DAVIDSON FRÓIS MADUREIRA

CINÉTICA DA EXPRESSÃO DE CITOCINAS NO LIGAMENTO PERIODONTAL E FLUIDO CREVICULAR GENGIVAL APÓS A APLICAÇÃO DE FORÇA ORTODÔNTICA

Instituto de Ciências Biológicas

Universidade Federal de Minas Gerais

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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia, do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de doutor em Ciências.

Área de concentração: Biologia Celular

Orientação: Profa. Dra. Tarcília Aparecida da Silva
Departamento de Clínica, Patologia e Cirurgia Odontológicas – Faculdade Odontologia da UFMG

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Instituto de Ciências Biológicas

Universidade Federal de Minas Gerais

Junho/2015

Universidade Federal de Minas Gerais - Instituto de Ciências Biológicas
Programa de Pós-Graduação em Biologia Celular

ATA DA DEFESA DE TESE DE DOUTORADO DE

DAVIDSON FROIS MADUREIRA

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Às **quatorze horas** do dia **09 de junho de 2015**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado de Programa, para julgar, em exame final, o trabalho final intitulado: **"CINÉTICA DA EXPRESSÃO DE CITOCINAS NO LIGAMENTO PERIODONTAL E FLUIDO CREVICULAR GENGIVAL APÓS A APLICAÇÃO DE FORÇA ORTODÔNTICA"**, requisito final para obtenção do grau de Doutor em Biologia Celular, área de concentração: **Biologia Celular.** Abrindo a sessão, a Presidente da Comissão, **Dra. Tarcilia Aparecida da Silva**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dra. Tarcilia Aparecida da Silva	UFMG	Aprovado
Dra. Elizabeth Maria Bastos Lages	UFMG	Aprovado
Dr. Rodrigo V. Soares	PUC Minas	Aprovado
Dr. Ildeu Andrade Junior	PUC Minas	Aprovado
Dr. Guilherme Araujo de Almeida	UFU	Aprovado
Dr. Gregory Thomas Kitten	UFMG	Aprovado

Pelas indicações, o candidato foi considerado: APROVADO

O resultado final foi comunicado publicamente ao candidato pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 09 de junho de 2015.**

Dra. Tarcilia Aparecida da Silva (Orientadora) Tarcilia Silva

Dra. Elizabeth Maria Bastos Lages (Co-Orientadora) [signature]

Dr. Rodrigo V. Soares [signature]

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Profa. Cristina Guatimosim Fonseca
Coordenadora do Programa de Pós Graduação
em Biologia Celular ICB / UFMG

Este estudo foi desenvolvido na **Clínica de Ortodontia** (Departamento de Odontopediatria e Ortodontia – Faculdade de Odontologia da UFMG), **Laboratório de Patologia Bucal** (Departamento de Clínica, Patologia e Cirurgia Odontológica – Faculdade de Odontologia da UFMG), **Laboratório de Imunofarmacologia** (Departamento de Bioquímica e Imunologia – Instituto de Ciências Biológicas da UFMG) e **Laboratório Interdisciplinar de Investigação Médica** (Departamento de Clínica Médica da Faculdade de Medicina da UFMG).

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RESUMO

A aplicação de força ortodôntica induz a ativação de vias metabólicas e liberação de mediadores envolvidos na remodelação dos tecidos periodontais. Dentre estes, as citocinas participam das diferentes fases desse processo. O objetivo deste trabalho foi: (i) avaliar a cinética de produção de citocinas no ligamento periodontal (LP) e fluido crevicular gengival (FCG) após aplicação de força ortodôntica; (ii) verificar se há correlação na produção destes mediadores no LP e FCG. A amostra consistiu de 23 pacientes com indicação de exodontia de pré-molares previamente ao tratamento ortodôntico. O total de 68 dentes de ambas as arcadas foram obtidos. O modelo de estudo foi do tipo boca dividida, sendo um pré-molar submedido à força ortodôntica de 0,980 N por períodos de 1, 3, 7, 14, 21 e 28 dias. O dente contralateral foi utilizado como controle. Amostras de FCG foram obtidas previamente a exodontia, e amostras de LP após a extração dentária. A análise da produção de citocinas [Interleucina-2 (IL-2), Interleucina-4 (IL-4), Interleucina-6 (IL-6), Interleucina-10 (IL-10), Interleucina-17A (IL-17A), Fator de necrose tumoral-alfa (TNF-α) e Interferon-gama (IFN-γ)] foi realizada empregando-se *Cytometric Bead Array* (CBA). Verificou-se que o estímulo mecânico não foi capaz de induzir mudanças significativas na maioria das moléculas investigadas no FCG ou LP. Entretanto, foi observado o aumento significativo da expressão de IL-6 no LP (1 dia após aplicação de força), e a redução da expressão de IL-6 no FCG (21 dias após), quando comparados ao respectivo controle. Correlações significativas positivas entre FCG e LP no grupo experimental puderam ser observadas nos dias 3 (IFN-γ), 7 (IL-10), 14 (IL-17A) e 28 (IL-17A e TNF- α). Observou-se ainda, correlações significativas negativas nos dias 14 (IFN-γ) e 21 (IL-2, IL-10). Os dados sugerem que não existe uma correlação direta clara sobre a contribuição de moléculas liberadas no LP para a composição do FCG. Além disso, observou-se que o estímulo mecânico está associado a um diferente padrão de expressão de IL-6 no LP e FCG.

ABSTRACT

Mechanical loading with orthodontic appliances induces activation of pathways and release of mediators that promote remodeling of periodontal tissues. Among these, cytokines are associated with different mechanisms in the periodontal remodeling process. The aim of this study was to: (i) assess the level of cytokines in the periodontal ligament (PDL) and gingival crevicular fluid (GCF) in response to orthodontic force; (ii) verify if there is a correlation in production of cytokines in PDL and GCF. 23 patients were included in this split-mouth study. A total of 68 premolars were included. While one premolar subjected to 0,980 N force for a specified period (days 1, 3, 7, 14, 21 and 28), the contralateral teeth were used as controls. Before surgery, GCF samples were collected and, after extraction, the PDL. Then, samples were analyzed for cytokine levels [Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-17A (IL-17A), Tumor necrosis factor-alfa (TNF-α) and Interferon-gama (IFN-γ)] by Cytometric Bead Array. Mechanical loading was not able to induce significant changes from most of cytokines investigated in PDL or GCF. However, after force application, increased expression of IL-6 in PDL (on day 1 after force application), and reduced expression of IL-6 in GCF (on day 21) were observed. Significant strong positive correlation between GCF and PDL in experimental group was seen on days 3 (IFN-γ), 7 (IL-10), 14 (IL-17A), 28 (IL-17A, TNF- α) and significant negative strong correlation on days 14 (IFN-γ) and 21 (IL-2, IL-10). This data suggest there is no clear direct correlation regarding the molecular release in PL for the contribution on the composition of GCF. Furthermore, mechanical stress has induced a differential expression of IL-6 in PDL and GCF.

LISTA DE ABREVIATURAS

CBA	*Cytometric bead array*
FCG	Fluido crevicular gengival
ICB	Instituto de Ciências Biológicas
IFN-ɣ	Interferon-gama
IL-1	Interleucina-1
IL-1β	Interleucina-1 Beta
IL-2	Interleucina-2
IL-4	Interleucina-4
IL-6	Interleucina-6
IL-8	Interleucina-8
IL-17A	Interleucina-17A
LP	Ligamento periodontal
MDO	Movimentação dentária ortodôntica
N	Newton
Ni-Ti	Níquel e Titânio
TMA	Titânio Molibidênio
TNF- α	Fator de Necrose Tumoral Alfa
VFCG	Volume de fluido crevicular gengival

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1 REFERENCIAL TEÓRICO

A movimentação dentária ortodôntica (MDO) é o resultado do processo de remodelação óssea. O estímulo mecânico gera uma resposta inflamatória asséptica e transitória nos tecidos periodontais. Há a ativação de vias metabólicas intracelulares e liberação de mediadores capazes de induzir reabsorção óssea nas áreas de compressão e aposição nas áreas de tensão do ligamento periodontal (LP). Esse processo de remodelação óssea resulta em dissipação da força e consequentemente na movimentação dentária (BUCK; CHURCH, 1972; RYGH, 1976; DAVIDOVITCH *et al.,* 1980; DAVIDOVITCH *et al.,* 1980; DAVIDOVITCH *et al.,* 1988; DAVIDOVITCH *et al.,* 1991; BRESNIAK *et al.,* 2002; KRISHNAN; DAVIDOVITCH *et al.,* 2006; HENNEMAN *et al.,* 2008; TADDEI *et al.,* 2012).

Entretanto, a heterogeneidade nas metodologias desses estudos dificulta a comparação dos resultados. Os estudos em animais geralmente utilizam amostras de tecidos periodontais (ALHASHIMI *et al.,* 1999; ALHASHIMI *et al.,* 2001; HAUG *et al.,* 2003; ANDRADE *et al.,* 2007; ANDRADE *et al.,* 2009), enquanto que os estudos em humanos utilizam amostras do fluido crevicular gengival (FCG) (UEMATSU *et al., 1996;* REN *et al.,* 2002; YAO *et al.,* 2003; LEE *et al.,* 2004; BASARAN *et al.,* 2006; REN *et al.,* 2007; REN *et al.,* 2008) e tecidos periodontais (OKADA *et al.,* 1997; GARLET *et al., 2007;* LEE *et al.,* 2007; GARLET *et al.,* 2008; KITASE *et al.,* 2009; GOTO *et al.,* 2011; MADUREIRA *et al.,* 2012) com tempos, métodos de aplicação de força e protocolos experimentais distintos. Apesar da utilização do FCG ser preconizada para o monitoramento não invasivo das alterações que ocorrem no LP, não existe informação disponível na literatura acerca da correlação entre os biomarcadores presentes no LP e FCG.

1.1 Fluido crevicular gengival (FCG)

O FCG é um transudato dos tecidos intersticiais produzidos por um gradiente osmótico (GRIFFITHS, 2003; PALUMBO, 2011; JAVED *et al., 2012;* BARBIERI *et al.,* 2013; PERINETTI *et al.,* 2013), que contém uma mistura de moléculas do sangue, tecidos e do biofilme, tais como eletrólitos, proteínas, pequenas moléculas, citocinas, anticorpos, enzimas e antígenos bacterianos (GRIFFITHS, 2003; UITTO, 2003; MALAMUD, 2006).

Em circunstâncias inflamatórias, o FCG tende a aumentar em volume e em permeabilidade capilar (REN *et al.* 2002; GRIFFITHS, 2003; BARBIERI *et al.,* 2013; PERINETTI *et al.,* 2013), tornando-se exsudativo (GRIFFITHS, 2003; GOODSON, 2003; PERINETTI *et al.,* 2013). Entretanto, a quantificação de diversos biomarcadores, tais como os de inflamação tecidual, necrose ou metabolismo ósseo, possui potencial para monitorar com sucesso os diferentes aspectos da resposta tecidual (REN & VISSINK, 2008; PERINETTI *et al.,* 2013; KAPORR *et al.,* 2014). A utilização do FCG é preconizada para o monitoramento não invasivo das alterações que ocorrem no LP em resposta a diferentes estímulos. As substâncias presentes no FCG têm potencial para serem utilizadas como indicadores da doença periodontal (UITTO 2003, JAVED *et al.,* 2012; KAPOOR *et al.,* 2014), auxiliar no monitoramento dessa doença (DELIMA & VAN DYKE, 2003), acompanhar o processo de regeneração pós-tratamento periodontal (UITTO, 2003) ou verificar a resposta tecidual frente ao estímulo mecânico (REN *et al.,* 2002; REN & VISSINK 2008; PERINETTI *et al.,* 2013; LUPPANAPORNLARP *et al.,* 2010; KUNII *et al.,* 2013). A base fisiológica para estas indicações do uso do FCG seria a liberação de mediadores inflamatórios no FCG durante o processo de remodelação óssea e periodontal (REN *et al.,* 2002; REN *et al.,* 2007; REN; VISSINK, 2008;

LUPPANAPORNLARP *et al.,* 2010; BARBIERI *et al.,* 2013; PERINETTI *et al.,* 2013). Essa informação encontra-se ainda limitada (REN; VISSINK, 2008; KUNII *et al.,* 2013) devido aos resultados inconclusivos e grande variação nas metodologias (BARBIERI *et al.,* 2013; PERINETTI *et al.,* 2013; KAPOOR *et al.,* 2014). Acredita-se que as alterações no FCG devam representar as mudanças indiretas que ocorram no LP (REN *et al.,* 2002; UITTO, 2003; REN; VISSINK, 2008; JAVED *et al.,* 2012; PERINETTI *et al.,* 2013). Entretanto, não estão ainda disponíveis na literatura, estudos que correlacionem os níveis de expressão de citocinas no FCG e LP. Essa informação poderia ajudar a esclarecer as alterações moleculares associadas ao processo de remodelação óssea, doença periodontal e MDO, e também a interação entre essas modificações moleculares nesses dois sítios.

1.2 Ligamento periodontal (LP)

O LP é um tecido conjuntivo especializado, localizado entre a raiz dentária e o osso cortical que forma a parede alveolar (NANCI, 2008), unindo assim, o dente ao osso alveolar (PALUMBO, 2011). O LP, similar a todos os outros tecidos conjuntivos, consiste de células, um compartimento extracelular de fibras colágenas e uma matriz extracelular não colágena, no qual estão presentes elementos celulares tais como osteoblastos, cementoblastos, osteoclastos, células tronco multipotentes, remanescentes epiteliais e fibroblastos, os quais são as células mais abundantes (NANCI, 2008; PALUMBO, 2011). Algumas das células mesenquimais presentes no LP, se estimuladas apropriadamente, são capazes de se diferenciar em um fenótipo de osteoblasto ou cementoblasto, ou seja, promover a formação de osso ou cemento. Além disso, fibroblastos perivasculares e endosteais também possuem a

capacidade de formar LP, cemento e osso (NANCI, 2008). No compartimento extracelular, existem feixes de fibras colágenas bem definidas, incluídas em um material amorfo, conhecido como substância fundamental, consistindo em glicosaminicanas, proteoglicanas, glicoproteínas e glicosídeos, entre outros.

O LP possui espessura média entre 0,15 a 0,38 mm, sendo a porção mais delgada em torno do terço médio da raiz. Essa espessura média reduz progressivamente com o avançar da idade: 0,21 mm dos 11 aos 16 anos, 0,18 mm dos 32 aos 52 anos, e 0,15 mm dos 51 aos 67 anos (NANCI, 2008). Além disso, o aumento da idade está associado a uma redução na vascularização, número de fibras, atividade celular mitótica e dos fibroblastos (PALUMBO, 2011). O LP é um tecido conjuntivo particularmente adaptado a suportar os dentes nos seus alvéolos, e ao mesmo tempo, resistir às forças da mastigação, sendo essas suas principais funções (NANCI, 2008; PALUMBO, 2011). O LP possui a importante função de articular o dente ao osso e possui ação sensorial indispensável para o posicionamento adequado dos maxilares durante a função normal (NANCI, 2008).

O LP tem um *turnorver* extremamente rápido e altas taxas de remodelação, propriedades importantes para a sua renovação, cura e reparo (KOOK *et al.,* 2011; PALUMBO, 2011). Acredita-se que seu potencial regenerativo esteja associado à presença dos fibroblastos do LP (KOOK*et al.,* 2011), que apresentam um nível excepcionalmente alto de renovação das proteínas do compartimento extracelular, particularmente do colágeno (NANCI, 2008). Estudos *in vitro* demonstraram que fibroblastos produzem constitutivamente citocinas que participam no processo de remodelação óssea fisiológica ou em processos patológicos (ARAUJO *et al.,* 2007; LEE *et al.,* 2007; KOOK *et al.,* 2011; LI *et al*. 2011; PAPADOPOLOU *et al.,*2013). As amostras de LP têm sido utilizadas para quantificar níveis de moléculas

sinalizadoras após aplicação de força ortodôntica. Os resultados demonstram que após o estresse mecânico, ocorreu aumento dos níveis IL-6 (MADUREIIRA *et al.,* 2012; BAKKER & JASPER, 2015); TNF-α, IL-10 (GARLET *et al.,* 2007), IFN-γ (ALASHIMI *et al.,* 2010), CCL-2 e CCL-3 (GARLET *et al.,* 2008). *In vitro,* os fibroblastos obtidos a partir do LP apresentaram aumento das seguintes moléculas: IL-6, CCL2 e CCL3 sob estiramento ou compressão (LEE *et al.,* 2007; GOTO *et al.,* 2011*);* IL-6 sob a influência de citocinas pró-inflamatórias (OKADA *et al.,* 1997; SHIMIZU *et al.,* 1992); e do receptor de quimiocina CCR2 sob hipóxia *(*KITASE *et al.,* 2009*)*.

1.3 Citocinas e movimentação dentária ortodôntica

As citocinas são proteínas regulatórias secretadas por leucócitos e uma variedade de outras células do organismo. A ação pleiotrópica das citocinas incluem inúmeros efeitos no sistema imune e modulação da resposta inflamatória (VISSINK, 2008; KOOK *et al.,* 2011; ALHASHIMI *et al.* 2001; REN; ANDRADE *et al.,* 2012). Estas moléculas estão envolvidas na iniciação, amplificação, manutenção e resolução da resposta inflamatória (BASARAN *et al.,* 2006), e podem ser classificadas de forma geral como efetoras (pró-inflamatórias) ou supressoras (anti-inflamatórias) (BASARAN *et al.,* 2006; BLETSA *et al.,* 2006; GARLET *et al.,* 2006; ANDRADE *et al.,* 2007; GARLET *et al.,* 2007; ANDRADE *et al.,* 2012; KOHARA *et al.,* 2012; TADDEI *et al.,* 2012; AZUMA *et al.,* 2013; TADDEI *et al.,* 2013). As citocinas pró-inflamatórias são “moléculas de alarme”, induzindo a dilatação vascular com aumento de permeabilidade e intensificação da resposta inflamatória (BASARAN *et al.,* 2006; GARLET *et al.,* 2006; KOHARA *et al.,* 2012). Novas vias

associadas à remodelação óssea induzida por força mecânica foram recentemente identificadas utilizando animais com deleção de receptores para citocinas associadas à ostoclastogênese, tais como receptor tipo I do fator de necrose tumoral (TNF-RI) (ANDRADE *et al.,* 2007), *Receptor* 5 de quimiocinas com o domínio *CC (*CCR5) (ANDRADE *et al.,* 2009), *Receptor* 2 de quimiocinas com o domínio *CC (*CCR2) (TADDEI *et al.,* 2012), *Receptor* 1 de quimiocinas com o domínio CC (*CCR1*) e *Receptor* 3 de quimiocinas com o domínio *CC (*CCR3) (TADDEI *et al.,* 2013), os quais apresentaram taxa de movimentação dentária reduzida e reduzidos níveis de Interleucina-10 (IL-10) e osteorotegerina (ANDRADE *et al.,* 2009). De modo contrário, a deleção de osteoprotegerina esteve associada à reabsorção óssea severa durante a movimentação dentária (TANAKA *et al.,* 2012; YABUMOTO *et al.,* 2013).

As citocinas são mediadores envolvidos no *turnover* fisiológico do osso (BANDEIRA *et al.,* 2014) e da remodelação óssea associada ao movimento dentário (BLETSA *et al.,* 2006; ALHASHIMI *et al.,* 2001; BASARAN *et al.,* 2006; REN *et al.,* 2007; GIANNAPOLOU *et al.,* 2008, ANDRADE *et al.,* 2012; TADDEI *et al.,* 2012; KUNII *et al.,* 2013). Por exemplo, TNF-α está associado ao recrutamento de osteoclastos e reabsorção óssea nas áreas de compressão do LP (AHUJA *et al.,* 2003; GARLET *et al.,* 2007; LI *et al.,* 2012; ANDRADE *et al.,* 2012), enquanto que IL-6 e IL-1β são capazes de potencializar a reabsorção óssea nessa região ao estimular, de modo sinérgico, a diferenciação osteoclástica (STEEVE *et al.,* 2004; YAMAGUCHI *et al.,* 2004; AZUMA *et al.,* 2013). De modo contrário, IL-10, IL-4, IFN--ɣ inibem a formação de osteoclastos e consequentemente a reabsorção óssea (MANGASHETTI *et al.,* 2005; GARLET *et al.,*2007; MERMUT *et al.,* 2007; IVASHKIV *et al.,*2011; KOHARA *et al.,* 2011; KOHARA *et al.,* 2012; TADDEI *et al.,* 2012;

TADDEI *et al.,*2013), enquanto que IL-17A apresenta papel osteoprotetor ao induzir o aumento de osteoprotegerina (KOONERMAN *et al.,* 2012).

A literatura demonstra que a aplicação de força ortodôntica é capaz de modificar a expressão de biomarcadores no FCG e LP (ALHASHIMI *et al.* 1999; REN *et al*. 2002; REN *et al*. 2007; GARLET *et al*. 2007; GARLET *et al.* 2008, REN & VISSINK, 2008; LUPPANAPORLARP *et al*. 2010; MADUREIRA *et al.,* 2012; BARBIERI *et al.* 2013; PERINETTI *et al*. 2013; TADDEI *et al*. 2013). Os resultados demonstraram picos de moléculas, tais como (IL-8), Interleucina 1-Beta (IL-1β), Interleucina-6 (IL-6), Interleucina-8 (IL-8) Fator de necrose tumoral-alfa (TNF-α), Fator quimiotático de eosinófilos (ECF), Fator de crescimento transformador beta-1 (TGFβ-1) e as quimiocinas CC 2 e 3 (CCL2 e CCL3) durante os estágios iniciais, seja nos dias 1 ou 3. Em seguida, há uma redução gradual dessas moléculas até atingirem níveis basais a partir dos dias 7, 10 ou 21 (UEMATSU *et al.* 1996; ALHASHIMI *et al*. 2001, REN *et al.* 2002; BASARAN *et al*. 2006; REN *et al.* 2007; REN & VISSINK 2008; LUPPANAPORNLARP *et al.* 2010; BARBIERI *et al*. 2013). De modo interessante, a Prostaglandina-2 (PGE_2) demonstrou um padrão de expressão semelhante independente do tipo de força aplicada (contínua ou interrompida) (REN & VISSINK 2008), enquanto que o aumento no grau da magnitude da força aplicada esteve associado ao aumento nos níveis de IL-1β (REN & VISSINK 2008, LUPPANAPORLARP *et al.* 2010). Além disso, a produção de IL-1β foi maior após a aplicação da força ortodôntica de curta duração (24 h) em comparação a forças de longa duração (REN Y *et al.,* 2007). Em estágios posteriores, um segundo influxo de citocinas pode ocorrer, como observado pelo aumento de IL-6, CCL2 e CCL3 no dia 12 (MADUREIRA *et al.,* 2012) e IL-8 após 30 dias (REN *et al*. 2007). Entretanto, a origem dessas citocinas ainda necessita ser determinada. A hipótese é de que estas

moléculas sejam provenientes das células presentes no LP, osso alveolar e tecido gengival (UEMATSU *et al.,*1996; OKADA *et al.,* 1997; ALHASHIMI *et al.,* 2001; BLETSA *et al.,* 2006 LEE *et al.,* 2007; LI *et al.,* 2011. KUNII *et al.,* 2013). A literatura demonstra que essas moléculas também são produzidas por leucócitos, fibroblastos e osteoblastos (UEMATSU *et al.,* 1996; OKADA *et al.,* 1997; ALHASHIMI *et al.,* 2001; LEE *et al.,* 2007, KUNII *et al.,* 2013). As citocinas presentes no FCG possivelmente refletem o microambiente dos tecidos periodontais (REN & VISSINK*,* 2008). Além disso, as moléculas identificadas no FGC apresentam papel na sinalização das células ósseas (BERTOLINI *et al.,* 1986*;* LACEY *et al.,* 1998; REN & VISSINK, 2008), sendo capazes de mediar a remodelação óssea mecanicamente induzida (DAVIDOVITCH *et al.,* 1988; REN & VISSINK, 2008). A contribuição do FGC neste processo necessita, entretanto, de melhor esclarecimento.

2 JUSTIFICATIVA

Os mecanismos envolvidos no processo de remodelação óssea e movimentação dentária ortodôntica estão associados à liberação de biomarcadores no periodonto, os quais são responsáveis por regular a resposta tecidual frente a diferentes estímulos. O modelo experimental utilizando força ortodôntica é um dos mais utilizados, pois induz um processo inflamatório asséptico e transitório no periodonto, que permite o estudo dos mecanismos associados à remodelação óssea sem a influência bacteriana. Nesse contexto, observamos, em um estudo prévio realizado pelo nosso grupo de pesquisa, aumento dos níveis de marcadores inflamatórios no ligamento periodontal humano (MADUREIRA *et al.,* 2012).

Uma vez que a análise do FCG apresenta a vantagem de ser não invasiva, ela permite sua coleta em diferentes momentos durante o tratamento ortodôntico (KRISHNAN *et al.,* 2006; REN *et al.,* 2008; VAN GASTEL *et al.,* 2011). Desta forma, esta metodologia poderia ser utilizada para monitorar as modificações ocorridas no LP, pois se postula que o FCG seja representativo das mudanças que ocorram nesse sítio (PERINETTI *et al.,* 2013). Existe uma grande diversidade nas metodologias empregadas nos estudos avaliando FCG, o que dificulta a interpretação. Novos estudos são necessários com o intuito de esclarecer o potencial do FCG como ferramenta de diagnóstico para o monitoramento clínico (REN & VISSINK, 2008). Além disso, ainda não existem estudos que comparem os níveis de citocinas entre FCG e LP em uma mesma amostra. Os resultados obtidos podem ser úteis na ampliação do conhecimento sobre a biologia do movimento dentário e possivelmente no monitoramento de efeitos colaterais relacionados ao tratamento ortodôntico.

3. OBJETIVOS

3.1Geral

Avaliar, no ligamento periodontal e fluido crevicular gengival, de dentes de humanos submetidos aplicação de força ortodôntica, a expressão de moléculas envolvidas na remodelação do tecido ósseo e ligamento periodontal.

3.2 Específicos:

3.2.1 Avaliar a expressão de Interleucina-2, Interleucina-4, Interleucina-6, Interleucina-10, Interleucina-17A, Fator de necrose tumoral-alfa e Interferon-gama no ligamento periodontal e fluido crevicular gengival de pré-molares de humanos extraídos após 1, 3, 7, 14, 21 e 28 dias de aplicação de força ortodôntica.

3.2.2 Correlacionar os níveis de expressão dos marcadores avaliados entre o ligamento periodontal e fluido crevicular gengival.

3.2.3 Realizar uma revisão sistemática da literatura sobre os níveis de TNF-α no FCG como marcador de alterações inflamatórias no ligamento periodontal.

4. MATERIAL E MÉTODOS

4.1 Delineamento experimental

A padronização metodológica para a coleta do LP ocorreu em um estudo anterior (MADUREIRA *et al.,* 2012). No presente, 23 pacientes foram incluídos. O total de 68 dentes de ambas as arcadas foram obtidos, sendo os estudo dentes experimentais foram submetidos à força ortodôntica de 0,980 N em direção apical por 1, 3, 7, 14, 21 ou 28 dias. Estes períodos foram selecionados com base nos dados obtidos anteriormente (UEMATSU et al. 1996; ALHASHIMI et al., 2001; REN et al. 2007; MADUREIRA *et al.,* 2012) e dados da literatura (BUCK & CHURCH, 1972). Os dentes contra-laterais, sem aparelhos ortodônticos, foram utilizados como controle. O FCG foi coletado imediatamente antes das exodontias. Após esse procedimento, foi realizada a coleta do LP em toda sua extensão. Devido ao pequeno volume de fluido gengival obtido nas coletas, a expressão de citocinas no LP e FCG foi avaliada empregando-se *cytometric bead array* (CBA). Essa metodologia permite análise de múltiplas moléculas utilizando um volume pequeno (1/7) de amostra em comparação Enzyme-Linked Immunosorbent Assay (ELISA) convencional.

4.2 Protocolo experimental

4.2.1 Amostra

Os pacientes do curso de especialização em ortodontia, do Departamento de Odontopediatria e Ortodontia da Faculdade de Odontologia da UFMG foram

selecionados para participar deste estudo. Após análise clínica e da documentação ortodôntica, os indivíduos com indicação de exodontia de pré-molares para a realização do tratamento ortodôntico foram selecionados. Foram considerados ainda como critérios de inclusão: (1) pacientes saudáveis sem alteração sistêmica, tais como diabetes *mellitus* ou osteoporose; (2) ausência do uso de antibióticos, anti-inflamatórios ou hormônios por até seis meses antes do início do estudo; (3) pacientes com boa saúde periodontal e nenhuma evidência radiográfica de perda óssea periodontal.

4.2.2 Aprovação pelo comitê de ética em pesquisa (COEP)

Este projeto de pesquisa possui aprovação pelo comitê de ética em pesquisa (COEP) da UFMG (protocolo número 175.320/2012) (**Anexo A**). O Termo de consentimento livre e esclarecido foi obtido de todos os participantes.

4.2.3 Avaliação periodontal

a) Índice de placa

Na consulta inicial, os índices de placa de Silness e Löe (PI) (Löe & Silness, 1964) e o de sangramento gengival (Ainamo & Bay,1975) foram obtidos para certificar que os participantes possuíam higiene oral satisfatória antes do início do estudo. O índice de placa de Silness & Loe (1964) possui os scores: (0) para ausência de depósitos de biofilme; (1) visualização de biofilme após a sua remoção com sonda; (2) placa clinicamente visível; (3) placa abundante (**Figura 1**). Foram obtidos os dados para as superfícies vestibular, lingual, mesial e distal de todos os dentes presentes, sendo registrado o maior valor encontrado nas superfícies examinadas. O valor médio do índice foi obtido por indivíduo.

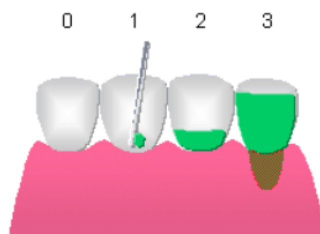

Figura 1. Índice de placa de Silness & Loe (1964).

b) Sangramento à sondagem

O índice de sangramento gengival de Ainamo & Bay (1975) foi realizado utilizando-se a sondagem suave do sulco gengival com sonda periodontal milimetrada (Sonda MM FOA – Williams 23 – Trinity, São Paulo, SP, BRA). Os dados concernentes ao sangramento à sondagem foram registrados para todas as superfícies (distal, vestibular, mesial e lingual). A leitura do sangramento foi realizada entre os 10 primeiros segundos após a sondagem. A ocorrência de sangramento após sondagem foi registrada em cada face de forma dicotômica, para sua presença (1) ou ausência (0). O número de sítios positivos é expresso pela porcentagem do número de sítios examinados. Esse índice tem correlação significante com o índice de sangramento gengival de Löe & Silness (1963) e são utilizados em estudos clínicos de curta duração.

c) Profundidade de sondagem

A profundidade de sondagem foi obtida mensurando-se, com sonda periodontal milimetrada (Sonda MM FOA – Williams 23 – Trinity, São Paulo, SP, BRA), a distância da margem gengival ao fundo do sulco ou bolsa gengival. A sondagem circunferencial foi realizada em todas as superfícies dos dentes incluídos no exame e os registros anotados no periodontograma.

d) Diagnóstico

Foram incluídos apenas os pacientes com uma média de índice de placa ≤ 1.0 e média de índice de sangramento gengival ≤ 0,20. A alteração periodontal foi considerada presente quando foram detectados quatro ou mais dentes com um ou mais sítios com profundidade de sondagem ≥ 4 mm e perda de inserção clínica ≥ 3 mm (LÓPEZ *et al* 2002) associada a sangramento à sondagem no mesmo sítio. Os pacientes que apresentaram alteração periodontal não foram incluídos no estudo, e foram orientados e referenciados para o tratamento da mesma.

4.2.4 Coleta do material

a) Aplicação de força ortodôntica

Em cada arcada, um pré-molar recebeu força ortodôntica utilizando um aparelho fixo composto por bráquetes (Aditek do Brasil Ldta, Cravinhos, SP, BRA) e tubos (Eurodonto, Curitiba, PR, BRA) Roth 22'' colados com Transbond XT (3M Unitek, Monrovia, CA, EUA). O outro pré-molar contra-lateral serviu como controle. A força foi aplicada empregando-se um cantiléver de Titânio Molibidênio (TMA) 0,017 × 0,025" (Morelli orthodontics, Sorocaba, SP, BRA). Essa força foi medida com um tensiômetro digital (modelo FGV-1X, Nidec-Shimpo, Itasca, ILL, EUA) com intensidade de 0,980 N em direção apical no dente experimental (**Figura 2**), enquanto que o dente contralateral foi utilizado como controle, sem aparelho. É importante ressaltar que esses dentes foram extraídos para que o tratamento ortodôntico fosse realizado e que a força aplicada nesses dentes não trouxe risco aos participantes. O aparelho foi mantido por períodos de 1, 3, 7, 14, 21 ou 28 dias.

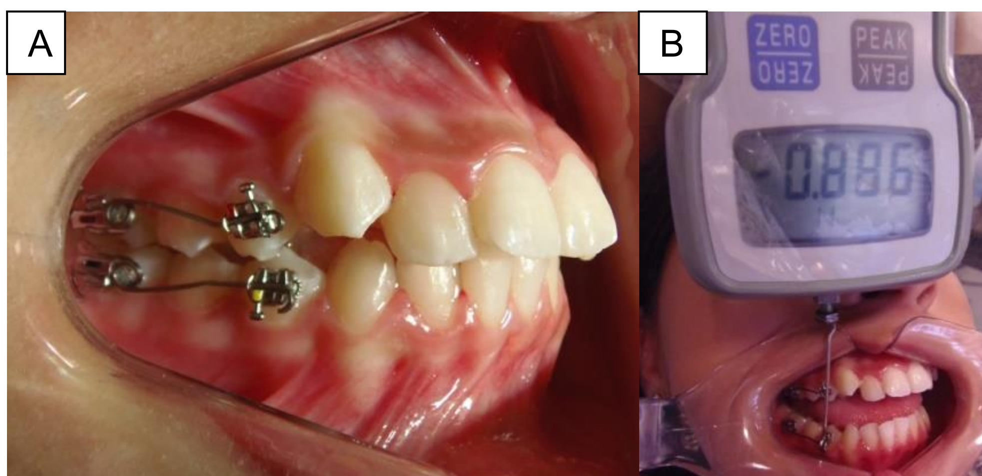

Figura 2. Aparelho ortodôntico ativado: **(A)** O aparelho ortodôntico consistiu de bráquetes e tubos colados com resina Transbond™ XT e cantiléver TMA de 0,017" ×0,025". (**B**) A magnitude da força aplicada foi de 0,980 N em direção apical, mensurada com tensiômetro digital (Nidec-Shimpo brand, modelo FGV-1X)

.

b) Coleta do fluido crevicular gengival e ligamento periodontal

Inicialmente o Periotron® 8000 (model 400 8000 Precision Gingival crevice Fluid Meter 8000. Oralflow Inc). foi calibrado com fitas de papel absorvente *Periopaper* (Periopaper™ Gingival Fluid Collection Strips, Oralflow Inc, Smithtown, NY, EUA) secas. Previamente a coleta do fluido gengival, para os pacientes que apresentarem acúmulo de placa nos sítios de coleta (índice ≥ 1 de Silness & Loe de 1964), a mesma foi removida com escova de Robinson apenas e aguardou-se 5 minutos para iniciar a coleta de fluido gengival. Foi realizado o isolamento relativo com afastador bucal, rolos de algodão e sugador. Os dentes foram secos gentilmente com gaze estéril, sem que ocorresse o pressionamento do sulco gengival e periodonto para evitar extravasamento do FCG. O FCG foi coletado com fitas de papel absorvente *periopaper* durante 30 segundos, inseridas 1 mm ou até

atingir resistência nos sítios mésio-vestibular e disto-vestibular. A quantidade de FCG coletada foi quantificada com o aparelho Periotron® 8000 (REN *et al.,*2002; YAO *et al.,* 2003; LEE *et al.,* 2004; KRISHNAN & DAVIDOVITCH *et al.,* 2006; REN *et al.,* 2007; REN *et al.,* 2008*)* (**Figura 3**). Ambos *periopapers* foram inseridos em *eppendorfs* estéreis, separados por indivíduos, e imersos em nitrogênio líquido até o seu armazenamento em freezer -80°C para posterior análise. As leituras do Periotron® 8000 foram convertidas para o volume em microlitros empregando-se uma curva padrão de soro humano (CHAPPLE *et al*. 1999; TOZUM *et al*. 2004). Amostras contaminadas com sangue ou saliva foram excluídas. Neste caso, os dentes foram lavados com jato de água e aguardou-se 10 minutos para uma nova coleta de fluido gengival.

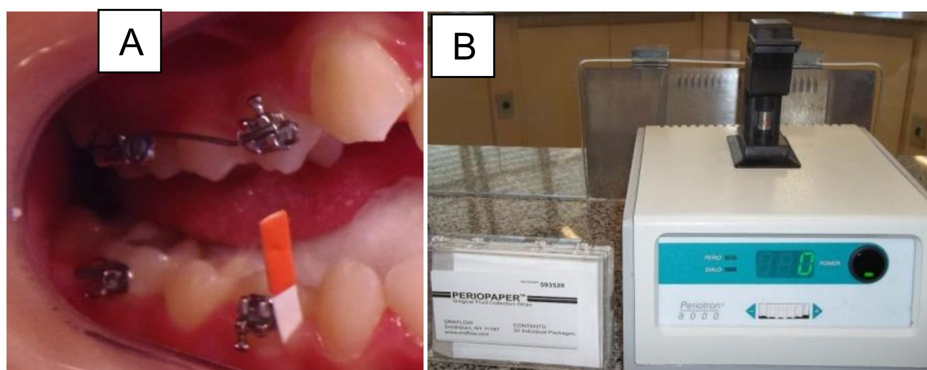

Figura 3. Coleta do fluido crevicular gengival: **(A)** O FCG foi coletado sob isolamento relativ utilizando-se fitas de *Periopaper*, inseridas 1 mm no sulco gengival, nas regiões mésio-vestibular e disto-vestibular durante 30 segundos. **(B)** Aparelho Periotron® 8000 utilizado para quantificar o FCG absorvido pelas fitas de *Periopaper.*

Em seguida, os pré-molares foram extraídos e todo LP foi coletado utilizando cureta periodontal Gracey (Maximus, Contagem, MG, BRA) (**Figura 4**).

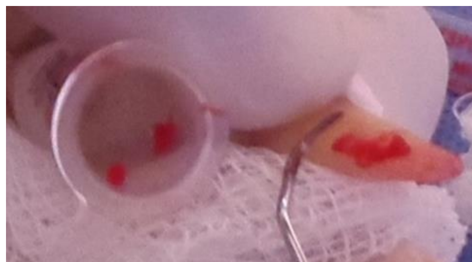

Figura 4. Coleta do ligamento periodontal.

4.2.5 Dosagem de citocinas

a) Processamento do Fluido crevicular gengival

Para as amostras de fluido crevicular gengival, foram adicionados 50 µL de solução de extração de citocinas [PBS (0,4 mM NaCl, 10 mM $NaPO_4$) com inibidores de proteases (0,1 mM PMSF, 0,1 mM Benzethonium clorídrico, 10 mM EDTA e 0,01 mg/mL Aprotinina A, pH 7.4) e Tween 20 (0,05%)], por *periopaper*. Em seguida os tubos foram levados ao vórtex e depois centrifugados por 10 min em 10.000 RPM a 4ºC. O sobrenadante foi armazenado em um *eppendorf* e o mesmo processo foi repetido com os demais *perioppappers*. Os sobrenadantes foram unidos em um único *eppendorf* e passados em vortex. Em seguida, foram separados 50 µL do sobrenadante para análise de citocinas por CBA e as demais amostras armazenadas em freezer a -80ºC.

b) Processamento do Ligamento Periodontal

Inicialmente, os tubos contendo as amostras de ligamento periodontal foram pesados. Para cada 100 mg de tecido, foi adicionado 1 mililitro (ml) de solução de extração de citocinas [PBS (0,4 mM NaCl, 10 mM $NaPO_4$) com inibidores de

proteases (0,1 mM PMSF, 0,1 mM Benzethonium clorídrico, 10 mM EDTA e 0,01 mg/mL Aprotinina A, pH 7.4) e Tween 20 (0,05%)], e homogeneizados utilizando-se de um triturador de tecidos (PowerGen 100, Fisher Scientific). Em seguida, as amostras foram centrifugadas a 10.000 RPM por 10 minutos, 4ºC. O sobrenadante foi separado e uma nova diluição foi realizada com a mesma quantidade de solução de extração de citocinas previamente adicionada (diluição final 1:2). Foram separados 50 µL do sobrenadante para análise de citocinas por CBA e as demais amostras armazenadas em freezer a -80ºC.

c) Cytometric Bead Array (CBA)

Análises do LP e FCG foram determinadas utilizando o kit de CBA Th1/Th2/Th17 CBA kit para humano (BD Biosciences, San Diego, CA, EUA) e analisados em BD FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA, EUA), seguindo as orientações do fabricante. O manual para *BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Kit 560484* está também disponível *on line* (http://www.bdbiosciences.com/external_files/Doc_Recon_2.0/pm/others/23-12381.pdf#search=instruction manual CBA. Para obtenção do resultado por concentração de proteína por volume de FCG, dividiu-se a concentração total de proteínas pela leitura do volume de fluido obtido, sendo o resultado final expresso em pg/µL. Para o LP, os resultados foram expressos em picogramas (pg) por 100 mg tecido.

5 RESULTADOS E DISCUSSÃO

Os resultados e discussão serão apresentados na forma de dois artigos científicos. O **Artigo I** "*Cytokine measurements in gingival crevicular fluid and periodontal ligament: are those correlated*?" foi recentemente aceito para publicação pelo periódico *American Journal of Orthodontics and Dentofacial Orthopaedics* – *Referência* AJODO-D-14-00708R2. O objetivo principal foi analisar a expressão das citocinas IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α, INF-γ no FCG e LP, após aplicação de força. Os resultados demonstram um aumento dos níveis de IL-6 no LP após 1 dia de aplicação de força. Observou-se também um padrão distinto de expressão de citocinas no LP e FGC após o estímulo mecânico. Os dados sugerem que não existe uma correlação direta clara sobre a contribuição de moléculas liberadas no LP para a composição do FGC.

O Artigo II entitulado "*TNF-α in GCF as a diagnostic marker for periodontal disease: a systematic review"* foi desenvolvido com o objetivo de avaliar se a produção de TNF-α no FCG pode ser utilizada como marcador para doença periodontal. O modelo de periodontite e não de movimentação ortodôntica foi escolhido, pois as oscilações de moléculas inflamatória são melhor caracterizadas na doença periodontal. Esse conhecimento poderia fortalecer o uso do FCG no monitoramento dessa doença, assim como ser extrapolado para a MDO. Entretanto, os dados da literatura apresentaram resultados conflitantes, não permitindo ainda utilização de TNF-α no FCG como ferramenta de monitoramento das alterações inflamatórias no ligamento periodontal.

5.1 Artigo científico I

Cytokine measurements in gingival crevicular fluid and periodontal ligament: are those correlated?

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Cytokine measurements in gingival crevicular fluid and periodontal ligament: are those correlated?

ABSTRACT

Introduction: Mechanical stress can induce molecular changes in gingival crevicular fluid (GCF) and periodontal ligament (PDL). It is still not clear if changes in PDL and GCF are linked. This article aimed to analyze the expression of cytokines in GCF and PDL after mechanical stress. **Methods**: 23 healthy patients were included. The experimental group consisted of premolars subjected to a force of 0.980 N for 1, 3, 7, 14, 21 or 28 days. The contralateral teeth were controls. GCF and PDL samples were collected at same time points for analysis of cytokines using cytometric bead array. **Results:** IL-6 production was significantly elevated in PDL on day 1 after force application. Significant strong positive correlations between GCF and PDL in experimental group were seen on days 3 (IFN-γ), 7 (IL-10), 14 (IL-17A), 28 (IL-17A, TNF- α) and significant negative strong correlation on days 14 (IFN-γ) and 21 (IL-2, IL-10). **Conclusions**: Different pattern of IL-6 expression was seen in PDL and GCF after mechanical stress. Despite occasional correlations between GCF and PDL, the molecular contribution of PDL to GCF changes could not be clearly defined by the purposed model.

INTRODUCTION

Gingival crevicular fluid (GCF) is a transudate of interstitial tissues produced by an osmotic gradient.[1,2] This initial pre-inflammatory fluid, on stimulation, becomes an inflammatory exudate, approaching serum concentrations.[3] It contains a mixture of molecules from blood, host tissue, and plaque biofilms, such as electrolytes, proteins, small molecules, cytokines, antibodies, enzymes and bacterial antigens.[3,4] Under conditions that induce bone and connective tissue remodeling, such as periodontitis and orthodontic tooth movement (OTM), there is a release of inflammatory mediators in GCF and periodontal ligament (PDL). In these circumstances, augmented levels of different cytokines have been reported.[1,2,4-7] GCF provides a unique window for analysis of periodontal condition. However, there is no practical and accurate periodontal indicator based on GCF due to extremely complex issues such as periodontal diseases, their microbial causes, cell regulation and tissue reactions during inflammation and healing.[3,8] In addition, the information regarding the release of molecules in GCF after OTM is still limited.[9]

Although GCF is considered to be associated with changes in PDL,[10] there are no studies available that concomitantly evaluated cytokines expression in GCF and PDL. It is hypothesized that mechanical stimuli might trigger similar molecular changes within PDL and GCF. It is still not clear if these changes are, in part, linked. The aim of this study was to estimate the contribution of PDL to GCF changes, concomitantly evaluating molecular profile in these two compartments along mechanical stimuli.

MATERIAL AND METHODS

Patients

Twenty-three patients (11 male, 12 female), aged 10 to 24 years (mean 13.43 ± 2,64 years), seen in the Department of Pediatric Dentistry and Orthodontics, Faculty of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, from August 2011 to July 2013, were selected to participate in this study. All patients required extraction of the premolars for orthodontic reasons, such as crowding, dental biprotrusion or class II malocclusion, prior the start of orthodontic treatment. The inclusion criteria were as follows: (1) healthy patients with no evidence of type 1 or type 2 diabetes mellitus or osteoporosis; (2) patients who had not taken systemic antibiotics, anti-inflammatory or hormonal drugs for 6 months before the study; (3) patients who required tooth extractions before treatment with fixed appliances; and (4) patients with good periodontal health and no radiographic evidence of periodontal bone loss.

This study was approved by institutional ethics committee (protocol number 175.320/2012) and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from each participant and their guardians when subject was less than 18 years of age. At the first appointment (baseline), the Silness and Löe Plaque Index (PI)[12] and Gingival Bleeding Index (GBI)[13] were recorded to ensure that patients had no periodontal alteration at start of study. The mandibular or maxillary premolars without orthodontic appliances were used as controls. The experimental group consisted of the contralateral teeth from the same arch that previously received orthodontic mechanical loading. The orthodontic appliance consisted of 0.022×0.028-in light Roth tubes (Eurodonto, Curitiba, PR, BRA) and brackets (Aditek do Brasil Ldta, Cravinhos, SP, BRA) was bonded with Transbond XT (3M Unitek, Monrovia, CA, USA). A

0.017×0.025-in beta-titanium alloy cantilever and a 0.010-inmetallic ligature (Morelli orthodontics, Sorocaba, SP, BRA) were placed between premolar and first molar on the same side by an orthodontist (D.F.M.) (**Figure 1A**). A force in apical direction was applied to premolar. The force magnitude was 0.980 N, measured by a digital tensiometer (model FGV-1X, Nidec-Shimpo, Itasca, Ill, USA) that was perpendicular to cantilever (**Figure 1B)**. No other forces were applied to teeth before or during this phase. The experimental teeth were randomly selected. If a patient had 4 premolars to be extracted, pairs of teeth were allocated to 2 time points. The patients were instructed about proper oral hygiene. After mechanical stimuli, the teeth were extracted at following time points: 1, 3, 7, 14, 21 or 28 days. Just before extraction, force magnitude was measured again. The experimental and control teeth were extracted at the same moment. Several time points were chosen to allow the evaluation of cytokine fluctuation after mechanical stimuli concomitantly in PDL and GCF. After the experimental period, the patient was referred to start orthodontic treatment.

Samples collection and analysis

GCF samples were collected using *periopaper* strips (Periopaper™ Gingival Fluid Collection Strips, Oralflow, Smithtown, NY, USA) at mesiobuccal and distobuccal sites of control and experimental premolars. Teeth were previously isolated with cotton rolls, cleaned of plaque deposits and dried gently with air before paper strips were carefully inserted 1 mm into the gingival crevice during 30 seconds. Samples of GCF absorbed by *periopapers* were measured using a calibrated Periotron® 8000 (model 400 8000 Precision Gingival crevice Fluid Meter 8000. Oralflow Inc). Readings from Periotron were converted to an actual volume (microliters) by reference to a standard curve calibrated with human serum.[14,15] PDL of each extracted tooth was taken from all root surfaces immediately scraped by using a 13/14 Gracey curette (Maximus, Contagem, MG, BRA) (**Figure 1C**). All tooth extractions and sample

collection were performed by a single professional (D.F.M). Samples of GCF and PDL were separately placed in sterile tubes that were immediately immersed in liquid nitrogen, and then kept frozen at -80°C for further analysis. Afterward, GCF and PDL samples were homogenized in phosphate-buffered saline solution (0.4 mmol/L of sodium chloride and 10 mmol/L of sodiumphosphate [NaPO4]) containing protease inhibitors (0.1 mmol/L of phenylmethylsulfonyl fluoride [PMSF],0.1 mmol/L of benzethonium chloride, 10 mmol/L of ethylenediaminetetraacetic acid [EDTA], and 0.01 mg/mL of aprotinin A and 0.05% Tween-20 at 1 mg/mL). Mixture was agitated on vortex for 30 seconds and then centrifuged (12.000 g) for 10 minutes at 4°C. Supernatants were collected and assayed with cytometric bead array (CBA). Analyses of PDL and GCF were determined using BD CBA human Th1/Th2/Th17 kit (BD Biosciences, San Diego, CA, USA) and analyzed on a BD FACSCalibur™ flow cytometer (Becton Dickinson and Company BD Biosciences, San Jose, CA, USA). The concentrations of analytes (IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF-α) were performed using a standard curve according to manufacturer's instructions. The sensitivity of the assays was 20 – 5000 pg/mL for all cytokines. Results were expressed in picogram (pg) per mL for GCF and pg per 100 mg of tissue for PDL.

Statistical analysis

Shapiro-Wilks test was used to assess quantitative variables ($P < 0.05$). For cytokines in which normality was seen, parametric tests were used, and for those that there was no normality of cytokines, nonparametric tests were performed. Mann-Whitney test was used to verify the influence of gender on cytokines. Kruskal-Wallis test was performed to compare cytokine levels and types of teeth. Paired *Student t*-test (parametric cytokines) and *Wilcoxon* test (nonparametric cytokines) were used to assess the influence of these molecules on experiment at each time point and to verify the difference of collected GCF volumes between

groups. *Pearson* (when both variables were parametric) and *Spearman* (when at least one variable was nonparametric) correlations were used to assess the association between age, plaque index, bleeding on probing and cytokines, and also the correlation between cytokine levels in PDL and GCF, stratified by experimental and control groups. The analysis was performed for each time point separately. Level of statistical significance was set at $P < 0.05$. All statistical evaluations were performed with SPSS software (version 19.0; SPSS, Chicago, Ill, USA).

RESULTS

A total of 68 premolars were obtained (36 maxillary first premolars, 2 maxillary second premolars, 28 mandibular first premolars and 2 mandibular second premolars). A mean of 5.67 (n=5-6) pairs of teeth was allocated at each time point. The appliances were well tolerated. Demographic description of participants is in **Table 1**. Clinical gingival condition was satisfactory at baseline and no significant difference was seen for PI or GBI indexes between groups. While no difference between GCF volume (VGCF) was seen on days 1, 3, 14 or 28 ($P > 0.05$), increased VGCF was seen on days 7 and 21 for experimental group ($P < 0.05$). The initially applied force magnitude of 0.980 N was gradually reduced to a mean of 0.866 ± 0.166 N just before experimental teeth extraction. Gender, type of tooth, age of participants and experimental force had no influence on concentrations of examined cytokines at any time points ($P > 0.05$).

Figure 2A shows cytokine concentrations of Th2 response: Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10). Th17: Interleukin 17-A (IL-17A); and Th1: Tumor necrosis factor-alpha (TNF-α) and Interferon-gama (IFN-γ) levels are demonstrated on **Figure 2B.** On day 1, a significant increase of IL-6 was seen in PDL

subjected to mechanical stress in relation to control (P = 0.043). Then, IL-6 levels in PDL were gradually reduced. In GCF, the peak of IL-6 was later than observed for PDL, on day 3, with a subsequent decrease. On day 21, IL-6 concentration in GCF of experimental group was lower than control (P = 0.028). Descriptive analysis shows a similar phenomenon in GCF for IL-10, IL-17-A and IFN-γ. Those cytokines seemed to increase at the beginning of OTM in experimental group, followed by a subsequent decrease on later stages, although there were no significant differences between the groups for any of these evaluated molecules ($P > 0.05$).

The correlation of cytokines in PDL and GCF are described on **Table2.** Interestingly, significant positive strong correlation in experimental group was observed on days 3 (IFN-γ), 7 (IL-10), 14 (IL-17A), 28 (IL-17A, TNF- α) and significant negative strong correlation on days 14 (IFN-γ) and 21 (IL-2, IL-10). For control group, significant negative strong correlation was seen on days 1 (IL-4), 21 (IFN-γ) and 28 (IL-2, IFN-γ).

DISCUSSION

Mechanotransduction during OTM leads to a release of several mediators such as cytokines, chemokines and growth factors in periodontium.[16-21] Mechanical stimuli also trigger molecular changes in GCF,[1,5,6,21] but it is not clear if these changes are linked to those in PDL. In this study, the entire PDL was collected for molecular analysis based on two major reasons: 1) As GCF continuously circulate in PDL, there is no clear cut-difference in the assessment of cytokines in GCF from pressure or tension areas;[21] 2) Regardless the type of tooth movement,[22-25] irregular patterns of stress occur due uneven strain distribution in PDL, making difficult to precisely define sites of tension and pressure.[25]

The Th1/Th2/Th17 cytokines have already been associated to periodontal T-cell responses under conditions of periodontal disease and bone cells regulation.[26] This paper demonstrated for the first time the kinetics of Th1 (TNF-α, IFN-γ), Th2 (IL-2, IL-4, IL-6, IL-10) and Th17 (IL-17A) cytokine release in human PDL and GCF after mechanical stimuli. The current findings provided data specific for orthodontic intrusion, in which strong correlations in cytokine production between PDL and GCF were only seen at few time points. However, mechanical stimulation did not induce significant changes in most of the parameters investigated, except for IL-6 on day 1. Accordingly, we have previously observed, for IL-6, a significant raised levels on day 12 in PDL subjected to orthodontic force.[27] Moreover*, in vitro* studies showed an up regulation of IL-6 after static compressive force[9,28] and, under compression, PDL fibroblasts were more responsive than gingival fibroblasts.[28] Surprisingly, in GCF, our results showed no alteration in IL-6 levels, which is in accordance with Ren *et al.,* (2002)[5] for adult population. However, this cytokine has been reported to be increased on day 1 after OTM.[5,30] In contrast, our results demonstrated reduced levels of this molecule in

GCF on day 21 in experimental group, probably associated with the activation of regulatory mechanisms. Furthermore, IL-6 itself amplifies and perpetuates its production by gingival and periodontal fibroblasts[16] and patients with severe root resorption due to OTM presented raised basal levels of IL-6.[9]

In this study, the other Th2 cytokines (IL-2, IL-4 and IL-10) showed no statistical differences between the groups associated with OTM, either in PDL or GCF. These results are in agreement with the literature, in which OTM have not changed the expression of IL-2 or IL-4 in GCF[10,31] or IL-4 in PDL.[32] However, increased IL-10 level in the experimental group was reported after OTM, especially at tension sites of PDL.[33]

Neither differences in Th17 (IL-17A) or Th1 (TNF-α and IFN-γ) response were seen associated to OTM in this article. The literature shows that IL-17A appears to initiate osteoprotective mechanisms, suggesting that IL-17A may activate a regulatory feedback mechanism in the PDL cells.[34] Differently from our results, the literature shows that IFN-ɣ increases on late stages of OTM,[35] controlling excessive osteoclastogenesis[36] and associated with an increase in trabecular bone volume.[37] Furthermore, as previously observed,[10] our results showed no differences in TNF-α levels, possibly because TNF-α is released at very early stages after mechanical stimuli and is followed by a negative-feedback mechanism that shuts down quickly the production of this cytokine.[20,38-41] or TNF-α is mainly released during force application and not produced de novo.[32] During OTM, this cytokine was reported to be more increased in the compressed than tension sites of human PDL[33] and raised levels on day 1 in GCF have already been described.[6,30] TNF-α, IL-6 and Interleukin-1 beta can stimulate osteoclast differentiation in a synergic fashion and this interaction may enhance bone resorption.[32,43] TNF-α induces osteoblasts-like cells to produce IL-6 in a dose dependent manner.[44] After TNF-α, IL-6 is produced during inflammation. Subsequently, IL-6 inhibits the secretion of TNF-α.[45]

GCF analysis is a non-invasive method[2,3,5,11,21] with potential for serving as indicators of disease and monitoring response during and after therapy[3,8] These factors led us to develop this model in which bacterial influences present in periodontal disease were avoided and controlled mechanical stimuli triggered inflammation in periodontal tissues. This purposed model tried to correlate two different sites of human periodontium. The results demonstrated that strong correlations between GCF and PDL were seen only at a few moments. GCF is composed by a mixture of substances derived from serum, leukocytes, bacteria, activated epithelial cells, connective tissue cells, and bone cells.[3] The inconstant correlation between the molecules expressed in GCF and PDL, seen in this paper, might be associated to different origin of these cytokines. It might be supposed that the cells around gingival sulcus contribute much more to GCF molecules than a distant PDL. The resultant cytokine level measured in GCF is possibly the result of sum of various cellular responses to mechanical stimuli to several locations, which is considered a limitation of *in vivo* experiments.[10] In this context, it is still not clear if the PDL produces cytokines in a sufficient amount to diffuse into the GCF. It would be overly simplistic to expect that the analytes in the GCF were exclusively linked to the PDL cells and extracellular matrix. Our results may suggest that a complex interaction in regulation process of cytokines is present, once GCF and PDL seemed to exhibit differential responses to mechanical stimuli and also during physiological condition over time.

Several studies tried to demonstrate cytokines production during OTM. There is a great difference in methodologies concerning the time, appliances, direction and magnitude of forces applied. In this study, an apical direction force was chosen to avoid the influence of secondary forces such as premature occlusal forces. Furthermore, according to the stimuli applied (heavy or light forces), results showed an up regulation or no differences in cytokine levels in GCF[1,2,5,6,7] and PDL[24,30,46-48] after mechanical loading. Usually, there are peaks of cytokine levels during early stages, either at 24h[1,5,7,30,32] or day 3,[32] followed by a decrease

towards baseline levels on late stages such as days 7,[10,30] 10[32] or 21.[27] In this study, levels of cytokines did not show any increase in GCF, although a tendency of increase of IL-2, IL-6, IL-10 and INF-Ɣ at the beginning of tooth movement (days 1-3) was detected, no statistical significance was reached. Perhaps it has occurred because our methods performed mechanical stimuli right after the placement of the orthodontic appliances. Controversially, the studies in which those cytokines were increased,[10,30,49] the patients were already undergoing orthodontic treatment with fixed appliances in control and experimental sites. We believe these differences might have influenced the results.

Finally, large individual variation in biological response to mechanical loading occurs.[50,51] These differences could be related to variation in age,[5] gender,[52] bone/mineral density, anatomic structures, cellular activity within the PDL and alveolar bone.[51] Most studies *in vivo* regarding OTM have concentrated on changes occurring within PDL. However, PDL can only provide a partial explanation of bone remodeling process, and more attention has focused lately on wider response.[53] Determining levels of various cytokines after mechanical stimuli may contribute to better understanding of PDL and GCF towards mechanical stress and their physiology. The differential cytokine expression seen in this paper might be associated with differences in composition of PDL and GCF or in biological response. Moreover, as GCF is sensitive to plaque and saliva contamination,[54] this bias must be considered during interpretation of the results. Other sources of cytokines such as bone, gingiva, blood vessels and external factors such as plaque accumulation might interfere with GCF and PDL microenvironments. Considering the wide range of biological responses and interfering factors in both PDL and GCF, the number of individuals participating in this study may be a limiting factor. In addition, due to the numerous pairwise comparisons and correlations performed in this study, it is assumed that there is a risk of type I error, thus data should be interpreted with caution. However, these results might be used as reference for

planning further studies once no previous research regarding this issue is available in the literature.

CONCLUSIONS

Differential expression of IL-6 in human periodontal ligament and gingival crevicular fluid were observed, indicating that IL-6 might play a role in bone remodeling process after mechanical stimuli. Despite punctual strong correlations between GCF and PDL, the purposed model could not clearly define the molecular contribution of PDL to GCF changes, once the source of GCF components is complex and not only derived from PDL.

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FIGURE LEGENDS

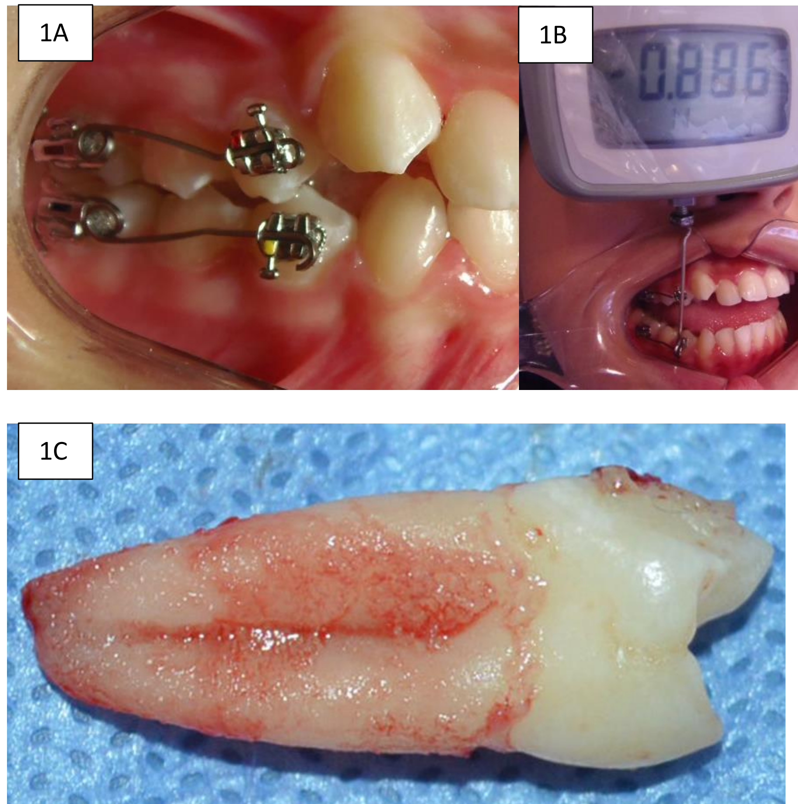

Figure 1. **A**, An activated orthodontic appliance consisting of a 0.022×0.028-in bracket and tube bonded with light cure adhesive and a 0.017×0.025-in beta-titanium alloy cantilever; **B**, the force was set at 0.980 N in the apical direction, and the force magnitude was measured with a digital tensiometer; **C,** PDL before scrapping.

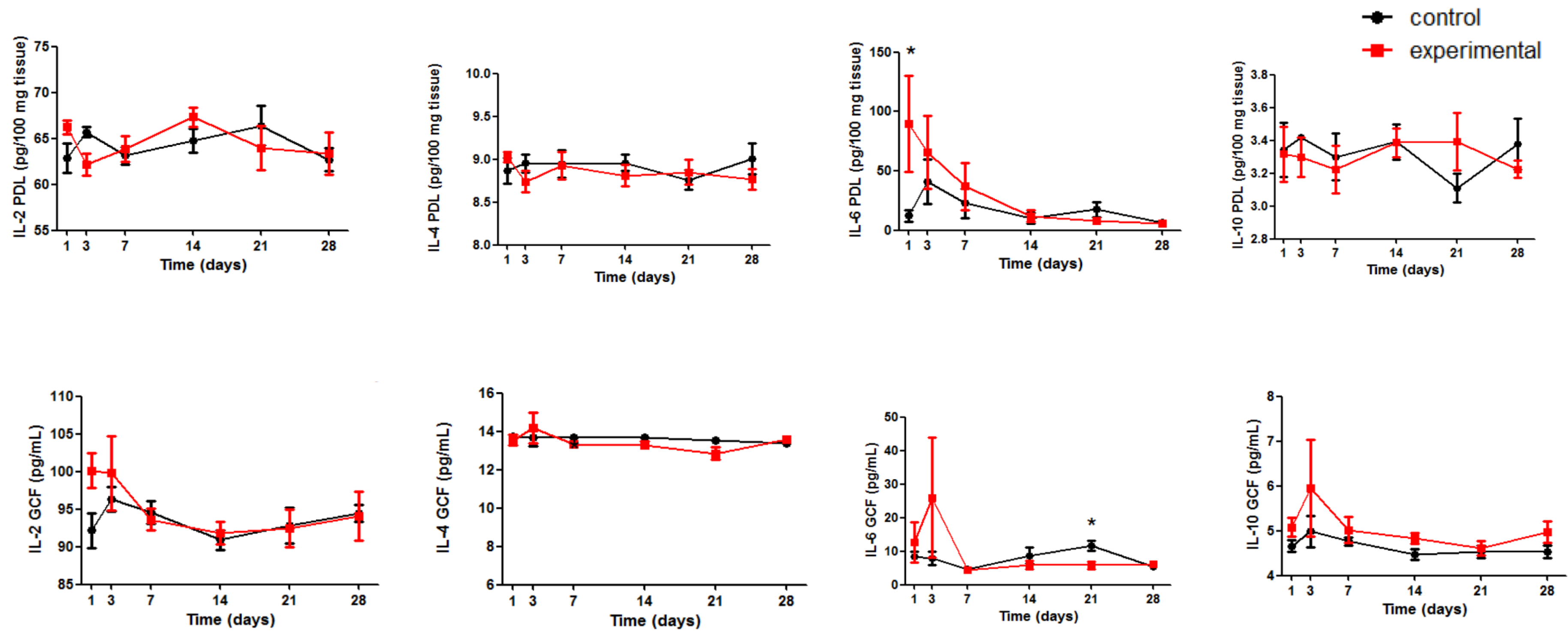

Figure 2A, Time course of Th2 (IL-2, IL-4, IL-6, IL-10) cytokines production in PDL and GCF. In the experimental group, increased levels of IL-6 in PDL on day 1 and reduced levels of IL-6 in GCF on day 21 were observed. The data were expressed as the mean ± standard deviations.**P* < 0.05 comparing the groups at the same timepoint. Paired *Student* t-test for IL-2 PDL, IL-2 GCF, IL-4 PDL and IL-10 PDL; *Wilcoxon* test for IL-4 GCF, IL-6 PDL, IL-6 GCF, IL-10 GCF;

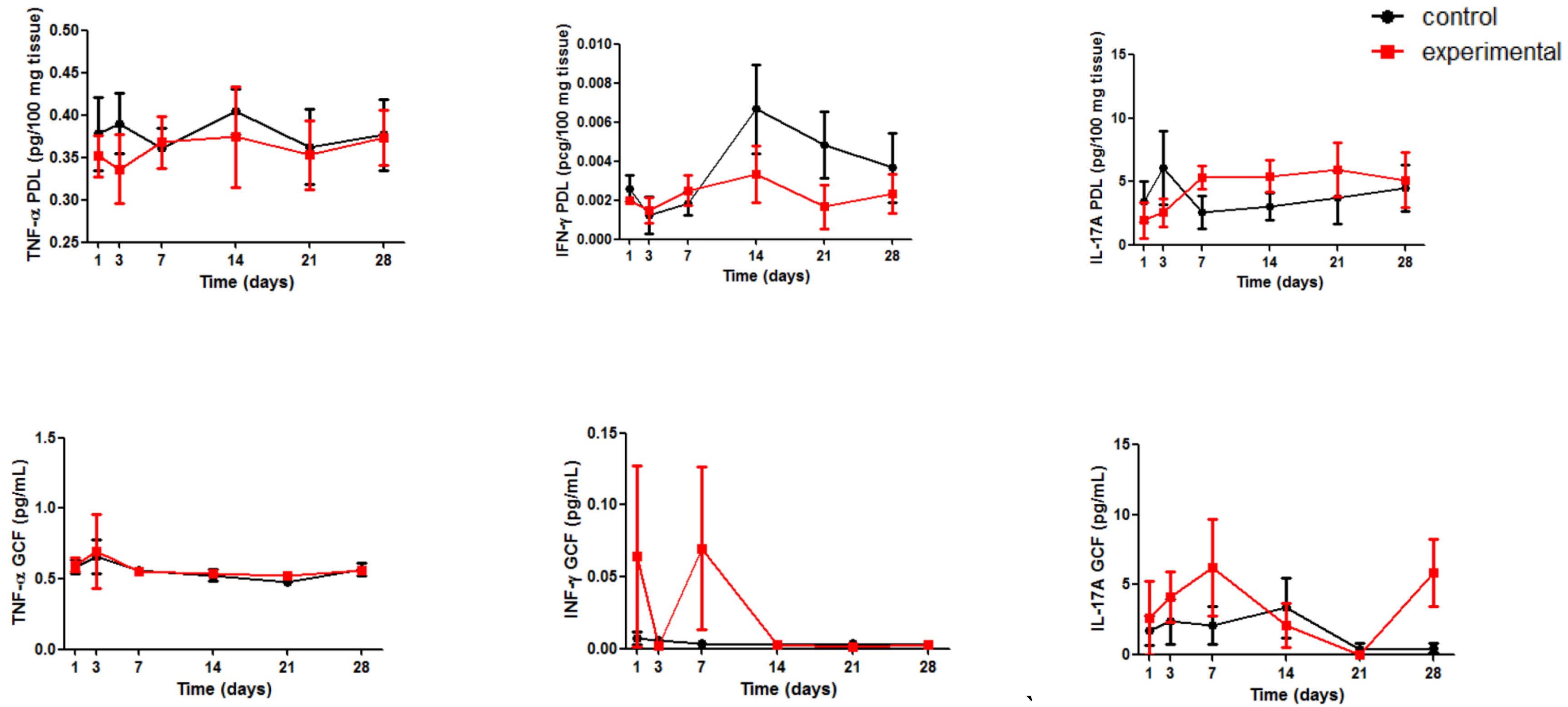

Figure 2B, Time course of Th1 (TNF-α and IFN-γ) and Th17 (IL-17A) cytokine production in PDL and GCF, in which no statistical difference was observed.*$P < 0.05$ comparing the groups at the same timepoint. Paired *Student* t-test for TNF-α PDL; *Wilcoxon* test for TNF-α; GCF, IFN-γ PDL, IFN-γ GCF, IL-17A PDL, IL-17A GCF.

Table 1. Demographic distribution of the participants (n=23) at the time points, gingival evaluation and forces applied.

Time point (day)	n	Gender	Age (y) (Mean ± SD)	PI (Mean ± SD)	GBI (Mean ± SD)	Control VGCF (μL) (Mean ± SD)	Experimental VGCF (μL) (Mean ± SD)	Initial force(N) (Mean ± SD)	Final force(N) (Mean ± SD)
1	5	1M, 4F	13.66 ± 3.44	0.09 ± 0.04	0.04 ± 0.02	0.47 ± 0.25	0.44 ± 0.10	0.98 ± 0.00	0.98 ± 0.00
3	5	2M, 3F	15.66 ± 5.22	0.08 ± 0.03	0.03 ± 0.02	0.48 ± 0.19	0.92 ± 0.30	0.98 ± 0.00	0.86 ± 0.13
7	6	3M, 3F	14.50 ± 21.17	0.14 ± 0.06	0.09 ± 0.04	0.47 ± 0.22	1.05 ± 0.30*	0.98 ± 0.00	0.92 ± 0.07
14	6	2M, 4F	12.67 ± 1.67	0.17 ± 0.05	0.10 ± 0.05	0.20 ± 0.13	0.33 ± 0.20	0.98 ± 0.00	0.90 ± 0.09
21	6	2M, 4F	12.17 ± 1.17	0.15 ± 0.04	0.13 ± 0.03	0.22 ±0.08	0.46 ± 0.14*	0.98 ± 0.00	0.81 ± 0.10
28	6	4M, 2F	12.33 ± 1.67	0.11 ± 0.03	0.06 ± 0.03	0.44 ± 0.19	0.52 ± 0.20	0.98 ± 0.00	0.73 ±0.05
Total	23	11 M, 12 F	13.43 ± 2.64	0.12 ± 0.05	0.07 ± 0.05	0.35 ± 0.18	0.57 ± 0.31	0.98 ± 0.00	0.87 ± 0.176

M, Male; F, female; Silness and Löe Plaque Index (PI), Gingival Bleeding Index (GBI), gingival crevicular fluid volume (VCGF); *$P < 0.05$.

Table 2. Correlation of cytokine levels between Periodontal Ligament (PDL) and Gingival Crevicular Fluid (GCF)

	Day 1	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
			Experimental			
			GCF *vs* PDL			
IL-2	- .355	.669	.209	- .115	**- .916***	- .216
IL-4	- .229	- .527	.714	.447	- .551	.103
IL-6	- .600	.600	.714	- .400	.029	.500
IL-10	- .224	.200	**.899***	- .359	**- .899***	- .600
IL-17A	- .395	- .289	- .185	**.894***	- .417	**.895***
IFN-γ	- .148	**.900***	.086	**- .949***	- .516	- .300
TNF-α	- .053	.400	.235	- .400	.058	**.900***
			Control			
			GCF *vs* PDL			
IL-2	.862	.251	.147	.737	.054	**- .891***
IL-4	**- 903***	.708	.800	.505	.549	.052
IL-6	.300	.400	.429	.400	.116	.800
IL-10	- .483	.141	.611	.392	.245	- .041
IL-17[a]	- .860	- .287	- .290	.112	-.417	-.447
IFN-γ	- .103	.700	.429	.205	**- .899***	-.800
TNF-α	- .414	- .095	- .168	- .140	.104	- .301

Correlations between PDL and GCF values for each time point. *Pearson´s* correlation for IL-2 experimental and IL-2, IL-4, IL-10, TNF-α control; *Spearman´*s correlation for IL-4, IL-6, IL-10, IL-17A, IFN-γ, TNF-α experimental and IL-6, IL-17A, IFN-γ control. *P < 0.05

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5.2 Artigo científico II:

TNF-α in GCF as a diagnostic marker for periodontal disease: a systematic review

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TNF-α in GCF as a diagnostic marker for periodontal disease: a systematic review

ABSTRACT

Introduction: Tumor necrosis-alpha (TNF-α) is a pro-inflammatory cytokine related to the innate immune response. It is suggested that TNF-α in gingival crevicular fluid (GCF) can be used to monitor periodontal disease progression. **Objectives:** The aim of this systematic review was to report the use of TNF-α level in human GCF from systemically healthy individuals to monitor periodontal inflammation (PROSPERO CRD42015020199). **Material and methods:** An electronic search for TNF-α levels in GCF of systemically health and non-smokers patients was conducted in three databases until May 2015. A search for gray literature was also conducted. Data from patients presenting periodontal health or any degree of periodontal disease were incorporated. Risk bias was assessed by Newcastle-Ottawa scale for case-control studies and Jadad scale for clinical trials. A qualitative synthesis was conducted. **Results:** 20 studies were included (9 case-control, 7 clinical trials and 4 randomized clinical trials). TNF-α levels from all studies were presented, however no clear pattern of cytokine levels could be established despite the periodontal condition, even when the same commercially kits were used. **Conclusion:** The evidence available still not support the use TNF-α in GCF as reliable tool to monitor periodontal disease, its progression and healing after therapy.

INTRODUCTION

In periodontitis, Tumor necrosis factor- alpha (TNF-α) is a pro-inflammatory cytokine able to stimulate the degradation of the connective tissue matrix and bone destruction (Tervahartiala et al. 2001; Santos *et al.,* 2010). The cytokines of TNF-α family play a critical role in the innate response against the periodontopathogenic bacteria (de Oliveira *et al.,* 2009). The chronic persistence of plaque and inappropriate cytokine response may result in inflammation in addition to tissue destruction and bone loss (Meikle *et al.,* 1989; Graves *et al.,* 2003).

Gingival crevicular fluid (GCF) is a transudate of interstitial tissues produced by an osmotic gradient (Barbieri *et al.,* 2013; Perinet *et al.,* 2013). It contains a mixture of molecules from blood, host tissue, and plaque biofilms, such as small molecules, cytokines, antibodies, electrolytes, proteins, enzymes and bacterial antigens (Uitto *et al.,* 2003; Khiste *et al.,* 2011). Under inflammatory conditions, such as periodontitis, GCF becomes exudative, approaching serum concentrations (Uitto *et al.,* 2003) and exhibits a variety of inflammatory mediators in its composition, including TNF-α (Khiste *et al.,* 2011; Barbieri, 2013; Perineti *et al.,* 2013).

The biochemical analysis of GCF allows a non-invasive approach to access the pathophysiological status of the periodontium in a site-specific manner (Uitto *et al.,* 2003; Javed *et al.,* 2012). These molecules in GCF have been used to evaluate the periodontal condition (Delima & Van Dyke 2003, Uitto 2003, Armitage 2004), although there is no practical and accurate periodontal indicator based on GCF. In this scenario, TNF-α may be a useful predictor of inflammation in periodontium (Rossomando *et al.,* 1990). However it is still not clear if TNF-α level in human GCF can precisely define periodontal health or disease. This paper aimed to perform a systematic review of literature to investigate, in systemically

health individuals and non-smokers, the use of TNF-α in human GCF as a diagnostic tool for periodontal disease diagnosis, treatment and progression. The PICO question was defined as follows: Can TNF-α in GCF be used to monitor periodontal disease in systemically healthy subjects? **Patients**: systemically health individuals and non-smokers; **Intervention/ exposure**: periodontal disease; **Comparison/ non-exposure**: sites with periodontal disease or healthy sites or healthy controls; **Outcome**: TNF-α levels in GCF.

MATERIAL AND METHODS

Search strategy

This systematic review (PROSPERO number CRD42015020199) was undertaken according with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher *et al.,* 2009). The inclusion criteria were epidemiological studies (cross-sectional, case-control, cohort, clinical trials) that evaluated the expression of TNF-α in human GCF as a diagnostic marker for periodontal disease in systemically health individuals and non-smokers. These patients were periodontally health or presented different gravity of periodontal disease; non-smokers; no systemic disease.

The exclusion criteria were: (1) reviews articles or other systematic reviews, case reports, case series, descriptive studies, opinion articles, abstracts, letters to the editor or laboratory and/or animal studies; (2) studies that included subjects with cleft lip or palate or both; other craniofacial deformities/syndromes; current smokers; under orthodontic treatment; under use of antibiotics or inflammatory drugs 2 month prior or during the experimental period; systemic disorders, and unrelated subjects; (3) studies regarding non-conventional periodontal therapy such as laser or phototherapy, gingival displacement or experimental

gingivitis. (4) absence of comparison (e.g. periodontally health individuals vs periodontal disease; healthy vs disease sites; or baseline vs post-treatment); (5) not mentioning if tobacco usage was in the exclusion criteria or for not splitting smokers and non-smokers in the groups.

Electronic searches were conducted, in June 2014 and updated in May 2015, in the databases: Medline through **PubMed** (http://www.pubmed.gov), **Cochrane Library** (http://www.cochrane.org/index.htm), **Web of Science** (http://www.isiknowledge.com). There were no restrictions regarding language or date of publication. The following search strategy was used on Medline: ((gingival crevicular fluid [Mesh] OR exudate gingival OR transudate gingival OR gingival crevice OR crevicular fluid biomarker) AND (TNF-α OR tumor necrosis factor-alpha [Mesh])) and was adapted using the same key words for the databases Web of Science and Cochrane Library. Moreover, the reference lists of the selected studies were manually searched for additional relevant publications that were not found through the electronic searches. A search for gray literature was also conducted.

The search retrieved 529 abstracts and titles. All references identified were compiled into Reference Manager Software® (Reference Manager, Thomson Reuters, version 12.0.3). After removal of duplicate references, 411 titles, abstracts or methods were read and analyzed by three independent reviewers (DFM, GCC and ILAL) who applied elegibility criteria. Discordances were resolved by consensus (n=10). After the first selection by titles, abstracts or methods, 29 potential studies were selected for full text analysis. After that, 20 studies were included in this systematic review (**Figure 1**). The list of studies excluded after full text analysis are presented in **Supplementary table I.**

Data extraction

The data were extracted and study's characteristics were tabulated in Word for Windows software (Microsoft, Redmond, WA, USA). A form used for data extraction recorded: author, year of publication, location, population (sample size, age of subjects and gender), clinical evaluation, method of GCF collection and measurement, technique for analysis, brand of reagents, TNF-α levels in permanent dentition (converted into picogram/mililiter), gingival crevicular fluid volume (VGCF) and conclusions (**Tables I, II, III and IV)**. 11 articles presented data in graphs had their mean values ± SD requested to the correspondence authors; 8 articles were excluded for not providing it until May 1st 2015. Not all studies described all variables. We have extracted only data that met the study criteria (e.g. if periodontal status were compared between diabetic and systemically healthy group, only data regarding the systemically health group was extracted at this point).

Methodological quality assessment

Risk bias was evaluated by methodological quality assessment using Newcastle-otawa scale for case-controls studies (http://www.ohri.ca/programs/clinical_epidemio-logy/oxford.asp). A system of points was given to eligible categories: Sample selection criteria (0-4); comparability (0-2) and exposure (0-3). The scale scores varied depending on the study design ranging from 0-9. For clinical trials and randomized clinical trials, the Jadad scale was used (Jadad *et al.,* 1996). A system of points was given to eligible categories: randomization (0-2), blinding (0-2) and an account of patients (0-1). The scale scores varied depending on the study design: ranging from 0-5. Disagreements were resolved by consensus.

RESULTS

Study characteristics

The study selection process is presented in **Figure 1.** Twenty studies were included in this systematic review: 9 case-control studies, 7 clinical trials and 4 randomized clinical trials. Characteristics of the 20 studies retrieved were presented in **supplementary Tables II and III,** and **Tables I, II, III, IV and V.** The population varied from **Asia** [China (n=1) (Yue *et al.,* 2013), India (n=1) (Gokul *et al.,* 2012), Iran (n=1) (Pourabbas *et al.,* 2014), Japan (n=2) (Fujita *et al.,* 2012; Shimada *et al.,* 2013), Turkey (n=05) (Erdermir *et al.,* 2004; Kurtis *et al.,* 2005; Fentoğlu *et al.,* 2012; Cetinkaya *et al.,* 2013; Ózgören *et al.,* 2014)]; **Europe** [Greece (n=1), (Kyriazis *et al.,* 2013); Romania (n=1) (Drugarin *et al.,* 1998), Spain (n=1) (Calderin *et al.,* 2013)]; **North America** [United States (n=3) (Engebretson *et al.,* 1999; Shaddox *et al.,* 2011; Alzahrani *et al.,* 2013)] and **South America** [Brazil (n=4) (Bastos *et al.,* 2009; Oliveira *et al.,* 2009; Oliveira *et al.,* 2012; Zimmermann et al 2013)].

Several parameters were used to classify periodontal disease: gingivitis (GI) (n=2) (Drugarin *et al.,* 1998; Gokul *et al.,* 2012); moderate gingivitis (MGI) (n=1); (Alzahrani *et al.,* 2013); severe gingivitis (SGV) (n=1) (Alzahrani *et al.,* 2013); chronic periodontitis (CP) (n=9) (Kurtis *et al.,* 2005; Fentoğlu *et al.,* 2012; Fujita *et al.,* 2012; Gokul *et al.,* 2012; Cetinkaya *et al.,* 2013; Shimada *et al.,* 2013; Zimermann *et al.,* 2013; Ózgören *et al.,* 2014; Pourabbas *et al.,* 2014); adult periodontitis (ADTP) (n=1) (Drugarin *et al.,* 1998); moderate to severe periodontitis (MD-SVP) (n=2) (Engebretson *et al.,* 1999; Erdermir *et al.,* 2004) or moderate to advanced periodontitis (MD-ADVP) (n=1) (Calderin et al, 2013); severe chronic periodontitis (SVCP) (n=1) (Kyriazis *et al.,* 2013); aggressive periodontitis (AGP) (n=6) (Drugarin *et al.,* 1998; Kurtis *et al.,* 2005; Bastos *et al.,* 2009; Oliveira *et al.,* 2009; Oliveira *et al.,* 2012; Yue *et al.,* 2013) and local aggressive periodontitis (LAGP) (n=1) (Shaddox *et al.,*

2011). In addition, only 8 studies presented data from periodontally health controls to be compared to periodontal disease (Kurtis *et al.,* 2005; Bastos *et al.,* 2009; Shaddox *et al.,* 2011; Gokul *et al.,* 2012; Oliveira *et al.,* 2012; Cetinkaya *et al.,* 2013; Zimmermann *et al.,* 2013; Yue *et al.,* 2013). Mean TNF-α levels are presented in **Tables II, III** and **IV**. Several methods were used to quantify TNF- α: ELISA (n=16) (Drugarin *et al.,* 1998; Engebretson *et al.,* 1999; Erdemir *et al.,* 2004; Kurtis *et al.,* 2005; Bastos *et al.,* 2009; Oliveira *et al.,* 2009; Fentoğlu *et al.,* 2012; Gokul *et al.,* 2012; Alzahrani *et al.,* 2013; Calderin *et al.,* 2013; Cetinkaya *et al.,* 2013; Kyriazis *et al.,* 2013; Yue *et al.,* 2013; Zimermann *et al.,* 2013; Ózgören *et al.,* 2014; Pourabbas *et al.,* 2014); Multiplex bead immunoassay (n=3) (Shaddox *et al.,* 2011; Fujita *et al.,* 2012; Shimada *et al.,* 2013) and human cytokine 7-plex immunoassay (n=1) (Oliveira *et al.,* 2012). The studies presented the TNF-α levels in GCF by concentration (n=11) (Drugarin *et al.,* 1998; Erdemir *et al.,* 2004; Kurtis *et al.,* 2005; Oliveira *et al.,* 2009; Shaddox *et al.,* 2011; Fentoglu *et al.,* 2012; Gokul *et al.,* 2012; Oliveira *et al.,* 2012; Alzahrani *et al.,* 2013; Calderin *et al.,* 2013; Pourabbas *et al.,* 2014), total amount (n=5) (Fujita *et al.,* 2012; Kyriazis *et al.,* 2013; Shimada *et al.,* 2013; Yue *et al.,* 2013; Ózgören *et al.,* 2014) or both (n=4) (Engebretson *et al.,* 1999; Bastos *et al.,* 2009; Cetinkaya *et al.,* 2013; Zimmermann *et al.,* 2013).

The Quality assessment of 9 case-control studies were scored as low (n=3), medium (n=2) and high (n=4) by Newcastle-otawwa. By Jadad scale, the quality assessment for clinical trials had scores 0 (n=5) and 1 (n=2); while the randomized clinical trials obtained scores 3 (n=1), 4 (n=1) and 5 (n=2) (**supplementary Tables I and II; Table I**).

Gingival crevicular fluid volumes and sample collection

The majority of studies performed GCF collection with filter paper strips (n=18) and measured its volume with a calibrated Periotron® (n=13) or weighted the volume absorbed

(n=1) (**Table I**). The other studies have collected GCF samples with syringe needle pore (n=1) or volumetric microcapillary pipetes (n=1). However, only 5 studies have reported the mean VGCF obtained by the filter paper strips (**Table V**). These volumes ranged from 0.12 ± 0.15 to 0.90 µl in periodontally health controls (Kurtis *et al.,* 2005; Bastos *et al.,* 2009; Zimmermann *et al.,* 2013); 0.17 ± 0.09 to 1.1 ± 0.2µl in healthy sites of patients with periodontal disease (Bastos *et al.,* 2009; Fujita *et al.,* 2012; Shimada *et al.,* 2013); 1.11 ± 0.56 to 4.7± 0.3 µl in CP (Kurtis *et al.,* 2005; Fujita *et al.,* 2012; Shimada *et al.,* 2013; Zimmermann *et al.,* 2011); and 0.83 ± 0.5 to 1.80 µl in AGP (Bastos *et al.,* 2009; Kurtis *et al.,* 2005). Five studies demonstrated an increase in VGCF in periodontal disease compared to control sites.

TNF-α levels in GCF

Mean TNF- α levels from the retrieved studies were presented in **Tables II, III** and **IV**. The great heterogeneity among studies impaired meta-analysis. Data extracted was presented according to study design:

Case Control studies

Nine case control studies were included (**Table II)**. 6 studies used healthy controls for comparisons to GI (Gokul *et al.,* 2012); CP (Kurtis *et al.,* 2005; Gokul *et al.,* 2012; Cetinkaya *et al.,* 2103; Zimermann *et al.,* 2013); AGP (Kurtis *et al.,* 2005; Bastos *et al.,* 2009) and LAGP (Shaddox *et al.,* 2011). Furthermore, 02 studies also used healthy sites in periodontal patients, besides the healthy subjects, as an additional control (Bastos *et al.,* 2009; Shaddox *et al.,* 2011). 2 studies have compared healthy vs disease sites in CP (Fujita *et al.,* 2012; Shimada *et al.,* 2013) and 1 study has performed comparisons only between different degrees of periodontal gravity: GI, ADTP and AGP (Drugarin *et al.,* 1998). The results demonstrates

an increase in TNF-α levels in periodontal disease for GI (Gokul *et al.,* 2012), CP (Kurtis *et al.,* 2005; Gokul *et al.,* 2012; Fujita *et al.,* 2012), ADTP (Drugarin *et al.,* 1998), AGP (Drugarin *et al.,* 1998; Kurtis *et al.,* 2005) and LAGP (Shaddox *et al.,* 2011) compared to GI (Drugarin *et al.,* 1998), healthy controls (Kurtis *et al.,* 2005; Shaddox *et al.,* 2011; Gokul *et al.,* 2012) or healthy sites (Shaddox *et al.,* 2011; Fujita *et al.,* 2012). Differently, no difference in TNF-α levels were seen in CP (Shimada *et al.,* 2013; Cetinkaya *et al.,* 2013; Zimmermann *et al.,* 2013) compared to control (Cetinkaya *et al.,* 2013; Zimmermann *et al.,* 2013) or healthy sites (Shimada *et al.,* 2013). In contrast, reduced TNF-α levels were seen in disease sites in CP (Cetinkaya *et al.,* 2013) and AGP (Bastos *et al.,* 2009) compared to healthy controls (Bastos *et al.,* 2009; Cetinkaya *et al.,* 2013) or healthy sites (Bastos *et al.,* 2009). Conflicting results were seen when comparisons were performed in concentration and total amount of TNF-α in GCF for CP vs controls (Cetinkaya *et al.,* 2013) and healthy sites of AGP (Bastos *et al.,* 2009).

Clinical trials

Seven clinical trials were included (**Table III)**. All studies have quantified TNF-α levels before/ after periodontal intervention [Scaling an root planning (SRP) (Engebretson *et al.,* 1999; Erdemir *et al.,* 2004; Oliveira *et al.,* 2009; Fentoglu *et al.,* 2012; Calderin *et al.,* 2013), oral hygiene instructions and prophylaxis (OHIP) (Alzahrani *et al.,* 2013) or periodontal treatment (not specified) (Yue *et al.,* 2013)]. Only 1 study included healthy control additionally for comparison (Yue *et al.,* 2013). These comparisons were performed from 21-45 days (Engebretson *et al.,* 1999; Alzahrani *et al.,* 2013; Calderin *et al.,* 2013), month 2 (Yue *et al.,* 2013), month 3 (Fentoglu *et al.,* 2012; Oliveira *et al.,* 2009) up to month 6 (Erdemir *et al.,* 2004). There were included patients with MGI and SGI (Alzahrani *et al.,*

2013), CP (Fentoglu *et al.,* 2012), AGP (Oliveira *et al.,* 2009; Yue *et al.,* 2013), MD-ADVP (Calderin *et al.,* 2013), MD-SVP (Engebretson *et al.,* 1999; Erdemir *et al.,* 2004). The results demonstrated that patients with AGP presented raised TNF-α levels compared to control (Yue *et al.,* 2013). After SRP no difference in TNF-α levels were seen in MD-SVP (Engebretson *et al.,* 1999; Erdemir *et al.,* 2004) or CP (Fentoglu *et al.,* 2012). In contrast, some reports demonstrated that SRP increased TNF-α levels in MD-ADVP (Calderin *et al.,* 2013) and reduced its levels in AGP (Oliveira *et al.,* 20009). Moreover, periodontal treatment (not specified) in AGP (Yue *et al.,* 2013) and OHIP in SGI (Alzahrani *et al.,* 2013) were also effective to reduce TNF-α levels.

Randomized clinical trials

Four randomized clinical trials were included (**Table IV)**. All studies have quantified TNF-α levels before/after periodontal intervention [Scaling an root planning (SRP) (Oliveira *et al.,* 2012; Pourabbas *et al.,* 2014; Ózgören *et al.,* 2014) or periodontal surgery (Kyriazis *et al.,* 2013)]. Only 1 study included healthy control additionally for comparison (Oliveira *et al.,* 2012). These comparisons were performed from month 1 (Ózgoren *et al.,* 2014), month 3 (Pourabbas *et al.,* 2014) up to month 6 (Oliveira *et al.,* 2012; Kyriazis *et al.,* 2013). The results demonstrated no difference in TNF-α levels between control and AGP at baseline (Oliveira *et al.,* 2012). SRP was not effective to reduce TNF-α levels in CP (Ózgören *et al.,* 2014) or AGP (Oliveira *et al.,* 2012), but reduced TNF-α levels were also seen after SRP in CP (Pourabbas *et al.,* 2014). Moreover, no difference in TNF-α levels were seen after periodontal surgery for SVCP (Kyriazis *et al.,* 2013).

DISCUSSION

As expected, mean TNFα levels varied according to methods used (e.g commercial kits, method of sample collection, gravity of periodontal disease and samples sites). Even when patients were classified with the same periodontal gravity, great variations were also seen within the same commercial kits (Bastos *et al.,* 2009; Oliveira *et al.,* 2009; Zimmermann *et al.,* 2013). There is a need of standardization of the methodologies to allow direct comparison between the studies, once the literature has already demonstrated that, for example, there were differences in the levels of IL-1β, IL-6 and TNF-α when buffer saline (PBS), Tris-HCL buffer, with and without protease inhibitors were used (Rios-Lugo MJ *et al.,* 2015). These divergences might be also due to heterogeneity of sample population, lack of information regarding participant characteristics, distinct periodontal classification systems, how data was presented (total amount or concentration), assay sensitivity, different methodologies and statistical methods that might have influenced the results. Therefore, it could not be established a reference value for TNF-α in GCF by this systematic review. Failure to matching GCF sample sites for relevant clinical parameters (i.e. probing depth, clinical attachment loss, plaque accumulation and bleeding on probing) are bias found in this review, as cytokines may be influenced by the periodontal disease severity, rather than the type of periodontitis (Duarte *et al.,* 2015). Furthermore, it should be considered that GCF collection can be sensitive to plaque accumulation, saliva contamination (Griffiths et all., 1992) and depends of the operator ability.

Regarding VGCF sample, 15 of the 20 studies have not presented this data. VGCF should be described all studies. The molecule comparisons based on total amount only should be interpreted with caution, as the total amount of these molecules may be an obvious consequence of VGCF sampled (Duarte *et al.,* 2015). The divergences between total amount

and concentration of TNF-α may be influenced by higher VGCF in disease sites (compared with healthy ones), that may result in decreased inflammatory mediator concentration in the GCF from inflamed sites (Bastos *et al.,* 2009). Interestingly, all studies that reported VGCF levels (n=5) demonstrated an increased VGCF in periodontal disease/disease sites compared to controls (Bastos *et al.,* 2009; Kurtis *et al.,* 2015; Fujita *et al.,* 2102, Shimada *et al.,* 2013; Zimmerman *et al.,* 2013).

TNF-α is related with the inflammatory condition of the periodontium. It may be synthesized and secreted by the local periodontal connective tissue cell (Gokul *et al.,* 2012). An increased release of some cytokines in patients with periodontal disease is expected (Ullbro C, 2004). An attempt is made to estimate the level of TNF-α in GCF and serum and to explore its relationship to periodontal disease (Gokul *et al.,* 2012). In this systematic review, the use of TNF-α levels in GCF for diagnosis of periodontal disease demonstrated raised levels in GI (Gokul *et al.,* 2012; Özçaka *et al.,* 2012), CP (Drugarin *et al.,* 1998; Kurtis *et al.,* 2005; Gokul K *et al.,* 2012), ADTP (Drugarin *et al.,* 1998), and AGP (Drugarin *et al.,* 1998; Kurtis *et al.,* 2005; Bastos *et al.,* 2009; Shaddox *et al.,* 2011) compared to healthy volunteers. Active periodontal disease sites also demonstrated raised TNF-α level than healthy sites in GI (Alzahrani *et al.,* 2013) and CP (Fujita *et al.,* 2012). In contrast, similar TNF-α levels were reported in patients with different periodontitis associated genotype (Engebretson *et al.,* 1999), periodontally health/gingivitis/chronic periodontitis (Fentoğlu *et al.,* 2011; Zimmermann *et al.,* 2013) and healthy/disease sites (Shimada *et al.,* 2013). Reduced levels of TNF-α were reported in CP compared to controls (Cetinkaya *et al.,* 2013) and in disease sites of AGP (Bastos *et al.,* 2009). The use TNF-α levels in GCF to monitor periodontal treatment also presented conflicting results: similar TNF-α levels were seen before/after treatment of GAGP (Oliveira *et al.,* 2012), MD-SVP (Engebretson *et al.,* 1999), SVCP (Kyriazis *et al.,* 2013), and CP (Fentoğlu *et al.,* 2012; Ozgören et al. 2014). Differently, some reports

demonstrated that TNF-α levels were decreased after conventional periodontal therapy (RSP) (Erdemir *et al.,* 2004; Oliveira *et al.,* 2009; Alzahrani *et al.,* 2013 Pourabbas *et al.,* 2014). In contrast, increased level of TNF-α was seen after treatment of MD-ADVP (Calderin *et al.,* 2013). Different pattern of TNF-α expression was described after treatment of AGP, with an increase on day 1 and a subsequent progressive decrease until day 90 (Oliveira *et al.,* 2009) and after periodontal surgery for SVCP, there was a reduction in TNF-α on month 3 and similar levels to baseline in month 6 (Kyriazis *et al.,* 2013). These data must be interpreted with caution, because the sensitivities of the assays were different and not specified in all studies, leading to bias of accuracy. The low expression of molecules in GCF requires high-sensitivity bioassays for measuring cytokines in GCF to ensure consistency of the results (Duarte *et al.,* 2014). Thus, it is still not clear if TNF-α levels were accurate or masked by the low-sensitivity method.

Concomitantly to TNF-α levels in GCF, the retrieved studies have also demonstrated that other GCF molecules were elevated in active periodontal disease or gingivitis, such as (1) Metaloproteinases (MMPs): MMP-1 (Shimada *et al.,* 2013), MMP-3 (Shimada *et al.,* 2013); MMP-8 (Pourabbas *et al.,* 2014; Ozgören *et al.,* 2014); MMP-9 (Pourabbas *et al.,* 2014); (2) adipokines: plasminogen activator inhibitor-1 (PAI-1) (Shimada et al, 2013), leptin (Shimada *et al.,* 2013); (3) Cytokines: interleukins (IL) such as IL-1 (Drugarin *et al.,* 1998); IL-1α (Shimada et al, 2013); IL-1β (Shaddox *et al.,* 2011; Oliveira *et al.,* 2012; Fujita *et al.,* 2012; Shimada *et al.,* 2013, Yue *et al.,* 2013; Pourabbas *et al.,* 2014), IL-2 (Shaddox *et al.,* 2011; Oliveira *et al.,* 2012; Yue *et al.,* 2013), IL-4 (Yue *et al.,* 2013), IL-6 (Drugarin *et al.,* 1998; Fujita *et al.,* 2012; Oliveira *et al.,* 2012; Yue *et al.,* 2013;), IL-8 (Fujita *et al.,* 2012; Shimada et al, 2013), IL-10 (Shaddox *et al.,* 2011; Fujita *et al.,* 2012); IL-12p40 (Shaddox *et al.,* 2011); IL-21 (Shimada et al, 2013), interferon-gama (IFN-Ɣ) (Drugarin *et al.,* 1998; Shaddox *et al.,* 2011; Yue *et al.,* 2013); RANKL (Oliveira *et al.,* 2009) (4) chemokines CC ligands (CCL):

CCL2 (Kurtis *et al.,* 2005; Shimada et al, 2013), CCL5 (Shimada et al, 2013), CCL7 (Shimada et al, 2013), interferon-inducible protein-10 (IP-10) (Shimada et al, 2013); (5) Cell adhesion molecule group: ICAM-1 (Shimada et al, 2013), vascular cell adhesion protein-1 (VCAM-1) (Shimada et al, 2013), E-selectin (Shimada et al, 2013); and (6) grow factors: fibroblast grow factor (FGF) (Shimada Y et al, 2013), PIGF (Shimada et al, 2013); vascular endothelial grow factor (VEGF) (Shimada et al, 2013), granulocyte-macrophage colony – smtimulating factor (GM-CSF) (Shaddox *et al.,* 2011; Oliveira *et al.,* 2012). (8) protein: pentraxin-3 (PTX3) (Fujita *et al.,* 2012); (9) hormones: resistin and leptin (Zimmerman *et al.,* 2013). In contrast, reduced levels of CCL2 (Shaddox *et al.,* 2011) and IL-4 (Bastos *et al.,* 2009; Shaddox *et al.,* 2011) were seen in disease sites. As reduced IL-1β (Fentoğlu *et al.,* 2012; Cetinkaya *et al.,* 2013), IL-4 (Cetinkaya *et al.,* 2013) IL-10 (Cetinkaya *et al.,* 2013) were seen in chronic periodontitis compared to controls. No significant differences for IL-1β (Fentoğlu *et al.,* 2012), IFN-Ɣ (Oliveira *et al.,* 2012), IL-6 (Zimmerman *et al.,* 2013; Fentoğlu *et al.,* 2012), IL-10 (Oliveira *et al.,* 2012), adiponectin (Zimmerman *et al.,* 2013) were observed between the disease/control or after treatment. Non detectable level of IL-6 in GCF was also reported (Takeichi *et al.,* 1994).

In this review, data retrieved demonstrated that, at some moments, it seems there is an increase in TNF-α levels in patients with periodontal disease compared to controls/healthy sites. However, when TNF-α was used to monitor periodontal healing after therapy, no difference was observed in the majority of studies, despite the improvement in clinical records. This systematic review does not support the use of TNF-α in GCF as a reliable tool for diagnosis in periodontal disease or to monitor healing after therapy. The conflicting results may be associated with different sampling technique (Ikezawa-Suzuki *et al.,* 2008) or be attributed to the differences in the accuracy of the methods to detect TNF-α (Santos *et al.,* 2010). Comparisons between the studies were hindered by methodological divergences, being

randomized clinical trials the minority of studied retrieved. There is a need of longitudinal studies concerning the periodontal health or disease activity/inactivity to allow the possibility of using TNF-α in GCF as a biochemical marker of periodontal disease (Gokul *et al.,* 2012), as well the standardization of methodological procedures to allow comparison between the results.

CONCLUSION

This systematic review presented conflicting results regarding TNF-α levels in human GCF. Thus, the evidence available still not support the use TNF-α in GCF as reliable tool to monitor periodontal disease, its progression and healing after therapy. Moreover, due to differences in populations, methodologies, techniques used and sensibility of the assays, this paper could not determine a TNF-α reference value in GCF to classify periodontal health/disease or activity/inactivity. More studies are necessary to investigate this issue. It is advisable to use healthy controls in further studies, as well the standardization of methods to allow comparisons between results.

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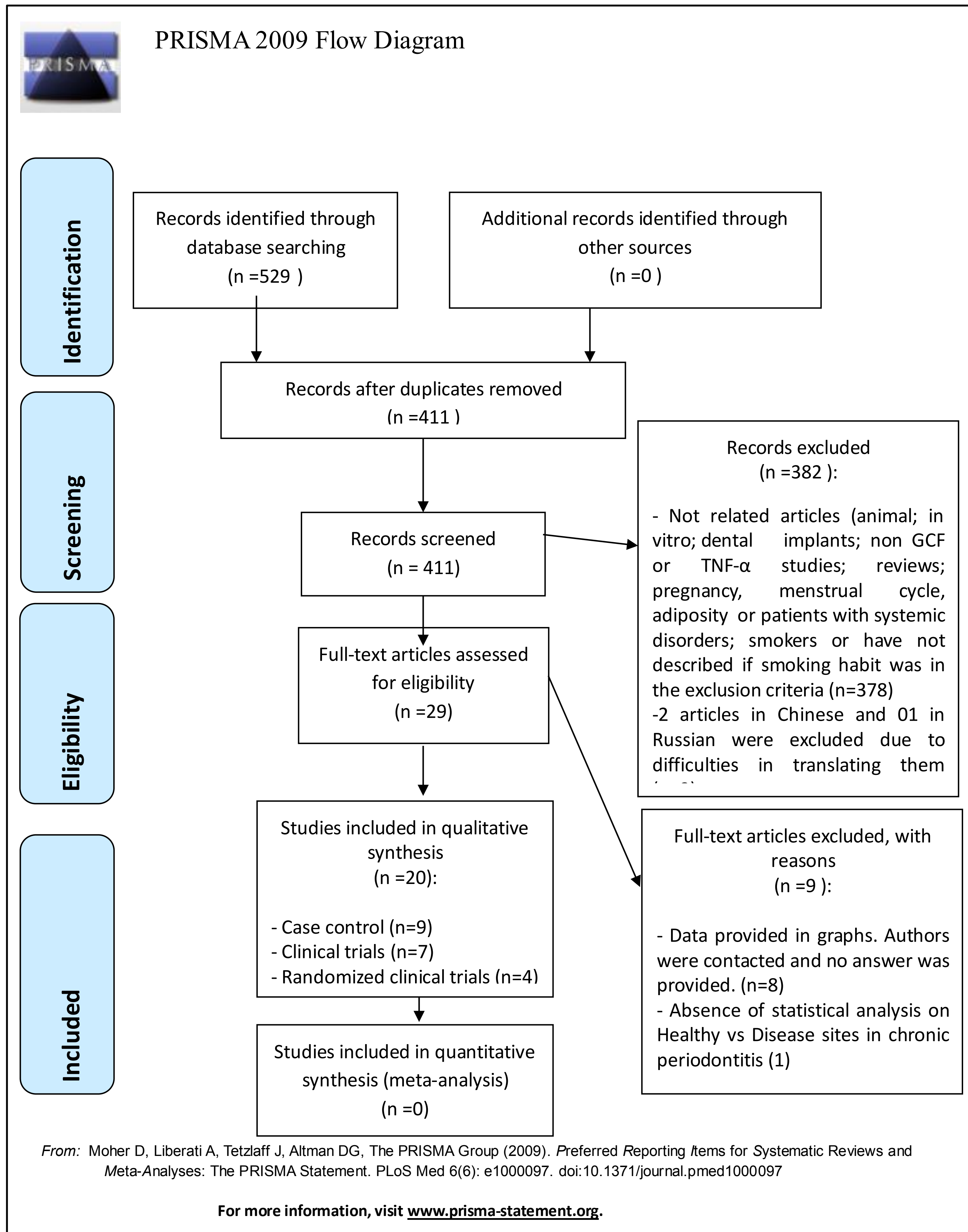

Figure 1. Inclusion and exclusion strategy by PRISMA 2009 flow diagram

Table I. Authors, Methods of GCF collection, quality evaluation and conclusion of the 20 retrieved studies.

Author, year, location	Method of 'GCF collection and its volume measurement	Quality evaluation	Conclusion regarding TNF-α levels in GCF
CASE CONTROL STUDIES			
Drugarin D *et al.,* 1998	FPS	2*	Significant increase of TNF-α in periodontitis (ADP and AGP) comparing to gingivitis
Kurtis B *et al.,* 2005	FPS; weight of accumulated fluid	7*	Raised TNF-α levels were seen between in AGP and CP compared to CC groups. No difference in TNF-α levels were seen between AGP and CP groups.
Bastos *et al.,* 2009	FPS; Periotron 8000	6*	TNF-α was lower for control sites. No differences among healthy and disease sites from AGP subjects.
Shaddox *et al.,*2011	FPS; Periotron 8000	8*	TNF-α were increased in diseased sites of LAGP compared to healthy sites or CC.
Fujita *et al.,* 2012	FPS; Periotron 8000	3*	TNF-α were higher in disease sites than in healthy sites in patients with CP.
Gokul K, 2012	VMCP	5*	Absence or low levels of TNF-α in GCF might indicate a stable lesion and elevated levels might indicate an active site.
Cetinkaya *et al.,* 2013	FPS; Periotron 8000	8*	Higher TNF-α levels in GCF were seen in CC compared to CP
Zimmermann*et al.,* 2013	FPS; Periotron 8000	8*	Similar TNF-α levels were seen in CC and CP.
Shimada Y *et al.,* 2013	FPS; Periotron 8000	3*	Similar TNF-α levels were seen in Healthy and disease sites in patients with CP .
CLINICAL TRIALS			
Engebretson et al 1999	FPS; Periotron 6000	1†	TNF-α was detected in GCF in 59% of samples at baseline and 49% at follow up.
Erdemir *et al.,* 2004	FPS; Periotron 8000	1†	No difference in cytokine levels after SRP in months 1, 3 and 6 in patients with MD-SVP.
Oliveira *et al.,* 2009	FPS; Periotron 8000	0†	Non-surgical periodontal treatment with SRP led to significant reductions in TNF-α levels 30 days following treatment
Fentoğlu *et al.,* 2012	FPS; Periotron 8000	0†	No significant difference in TNF-α was seen after SRP in CP at baseline and 3 months after treatment
Alzabrani *et al.,* 2013	FPS; Periotron	0†	Reduced levels of TNF-α after treatment of severe gingivitis
Calderin *et al.,* 2013	FPS; Periotron 8000	0†	Raised TNF-α levels in MD-ADVP after SRP at month 2.
Yue *et al.,* 2013	SNP;	0†	TNF-α was significantly higher in AP patients than in CC and decreased after periodontal treatment.
RANDOMIZED CLINICAL TRIALS			
Oliveira *et al.,* 2012	FPS; Periotron 8000	5†	No significant difference in TNF-α were seen after SRP in AGP at baseline and 6 months after treatment.
Kyriazis *et al.,* 2013	FPS	3†	No difference in TNF-α was noticed for CP patients treated with MWFS or ARFS
Pourabbas *et al.,* 2014	FPS;	5†	Significant improvement was noted for TNF-α due to SRP from baseline to 3-month follow up in CP
Ozgoren *et al.,* 2014	FPS	4†	No significant difference in TNF-α were seen after SRP in CP at baseline and 1 month after treatment.

ADP: Adult periodontitis; AGP: aggressive periodontitis; ARFS: Apical repositioning flap surgery; CC: control group with periodontally health individuals; CP: chronic periodontitis; FPS: Filter paper strips; GCF: gingival crevicular fluid; LAGP: Local aggressive periodontitis; MD-ADVP: moderate to advanced periodontits; MD-SVP: moderate to severe periodontitis; MWFS: Modifies widman flap surgery; SNP: syringe with a needle pore of 150 µm; SRP Scaling and root planning treatment; TNF-α: Tumor necrosis factor-alpha; VMCP: volumetric microcapillary pipettes. *Quality evaluation by Newcastle-otawa (scores 0-9); †Quality evaluation by Jaddad (scores 0-5).

Table II - TNF-α Levels in GCF in nine case control studies

Author., Year (Location)	Method of analysis (Brand)	Control (all healthy individuals) n (f/m)	Mean age ± SD (range) years	Mean TNF-α level	Periodontal disease group N(f/m)	Mean age ± SD (range) years	Classification	Mean TNF-α level	P value
							CONCENTRATION pg/mL		**P value**
Drugarin *et al.*, 1998 (Romania)	ELISA (R&D system)				9 5 8	15-70 15-70 15-70	GI ADTP AGP	4.1 ± 1.2 12.1 ± 4.3* 16.3 ± 2.3*	*P < 0.01 to GI
Kurtis B *et al.*, 2005 (Turkey)	ELISA (CYTELISA)	20 (10,10)	27.6 (23-34)	340000 (250000 - 480000)	25 (14,11) 20 (12,8)	41.24 (30-75) 28.6 (21-38)	CP AGP	710000 (550000-3580000* 1030000 (170000-3020000)*	*P < 0.001 to CC
†Bastos *et al.*, 2009 (Brazil)	ELISA (R&D systems)	13 (7,6)	28.5 ± 5.0	10800 ± 11000	14 (8,6)	23.4 ± 3.5	AGP HS AGP MS AGP AS	14000 ± 13100 5100 ± 6400* 2700 ± 2300*	*P < 0.05 to HS
Shaddox et al, 2011 (USA)	MBI (Milipore)	9 10	13.33 ± 4.61 12.56 ± 5.25	80.24 ± 45.33 2012 ± 4604	34	14 ± 4.09	LAGP HS LAGP DS	146.8 ± 191.7 9931 ± 1822*	*P < 0.0001 to CC or HS
Gokul K., 2012 (India)	ELISA (Antigenix)	20	(35-50)	3.02	20 20	35-50 35-50	GI CP	90.22* 82.94*	*p < 0.05 to CC
†Cetinkaya *et al.*, 2013 (Turkey)	ELISA (Biosource)	16 (8,8)	28.06 ± 6.18	71920±45530	16 (6,10)	44.00 ± 7.00	CP	9410 ± 4260*	*P < 0.05 to CC
†Zimmermann *et al.*, 2013 (Brazil)	ELISA (R&D systems)	20 (14,6)	42.9 ± 7.2	200 ± 500	20 (15/5)	47.8 ± 7.7	CP	120 ± 320	
							TOTAL AMOUNT (pg/site)		
†Bastos *et al.*, 2009 (Brazil)	ELISA (R&D systems)	13 (7,6)	28.5 ± 5.0	0.9 ± 1.2	14 (8,6)	23.4 ± 3.5	AGP HS AGP MS AGP AS	2.2 ± 1.6* 2.0 ± 1.9* 2.4 ± 1.7*	*P < 0.05 to CC
Fujita *et al.*, 2012 (Japan)	MBI (Millipore)				50 (27,23)	59.8 (40-72)	CP HS CP DS	0.17 ± 0.31 0.33 ± 0.33*	*P < 0.01 to HS
†Cetinkaya *et al.*, 2013 (Turkey)	ELISA (Biosource)	16 (8,8)	28.06 ± 6.18	11.55 ± 0.85	16 (6,10)	44.00 ± 7.00	CP	11.47 ± 0.33	
Shimada Y *et al.*, 2013 (Japan)	MBI (Bio-Rad)				11		CP HS CP DS	0.70 ± 0.13 0.99 ± 0.15	
†Zimmermann *et al.*, 2013(Brazil)	ELISA (R&D systems)	20 (14,6)	42.9 ± 7.2	0.01 ± 0.04	20 (15/5)	47.8 ± 7.7	CP	0.10 ± 0.23	

There are nine case-control studies. †Studies that presented data in concentration (pg/ml) and total amount (pg); n: number of participants; f: female; m: male; ADTP: Adult periodontitis; AGP: Aggressive periodontitis;; CC: controls (all healthy individuals); CP: Chronic periodontitis; GI: Gingivitis; LAG: Local aggressive periodontitis; AS: Advanced sites; DS: Disease site; HS: Healthy site; MS: Moderate sites; ELISA: Enzime linked immunosorbent assay; MBI – Multiplex beads immunoassay; pg: picogram; TNF-α: Tumor necrosis factor-alfa

Table III –TNF-α Levels in GCF in seven clinical trials

Author, Year (location)	Method of analysis (Brand)	n(f/m)	Mean age (range) years	Classification	Mean TNF-α at Baseline	Type of interven tion	Time after treatment d=days m=months Y=years	Mean TNF-α levels after intervention	P value
					CONCENTRATION (pg/mL)				
[T]Engebretson et al 1999 (USA)	ELISA (Cistron Biotechnology)	15 7	45.5 46.4	MD-SVP/Polim.PAG- MD-SVP/Polim.PAG+	2700 ± 520 1820 ± 270	SRP SRP	21d 21d	3200 ± 610 2100 ± 400	
[T]Erdemir *et al.*, 2004 (Turkey)	ELISA (Immunotech)	19	46.94 ± 6.07	MD-SVP		SRP	1 m 3 m 6 m	510 ± 810 490 ± 640 270 ± 410	
Oliveira *et al.*, 2009 (Brazil)	ELISA (R&D systems)	10 (8/2)	31.01 ± 4.43	AGP	410 ± 20	SRP	0 d 1 d 7d 30 d 90 d	400 ±20 520 ±10* 380 ± 20 300 ± 20* 220 ± 20*	*p < 0.05 to BS
Fentoğlu *et al.*, 2012 (Turkey)	ELISA (Orgenium)	28(14,14)	31-54	CP	0.52(0.52-0.52)	SRP	3m	0.58(0.54 – 0.60)	
Alzahrani et al., 2013 (USA)	ELISA (eBioscience)	18 23		MGI SGI	54.45±7.36 58.08±14.66	OHIP OHIP	1-1.5 m 1-1.5 m	50.5±6.92 46.6±11.50*	*P = 0.006 to SGI at baseline
Calderin *et al.*, 2013 (Spain)	ELISA (Biolink)	9	50.44 ±15.91	MD-ADVP	18970±8550	SRP	1 m 2 m	28240±9400 41830±11800*	*P < 0.05 to BS
					TOTAL AMOUNT (pg)				
[T]Engebretson et al 1999 (USA)	ELISA (Cistron Biotechnology)	15 7	45.5 46.4	MD-SVP/Polim.PAG- MD-SVP/Polim.PAG+	2.0±0.29 2.2±0.32	SRP SRP	21d 21d	4.5 ± 0.9 3.1 ± 0.65	
[T]Erdemir *et al.*, 2004 (Turkey)	ELISA (Immunotech)	19	46.94 ± 6.07	MD-SVP		SRP	1 m 3 m 6 m	0.13 ± 0.16 0.13 ± 0.16 0.10 ± 0.11	
Yue *et al.*, 2013 (China)	ELISA (Diaclone)	40 (15,25) 40(13,27)	24.33±2.70 24.43±2.61	CC AGP	61.52 ± 11.00 653.00 ± 171.62*	PT	1 m	293.85 ± 77.23*#	*P<0.05 to CC #P<0.05 to AGP BS

There are seven clinical trials. [T]Studies that presented data in concentration (pg/ml) and total amount (pg);); n: number of participants; f: female; m: male; ADTP: Adult periodontitis; AGP: Aggressive periodontitis; CC: control group (all healthy individuals); CP: Chronic periodontitis; GI: Gingivitis; LAG: Local aggressive periodontitis; MD-ADVP: Moderate to advanced periodontitis; MGI: Moderate gingitis; MD-SVP: Moderate to severe periodontitisMS: Moderate sites; PlimPAG+: Polymorphism of Periodontitis associated genotipe positive; PolymPAG-: Polymorphism of Periodontitis associated genotipe negative; SGI: Severe gingivitis; AS: Advanced sites; BS: baseline; DS: Disease site; HS: Healthy site; OHIP: oral hygiene instructions and prophylaxis; SRP: Scaling and root planning; PT: Periodontal treatment (not specified); ELISA: Enzime linked immunosorbent assay; MBI – Multiplex beads immunoassay; pg: picogram; TNF-α: Tumor necrosis factor-alfa.

Table IV – TNF-α Levels in four randomized clinical trials

Author, Year (location)	Method of analysis (Brand)	n(f/m)	Mean age (range) years	Classifi cation	Mean TNF-α at baseline	Type of intervent ion	Time after treatment d=days m=months Y=years	Mean TNF-a levels after intervention	P value
					CONCENTRATION (pg/mL)				
Oliveira *et al.*, 2012 (Brazil)	human cytokine 7-plex/Millipore	25 (14,11)	26 ± 3	CC	1.9 ± 1.4				
		12 (8,4)	26 ± 3	AGP	2.2 ± 2.4	RSP	6 m	3.7 ± 5.2	
Pourabbas *et al.*, 2014 (Iran)	ELISA (USCN life science)	24 (14,10)	46 ± 8	CP	0.16 (0.09 – 1.04)	RSP	3 m	0.09 (0.03 – 0.63)*	*P < 0.001 to BS
					TOTAL AMOUNT (pg)				
Kyriazis *et al.*, 2013 (Greece)	ELISA (R&D systems)	14 (8/6)	50.3 ± 13.2	SVCP	27.5 ± 1.08	ARFS	1 ½ m	24.43 ± 1.19	*P < 0.05 to ARFS at BS
							3 m	23.38 ± 1.04*	
							6 m	25.48 ± 1.13	
		16(9/7)	51.7±14.6	SVCP	27.15 ± 1.09	MWFS	1 ½ m	16.15 ± 1.42	
							3 m	25.91 ± 1.52	
							6 m	25.22 ± 1.38	
Ózgören *et al.*, 2014 (Turkey)	ELISA (Invitrogen)	16 (7,9)	42.3 ± 7.3	CP	4.07±1.58	RSP	1 m	3.84±2.29	

There are four randomized clinical trials.); n: number of participants; f: female; m: male; AGP: Aggressive periodontitis; CC: control group (all healthy individuals); CP: chronic periodontitis; SVCP: Severe chronic periodontitis; ARFS: Apical repositioning flap surgery; MWFS: Modifies widman flap surgery; RSP: Root scalling and planning; ELISA: Enzime linked immunosorbent assay; TNF-α: Tumor necrosis factor-alfa.

Table V - Gingival crevicular fluid volume (µL)

	Group	time	VGCF (µl)	P value
Kurtis B *et al.,* 2005	CC	BS	0.90 (0.80 -1.00)	*P < 0.001
	CP	BS	2.10 (1.70 - 3.00)*	
	AGP	BS	1.80 (1.35 - 2.35)*	
Bastos *et al.,* 2009	CC	BS	0.12 ± 0.15	*P < 0.05 to CC and AGP HS
	AGP HS	BS	0.17 ± 0.09	
	AGP MS	BS	0.83 ± 0.5*	
	AGP AS	BS	1.08 ± 0.5*	
Fujita *et al.,* 2012	CP HS	BS	0.43 ± 0.21	*P < 0.01
	CP DS	BS	2.48 ± 0.90*	
Shimada Y *et al.,* 2013	CP: HS	BS	1.1± 0.2	*P < 0.0001
	CP: DS		4.7 ± 0.3*	
Zimmermann *et al.,* 2013	CC	BS	0.61 ± 0.25	*P < 0.05
	CP	BS	1.11 ± 0.56*	

AGP: Aggressive periodontitis; CC: control group (all healthy individuals); CP: Chronic periodontitis; AS: Advanced sites; BS: Baseline; DS: Disease sites; HS: Healthy site; MS: Moderate sites; VGCF: Gingival crevicular fluid volume

Supplementary Table I – Reason for exclusion of potential articles

EXCLUSION CRITERIA	AUTORS
Have not provided additional information (data was presented in graphs)	Ikezawa-susuki *et al.,* 2008; Dominguez *et al.,* 2010; Holzhausen *et al.,* 2010; Gomez *et al.,* 2011; Fagundes *et al.,* 2011; Pereira *et al.,* 2012; Ertugrul *et al.,* 2013; Da silva *et al.,* 2014
Absence of statistical analysis on healthy sites vs disease sites in systemically health group.	Duarte *et al.,* 2014

Supplementary Table II – Quality assessment criteria used for case-control studies throught a modified Newcastle-Otatawa Scale

	Drugarin D *et al.*, 1998	Kurtis B *et al.*, 2005	Bastos *et al.*, 2009	Shaddox *et al.*,2011	Fujita *et al.*, 2012	Gokul K, 2012	Cetinkaya *et al.*, 2013	Shimada *et al.*, 2013	Zimmermannet *al.*, 2013
SAMPLE SELECTION CRITERIA									
1) Is the case definition adequate? a) yes, with independent validation ★; b) yes, eg record linkage or based on self reports; c) no description of clinical parameters for diagnosis	c	★	★	★	★	★	★	★	★
2) Representativeness of the Cases; a) All eligible cases with outcome of interest over a defined period of time, all cases in a defined catchment area, all cases in a defined hospital or clinic, group of hospitals, health maintenance organisation, or an appropriate sample of those cases (e.g. random sample); ★ b) Not satisfying requirements in part (a), or not stated	★	★	b	★	★	b	★	★	★
3) Selection of Controls; This item assesses whether the control series used in the study is derived from the same population as the cases and essentially would have been cases had the outcome been present. a) Community controls (i.e. same community as cases and would be cases if had outcome) ★ b) Hospital controls, within same community as cases (i.e. not another city) but derived from a hospitalised population c) No description; d) no healthy controls or controls were healthy sites in patients with periodontal disease	d	★	★	★	d	★	★	d	★
4) Definition of Controls; a) no history of disease (endpoint) ★ b) no description of source c) no healthy controls or controls were healthy sites in patients with periodontal disease	c	★	★	★	c	★	★	c	★
COMPARABILITY									
1) Comparability of Cases and Controls on the Basis of the Design or Analysis; A maximum of 2 stars can be allotted in this category Either cases and controls must be matched in the design and/or confounders must be adjusted for in the analysis. Statements of no differences between groups or that differences were not statistically significant are not sufficient for establishing comparability. Note: If the odds ratio for the exposure of interest is adjusted for the confounders listed, then the groups will be considered to be comparable on each variable used in the adjustment. There may be multiple ratings for this item for different categories of exposure (e.g. ever vs. never, current vs. previous or never) Age = ★ , Other controlled factors = ★ b) not information available; c) comparisons were between healthy and disease sites in patients with periodontal disease	c	★	★	★★	c	b	★★	c	★★
EXPOSURE									
1) Ascertainment of exposure; a) secure record ★ b) blind to case/control status ★ c) not blinded to case/control status	★	★	★	★	★	★	★	★	★
2) Same method of ascertainment for cases and controls; a) yes ★ b) no c) absence of healthy controls or controls were healthy sites in patients with periodontal disease	c	★	★	★	c	★	★	c	★
SUMARY SCORE (Stars) maximum: 9	2 low	7 high	6 medium	8 high	3 low	5 medium	8 high	3 low	8 high

Not all studies described al variables. Confounders were extracted and described as if they were evaluated in multivariate analysis.

Supplementary Table III - Jaddad quality evaluation for clinical trials and Randomized clinical trials

Author	**Randomization**			**Blinding**			**Fate**	**Total**
	Mentioned (1pt)	Appropriate methods (1pt)	Inappropriate Method (-1)	Mentioned (1pt)	Correct blinding method (1pt)	Incorrect blinding method (-1pt)	Fate of all patients is known (1pt)	Maximum (5pts)
CLINICAL TRIALS								
Engebretson et al 1999	0	0	0	0	0	0	1	1
Erdemir *et al.,* 2004	0	0	0	0	0	0	1	1
Oliveira *et al.,* 2009	0	0	0	0	0	0	0	0
Fentoğlu *et al.,* 2012	0	0	0	0	0	0	1	1
Alzahrani *et al.,* 2013	0	0	0	0	0	0	0	0
Calderin *et al.,* 2013	0	0	0	0	0	0	0	0
Yue *et al.,* 2013	0	0	0	0	0	0	0	0
RANDOMIZED CLINICAL TRIAL								
Oliveira *et al.,* 2012	1	1	0	1	1	0	1	5
Kyriazis *et al.,* 2013	1	1	0	0	0	0	1	3
Ózgören *et al.,* 2014	1	1	0	1	1	0	0	4
Pourabbas *et al.,* 2014	1	1	0	1	1	0	1	5

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6 CONSIDERAÇÕES FINAIS

A aplicação de força ortodôntica sobre o dente está associada ao estiramento/compressão da matriz, mudanças no fluxo do fluido no LP e osso, e deformação das células locais (HENNEMAN *et al.,*2008). Há também uma redução do fluxo sanguíneo que é capaz de alterar, em poucas horas, o ambiente químico e atividade celular local (DAVIDOVITCH & SHAMFIELD,1975). Como resultado, desencadeia-se uma resposta inflamatória asséptica e transitória nos tecidos periodontais com a ativação de vias de sinalização responsáveis por ativar fibroblastos, osteoblastos, osteócitos e osteoclastos, os quais estão envolvidos no processo de remodelação óssea durante a MDO (HENNEMAN *et al.,* 2008; DIERCKE *et al.,* 2011). Moléculas sinalizadoras, como citocinas e quimiocinas, apresentam papel chave na reposta do periodonto humano à força mecânica (DAVIDOVITCH *et al.,* 1980; DAVIDOVITCH *et al.,* 1980; KRISHNAN; DAVIDOVITCH *et al.,* 2006; MEIKLE, 2006). Nesse contexto, esse trabalho objetivou correlacionar, pela primeira vez na literatura, a cinética de expressão das citocinas IL-2, IL-4, IL-6, IL-10, IL17A, IFN-γ e TNF-α no LP e FCG após a aplicação de força ortodôntica durante diferentes períodos (dias 1, 3, 7, 14, 21 e 28). Optou-se pelo modelo tipo boca-dividida, para minimizar os efeitos das variações interindividuais. Os resultados demonstram padrões de expressão diferente dessas moléculas ao longo do tempo, assim como entre o LP e FCG, impossibilitando estabelecer uma correlação direta entre os eventos moleculares que ocorrem nesses sítios após o estresse mecânico.

O grupo experimental apresentou o mesmo volume FCG que o grupo controle nos dias 1, 3, 14, 28. Entretanto, aumento no volume de FCG foi observado nos dias

7 e 21 no grupo experimental. de VFCG. A literatura demonstra pouca ou nenhuma alteração no volume de FCG em resposta à MDO (UEMATSU *et al.,* 1996; REN *et al.,* 2002; LUPPANAPORNLAP *et al.,* 2010; PERINETTI *et al.,* 2013), embora haja também relatos de aumento neste volume nos grupos experimentais (PERINETTI *et al.,* 2002; SERRA *et al.,* 2003; BASARAN *et al.,*2006). Dessa forma, a alteração no volume do FCG não deve ser utilizada como referência para o estudo da remodelação tecidual associada à MDO.

O processo de remodelação óssea está relacionado à aposição óssea nos locais onde ocorre o estiramento do LP, e reabsorção óssea (frontal e/ou a distância) nos locais onde ocorre a compressão do LP (BUCK *et al.,* 1972). A reabsorção frontal, mais desejável por permitir maior preservação das células do tecido, maior permeabilidade vascular, movimento dentário mais suave e gradual com menor risco de reabsorção radicular apical externa, geralmente está associada à aplicação de forças mais leves, ou seja, em torno de 0.490-0.890 N. A reabsorção óssea à distância está mais associada a forças pesadas (necrosantes) que causam o esmagamento do LP, morte celular, e consequente ausência de células no LP e osso alveolar adjacente (MASELLA & MEISTER, 2006). As áreas de hialinização permanecem por cerca de 4 a 49 dias dependendo da sua extensão (BUCK *et al.,* 1972; KRISHNAN & DAVIDOVITCH, 2006; KUROL & OWMAN-MOLL, 1998; MEIKLE, 2006; REITAN, 1957) e são responsáveis por um atraso no movimento dentário. Assim, uma considerável espessura de tecido ósseo precisa ser removida antes que qualquer movimento dentário ocorra. Na reabsorção a distância há um maior risco de reabsorção radicular apical externa (BUCK *et al.,* 1972; DAVIDOVITCH, 1991; KRISHNAN; DAVIDOVITCH *et al.,* 2006; HENNEMAN, 2008). Além disso, há uma grande variação interindividual na resposta biológica ao

estresse mecânico, não existindo uma clara relação entre o nível de força aplicada, o tempo, extensão e duração da hialinização (REITAN, 1960; REITAN, 1967; KUROL & OWMAN-MOL, 1998; VON BOHL & KUIJIPERS-JAGTMAN, 2009). O tempo necessário para ocorrer a remodelação óssea parece ser independente do nível de força aplicada, uma vez que forças como 50 cN também foram capazes de induzir áreas de hialinização (KUROL & OWMAN-MOLL, 1998; VON BOHL & KUIJIPERS-JAGTMAN, 2009). A relação de que a aplicação de forças mais pesadas induziriam mais áreas de hialinização também não pode ser estabelecida, de acordo com dados de uma revisão sistemática publicada em 2009 (VON BOHL & KUIJIPERS-JAGTMAN, 2009). De modo interessante, áreas de hialinização são também observadas na migração fisiológica do dente humano (KUROL & OWMAN-MOLL, 1998) e durante a recidiva do tratamento ortodôntico (REITAN, 1960; REITAN, 1967, KUROL & OWMAN-MOLL, 1998). Forças de alta magnitude, *in vitro*, foram capazes de inibir a diferenciação de células formadoras de tecido ósseo. Dessa forma, durante as diferentes fases da movimentação dentária, mudanças estruturais no osso e tecido periodontal ocorrem (BUCK *et al.,* 1972; VON BOHL & KUIJPERS-JAGTMAN, 2009). Como resultado, há um diferente padrão de expressão moléculas sinalizadoras, que deve estar associado ao método empregado e influenciado pela susceptibilidade individual.

Sendo assim, o fator força aplicada foi considerado no planejamento desse estudo. Não está estabelecido na literatura valor médio ideal para aplicação de força devido à heterogeneidade nas metodologias disponíveis (REN *et al.,* 2003). Optamos pela aplicação da força de 0.980 N devido à padronização prévia do método realizado pelo nosso grupo (MADUREIRA *et al.,* 2012). A aplicação de forças maiores poderia resultar em uma *lag phase* de aproximadamente 21 dias

antes que qualquer movimento dentário ocorresse (VON BOHL & KUIJPERS-JAGTMAN, 2009; REITAN *et al.,*1957; REN). A literatura também demonstra que forças mais suaves (0.588 – 0.686 N) também são eficazes para induzir a MDO (GIANELLY, 1971; REITAN *et al.,* 1957*).* Era esperado um decréscimo da força aplicada devido aos possíveis danos ocorridos na mola de titânio molibidênio (TMA) durante a mastigação. Como resultado, obtivemos uma força média final de 0.87 ± 0.176 N, ainda efetiva para induzir o processo de remodelação óssea. Ressalta-se que a diferença entre os valores médios das forças finais, nos diferentes tempos observados nesse estudo, não apresentaram significância estatística e, portanto não influenciaram os resultados.

O modelo de remodelação óssea induzido pela MDO está bem estabelecido na literatura. Entretanto, há uma vasta variabilidade nos métodos como características dos participantes, modo, tipo de aparelho, tempo de aplicação de força e mediadores alvos estudados. Tais fatores dificultam a comparação desses resultados. Portanto, a partir de um estudo realizado pelo nosso grupo (MADUREIRA *et al.,* 2012), no qual foi realizada análise do LP em cinco tempos após o estresse mecânico, optamos, nesse novo estudo, realizar uma análise concomitante das citocinas presentes no LP e FCG humano. Devido à complexidade do processo de remodelação óssea durante a MDO, esperávamos uma mudança nos padrões de citocinas nas suas diferentes fases da remodelação óssea, pois diversos estudos já apresentaram a alteração expressão de moléculas nos sítios periodontais submetidos ao estresse mecânico. TNF-α (BLETSA *et al.,* 2006; REN *et al.,* 2007, GARLET *et al.,* 2007; ANDRADE *et al.,* 2007; ANDRADE *et al.,* 2012; TADDEI *et al.,* 2012; KUNII *et al.,* 2013) e IL-6 (ALHASHIMI *et al.,* 2001; BASARAN *et al.,* 22006; REN *et al.,* 2007; KUNI & YAMAGUCHI, 2013) são citocinas

comumente associadas a MDO, as quais são produzidas por células epiteliais, endoteliais, macrófagos (MATSUKI *et al.,* 1992; OKADA *et al.,* 1997; TADDEI *et al.,* 2013), fibroblastos (MATSUKI *et al.,* 1992; BLETSA *et al.,* 2006; MATARASE *et al.,* 2006; LEE *et al.,* 2007; AZUMA *et al.,* 2013; KUNII & YAMAGUCHI, 2013) e osteoblastos (AGGARWAL 2000; ANDRADE *et al.,* 2012; GARCIA-LOPEZ *et al.,* 2013; KUNII *et al.,* 2013). A IL-2 é derivada da célula *T-helper.* IL-2 (REN *et al.,* 2002), IL-6 (ROODMAN, 1992; OKADA *et al.,* 1997; ALHASHIMI *et al.,* 2001; BASARAN *et al.,* 2006; AZUMA *et al.,* 2013) e TNF-α (YANO *et al.,* 2005; KOHARA *et al.,* 2011) são moléculas capazes de induzir osteoclastogênese. Além disso, TNF-α pode induzir apoptose dos osteócitos, gerando um novo sinal para o recrutamento de osteoclastos e reabsorção óssea nas áreas de compressão do LP submetido à MDO, e também, inibindo os osteoblastos nessa região (AHUJA *et al.,* 2003; GARLET *et al.,* 2007; LI *et al.,* 2012; ANDRADE *et al.,* 2012). IL-6 também possui papel chave no metabolismo ósseo (BAKKER & JASPERS, 2015). TNF-α, IL-6 e IL-1β beta podem estimular a diferenciação osteoclástica de modo sinérgico e essa interação é capaz de potencializar a reabsorção óssea (STEEVE *et al.,* 2004; YAMAGUCHI *et al.,* 2004; AZUMA *et al.,* 2013). Além disso, TNF-α é capaz de induzir osteoblastos a produzirem IL-6 de maneira dose-dependente (KOZAWA *et al.,* 1997). Após a secreção de TNF-α e IL-1 (UEMATSU *et al.,* 1996; AZUMA *et al.,* 2013), IL-6 é produzida. Em seguida, IL-6 inibe a produção de TNF-α e IL-1 (SCHINDER *et al.,* 1990; AZUMA *et al., 2013).* Estudos *in vitro* demonstram que IL-6 é produzida após a aplicação de força estática compressiva (LEE *et al.,* 2007; KUNII *et al.,* 2013) e deve ser auto-estimulatória, o que significa que IL-6 é capaz de amplificar sua própria produção por fibroblastos (gengivais e periodontais) na presença de receptores nessas células para IL-6 (IL-6sR) (OKADA *et al.,* 1997). De

modo interessante, pacientes que apresentam a reabsorção radicular apical externa devido a MDO apresentaram níveis basais aumentados para IL-6 no FCG (KUNII *et al.,* 2013), tornando essa citocina um potencial marcador para a susceptibilidade individual para esse efeito colateral indesejável. Entretanto, ainda não está estabelecido um nível de referência para se determinar esse prognóstico.

Nesse estudo, embora não tenha sido observada diferença estatística entre os níveis de IL-2, tanto no LP ou no FCG, esse dado é similar aos resultados de BASARAN *et al* (2006) que também não observaram diferença nos níveis de IL-2 no FCG nos dias 7, 21 e mês 6. Níveis de TNF-α aumentados também não foram observados nesse estudo, possivelmente porque TNF-α é liberado em estágios muito iniciais da MOD, seguido por um *feedback* negativo que inibe rapidamente a sua produção (BLETSA *et al.,* 2006). Esta citocina é principalmente liberada durante a aplicação de força mecânica (ALHASHIMI *et al.,* 2001). Níveis maiores de TNF-α foram observados nos sítios de compressão do que de tensão do LP humano (GARLET *et al.,* 2007; TADDEI *et al.,* 2012), assim como níveis aumentados no FCG também puderam ser detectados após o dia 1 da MDO (UEMATSU *et al.,* 1996; REN *et al.,* 2007), conflitando com nossos resultados. Estudos *in vitro* demonstram um aumento dos níveis de IL-6 após a aplicação de força estática compressiva em 12 horas (LEE *et al.,* 2007). Níveis aumentados de IL-6 no FCG foram descritos no dia 1 após MDO em jovens (UEMATSU *et al.,* 1996; REN *et al.,* 2002) e adultos jovens (REN *et al.,* 2007), o que não está de acordo com nossos resultados, porém corrobora com REN *et al.,* (2012), que não observou diferenças na concentração de IL-6 no FCG em população adulta. Por outro lado, foram observados níveis reduzidos de IL-6 no FCG no dia 21 nesse estudo, provavelmente devido a um mecanismo de *feedback* negativo. Em nossos resultados foram detectados níveis

aumentados de IL-6 no LP no dia 1, assim como no dia 12 em um estudo anterior realizado pelo nosso grupo (MADUREIRA *et al.,* 2012). Em suma, IL-6 parece ter um papel relevante na regulação da remodelação óssea (BAKKER & JASPER, 2015) após estímulo mecânico. Além disso, enquanto que os fibroblastos do LP apresentam aumento na expressão de indutores de osteoclastogênese, os fibroblastos gengivais (LI *et al.,* 2011) e precursores de osteoclastos (KUNII *et al.,* 2013) demonstraram quase que nenhuma indução sob compressão *in vitro* (LI *et al.,* 2011). Esses resultados talvez ajudem a explicar as diferenças nos níveis de IL-6 no LP e FCG, embora outros fatores também devam ser considerados como diferenças na composição celular, função e capacidade de resposta a diferentes estímulos (GRIFFITHS, 2003; UITTO, 2003; MALAMUD, 2006; NANCI, 2008; PALUMBO, 2011).

IL-10 (GARLET *et al.,*2007; IVASHKIV *et al.,*2011; TADDEI *et al.,* 2012; TADDEI *et al.,*2013) e IFN-ɣ (MERMUT *et al.,* 2007; KOHARA *et al.,* 2011; KOHARA *et al.,* 2012) inibem a formação de osteoclastos e consequentemente a reabsorção óssea. IL-17A, em algumas situações, também aparenta iniciar um processo “osteoprotetivo” por meio do aumento de osteoprotegerina, (KOONERMAN *et al.,* 2012). IL-10 apresentou um maior aumento nos sítios de compressão que tensão do LP humano (GARLET *et al.,* 2007) e a sua redução afetou negativamente o reparo ósseo (BRITO *et al.,*2012; AZUMA *et al.,*2013). IFN-ɣ pode funcionar inibindo a osteoclastogênese excessiva (KOHARA *et al.,*2011; KOHARA *et al.,* 2012) durante a MOD (KORHARA *et al.,* 2012), sendo expressa mais no lado de compressão do LP (ALHASHIMI *et al.,* 2000) e associada a um aumento no volume de osso trabecular (MERMUT *et al.,* 2007). Geralmente, IFN-ɣ esta aumentado em estágios tardios da MOD (ALHASHIMI *et al.,* 2000). Entretanto, os nossos resultados não apresentaram

qualquer modificação em IFN-ɣ após a MDO, assim como nenhuma diferença estatística para os níveis de IL-17A no LP ou FCG. Apenas uma tendência de aumento pode ser observada no FCG no dia 28, embora não significante.

O volume FCG (VFCG) geralmente apresenta pouca ou nenhuma diferença durante a MDO (UEMATSU *et al.,* 1996; REN *et al.,* 2002; LUPPANAPORNLAP *et al.,* 2010; PERINETTI *et al.,* 2013) como observado nos dias 1, 3, 14 e 28. Entretanto, assim como visto também nesse estudo (dias 7 e 21), outros trabalhos demonstraram um aumento no VFCG nos grupos experimentais (PERINETI *et al.,* 2002; SERRA *et al.,* 2003; BASARAN *et al.,* 2006).

Após MDO, diversos trabalhos demonstram diferenças na concentração de alguns biomarcadores presentes no FCG (UEMATSU *et al.,* 1996; REN *et al.,* 2002; REN *et al.,* 2007; LUPPANAPORLARP *et al.,* 2010; PERINETTI *et al.,* 2013; BARBIERI *et al.,* 2013) e LP (ALASHIMI *et al.,* 1999; GARLET *et al.,* 2007; GARLET *et al.,* 2008; MADUREIRA *et al.,* 2012; TADDEI *et al.,*2013). Com relação ao tempo de tratamento ativo, a literatura demonstra que variou de 5 minutos a 6 meses, sendo 1h e 24 h os tempos mais frequentemente estudados (UEMATSU *et al.,* 1996; REN *et al.,* 2002; BASARAN *et al.,* 2006; LUPPANAPORLAP *et al.,* 2010). Os resultados demonstram aumento dos níveis de citocinas no FCG após o estímulo ortodôntico (KAPOOR *et al.,* 2014). Foram observados picos de prostagladina-2 (REN *et al.,* 2002; REN & VISSINK, 2008), IL-8 (BASRARAN *et al.,* 2006), IL-1β, TNF-α, IL-6, fator quimiotático de eosinófilos (UEMATSU *et al.,* 1996; REN *et al.,* 2002; REN *et al.,* 2007; REN & VISSINK, 2008; LUPPANAPORLARP *et al.,* 2010), fator transformador de crescimento-1 beta (BARBIERI *et al.,* 2013) e fator estimulador de colônia (REN *et al.,* 2002) durante os estágios iniciais, tanto nos dias

1 ou 3 (UEMATSU *et al.,* 1996; REN *et al.,* 2002; REN & VISSINK, 2008; LUPPANAPORNLARP *et al.,* 2010), seguido por uma redução em direção a níveis basais nos dias 7 ou 10 (UEMATSU *et al.,* 1996; BASARAN *et al.,* 2006; REN & VISSINK, 2008;). De modo interessante, enquanto a prostaglandina-2 apresentou padrões similares de expressão independente da mecânica aplicada (REN & VISSINK, 2008), a IL-1β apresentou diferentes padrões regulatórios de acordo com o estímulo exercido (REN & VISSINK, 2008; LUPPANAPORNLARP *et al.,* 2010). Um segundo influxo de citocinas deve ocorrer (REN *et al.,* 2007). No ligamento periodontal, estudos de MDO em animais demonstraram aumento dos níveis de IL-6 (ALHASHIMI *et al.,* 2001) e IFN-γ (ALHASHIMI *et al.,* 2000) no dia 3, seguido de uma redução gradual até níveis basais no dia 10, enquanto que as citocinas IL-4 (GIANNOPOLOU et al. 2008; ALHASHIMI *et al.,* 2000) e IL-10 (ALHASHIMI *et al.,* 2000) apresentaram os mesmos níveis nos grupos experimentais e controle. No LP, estudos referentes à MDO em humanos demonstraram aumento dos níveis de IL-6 12 dias após o estímulo mecânico (MADUREIRA *et al.,* 2012). Além disso, observou-se que houve um maior aumento de TNF-α, IL-10 (GARLET *et al.,* 2007), CCL2 e CCL3 (GARLET *et al.,* 2008) nos sítios de compressão do que de tensão do LP. Entretanto, no presente estudo, a maioria das citocinas não apresentou alterações. Talvez seja porque o método empregado realizou a quantificação das citocinas após um período de tempo menor (até 28 dias) da instalação de um aparelho ortodôntico fixo parcial. Diferentemente, os estudos que apresentaram um aumento dos níveis dessas moléculas no FCG após estímulo mecânico (UEMATSU *et al.,* 1996; BASARAN *et al.,* 2006) selecionaram pacientes que já estavam em tratamento ortodôntico fixo total em ambos dentes experimental e controle por um período de tempo maior. Essa diferença no método pode ter influenciado os resultados, uma

vez que uma inflamação crônica deve ocorrer durante o tratamento ortodôntico, aumentando a chance de detecção de alterações moleculares.

As diferenças individuais na MDO deve estar relacionada a uma variação individual na idade (REN *et al.,* 2002), densidade óssea/mineral estrutura anatômica, atividade celular no LP e osso alveolar (REITAN, 1967; VON BOHL & KUIJPERS-JAGTMAN, 2009) ou mesmo variações na capacidade metabólica, o que determina a taxa de renovação e reação do tecido conjuntivo (VON BOHL & KUIJPERS-JAGTMAN, 2009; SHIAU *et al.,* 2014). Esses fatores devem explicar a diferença nos níveis de citocinas no LP e FCG das pessoas incluídas nesse estudo. No LP e FCG, a produção de citocinas está ligada a diversos eventos celulares que ocorrem durante a MDO. A determinação dos níveis de várias citocinas em diversas fases da MDO é desafiadora e deverá contribuir para um melhor entendimento dos mecanismos que controlam a remodelação óssea (REN *et al.,* 2002; BASARAN *et al.,* 2006; REN *et al.,* 2007; KUNII *et al.,* 2013). Os aspectos biológicos da movimentação dentária devem ser claramente elucidados, pois pode contribuir para a determinação de forças ideais nos diferentes tipos de mecânicas aplicadas (BASARAN *et al.,* 2006; GARLET *et al.,* 2006; GARLET *et al.,* 2008).

Em geral, os estudos *in vivo* relacionados à MDO focam nas mudanças que ocorrem no LP. Entretanto, o LP fornece apenas uma explicação parcial do processo (MILNE *et al.,* 2009). Nesse estudo, padrões similares de expressão de citocinas, embora não correlacionáveis, foram vistas entre o LP e FCG. Portanto, outras fontes de citocinas como osso, gengiva, vasos sanguíneos e fatores externos como acúmulo de biofilme devem ser também investigados para ajudar a esclarecer as mudanças que ocorrem no LP e FCG. A partir dos resultados obtidos, observamos

que FCG não é representativo do microambiente do LP. Sendo assim, o método proposto não nos permite utilizar o FCG como referência para as alterações moleculares que ocorrem no LP. Além disso, ressalta-se que as interleucinas liberadas no LP podem ficar retidas em seu sítio de secreção devido a interação com proteínas da matriz extracelular e das superfícies celulares (RAMSDEM & RAIDER 1992; COOMBER, 2008). Esse fator pode ter interferido nos resultados finais desse estudo, pois na maioria dos tempos, pouca ou nenhuma diferença entre as citocinas alvo no grupo experimental e controle foram observadas.

Ressalta-se que os estudos em humanos relacionados à remodelação óssea apresentam algumas limitações: (1) A análise histológica de material humano é limitada pelo fato de que os dentes submetidos à força que foram extraídos, tiveram o PDL parcialmente danificado, enquanto que o osso alveolar adjacente não pode ser analisado por questões éticas (VON BOHL & KUIJPERS-JAGTMAN, 2009); (2) Embora o presente trabalho propõe avaliar as alterações que ocorrem no LP e FCG em um modelo de estresse mecânico, supostamente sem a influência de fatores externos como a infecção periodontal, a análise dos resultados ainda é limitada devido aos diversos fatores inerentes ao indivíduo (como por exemplo gênero, idade, grau de higiene bucal, susceptibilidade individual e variabilidade genética). Essas diferenças são, provavelmente, devido às diferenças na população das células do periodonto, características hereditárias e padrão de expressão destas moléculas (MASELLA & MEISTER, 2006); (3) Existem poucos estudos disponíveis (com aplicação de protocolos experimentais distintos) que demonstram a expressão de citocinas/quimiocinas com amostra de LP humano durante a MDO; (4) Devido as diferenças nas composições do FCG e LP, talvez a coleta de mais sítios e de um volume maior de amostra, ou mesmo um número maior de indivíduos participantes,

poderiam ser mais representativos; (5) Embora todo o ligamento periodontal tenha sido coletado, o volume obtido para amostra foi pequeno, impossibilitando ampliar a investigação para outros mediadores; (6) A força aplicada neste estudo foi na face vestibular em sentido apical, porém a distribuição desta força não ocorre de forma homogênea no LP (TANNE *et al.,* 1987) e, portanto são criadas áreas de compressão e tensão dentro do LP que não podem ser visualizadas clinicamente. Dessa forma, todo o ligamento periodontal foi coletado. É possível que a concentração das citocinas no lado de pressão e tensão tenham sido realmente diferentes, como previamente descrito na literatura, porém devem se diluir no volume total quando o ligamento é coletado como um todo; (7) A contínua circulação das moléculas presentes do FCG no sulco gengival potencialmente mistura as proteínas originadas do sítio de compressão e tensão. Somente empregando-se a coleta de todo LP que essa comparação poderia ser realizada. Para minimizar tal efeito, foi utilizado o dente contralateral, sem aparelho ortodôntico, como controle; (8) O FCG é mais sensível à contaminação por saliva e biofilme (GRIFFTHS *et al.,* 1992), portanto um possível erro de manipulação deve ser considerado como viés durante a interpretação dos resultados; (9) Seis tempos de aplicação de força durante MDO foram avaliados (dias 1, 3, 6, 14, 21 e 28). Porém, ainda há necessidade de investigação em outros tempos, assim como outros mediadores, uma vez que o processo de remodelação óssea é contínuo e dividido em diferentes fases (KRISHNAN; DAVIDOVITCH, 2006); (10) Por se tratar de uma amostra de conveniência, o número de indivíduos participantes nesse estudo é um fator limitador, e deve-se assumir que erro do Tipo I possa estar presente.

O esclarecimento do processo de remodelação óssea durante a MDO é necessário para o planejamento, conduta terapêutica e prevenção de efeitos

colaterais como reabsorção radicular apical externa, dor, movimentos indesejáveis, dentre outros. Embora a literatura apresente diversos estudos relacionados a esse assunto, a interação entre as moléculas de sinalização e as células alvo na remodelação óssea ainda não foi totalmente esclarecida. Além disso, seria uma dedução muito simplista esperar que todas as alterações que ocorreram no LP estivessem diretamente refletidas no FCG, uma vez que as células gengivais (fibroblastos, células epiteliais, endoteliais e inflamatórias) devem contribuir muito mais para as moléculas constituintes do FCG do que as do LP, que estão mais distantes. Confirmando essa suposição, os resultados deste trabalho sugerem que ocorre uma complexa interação entre FCG e LP, uma vez que estes apresentaram respostas diferentes ao estímulo mecânico e mesmo em condições fisiológicas. O conhecimento obtido com esse estudo traz pela primeira vez, uma direção referente à relação entre as mudanças moleculares que ocorrem no LP e FCG, possibilitando assim o planejamento de estudos futuros que tentem correlacionar esses dois sítios empregando-se diferentes métodos.

7 CONCLUSÕES

1 - A interleucina-6 parece apresentar um papel importante no processo de remodelação óssea e periodontal associados ao estresse mecânico;

2 – A ausência de correlação direta entre a expressão de citocinas no fluido crevicular gengival e ligamento periodontal não permite determinar a contribuição de moléculas liberadas no ligamento periodontal para a composição do fluido crevicular gengival. Os dados sugerem que estes microambientes são distintos e apresentam interação complexa apesar da aparente interdependência.

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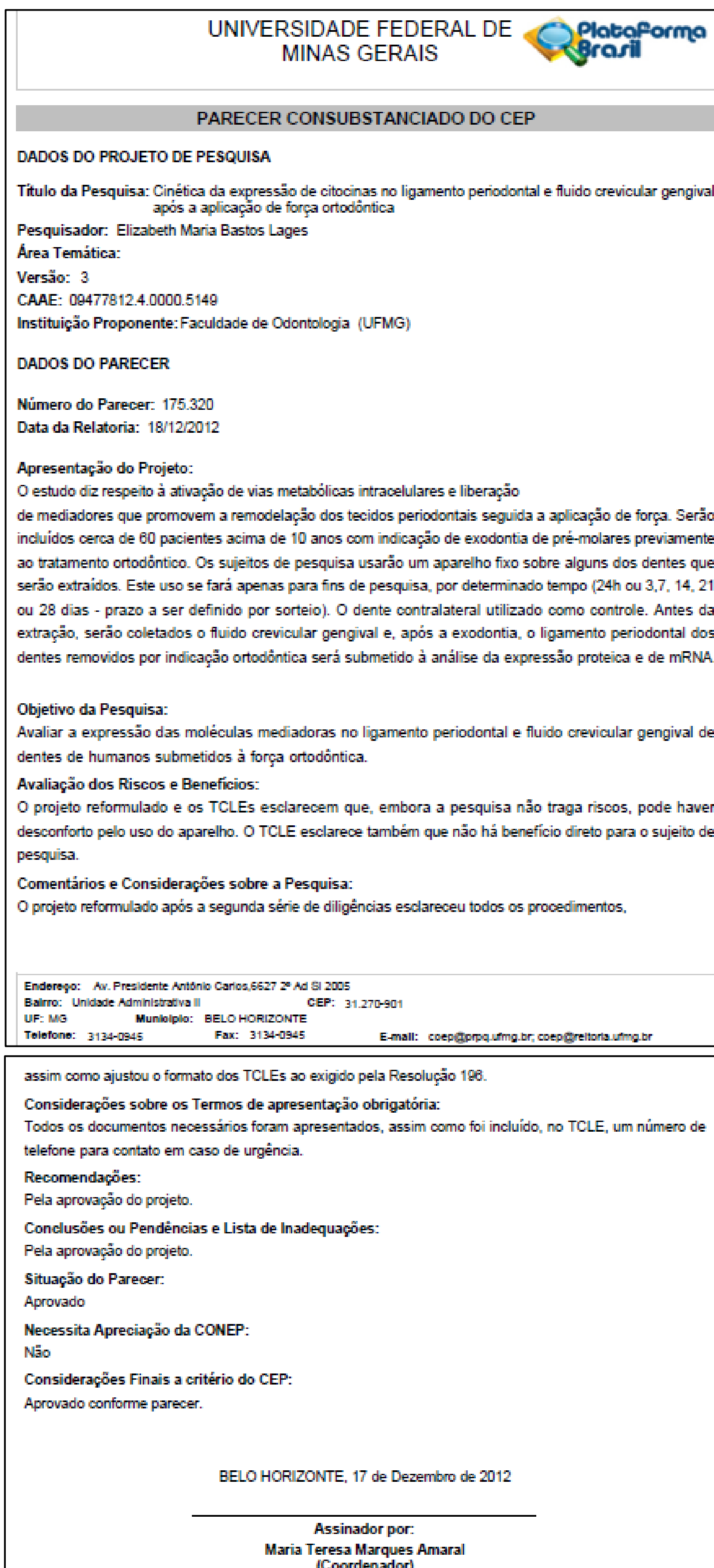
ANEXOS

Anexo A – Parecer do Comitê de Ética em Pesquisa (COEP/UFMG)



UNIVERSIDADE FEDERAL DE MINAS GERAIS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Cinética da expressão de citocinas no ligamento periodontal e fluido crevicular gengival após a aplicação de força ortodôntica

Pesquisador: Elizabeth Maria Bastos Lages

Área Temática:

Versão: 3

CAAE: 09477812.4.0000.5149

Instituição Proponente: Faculdade de Odontologia (UFMG)

DADOS DO PARECER

Número do Parecer: 175.320

Data da Relatoria: 18/12/2012

Apresentação do Projeto:

O estudo diz respeito à ativação de vias metabólicas intracelulares e liberação de mediadores que promovem a remodelação dos tecidos periodontais seguida a aplicação de força. Serão incluídos cerca de 60 pacientes acima de 10 anos com indicação de exodontia de pré-molares previamente ao tratamento ortodôntico. Os sujeitos de pesquisa usarão um aparelho fixo sobre alguns dos dentes que serão extraídos. Este uso se fará apenas para fins de pesquisa, por determinado tempo (24h ou 3,7, 14, 21 ou 28 dias - prazo a ser definido por sorteio). O dente contralateral utilizado como controle. Antes da extração, serão coletados o fluido crevicular gengival e, após a exodontia, o ligamento periodontal dos dentes removidos por indicação ortodôntica será submetido à análise da expressão proteica e de mRNA.

Objetivo da Pesquisa:

Avaliar a expressão das moléculas mediadoras no ligamento periodontal e fluido crevicular gengival de dentes de humanos submetidos à força ortodôntica.

Avaliação dos Riscos e Benefícios:

O projeto reformulado e os TCLEs esclarecem que, embora a pesquisa não traga riscos, pode haver desconforto pelo uso do aparelho. O TCLE esclarece também que não há benefício direto para o sujeito de pesquisa.

Comentários e Considerações sobre a Pesquisa:

O projeto reformulado após a segunda série de diligências esclareceu todos os procedimentos,

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assim como ajustou o formato dos TCLEs ao exigido pela Resolução 196.

Considerações sobre os Termos de apresentação obrigatória:

Todos os documentos necessários foram apresentados, assim como foi incluído, no TCLE, um número de telefone para contato em caso de urgência.

Recomendações:

Pela aprovação do projeto.

Conclusões ou Pendências e Lista de Inadequações:

Pela aprovação do projeto.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Aprovado conforme parecer.

BELO HORIZONTE, 17 de Dezembro de 2012

Assinador por:
Maria Teresa Marques Amaral
(Coordenador)

Anexo B – Dados suplementares do artigo científico I

Table 1. Experimental and control sites paired differences for cytokines with normal distribution.

	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28
	Paired differences					
IL-2 PDL (EX – CC)	3.362 ± 4.321	-3.533 ± 2.996	0.707 ± 2.600	2.562 ± 3.510	-1.240 ± 3.397*	0.615 ± 6.677
IL-2 GCF (EX – CC)	7.953 ± 8.907	3.480 ± 10.848	-0.982 ± 4.616	0.950 ± 5.515	-0.409 ± 7.871	-0.298 ± 7.372
IL-4 PDL (EX – CC)	0.132 ± 0.234	-0.124 ± 0.333	-0.024 ± 0.391	-0.149 ± 0.258	0.090 ± 0.215	-0.233 ± 0.499
IL-10 PDL (EX – CC)	-0.241 ± 0.456	-0.119 ± 0.272	-0.074 ± 0.305	-0.006 ± 0.277	0.285 ± 0.544	-0.152 ± 0.403
TNF-α PDL (EX- CC)	-0.026 ± 0.049	-0.053 ± 0.057	-0.007 ± 0.083	-0.030 ±0.091	-0.009 ± 0.125	0.003 ± 0.109

Paired differences levels of cytokines for each time point. Mean levels ± standard deviation expressing aired difference between experimental and control samples. Test t-*student* was used for comparisons; *Significant values $P<0.05$.

Table 2. Experimental and control site levels for cytokines with non-normal distribution.

	Day 1		Day 3		Day 7		Day 14		Day 21		Day 28	
	Min.-Max.	Median	Min.-Max.	Median	Min.-Max.	Median	Min.-Max.	Median	Min.-Max.	Median	Min.-Max.	Median
IL-4 GCF EX	13.084-14.732	13.084	13.243-17.430	13.520	12.807-13.738	13.381	12.668-13.520	13.243	11.484-13.659	13.084	13.025-13.957	13.520
IL-4 GCF CC	13.302-14.116	13.818	13.520-15.051	13.441	13.243-14.116	13.669	13.520-13.818	13.738	13.084-14.116	13.480	13.084-13.818	13.243
IL-6 PDL EX	22.366--37.471	40.383*	12.822-172.403	24.012	7.751-131.113	13.295	4.093-58.106	8.594	3.779-15.674	6.552	4.093-5.790	5.256
IL-6 PDL CC	5.628-231.025	7.391	3.711-159.447	85.033	2.603-81.782	9.007	4.093-145.585	7.417	2.995-36.778	14.212	2.559-16.942	4.895
IL-6 GCF EX	3.498-36.017	9588	3.667-37.945	10.688	3.073-6.489	3.721	3.073-21.071	6.060	2.861-9.117	5.739*	5.566-8.443	5.669
IL-6 GCF CC	5.041-13.946	6.681	3.232-113.258	8.024	3.333-6.681	4.626	5.338-100.286	8.685	7.440-35.396	12.415	3.970-7.440	4.609
IL-10 GCF EX	4.792-5.870	4.792	4.398-10.229	4.918	4.220-6.336	4.855	4.398-5.136	4.846	4.043-5.191	4.630	4.344-5.704	4.792
IL-10 GCF CC	4.291-4.918	4.792	4.344-6.298	4.666	4.666-5.191	4.666	4.079-4.792	4.469	4.043-5.063	4.594	4.043-4.846	4.666
IL-17A PDL EX	0.000-7.280	0.000	0.000-5.380	3.080	1.66-7.760	5.380	0.000-8.26	6.560	0.000-14.020	5.405	0.000-11.590	4.000
IL-17A PDL CC	0.000-8.260	3.530	0.000-15.700	7.040	0.000-7.760	1.765	0.000-6.32	3.760	0.000-12.660	2.225	0.000-12.380	3.535
IL-17A GCF EX	0.000-13.13	0.000	0.000-8.770	6.680	0.000-21.03	2.650	0.000-8.070	0.000	0.000-0.000	0.000	0.000-10.920	8.800
IL-17A GCF CC	0.00-4.620	0.000	0.000-8.070	0.000	0.000-8.070	0.320	0.000-10.200	0.000	0.000-2.500	0.000	0.000-1.710	0.000
IFN-γ PDL EX	0.002-0.002	0.002	0.000-0.005	0.002	0.000-0.005	0.003	0.000-0.009	0.002	0.000-0.007	0.001	0.000-0.007	0.002
IFN-γ PDL CC	0.001-0.005	0.002	0.000-0.005	0.001	0.000-0.004	0.001	0.000-0.012	0.007	0.001-0.011	0.003	0.000-0.011	0.001
IFN-γ GCF EX	0.000-0.316	0.000	0.000-1.401	0.003	0.000-0.350	0.005	0.001-0.007	0.002	0.000-0.004	0.001	0.000-0.011	0.001
IFN-γ GCF CC	0.001-0.026	0.003	0.001-0.245	0.009	0.000-0.011	0.002	0.000-0.011	0.001	0.000-0.011	0.001	0.001-0.006	0.002
TNF-α GCF EX	0.490-0.781	0.587	0.390-1.734	0.417	0.490-0.609	0.562	0.490-0.587	0.537	0.450-0.609	0.516	0.490-0.609	0.559
TNF-α GCF CC	0.450-0.724	0.548	0.408-1.106	0.587	0.490-0.680	0.553	0.408-0.638	0.537	0.431-0.609	0.450	0.417-0.693	0.559

Minimum, maximum and median values of cytokines for each time point. Wilcoxon test was used for comparisons. *Significant values $P<0.05$