarette smoke presented higher levels of bone loss, matrix metalloproteinase 2 (MMP-2), IL-6 and interferon gamma (IFN-γ) (Cesar-Neto et al., 2006).

Smoking patients with periodontitis tended to present lower levels of the receptor activator of nuclear factor kappa-B (RANK) decoy osteoprotegerin (OPG) compared to nonsmoking periodontitis patients in blood stream (Lappin et al., 2007). In addition, RANKL:OPG ratio was found higher in smokers possibly explaining the higher attachment loss observed in smokers.

However, some authors failed to observe *in vitro* a tendency of increase in inflammatory genes expression in smoking periodontal patients after smoking cessation (Morozumi et al., 2004, Ouyang et al., 2000). It is important to emphasize that most of the studies that evaluated periodontal disease pathogenesis associated with smoking habits evaluated crevicular fluid samples, cell cultures or processed tissues.

Thus, this study evaluated differences in RANKL and OPG immunohistochemical detection in periodontal pockets from smoking and not smoking patients with periodontitis. In addition, Nod1 and Nod2 expression was evaluated in healthy and periodontitis-affected gingival tissues.

Material and methods

The study was approved by the Federal University of Pelotas institutional ethical committee (#038/2008).

All subjects were submitted to anamnesis and to periodontal and radiographic examination. Supragingival calculus was removed to allow periodontal probing. Patients were categorized according to the classification of the American Academy of Periodontology (Armitage, 1999) into healthy and chronic periodontitis groups.

Inclusion criteria included dentate patients (at least 14 natural teeth excluding third molars), systemically healthy with no evidence of systemic periodontal modifiers other than smoking (diabetes mellitus, osteoporosis, and medications known to influence periodontal tissues) (Garlet et al., 2003). Smokers should use at least 20 cigarettes/day for at least 5 years and nonsmokers should never had smoked.

Exclusion criteria included patients with systemic modifiers of periodontal disease as cited above; who had taken systemic antibiotic, anti-inflammatory, or other drug therapy in the last 3 months; who had received previous periodontal therapy in the last 2 years or pregnant/lactating women (Garlet et al., 2003).

Chronic periodontitis subjects presented moderate to advanced chronic periodontitis ($\geq$1 tooth per sextant with probing depth >5 mm and attachment loss $\geq$3 mm, and extensive radiographic bone loss) (Garlet et al., 2003). Gingival biopsy sites in healthy control subjects did not exhibit radiographic bone destruction, as well as having clinical probing depths less than 3 mm without sulcular bleeding on probing.

Gingival biopsies

Samples were surgically obtained from subjects with teeth to be extracted, root scaled, or having their crown lengthened for restoration. Gingival tissues were obtained from

periodontally health subjects (n=10), and patients with chronic periodontitis smokers (n=15) and nonsmokers (n=16). Probing depth (PD), gingival margin level (GM) and bleeding on probing (BP) were recorded (Garlet et al., 2003).

Biopsies were obtained of the proximal face under the contact point using intrasulcular incisions. In case of isolated teeth two parallel 5mm incisions connected by a perpendicular incision (fig. 1 and 2).

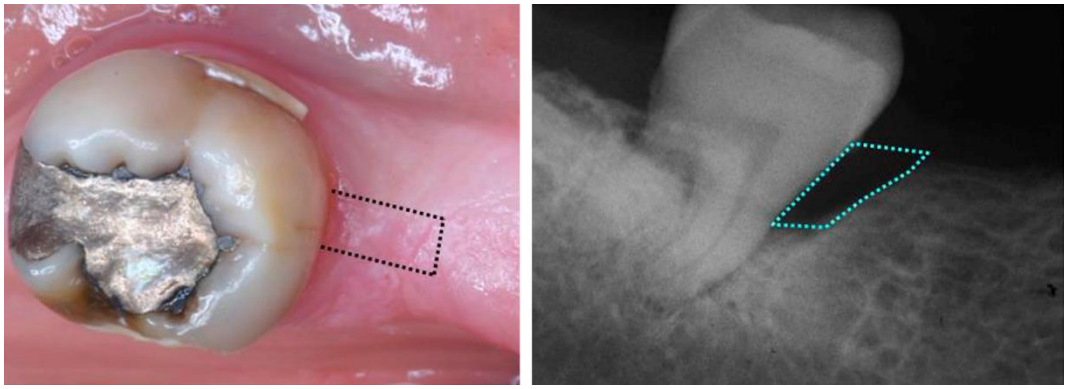

Figure 1. Representation of sample removal for an isolated tooth. Oclusal view with incision scheme and the radiographic image representing the depth of the incision.

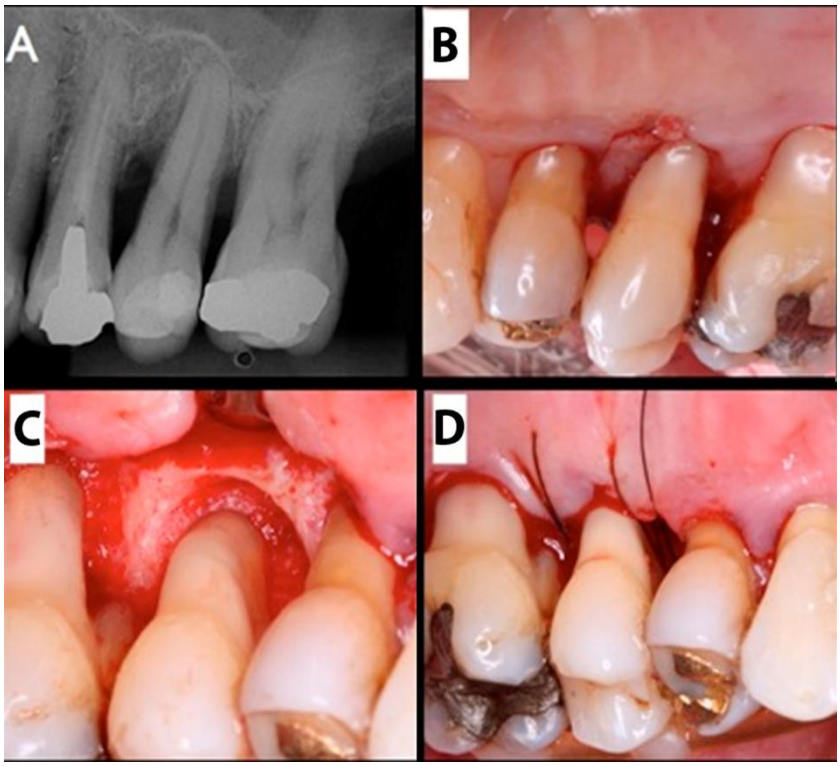

Figure 2. Operative procedure for proximal surface under contact point. **A:** Bone defect between the premolars; **B:** Intrasulcular incisions delimiting the area. **C:** Full thickness flap; **D:** Immediate post-operative.

Immunohistochemistry (IHC)

Gingival tissue were fixed in 10% buffered formalin, serially cut in the coronal plane, and embedded in paraffin for sectioning. The formalin-fixed, paraffin-embedded tissue specimens were sectioned at 5 μm. Sections were mounted on silanized slides, deparaffinized in xylene, blocked in 1:1 H_2O_2 in absolute methanol, and processed for antigen retrieval.

The sections were reacted with specific antibodies as shown in Table 1. After being washed with TRIS-HCl, the sections were incubated with biotinylated immunoglobulins (Universal Ab, Dako Corporation, CA, USA) for 20 min at room temperature and washed with TRIS-HCl to remove any unreacted antibodies. The sections were then treated with peroxidase-conjugated streptavidin (DAKO) for 10 min, and washed and reacted with DAB (3,3-diaminobenzidine tetrahydrochloride; DAKO) in the presence of 3% H_2O_2 to develop color. The sections were counterstained with Mayer´s hematoxylin and mounted with Entellan (Merck; Darmstadt, Germany).

Table 1. Antibodies and parameters used for immunohistochemistry reactions

ANTIBODY	***CLONE***	***BRAND***	***HOST***	***DILUTION***	***INCUBATION***
CD20	7D1	Novocastra	Mouse	1:200	60’
CD68	PG-M1	Dako	Mouse	1:50	60’
CD45RO	UCHL1	Dako	Mouse	1:100	60’
RANKL	N-19	Santa Cruz	Goat	1:100	60’
OPG	N-20	Santa Cruz	Goat	1:50	60’
NOD1	bs-7085R	Bioss	Rabbit	1:800	12h
NOD2	bs-7084R	Bioss	Rabbit	1:800	12h

Novocastra laboratories Ltd, Newcastle, UK; Dako Cytomation, Glostrup, DN; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Bioss, Woburn, MA, USA

Slide evaluation was independently performed by two blinded examiners. In case of disagreement consensus was reached by discussion with a pathologist. For RANKL, OPG, CD20 (B-cells), CD68 (monocytes/macrophages) and CD45RO (T-cells) positive cells were

counted along the periodontal pocket with a grid. The optical parameters were adjusted in such a way (magnification factor 200) that one square of the counting grid corresponded to 0.0025 (mm^2) (Garcia de Aquino et al., 2009). Sample was divided in coronal, medium and apical thirds (fig. 3). Cells expressing Nod1 and Nod2 were counted with the grid by the hot spot technique (Shimura et al., 2000).

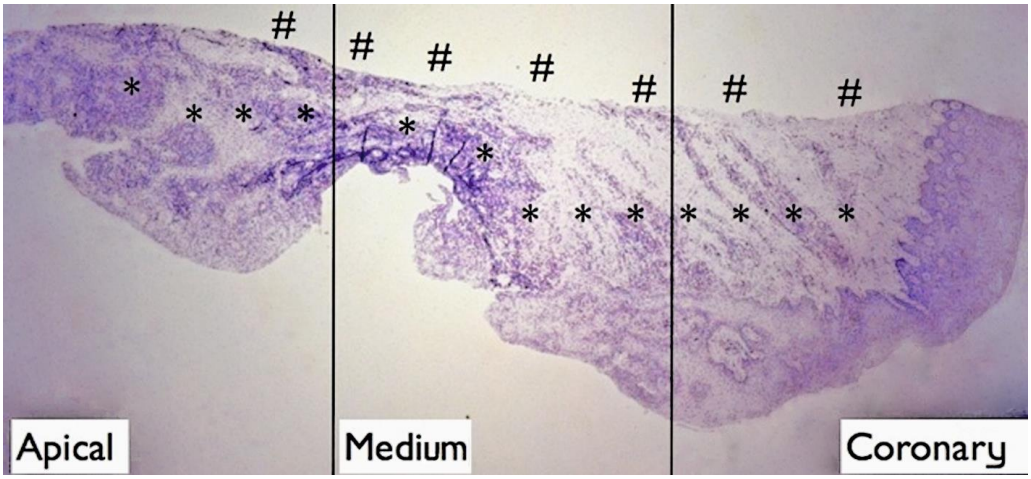

Figure 3. Illustration of an immunohistochemistry reaction divided in coronal, medium and apical thirds. # tissue in direct contact with tooth; * area of the connective tissue where cells were counted. Magnification 100X.

Gelatin zymography

MMP-2 and MMP-9 activity were assessed by zymography. Part of the gingival tissue were dissected immediately following excision, pooled and washed (at 24°C for 30 min) in Dulbecco's modified Eagle's medium (DMEM) containing 80 mg ml^{-1} of gentamicin. After 24h the supernatant was frozen at −70°C until analysis for enzyme activity. Proteolytic activity was examined on 10% polyacrylamide gels containing 0.05% gelatin. The conditioned medium was mixed with an equal volume of non-reducing sample buffer [2% sodium dodecyl sulfate (SDS); 125 mM Tris–HCl (pH 6.8), 10% glycerol, and 0.001% Bromophenol Blue) and then electrophoresed. Gels were washed in 2% Triton X-100 for 60 min at room temperature and then incubated at 37°C for 24 h in 50 mM Tris–HCl buffer,

pH 7.4, containing 5 mM Tris–$CaCl_2$. Gels were stained with 0.05% Coomassie Brilliant Blue G-250 (Bio-Rad, Richmond, CA, USA). The gelatinolytic activity was detected as unstained bands. To assess the identity of the lytic bands present in the conditioned media, parallel experiments using proteinase inhibitions were performed. Gelatin-containing gels were incubated in Tris–$CaCl_2$ buffer at 37°C for 24 h with the addition of 0.5 mM EDTA (Reagen, São Paulo, SP, Brazil) to inhibit lytic activities caused by MMP, while 0.5 mM *N*-ethyl-maleimide (NEM) was used to inhibit activities caused by serine proteinases. MMP-2 and -9 were identified by comparing the molecular weight (Perfect Protein™ AP Kit, Novagen, Darmstadt, Germany). Images were analyzed densitometrically after digital imaging capture (Image Quant 100 – GE Healthcare), using ImageJ 1.45 software (Wayne Rasband, NIH, USA).

Scoring System and Statistics

Statistical significance was analyzed with t-test for data with normal distribution and Mann-Whitney followed by Dunn test for non-parametric; $P \leq 0.05$ were considered significant. The spearman coefficient was used to study correlations between continuous variables. A statistical software was used to perform the statistical analysis (GraphPad Prism 5.0 Software, San Diego, CA, USA).

Results

Forty-one patients, which included 20 men participated in this study. No statistical differences were observed for age and gender among the three groups, and for pocket depth (PD) and clinical attachment loss (CAL) between the two periodontitis groups. Table 2 shows the sample distribution into the groups according to demographic and clinical variables.

Table 2. Patients´ demographic data and clinical parameters of the biopsied sites in each group (mean values and standard deviation)

	Healthy	**Periodontitis Nonsmoking**	**Periodontitis Smoking**
Age (years)	39.8 (±5.4)	42.3 (±6.7)	46.4 (±7.7)
Gender	F= 5/10 M=5/10	F= 8/15 M= 7/15	F= 7/15 M=8/15
Pocket depth (mm)	2.1 (±0.8)	7.8 (±1.4)	7.9 (±1.2)
Attachment loss (mm)	0	7.6 (±1.6)	7.3 (±2.2)
Cigarettes smoked/day	-	-	17 (±4.1)
Years of smoking	-	-	22.9 (±12.9)

A significant statistical difference was observed between the healthy and both periodontitis affected groups for all IHC and gelatin zymography reactions ($P < 0.001$). However, no differences were seen between smokers and nonsmokers (Table 3).

Table 3. Cell counting for each antibody marker by immunohistochemistry in smokers and nonsmokers affected by periodontitis

IHC parameter	**Nonsmokers** Positive cells (mean ± SD)	**Smokers** Positive cells (mean ± SD)	P value
CD20	173.25 ± 330.35	162.27 ± 171.20	0.745
CD45RO	614.31 ± 901.82	586.60 ± 626.38	0.887
CD68	155.10 ± 182.78	145.53 ± 106.40	0.174
Total (CD20 + CD68 + CD45RO)	942.56 ± 1196.11	894.40 ± 765.02	0.267
OPG	193.31 ± 89.36	142.31 ± 72.33	0.494
RANKL	566.59 ± 277.36	614.30 ± 270.58	0.824
NOD1	423.60 ± 188.26	531.15 ± 222.76	0.247
NOD2	346.54 ± 134.25	370.19 ± 179.01	0.564

Inflammatory cell population was concentrated at the apical compared with the coronal third of the periodontal pocket for both groups (Table 4). Healthy samples were not split in thirds since PD was very short to be divided and the inflammatory cells scarce.

Table 4. Cell counting along the periodontal pocket split in thirds for each antibody marker by immunohistochemistry in smokers and nonsmokers affected by periodontitis.

Labelling	**Sample region** (thirds)	**Nonsmokers** Positive cells (mean ± SD)	**Smokers** Positive cells (mean ± SD)
CD20	Coronal	21.31 ± 21.57^{a}	22.40 ± 38.10^{a}
	Average	56.50 ± 88.12^{ab}	58.46 ± 70.25^{ab}
	Apical	95.43 ± 249.67^{b}	81.40 ± 89.98^{b}
CD45RO	Coronal	54.93 ± 85.84^{a}	71.27 ± 70.01^{a}
	Average	227.25 ± 335.56^{ab}	160.06 ± 167.18^{ab}
	Apical	332.12 ± 549.18^{b}	335.26 ± 500.17^{b}
CD68	Coronal	53.37 ± 89.73^{a}	25.2 ± 23.81^{a}
	Average	62.81 ± 94.98^{a}	60.00 ± 61.82^{b}
	Apical	38.81 ± 40.20^{a}	60.33 ± 54.32^{ab}
OPG	Coronal	21.31 ± 11.57^{a}	22.40 ± 12.10^{a}
	Average	76.57 ± 28.12^{b}	58.46 ± 20.25^{ab}
	Apical	95.43 ± 49.67^{b}	61.45 ± 39.98^{b}
RANKL	Coronal	71.27 ± 70.01^{a}	54.93 ± 15.84^{a}
	Average	160.06 ± 107.18^{ab}	227.25 ± 105.56^{b}
	Apical	335.26 ± 100.17^{b}	332.12 ± 149.18^{b}

*Different lowercase letters indicate statistical significant difference

A statistically significant difference was observed between the number of cells positive for Nod1 and Nod2 in healthy and periodontitis affected samples ($P < 0.001$). However, no differences were observed in Nod1 and Nod2 expression between smokers and nonsmokers ($P = 0.564$ and $P = 0.856$, respectively).

Illustrative images of the reactions in the middle third of the samples for smoking patients are presented in Figure 4.

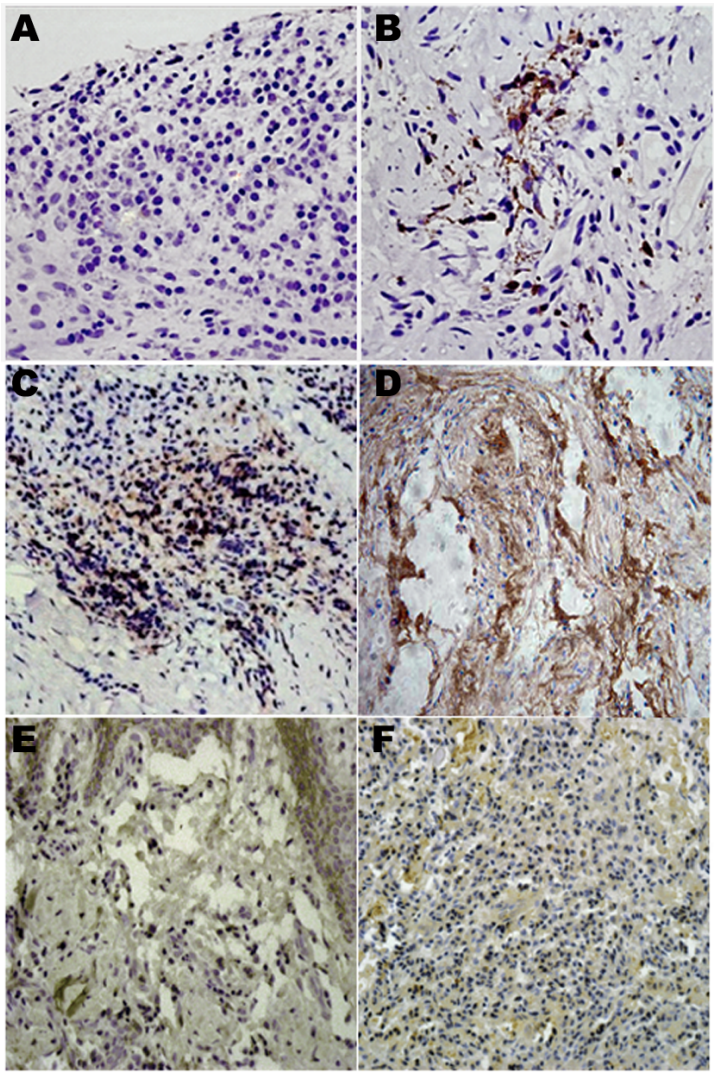

Figure 4 - Increased in immunoreactive cells number in chronic periodontitis. Periodontal tissue sections were submitted to immunohistochemical reaction for CD20 (A), CD45RO (B), CD68 (C), RANKL (D), Nod1 (E), Nod2 (F). Illustrative image of the middle third section of the smoking group. Magnification 400Xnão é 40x?.

In addition, no differences were seen in MMP-2 and MMP-9 expression (P = 0.951 and 0.183, respectively) between smokers and nonsmokers with periodontitis. Moreover, gelatinases activity were not different between smokers and nonsmokers (P = 0.671).

Discussion

The present study was the first to analyze by IHC biopsies from smoking and nonsmoking patients with periodontitis in order to evaluate the distribution of inflammatory cells and markers along the periodontal pocket to justify a possible increase in CAL in smokers.

As expected, our results suggest that a chronic inflammatory infiltrate is observed after periodontitis development. However, no differences were seen on cell concentration or cell distribution along the periodontal pocket between the smoking and nonsmoking groups. Hypothesizing that smokers with periodontitis have higher CAL, previous studies in rats (Cesar-Neto et al., 2006, Cesar-Neto et al., 2007) showed that some cigarette components could stimulate a higher expression of inflammatory cytokines and that CAL was more associated with the mediators expressed than with cell population and concentration.

Lymphocytes play a major role in periodontitis pathogenesis (Khalaf and Bengtsson, 2012). The nature of adaptive immune response is controlled by T-cells, which regulate the B-cells population and immunoglobulin release. T-cells are responsible for the production of different mediators, such as interferon-gamma, IL-1, -6, RANKL, adhesion molecules and chemokines (Khalaf and Bengtsson, 2012). These mediators direct the immune response from gingivitis to periodontitis directly or indirectly stimulating bone resorption and CAL (Loos et al., 2004, Gemmell and Seymour, 2004).

Higher T-cell proliferation was observed in heavy smokers (Loos et al., 2004). Authors concluded that the increase in T-cells responsiveness and number would be one of many factors that explain why smoking is a risk factor for periodontitis. Our results failed to show an increased number of T-cells in smokers than nonsmokers. However, the present lymphocytes should be checked for their activity since an increase in cytokines release could induce RANKL expression.

Osteoclastogenesis is mediated by the interaction among three molecules RANK, RANKL and OPG. OPG was the first protein to be described of this metabolic axis as an osteoclast inhibitor (Tsuda et al., 1997, Simonet et al., 1997). A few months later, RANKL was reported as a tumor necrosis factor capable of stimulating dendritic cells to induce T-cells proliferation (Anderson et al., 1997, Wong et al., 1997). RANK was cloned from a dendritic cell cDNA library because when activated it extended the cell survival (Anderson et al.,

1997). At the same time, it was observed that it was involved in osteoclast differentiation (Nakagawa et al., 1998). In sum, the RANK/RANKL/OPG axis acts by the interaction of RANKL-RANK, which positively regulates osteoclastogenesis and is counterbalanced by OPG, a false natural decoy for RANKL (Hofbauer and Heufelder, 2001).

According to our data, periodontitis affected patients presented higher levels of RANKL, OPG, Nod1 e Nod2 when compared with healthy ones, which is proportional to the levels of CD20, CD68, CD45RO positive cells. (Tang et al., 2009) corroborate the fact that RANKL levels are not affected by smoking, but OPG levels slight reduce causing bone resorption. For the first time, we show that RANKL and OPG levels are higher at the apical portion of the pocket, where a high gram-negative bacterial load is present and closer to alveolar bone.

Various stimuli can induce increased expression of RANKL specially in osteoblasts, such as parathyroid hormone, 1,25-dihydroxyvitamin D , interleukin- 1 and lipopolysaccharide (Gallagher, 2008). Intracellular signaling by TLRs or bacterial stimulation via IL-1R induced by interleukin-1 requires the activation of intracellular proteins like p38 MAPK and especially NF-kB (O'Neill and Bowie, 2007, Stone et al., 1988). The role of MyD88 adapter protein in the expression of proinflammatory genes induced via activation of TLRs involves the recruitment of signaling proteins common to both paths located upstream. It is already known that IRAKs (IL-1 receptor-associated kinase) activate TRAF6 (tumor necrosis factor receptor-associated factor 6-associated), which subsequently recruits TAK1 (Transforming growth factor β-activated-kinase-1) and TABs (TAK1 binding proteins). From this point two signaling pathways can be distinctly activate through the recruitment and activation of IKK (IκB kinase) complex and MAP3Ks (as MKK3 and MKK6, upstream activators of p38 MAPK) (Adhikari et al., 2007, Kobayashi et al., 2002).

Alternatively, activation of p38 MAPK and NF-kB can also be induced by two other adapter proteins, TRIF (TIR domain-containing adapter protein inducing IFN-b) and TRAM (-related adapter molecule) (Krishnan et al., 2007). Thus, the same intracellular signaling pathway through branching and plasticity regulate distinct genes in different cell types or, alternatively, in the distinct regulation of a single gene in different cell types.

It is speculated that Nod2 and probably Nod1 binds to the serine / threonine kinase RIP2 (also known as RICK or Cardiak), which is involved in signal transduction via NF-kB (Hasegawa et al., 2008). Results indicate that Nod1 and Nod2 signaling pathway is required for expression of RANKL induced by agonists of TLR2 and 4, but only Nod1 is required for expression of RANKL after stimulation by IL-1β (Hasegawa et al., 2008). In this study, RANKL, Nod1 and Nod2 levels were higher in periodontitis affected patients, in which alveolar bone resorption is present.

Nod proteins effect seems to be potentiated by the presence of IFN-γ and TNF-α (Hosokawa et al., 2010). Kawai et al. (2000) observed that Th1-type cells migrated to periodontitis sites induced by MDP. Takahashi et al. (2006) reported that mononuclear cells and fibroblasts expressed Nod1 and Nod2 in sites affected by periodontitis, as observed in this study, and Nod expression was enhanced by IFN-γ, TNF-α and LPS.

1.1.1. Importantly, the information concerning the role of Nod proteins as inflammatory signaling mediators involved in the innate immune response is still scarce. However, there is evidence highlighting their role in the expression of several inflammatory cytokines, including IL-1β, IL-6, IL-8 and TNF-α both in monocytic cells and epithelial cells (Uehara et al., 2007, Kim et al., 2004, Kobayashi et al., 2005) and modulation of cytokine expression via activation of TLRs (Netea et al., 2005).

Since a mutation in Nod2 protein is associated with Crohn´s disease in humans with a hyperinflammatory phenotype (Inohara et al., 2003) and considering that Nod higher expression is induced by proinflammatory cytokines and LPS (Gutierrez et al., 2002), Nod

proteins could be a potential pharmacologic target for new locally delivery drugs in periodontal pockets.

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5. Conclusões

Não foram observadas diferenças estatisticamente significantes entre as amostras de pacientes com periodontite crônica fumantes e não fumantes quanto à contagem de células inflamatórias, marcação para RANKL, OPG, Nod1 e Nod2, e também para atividade das metaloproteinases 2 e 9. As diferenças foram observadas entre os pacientes periodontalmente saudáveis e aqueles com periodontite crônica.

Interessante observar que todas as marcações foram observadas com maior intensidade nas porções mais profundas das bolsas periodontais, um local com maior dificuldade de acesso para remoção de fatores etiológicos da periodontite, como bactérias gram-negativas anaeróbias, potentes indutores de resposta inflamatória. Denotando a importância do tratamento periodontal englobando raspagem subgengival e, prinipalmente, a necessidade de ressaltar ao paciente os benefícios da terapia periodontal de suporte e manutenção.

Assim, presume-se que o processo de etiopatogênese da doença periodontal em pacientes fumantes e não fumantes é semelhante em termos de destruição de matriz extracelular por gelatinases e de reabsorção óssea. Portanto, a influência do fumo na instalação, progressão e reparo periodontal ainda devem ser explorados em estudos futuros.

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Journal of Clinical Periodontology publishes original research articles, reviews, clinical innovation reports and case reports. The latter will be published only if they provide new fundamental knowledge and if they use language understandable to the clinician. It is expected that any manuscript submitted represents unpublished original research.

Original Research Articles must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically

evaluated and, if necessary, repeated. Original articles will be published under the heading of clinical periodontology, implant dentistry or pre-clinical sciences and must conform to the highest international standards in the field.

Clinical Innovation Reports are suited to describe significant improvements in clinical practice such as the report of a novel surgical technique, a breakthrough in technology or practical approaches to recognized clinical challenges. They should conform to the highest scientific and clinical practice standards.

Case Reports illustrating unusual and clinically relevant observations are acceptable but their merit needs to provide high priority for publication in the Journal. On rare occasions, completed cases displaying non-obvious solutions to significant clinical challenges will be considered.

Reviews are selected for their broad general interest; all are refereed by experts in the field who are asked to comment on issues such as timeliness, general interest and balanced treatment of controversies, as well as on scientific accuracy. Reviews should take a broad view of the field rather than merely summarizing the authors´ own previous work, so extensive citation of the authors´ own publications is discouraged. The use of state-of-the-art evidence-based systematic approaches is expected. Reviews are frequently commissioned by the editors and, as such, authors are encouraged to submit a proposal to the Journal. Review proposals should include a full-page summary of the proposed contents with key references.

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format Language: The language of publication is English. Authors for whom English is a second language may choose to have their manuscript professionally edited before submission to improve the English. It is preferred that manuscript is professionally edited. Please refer to English Language Editing Services offered by Wiley at http://wileyeditingservices.com/en/. Japanese authors can also find a list of local English improvement services at http://www.wiley.co.jp/journals/editcontribute.html. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Abbreviations, Symbols and Nomenclature: Journal of Clinical Periodontology adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. Abbreviations

should be kept to a minimum, particularly those that are not standard. Non-standard abbreviations must be used three or more times and written out completely in the text when first used.

5.2. Structure All articles submitted to Journal of Clinical Periodontology should include Title Page, Abstract, and References. In addition, Journal of Clinical Periodontology requires that all articles include a section on Clinical Relevance and disclose Source of Funding and Conflict of Interests. Figures, Figure Legends and Tables should be included where appropriate. All manuscripts should emphasize clarity and brevity. Authors should pay special attention to the presentation of their findings so that they may be communicated clearly. Technical jargon should be avoided as much as possible and be clearly explained where its use is unavoidable.

Title Page: The title must be concise and contain no more than 100 characters including spaces. The title page should include a running title of no more than 40 characters; 5-10 key words, complete names of institutions for each author, and the name, address, telephone number, fax number and e-mail address for the corresponding author.

Conflict of Interest and Source of Funding: Authors are required to disclose all sources of institutional, private and corporate financial support for their study. Suppliers of materials (for free or at a discount from current rates) should be named in the source of funding and their location (town, state/county, country) included. Other suppliers will be identified in the text. If no funding has been available other than that of the author's institution, this should be specified upon submission. Authors are also required to disclose any potential conflict of interest. These include financial interests (for example patent, ownership, stock ownership, consultancies, speaker's fee,) or provision of study materials by their manufacturer for free or at a discount from current rates. Author's conflict of interest (or information specifying the absence of conflicts of interest) and the sources of funding for the research will be published under a separate heading entitled "Conflict of Interest and Source of Funding Statement". See Editor-in-Chief Maurizio Tonetti'sEditorial on Conflict of Interest and Source of Funding and www.icmje.org/#conflicts for generally accepted definitions.

Abstract: is limited to 200 words in length and should not contain abbreviations or references. The abstract should be organized according to the content of the paper. For Original Research Articles the abstract should be organized with aim,

materials and methods, results and conclusions. For clinical trials, it is encouraged that the abstract finish with the clinical trial registration number on a free public database such as clinicaltrials.gov.

Clinical Relevance: This section is aimed at giving clinicians a reading light to put the present research in perspective. It should be no more than 100 words and should not be a repetition of the abstract. It should provide a clear and concise explanation of the rationale for the study, of what was known before and of how the present results advance knowledge of this field. If appropriate, it may also contain suggestions for clinical practice. It should be structured with the following headings: scientific rationale for study, principal findings, and practical implications. Authors should pay particular attention to this text as it will be published in a highlighted box within their manuscript; ideally, reading this section should leave clinicians wishing to learn more about the topic and encourage them to read the full article.

Acknowledgements: Under acknowledgements please specify contributors to the article other than the authors accredited.

5.3. Original Research Articles These must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically evaluated and, if necessary, repeated. Original articles will be published under the heading of clinical periodontology, implant dentistry or pre-clinical sciences and must conform to the highest international standards in the field.

The word limit for original research articles is 3500 words, and up to 7 items (figures and tables) may be included. Additional items can be included as supplementary files online (please see 5.9 below). Main Text of Original Research Articles should be organized with Introduction, Materials and Methods, Results and Discussion. The background and hypotheses underlying the study, as well as its main conclusions, should be clearly explained. Please see Sample Manuscript.

Introduction: should be focused, outlining the historical or logical origins of the study and not summarize the results; exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation.

Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully

reproduced. As a condition of publication, authors are required to make materials and methods used freely available to academic researchers for their own use. This includes antibodies and the constructs used to make transgenic animals, although not the animals themselves.

(a) Clinical trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist should also be included in the submission material. If your study is a randomized clinical trial, you will need to fill in all sections of the CONSORT Checklist. If your study is not a randomized trial, not all sections of the checklist might apply to your manuscript, in which case you simply fill in N/A. Journal of Clinical Periodontology encourages authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials/.

The clinical trial registration number and name of the trial register will then be published with the paper.

(b) Statistical Analysis: As papers frequently provide insufficient detail as to the performed statistical analyses, please describe with adequate detail. For clinical trials intention to treat analyses are encouraged (the reasons for choosing other types of analysis should be highlighted in the submission letter and clarified in the manuscript).

(c) DNA Sequences and Crystallographic Structure Determinations: Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors directly.

(d) Experimental Subjects: Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included.

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study, if applicable. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

Results: should present the observations with minimal reference to earlier literature or to possible interpretations. Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The discussion section should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references. The discussion may usefully be structured with the following points in mind (modified from the proposal by Richard Horton (2002), The Hidden Research Paper, The Journal of the American Medical Association, 287, 2775-2778). Not all points will apply to all studies and its use is optional, but we believe it will improve the discussion section to keep these points in mind. Summary of key finding

* Primary outcome measure(s)

* Secondary outcome measure(s)

* Results as they relate to a prior hypothesis Strengths and Limitations of the Study.

* Study Question

* Study Design

* Data Collection

* Analysis

* Interpretation

* Possible effects of bias on outcomes Interpretation and Implications in the Context of the Totality of Evidence

* Is there a systematic review to refer to?

* If not, could one be reasonably done here and now?

* What this study adds to the available evidence

* Effects on patient care and health policy

* Possible mechanisms Controversies Raised by This Study Future Research Directions

* For this particular research collaboration

* Underlying mechanisms

* Clinical research

5.4. Clinical Innovation Reports

These are suited to describe significant improvements in clinical practice such as the report of a novel surgical technique, a breakthrough in technology or practical approaches to recognized clinical challenges. They should conform to the highest scientific and clinical practice standards.

The word limit for clinical innovation reports is 3000 words, and up to 12 items (figures and tables) may be included. Additional items can be included as supplementary files online (please see 5.9 below).

The main text of Clinical Innovation Reports should be organized with Introduction, Clinical Innovation Report, Discussion and Conclusion.

5.5. Case Reports

Case reports illustrating unusual and clinically relevant observations are acceptable but their merit needs to provide high priority for publication in the Journal. On rare occasions, completed cases displaying non-obvious solutions to significant clinical challenges will be considered.

The main text of Case Reports should be organized with Introduction, Case report, Discussion and Conclusion.

5.6. Reviews

Reviews are selected for their broad general interest; all are refereed by experts in the field who are asked to comment on issues such as timeliness, general interest and balanced treatment of controversies, as well as on scientific accuracy. Reviews should take a broad view of the field rather than merely summarizing the

authors´ own previous work, so extensive citation of the authors´ own publications is discouraged. The use of state-of-the-art evidence-based systematic approaches is expected. Reviews are frequently commissioned by the editors and, as such, authors are encouraged to submit a proposal to the Journal. Review proposals should include a full-page summary of the proposed contents with key references.

The word limit for reviews is 4000 words. The main text of Reviews should be organized with Introduction, Review of Current Literature, Discussion and Conclusion.

5.7. References

It is the policy of the Journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

We recommend the use of a tool such as Reference Manager for reference management and formatting. Reference Manager reference styles can be searched or here: http://www.refman.com/support/rmstyles.asp

Please note that all unpublished papers (submitted or in press) included in the reference list should be provided in a digital version at submission. The unpublished paper should be uploaded as a supplementary file for review.

Reference style (Harvard):

References in the text should quote the last name(s) of the author(s) and the year of publication (Brown & Smith 1966). Three or more authors should always be referred to as, for example, Brown et al. 1966. A list of references should be given at the end of the paper and should follow the recommendations in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors, (1975), p. 36. London: The Royal Society of Medicine.

a) The arrangement of the references should be alphabetical by first author's surname.

b) The order of the items in each reference should be: (i) for journal references: name(s) of author(s), year, title of paper, title of journal, volume number, first and last page numbers. (ii) for book references: name(s) of author(s), year, chapter title, title of book in italics, edition, volume, page number(s), town of publication, publisher.

c) Authors' names should be arranged thus: Smith, A. B., Jones, D. E. & Robinson, F. C. Note the use of the ampersand and omission of comma before it. Authors' names when repeated in the next reference are always spelled out in full.

d) The year of publication should be surrounded by parentheses: (1967).

e) The title of the paper should be included without quotation marks.

f) The journal title should be written in full, italicised (single underlining in typescript), and followed by volume number in bold type (double underlining on typescript) and page numbers.

Examples: Botticelli, D., Berglundh, T. & Lindhe, J. (2004) Hard-tissue alterations following immediate implant placement in extraction sites. Journal of Clinical Periodontology 10, 820-828. doi:10.1111/j.1600-051X.2004.00565.x Lindhe, J., Lang, N.P. & Karring, K. (2003) Periodontology and Implant Dentistry. 4th edition, p. 1014, Oxford. Blackwell Munksgaard. Bodansky, O. (1960) Enzymes in tumour growth with special reference to serum enzymes in cancer. In Enzymes in Health and Disease, eds. Greenberg, D. & Harper, . A., pp. 269-278. Springfield: Thomas. URL: Full reference details must be given along with the URL, i.e. authorship, year, title of document/report and URL. If this information is not available, the reference should be removed and only the web address cited in the text. Example: Smith A. (1999) Select Committee Report into Social Care in the Community [WWW document]. URL http://www.dhss.gov.uk/reports/report0394498.html [accessed on 7 November 2003]

5.8. Tables, Figures and Figure Legends

Tables: should be double-spaced with no vertical rulings, with a single bold ruling beneath the column titles. Units of measurements must be included in the column title.

Figures: All figures should be planned to fit within either 1 column width (8.0 cm), 1.5 column widths (13.0 cm) or 2 column widths (17.0 cm), and must be suitable for photocopy reproduction from the printed version of the manuscript. Lettering on figures should be in a clear, sans serif typeface (e.g. Helvetica); if possible, the same typeface should be used for all figures in a paper. After reduction for publication, upper-case text and numbers should be at least 1.5-2.0 mm high (10 point Helvetica). After reduction symbols should be at least 2.0-3.0 mm high (10 point). All half-tone photographs should be submitted at final reproduction size. In general,

multi-part figures should be arranged as they would appear in the final version. Each copy should be marked with the figure number and the corresponding author's name. Reduction to the scale that will be used on the page is not necessary, but any special requirements (such as the separation distance of stereo pairs) should be clearly specified.

Unnecessary figures and parts (panels) of figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected; each panel of a multipart figure should be sized so that the whole figure can be reduced by the same amount and reproduced on the printed page at the smallest size at which essential details are visible.

Figures should be on a white background, and should avoid excessive boxing, unnecessary colour, shading and/or decorative effects (e.g. 3-dimensional skyscraper histograms) and highly pixelated computer drawings. The vertical axis of histograms should not be truncated to exaggerate small differences. The line spacing should be wide enough to remain clear on reduction to the minimum acceptable printed size. Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same typesize as used elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field. Thousands should be separated by thin spaces (1 000). Unusual units or abbreviations should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general, visual cues (on the figures themselves) are preferred to verbal explanations in the legend (e.g. broken line, open red triangles etc.).

Preparation of Electronic Figures for Publication Although low quality images are adequate for review purposes, print publication requires high quality images to prevent the final product being blurred or fuzzy. Submit EPS (lineart) or TIFF (halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable

for printed pictures. Do not use pixel-oriented programmes. Scans (TIFF only) should have a resolution of 300 dpi (halftone) or 600 to 1200 dpi (line drawings) in relation to the reproduction size (see below). EPS files should be saved with fonts embedded (and with a TIFF preview if possible). For scanned images, the scanning resolution (at final image size) should be as follows to ensure good reproduction: lineart: >600 dpi; half-tones (including gel photographs): >300 dpi; figures containing both halftone and line images: >600 dpi. Detailed information on our digital illustration standards can be found athttp://authorservices.wiley.com/bauthor/illustration.asp.

Check your electronic artwork before submitting it: http://authorservices.wiley.com/bauthor/eachecklist.asp.

Permissions: If all or parts of previously published illustrations are used, permission must be obtained from the copyright holder concerned. It is the author's responsibility to obtain these in writing and provide copies to the Publishers.

Figure Legends: should be a separate section of the manuscript, and should begin with a brief title for the whole figure and continue with a short description of each panel and the symbols used; they should not contain any details of methods.

5.9. Supplementary Material

Supplementary material, such as data sets or additional figures or tables that will not be published in the print edition of the Journal but which will be viewable in the online edition, can be uploaded as 'Supporting information for review and online publication only'. Please see http://authorservices.wiley.com/bauthor/suppmat.asp for further information on the submission of Supplementary Materials.

6. AFTER ACCEPTANCE

Upon acceptance of a paper for publication, the manuscript will be forwarded to the Production Editor who is responsible for the production of the journal.

6.1 Proof Corrections

The corresponding author will receive an email alert containing a link to a web site. A working email address must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site. Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from the following Web site: www.adobe.com/products/acrobat/readstep2.html . This will enable the file to be opened, read on screen, and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no e-mail address is available; in your absence, please arrange for a colleague to access your e-mail to retrieve the proofs. Proofs must be returned to the Production Editor within three days of receipt. As changes to proofs are costly, we ask that you only correct typesetting errors. Excessive changes made by the author in the proofs, excluding typesetting errors, will be charged separately. Other than in exceptional circumstances, all illustrations are retained by the publisher. Please note that the author is responsible for all statements made in his work, including changes made by the copy editor.

6.2 Early View (Publication Prior to Print)

The Journal of Clinical Periodontology is covered by Wiley-Blackwell's Early View service. Early View articles are complete full-text articles published online in advance of their publication in a printed issue. Early View articles are complete and final. They have been fully reviewed, revised and edited for publication, and the authors' final corrections have been incorporated. Because they are in final form, no changes can be made after online publication. The nature of Early View articles means that they do not yet have volume, issue or page numbers, so Early View articles cannot be cited in the traditional way. They are therefore given a Digital Object Identifier (DOI), which allows the article to be cited and tracked before it is allocated to an issue. After print publication, the DOI remains valid and can continue to be used to cite and access the article.

6.3 Production Tracking Online production tracking is available for your article once it is accepted by registering with Wiley-Blackwell's Author Services.

6.4 Accepted Articles 'Accepted Articles' have been accepted for publication and undergone full peer review but have not been through the copyediting,

typesetting, pagination and proofreading process. Accepted Articles are published online a few days after final acceptance, appear in PDF format only (without the accompanying full-text HTML) and are given a Digital Object Identifier (DOI), which allows them to be cited and tracked. The DOI remains unique to a given article in perpetuity. More information about DOIs can be found online at http://www.doi.org/faq.html. Given that Accepted Articles are not considered to be final, please note that changes will be made to an article after Accepted Article online publication, which may lead to differences between this version and the Version of Record. The Accepted Articles service has been designed to ensure the earliest possible circulation of research papers after acceptance. Given that copyright licensing is a condition of publication, a completed copyright form is required before a manuscript can be processed as an Accepted Article.

Accepted articles will be indexed by PubMed; therefore the submitting author must carefully check the names and affiliations of all authors provided in the cover page of the manuscript, as it will not be possible to alter these once a paper is made available online in Accepted Article format. Subsequently the final copyedited and proofed articles will appear either as Early View articles in a matter of weeks or in an issue on Wiley Online Library; the link to the article in PubMed will automatically be updated.