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EFEITO DA TERAPIA CIRÚRGICA ANTI-INFECCIOSA SOBRE BIOMARCADORES IMUNOLÓGICOS EM PACIENTES PORTADORES DE PERI-IMPLANTITE

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PORTO ALEGRE

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RESUMO

O presente estudo tem por objetivo avaliar o efeito da terapia cirúrgica anti-infecciosa sobre 20 biomarcadores imunológicos em pacientes com peri-implantite, e comparar esse perfil imunológico com os parâmetros clínicos.

Foram selecionados 52 pacientes que apresentavam implantes com peri-implantite, os quais foram divididos em 4 grupos de acordo com a profundidade de sondagem e presença de sangramento e/ou supuração no sítio mésio-vestibular de cada implante com peri-implantite. Os parâmetros clínicos foram mensurados e amostras de fluido crevicular peri-implantar, coletadas do sítio mésio-vestibular de cada implante. Todos pacientes foram submetidos à terapia cirúrgica anti-infecciosa e reavaliados 6 e 12 meses após o tratamento. As amostras de fluido crevicular foram analisadas através do ensaio imunológico Luminex (multi-análise com micro-esferas) para detecção de 20 biomarcadores. Diferenças entre os grupos em relação aos níveis desses biomarcadores ao longo do tempo foram analisadas estatisticamente através das equações de estimação generalizadas. O nível de significância estabelecido foi de 5% ($p < 0,05$).

Os resultados demonstraram reduções significativas dos parâmetros clínicos, entretanto mostraram aumentos significativos dos níveis de alguns biomarcadores pró-inflamatórios (IL-1β, IL-6, IL-12p40, IL-12p70, IL-17a e sCD-40L) após a terapia cirúrgica. A IL-13 foi a única citocina anti-inflamatória que mostrou aumento significativo após o tratamento.

Palavras chave: peri-implantite, implantes dentários, citocinas, biomarcadores farmacológicos, líquido do sulco gengival.

ABSTRACT

The objective of the present study was to evaluate the effects of an anti-infective surgical therapy over 20 peri-implant crevicular fluid biomarkers in patients with peri-implantitis and to compare these immunologic profiles with the clinical parameters.

Fifty-two patients presenting implants affected by peri-implantitis were classified into 4 groups according with probing depth and the presence of bleeding and/or suppuration at mesio-buccal site of each implant with peri-implantitis. Clinical parameters were measured and PICF samples were collected from the mesio-buccal site of every implant. All subjects were submitted to anti-infective surgical therapy. Clinical parameters and PICF biomarkers were re-measured at 6 and 12 months after treatment. PICF samples were analyzed using multiplex bead immunoassay for 20 biomarkers. Differences over time were analyzed using generalized estimating equations. The significance level established for all analyses was 5% ($p < 0.05$).

The results demonstrated a significant reduction of the clinical parameters post-therapy. However, it was observed a significant increase of some pro-inflammatory biomarkers levels (IL-1β, IL-6, IL-12p40, IL-12p70, IL-17a e sCD-40L). And IL-13 was the only anti-inflammatory cytokine, which had a significant increase in its levels after anti-infective surgical therapy.

Key words: peri-implantitis, dental implants, biological markers, cytokines, therapy.

SUMÁRIO

1. INTRODUÇÃO

O tratamento com implantes dentários é um método bem estabelecido na reposição de dentes ausentes. Resultados excelentes são alcançados, porém algumas falhas podem ocorrer (RENVERT, POLYZOIS & CLAFFEY, 2012). A falha precoce está associada a fatores inerentes ao hospedeiro (doenças sistêmicas, fumo, qualidade óssea), ao procedimento cirúrgico (trauma cirúrgico excessivo, contaminação bacteriana na cirurgia, estabilidade inicial do implante, habilidade técnica do profissional) e ao tipo de implante (tipo de superfície e desenho). Enquanto que a falha tardia está associada à sobrecarga oclusal e/ou infecção crônica marginal progressiva, denominada de peri-implantite (ESPOSITO *et al.,*1998; LACHMANN *et al.*, 2007). Entre as complicações, a peri-implantite é a mais comum, ocorrendo em 1 a cada 5 pacientes (KLINGE & MEYLE, 2012).

1.1 Doenças peri-implantares

A resposta do hospeiro à formação do biofilme nos implantes inclui uma série de reações inflamatórias, as quais inicialmente ocorrem no tecido mole, podendo subsequentemente progredir e levar à perda de suporte ósseo (BERGLUNDH *et al.,* 2003). Quando a inflamação é reversível e está confinada apenas aos tecidos moles, a doença peri-implantar é denominada mucosite peri-implantar. Já a peri-implantite é uma doença infecciosa induzida pelo biofilme que afeta os tecidos ao redor de um implante osseointegrado em função, resultando na perda progressiva de suporte ósseo (ESPOSITO *et al.,* 1999).

A prevalência da peri-implantite pode variar entre 28 - 56% nos indivíduos, acometendo de 12 - 43% dos sítios, após 5 anos da instalação do implante (ZITZMANN & BERGLUNDH, 2008). Outro estudo relata que a prevalência da peri-implantite ocorre em 10% dos implantes e em 20% dos pacientes, durante um período de 5 a 10 anos após a instalação do implante (KLINGE & MEYLE, 2012). A grande variação, nesses valores em relação à prevalência desta doença, deve-se às diferenças na definição de peri-implantite. Essas diferenças incluem os parâmetros clínicos inflamatórios (sangramento à sondagem, profundidade de sondagem), os limites relacionados à perda óssea e diferenças na combinação de ambos. Alguns fatores têm aumentado a prevalência da peri-implantite como o fumo, a higiene oral deficiente e a história prévia de periodontite (KLINGE & MEYLE, 2012).

A peri-implantite é clinicamente caracterizada pela profundidade de sondagem ≥5 mm, apresentando, concomitantemente, sangramento à sondagem e/ou supuração e pela presença de perda óssea da crista marginal >4 mm em pelo menos um sítio, após o implante estar em função por 1 ano (SHIBLI *et al.,* 2008*;* SARLATI *et al.,* 2010; CHARALAMPAKIS *et al.,* 2012).

A resposta inflamatória que ocorre na peri-implantite é similar àquela presente na periodontite, devido às similaridades microbiológicas e clínicas entre as doenças periodontal e peri-implantar (HULTIN *et al*., 2002). Os patógenos e seus produtos induzem a resposta imune do hospedeiro, ativando o sistema imune inato e adaptativo (DUARTE *et al.,* 2009).

As citocinas induzidas durante a resposta do hospedeiro frente ao desafio bacteriano estão associadas com o início e progressão da doença. A

resposta imune e inflamatória geralmente pode ser mais agressiva ao hospedeiro do que a própria virulência da atividade dos patógenos (MEDZHITOV, 2007).

1.2 Fatores predisponentes na peri-implantite

A peri-implantite poderá ser iniciada ou mantida por fatores iatrogênicos como: excesso de cimento remanescente, assentamento inadequado da restauração sobre o pilar, sobrecontorno das restaurações, implantes mal posicionados, complicações técnicas. Implantes com plataforma *"switching"* e/ou desenhos de pilares podem dificultar a sondagem, sub estimando a extensão da lesão (LANG & BERGLUNDH, 2011). A peri-implantite é mais frequentemente encontrada em pacientes com história prévia de periodontite (*American Academy of Periodontology*, 2013). Além disso, existe um risco aumentado para o desenvolvimento de peri-implantite em pacientes fumantes (STRIETZEL *et al*., 2007).

1.3 Resposta imunológica

O sistema imune protege o organismo da invasão de microrganismos infecciosos, produzindo uma enorme variedade de células e moléculas capazes de reconhecer especificamente e eliminar uma infinidade de substâncias estranhas. A defesa contra microrganismos é mediada pelas reações iniciais da imunidade inata e pelas respostas tardias da imunidade adaptativa (FOKKEMA; LOOS & VAN DER VELDEN, 2003).

A resposta imunológica inata e adaptativa são os componentes de um sistema integrado de defesa do hospedeiro, no qual várias células e moléculas

funcionam em cooperação. Os mecanismos da imunidade inata proporcionam uma defesa eficaz contra as infecções. Entretanto, muitos microrganismos patogênicos desenvolveram uma resistência à imunidade inata, e a sua eliminação requer os mecanismos da imunidade adaptativa. As moléculas produzidas durante a resposta imunológica inata estimulam a imunidade adaptativa e influenciam a natureza das respostas imunológicas adaptativas. Os principais componentes do sistema imunológico inato são barreiras físicas e químicas, tais como o epitélio e as substâncias antibacterianas nas superfícies epiteliais; células fagocitárias (neutrófilos, macrófagos) e células NK (*natural killer*); proteínas do sangue, incluindo frações do sistema complemento e outros mediadores da inflamação, e proteínas denominadas citocinas, que regulam e coordenam várias atividades das células da imunidade inata. Por sua vez, os principais componentes da imunidade adaptativa incluem os linfócitos e seus produtos, como os anticorpos (ABBAS; LICHTMAN & PILLAI, 2008).

Existem dois tipos de resposta imunológica adaptativa, a imunidade humoral e a imunidade celular, que são mediadas por diferentes componentes do sistema imunológico, cuja função é eliminar os diversos tipos de microrganismos. A imunidade humoral é mediada pelos anticorpos, que são produzidos pelos linfócitos B e a imunidade celular é mediada pelos linfócitos T (também chamados de células T). Os dois subconjuntos principais de células T são os linfócitos T auxiliares (Th) e linfócitos T citotóxicos (KINDT; GOLDSBY & OSBORNE, 2008). As células T auxiliares, Th1 e Th2, estão envolvidas também na imuno-patogênese da doença periodontal. A resposta imune pode exibir um padrão Th1, consistindo predominantemente de uma resposta celular pró-inflamatória, ou um padrão Th2, com características anti-inflamatórias, ou

ainda uma resposta imune predominantemente humoral (GARLET *et al.,* 2003). A resposta imunológica à infecção é regulada pelo equilíbrio entre citocinas Th1 e Th2, determinando assim o efeito da mesma (GEMMELL; MARSHALL & SEYMOUR, 1997).

Recentemente, foi identificado um terceiro subconjunto de células T, chamadas de células Th17, as quais secretam a inteleucina-17 (IL-17), também conhecida como IL-17a. A função principal das células Th17 está relacionada à indução de reações inflamatórias, sendo consideradas importantes mediadoras de dano tecidual em doenças inflamatórias imunomediadas (SEVERINO; NAPIMOGA & PEREIRA, 2011).

1.4 Atividades biológicas de alguns biomarcadores

1.4.1 Citocinas pró- e anti-inflamatórias

A resposta imune é regulada pelo equilíbrio entre as citocinas pró- e anti-inflamatórias (SEVERINO; NAPIMOGA & PEREIRA, 2011). A produção das citocinas, de forma apropriada, é essencial para o desenvolvimento da imunidade protetora, porém se houver um desequilíbrio entre elas, consequentemente, resultará em doença destrutiva e progressiva, determinando assim a severidade da doença (GEMMELL; MARSHALL & SEYMOUR, 1997).

A interleucina-1 (IL-1) é uma citocina pró-inflamatória que permite o recrutamento de células em direção aos sítios infectados, promovendo reabsorção óssea e estimulando prostaglandinas, liberadas por monócitos e fibroblastos, e liberando metaloproteinases que degradam as proteínas da matriz extracelular. A IL-1 é a mais potente indutora de desmineralização

óssea, agindo sinergicamente com o fator de necrose tumoral-alfa (TNF-α) no estímulo de reabsorção óssea e nas principais mudanças da matriz do tecido conjuntivo.

A forma predominante de IL-1 encontrada nos tecidos periodontais é a interleucina-1 beta (IL-1β), a qual é primariamente produzida por macrófagos. Na literatura, é relatado o aumento dos níveis de IL-1β no fluido crevicular gengival em pacientes com periodontite. Esses níveis elevados de IL-1β foram associados a sítios ativos e com progressão de doença (REINHARDT *et al.*, 1993).

A IL-17 é uma citocina pró-inflamatória, a qual estimula alguns tipos de células a produzirem mediadores inflamatórios, como por exemplo, IL-1, IL-6, TNF-α, metaloproteinases e quimiocinas, induzindo à atividade osteoclástica (BEKLEN *et al.*, 2007).

As citocinas anti-inflamatórias, como a interleucina-10 (IL-10) e o fator de crescimento transformador-beta (TGF-β), inibem a produção de citocinas pró-inflamatórias por diversas células, consequentemente, contra balanceando a resposta imune e evitando assim a reabsorção óssea na doença periodontal (KITAMURA *et al.,* 1996).

O receptor antagônico de interleucina-1 (IL-1ra) é uma citocina anti-inflamatória, que se une à superfície das células do hospedeiro, ligando-se à IL-1, bloqueando a sua ação. As citocinas IL-1α, IL-1β e IL-1ra são potenciais candidatas para marcadores genéticos na peri-implantite, devido ao papel crítico desses biomarcadores na inflamação (LAINE *et al.*, 2006).

A colonização bacteriana ao redor dos implantes, por microrganismos presentes na cavidade oral, ocorre em poucas semanas após a sua instalação.

Produtos bacterianos dos periodonto-patógenos estimulam a produção de mediadores inflamatórios, secretados no fluido crevicular peri-implantar, os quais causam a destruição dos tecidos peri-implantares, quando houver um desequilíbrio na resposta imune (MELO *et al.*, 2012).

No estudo de LUO *et al.* (2011), foi relatado que sítios com peri-implantite apresentaram níveis mais elevados de IL-1β, IL-6, IL-8 e TNF-α no fluido crevicular peri-implantar. Corroborando com o estudo de MENDONÇA *et al.* (2009), o qual mostrou que níveis mais elevados de TNF-α, no fluido crevicular peri-implantar, podem contribuir significativamente na patogênese da peri-implantite.

1.4.2 Quimiocinas

As quimiocinas são pequenas proteínas (8 a 11 KDa) que são capazes de ativar e promover a migração de uma grande variedade de leucócitos (DEZEREGA *et al.,* 2010a). Além da capacidade de recrutar leucócitos, também estimulam a liberação de mediadores inflamatórios e modulam a vascularização, exarcebando o processo inflamatório (DEZEREGA *et al.,* 2010b).

No estudo de DEZEREZA *et al.* (2010a), foi demonstrado que pacientes com periodontite crônica apresentaram níveis mais elevados da quimiocina, chamada proteína-3 quimiotática de monócito (MCP-3), no fluido crevicular gengival, quando comparados aos pacientes saudáveis. O estudo de PETKOVIC *et al.* (2010) mostrou que a concentração da quimiocina, proteína-1alfa inflamatória de macrófago (MIP-1α), foi significativamente mais elevada, no fluido crevicular peri-implantar, em pacientes com peri-implantite.

1.4.3 Fatores de crescimento

Por sua vez, os fatores de crescimento aceleram o processo de cicatrização, promovendo a regeneração óssea e tecidual (KAIGLER *et al.* 2011). Entre eles, está o fator de crescimento de fibroblasto - básico (FGF-2), que induz à forte atividade angiogênica e à capacidade proliferativa nas células mesenquimais indiferenciadas dentro do ligamento periodontal, possuindo, portanto, um papel importante no reparo tecidual (MURAKAMI, 2011; KITAMURA *et al.*, 2011).

O fator de crescimento derivado das plaquetas (PDGF) possui um potencial previsível na promoção de regeneração óssea na clínica diária. É uma proteína natural encontrada na matriz óssea e apresenta-se em três diferentes formas: PDGF-AA, -AB, -BB. O PDGF é liberado pelas plaquetas durante a coagulação, após uma injúria aos tecidos moles e duros, possuindo um potente fator quimiotático e mitogênico para os fibroblastos do ligamento periodontal, cementoblastos e osteoblastos (KAIGLER *et al*, 2011).

Há relatos na literatura que o fator de crescimento vascular endotelial (VEGF) estimula a proliferação endotelial *in vitro* e também possui atividade angiogênica *in vivo*. No estudo de CORNELINI *et al.* (2001), a expressão de VEGF foi mais baixa nas biópsias removidas de tecido mole ao redor de implantes com peri-implantite do que de implantes saudáveis.

Os mecanismos da resposta imunológica do hospedeiro frente à agressão bacteriana na doença peri-implantar têm sido explorados, porém os estudos ainda são restritos.

1.5 Tratamento das doenças peri-implantares

A terapia mecânica não cirúrgica mostrou-se efetiva apenas no tratamento das lesões de mucosite peri-implantar. A associação de enxaguatórios antimicrobianos tem melhorado o resultado deste tratamento. Portanto, a terapia mecânica não cirúrgica não é indicada para as lesões de peri-implantite (RENVERT; ROOS-JANSAKER & CLAFFEY, 2008).

Estudos têm sido realizados na tentativa de determinar o melhor protocolo de tratamento para a peri-implantite. O principal objetivo do tratamento cirúrgico para essa doença é acessar a superfície do implante, para ser realizado o debridamento mecânico e a descontaminação do mesmo, a fim de alcançar a resolução da lesão inflamatória (LINDHE & MEYLE, 2008).

Diversos agentes químicos e mecânicos têm sido usados para a descontaminação da superfície do implante como jato de bicarbonato, irrigação com soro fisiológico e clorexidine, aplicação de ácido cítrico, uso de peróxido, *laser* terapia, debridamento manual ou com ultrassom e associação de medicação tópica (RENVERT; POLYZOIS & CLAFFEY, 2012). A combinação de técnicas de descontaminação mecânica e química deve ser aplicada. O biofilme bacteriano, depósitos de cálculo e tecido de granulação devem ser removidos sem alterar a superfície do implante (WISMEIJER & SUBRAMANI, 2012). Curetas metálicas e pontas de ultrassom, embora sejam mais efetivas para alcançar um adequado debridamento, podem danificar a superfície do implante (MANN *et al.,* 2012).

Os procedimentos regenerativos, como as técnicas de enxerto ósseo associadas ou não à utilização de membranas, têm sido realizados com o objetivo de preencher o defeito ósseo causado pela peri-implantite, entretanto

não há evidência de que haja benefícios no resultado final do tratamento (CLAFFEY *et al.,* 2008).

A re-osseointegração vai depender principalmente do tipo de técnica de descontaminação, do material de regeneração óssea e da superfície do implante. A re-osseointegração não pode ser alcançada somente com a descontaminação da superfície (WISMEIJER & SUBRAMANI, 2012).

A maioria dos trabalhos descritos na literatura objetivou a avaliação dos efeitos clínicos, radiográficos e microbiológicos após o tratamento da peri-implantite, havendo poucos estudos que observaram os efeitos imunológicos nos sítios peri-implantares após a terapia. Portanto, o presente trabalho tem como objetivo avaliar os parâmetros clínicos e 20 biomarcadores imunológicos em sítios peri-implantares de pacientes com peri-implantite após a terapia cirúrgica anti-infecciosa.

2. CAPÍTULO I

Artigo 1

Relationship between PICF biomarkers and clinical parameters in peri-implant sites

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Relationship between PICF Biomarkers and Clinical Parameters in Peri-implant Sites

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Abstract

Background: The objectives of this study were to assess profiles of peri-implant crevicular fluid (PICF) biomarkers in different groups of implant sites and to compare these levels with the clinical parameters.

Methods: Clinical parameters were measured and the peri-implant sites were classified in 4 groups, according to probing depth (PD) and presence of bleeding on probing (BoP) and/or suppuration (SUP). PICF samples were collected from the mesio-buccal site of every implant. PICF profiles of 20 biomarkers were measured using multiplex bead immunoassay. The significance of the differences between groups was determined using generalized estimating equations. Multiple comparisons were adjusted to control false discovery rate and the significance level established for all analyses was 5% ($p < 0.05$).

Results: There were positive correlations between clinical parameters and mean levels of biomarkers ($p < 0.05$). Deep sites associated with BoP and/or SUP showed statistically significantly higher levels of IL-1β, IL-17a, TNF-α, sCD-40L, MDC, FGF-2, GM-CSF and PDGF-BB ($p < 0.05$).

Conclusion: The results suggest that the expression of several biomarkers may play an important role in the onset and progression of peri-implantitis.

Kew Words: biological markers; periimplantitis; cytokines; dental implant

Introduction

The clinical application of implant-supported rehabilitations has increased significantly in the past decades. It has been estimated that more than 2 million dental implants are installed worldwide every year, and bacterial induced peri-implant inflammation with loss of bone support, so called peri-implantitis, has been diagnosed in an average of 28-56% of subjects and in 12-43% of implant sites after 5 years of function.[1,2]

Peri-implantitis is defined as an inflammatory reaction affecting the tissues surrounding osseointegrated dental implants in occlusal function, resulting ultimately in loss of supporting bone.[3] This disease is usually diagnosed by assessing the presence of deep pockets, bleeding on probing, suppuration and radiographic measurements of bone loss.[4]

The inflammatory response observed in peri-implantitis is similar to periodontitis. Bacterial products from periodontal pathogens may stimulate the production of inflammatory mediators leading to damage of hard tissues around implants.[5] The cytokines induced by the host response to this bacterial challenge are associated with both the beginning and progression of the disease. Also, it is accepted that the host immune and inflammatory responses are more damaging to the implant surrounding tissues than the very virulence of the pathogen that elicit them.[6]

Gingival crevicular fluid is a non-invasive collectable fluid used for the evaluation of the local cellular metabolism that reflects the periodontal heath status.[7] Patients presenting peri-implantitis usually show an increase in volume of peri-implant crevicular fluid (PICF).[8] The assessment of levels of certain inflammatory mediators in PICF has been propose as a measure of active peri-implantitis, which may be useful for early diagnosis and prevention of this disease.[9]

A biomarker is an indicator of a biological state and might help to distinguish between normal and pathologic processes[10].The presence of biomarkers such as inflammatory mediators in the PICF has been investigated in some studies.[11-14] However, few of these studies demonstrated the presence of more than 5 biomarkers in PICF, failing to analyze a broad spectrum of biomarkers that may directly influence the local inflammatory response. Therefore, the main objective of the present cross-sectional study was to examine the profiles of 20 PICF biomarkers in 4 different groups of implant sites. The hypothesis to be tested was that sites with deep probing depth associated with presence of bleeding on probing and/or suppuration would differ in PICF biomarkers profiles from those shallow probing depth sites without any other clinical signs of inflammation.

Materials and Methods

Subject Population and Clinical Examination

Fifty-two patients who had been rehabilitated with implants were recruited at the Guarulhos University Oral Implantology Clinic (Guarulhos, SP, Brazil). The study population included 41 women and 11 men with mean age of 55.7 years, 80% were Caucasian and 21% were smokers. Also, to be included in the present investigation, all subjects should present loaded implants for at least 12 months and should not have medical history of any chronic diseases, recent use of mouthwashes and history of antibiotic therapy and/or steroid or non-steroid anti-inflammatory medications in the 6 months preceding the study. Individuals who had received any previous peri-implantitis therapy or were either pregnant or nursing were also excluded. All subjects signed an informed consent form prior to entering the study. The Guarulhos University's Ethics Committee in Clinical Research has approved the present study protocol including the periodontal examination and the collection of peri-implant crevicular fluid (PICF) of each patient.

A calibrated clinician assessed every clinical parameter in each patient, at 6 specific sites equally around implants and natural teeth (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual and disto-lingual). The clinical parameters included visible plaque index - PI (0 or 1), gingival bleeding - GBI

(0 or 1), probing depth - PD (mm), attachment loss - AL (mm), bleeding on probing - BoP (0 or 1) and suppuration - SUP (0 or 1). PD and AL were recorded to the nearest millimeter using a North Carolina periodontal probe (PCPUNC 15 Hu-friedy Mfg Co, Chicago, IL). Intraexaminer variability of sites was determined by means of standard error of measurement[15] for PD and AL parameters (SE = 0.2mm for PD and SE = 0.3mm for AL). *Kappa* Test was performed for other parameters (*K* > 93% for PI, GBI, BoP and SUP).

The implant sites of the patients were distributed into 4 groups according with PD, BoP and/or SUP parameters at mesio-buccal site, due to the PICF samples that had been collected from the mesio-buccal site as well. Group 0 had PD ≤ 4mm and no bleeding on probing and no suppuration, group 1 had PD > 4 mm and BoP and SUP=0, group 2 had PD ≤ 4mm and BoP or SUP=1 and group 3 had PD > 4 mm and BoP or SUP=1.

Peri-implant Crevicular Fluid Collection

The samples were collected before the clinical measurements, in order to avoid the presence of bleeding on probing contaminating the sample. PICF samples were obtained from the mesio-buccal site of all implants. After removing the supramucosal biofilm, the sites were isolated with cotton rolls and gently dried with an air syringe to eliminate a possible contamination with saliva. PICF samples were collected by inserting filter paper strips (Periopaper®, Interstate Drug Exchange, Amityville, NY, USA) 1-2mm into the peri-implant sulcus or pocket for 30 seconds. Samples were immediately placed in Eppendorf tubes on ice, transported to the laboratory and stored at -80ºC. Samples visually diagnosed as contaminated with blood were discarded. All PICF samples were then shipped to the laboratory for analysis (The Forsyth Institute, Cambridge, MA, USA). Only the PICF samples from implants which presented peri-implantitis at some site were processed.

Quantification of biomarkers using multiplexed bead immunoassay (Luminex)

Biomarkers levels were determined using the human cytokine 20-plex (magnetic bead panel) Millipore kit (Millipore Corporation, Billerica, MA, USA). Before the assay, PICF samples were eluted using 60µl of the assay buffer provided in the Millipore kit by vortexing for 30 min. and then centrifuged for 10 min. at 10,000 rpm. Twenty biomarkers, meaning interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 p40 (IL-12p40), interleukin-12 p70 (IL-12p70), interleukin-15 (IL-15), interleukin-17 (IL-17a), tumor necrosis factor alpha (TNF-α), soluble human CD40 ligand (sCD-40L), interleukin-10 (IL-10), interleukin-13 (IL-13), interleukin-1 receptor antagonist (IL-1ra), eotaxin, monocyte chemoattractant protein-3 (MCP-3), macrophage-derived chemoattractant (MDC), fibroblast growth factor-2 (FGF-2), fms-like tyrosine kinase-3 ligand (Flt-3L), granulocyte-macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor BB (PDGF-BB) and vascular endothelial growth factor (VEGF) were measured. The assays were performed in 96-well solid plate following the manufacturer's instructions. Briefly, the plate was washed and the controls and standards (ranging from 3.2 to 10,000 pg/ml) were added into the wells in duplicate. The samples were then loaded in the remaining wells and microsphere beads coated with monoclonal antibodies against the twenty different target analytes were added to all wells. The plate was wrapped with aluminum foil and incubated with agitation on a plate shaker overnight at 4ºC. The well contents were removed using a hand-held magnetic plate and the plate was washed twice, and a mixture of biotinylated secondary antibodies was added into each well. After incubation for 1 hour with agitation on a plate shaker at room temperature, streptavidin conjugated to the fluorescent protein, R-phycoerythrin (streptavidin-RPE) was added to the beads and incubated for 30 min. with agitation on a plate shaker at room temperature. After washing to remove the unbound reagents, sheath fluid (Luminex Corporation, Austin, TX, USA) was added to the wells and the beads (minimum of 50 per analyte) were immediately analyzed in the Luminex 200^{TM} instrument (Luminex Corporation, Austin, TX, USA). The Luminex 200^{TM} monitors the spectral properties of the beads to distinguish the different analytes, while

simultaneously measuring the amount of fluorescence associated with R-phycoerythrin, reported as median fluorescence intensity. The concentrations of the unknown samples (antigens in PICF samples) were estimated from the standard curve using the Bio-Plex® software (Bio-Rad Laboratories, Hercules, CA, USA) and expressed as picograms/30seconds (pg/30s). Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification of the standard curves were assigned the highest value of the curve.

Data analysis

Clinical parameters were compared among groups using Pearson Chi-Square Test or ANOVA, accordingly. The mean values for the profiles of 20 PICF biomarkers in each group were compared by generalized estimating equations. Log-transformation was applied to the measurements of biomarkers. Generalized estimating equations were used to model this association while accounting for the clustering of implant sites within subjects.[16] The mesio-buccal site was used as the unit of analysis. Generalized estimating equations with an exchangeable working correlation, normal link and semirobust standard errors were used to estimate mean ratios and their respective 95% confidence intervals. Multiple comparisons were adjusted to control false discovery rate. Statistical analysis was performed using statistical software (SPSS version 22.0). The significance level established for all analyses was 5% ($p<0.05$).

Results

Clinical findings

Table 1 presents the mean clinical parameters for each analyzed group, showing statistically significant difference among the groups for PD, AL, BoP and SUP parameters.

Peri-implant Crevicular Fluid Biomarkers

A total of 150 PICF samples from 150 implants presenting peri-implantitis were processed as part of the study. Table 2 and 3 present the geometric mean of each biomarker (pg/30s) for all groups and the comparisons between the

groups, respectively. The multiple comparisons were adjusted to control false discovery rate. Table 2 presents 11 biomarkers presenting significant differences between them. Table 3 presents PDGF-BB as the only biomarker that had a statistically significant difference when comparing group 1 with group 0, showing that deeper PD sites (PD > 4 mm) had more PDGF-BB concentration. However, when inflammation was examined (BoP or SUP=1), group 2 had a statistically significant difference in some pro-inflammatory cytokines (IL-1β, IL-17a and sCD-40L) in relation to group 0. Group 2 had a statistically significant difference in IL-1ra (anti-inflammatory cytokine), MDC (chemokine), FGF-2 and GM-CSF (growth factors) when compared to group 0. Differences were also present when sites with deep pockets (group 3) were compared to those with shallow PD without inflammation (group 0). There was a statistically significant increase in IL-1β, IL-17a, TNF-α, sCD-40L (pro-inflammatory cytokines), MDC (chemokine), FGF-2, GM-CSF and PDGF-BB (growth factors) concentrations.

Discussion

The main goal of the present study was to examine the relationship between biomarkers profiles in 4 different groups of implant sites in patients presenting clinically assessed signs of peri-implantitis. In order to achieve this goal, a multiplex bead immunoassay was used to determine the PICF biomarker expression profile in peri-implant sites. Other standard techniques have been shown to be reliable in investigating biomarkers levels in PICF for the same purpose, mainly involving enzyme link immunosorbent assay (ELISA) and western blotting[17], however the use of the multianalyte microsphere assay allowed the simultaneous quantification of several targets in a large number of samples at the same assay, improving the quality and the spectrum of the analysis.[18]

The results demonstrated that sites presenting inflammation (BoP=1 or SUP=1) with deep pockets (PD >4mm) had significantly higher levels of 4 pro-inflammatory cytokines, showing positive associations between the clinical parameters of peri-implant sites and the profiles of these biomarkers (see Table 3).

Results of the present study are in accord with Luo et al.,[19] who reported that sites with diagnosed peri-implantitis presented higher levels of IL-1β, lL-6, IL-8 and TNF-α in PICF, even though other reports analyzing the mean concentration of IL-1β and IL-6 between healthy and sites with peri-implantitis presented no different levels of these assessed biomarkers.[14] Interleukins have been said to present a role in regulating bone resorption and formation, although loss of implant osseointegration, marginal bone loss or implant failure, might be linked to an increased level of these cytokines in PICF.[20] Also, some of our findings are in agreement with Darabi et al.[21] study which demonstrated an increased presence of IL-17 in patients with peri-implantitis. This biomarker has been said to influence the production of other inflammatory cytokines and thus contribute significantly to the peri-implant bone loss. Mendonça et al.[4] showed that high levels of TNF-α in the PICF might be related to the presence of clinical signs of peri-implant inflammation, indicating that TNF-α may also play an important role in the pathogenesis of peri-implantitis. Also, other reports supported these results, where higher levels of IL-12 and TNF-α were verified in sites with PD > 4 with BoP and/or SUP and bone loss > 4 mm.[11,22]

As part of the large spectrum of biomarkers investigated in the present study, we could observe higher levels of sCD-40L, MDC, FGF-2, GM-CSF and PDGF-BB present in peri-implant pockets. To the best of our knowledge, this possible correlation, although yet to be confirmed by further investigations, has never been mentioned before in the literature.

The pro-inflammatory cytokine IL-1β is used as a biochemical marker of periodontal disease by several investigations because of its high crevicular concentrations in periodontal and peri-implant diseased sites.[23-25] The inflammatory response that occurs in peri-implantitis has been said to be similar to those patients with periodontitis.[26] Higher concentrations of pro-inflammatory cytokines may up-regulate a bone biomarker called receptor activator nuclear factor kappa-b (RANK), increasing the osteoclastogenesis reported in peri-implantitis.[27] Previous studies suggested that generalized aggressive periodontitis might be associated with higher gingival crevicular fluid levels of IL-1β, GM-CSF and IL-1β/IL-10 ratio when compared to the contents of gingival fluids from a periodontally healthy subject group.[28-30] In our study, we

found higher levels of IL-1β and GM-CSF in sites with PD > 4mm associated with BoP and/or SUP when compared to sites without inflammation.

There are limited data regarding the presence and specific levels of biomarkers in peri-implant crevicular fluid, along with their precise role in the establishment and progression of the peri-implant inflammatory diseases. Therefore, further studies are needed to investigate the very importance, expression and specific part played by a large number of biomarkers in peri-implantitis. Since peri-implantitis is usually latent in early stages after implant placement, biomarkers analysis in PICF might act as an important tool aiming at prediction at the site level and/or determination of patient susceptibility. The identification of these specific biomarkers that might directly influence the establishment and progression of peri-implant diseases may also contribute to a new method of evaluation of the general prognosis related to the disease, and even influence distinct treatment approaches for a particular group of individuals.

Conclusion

The present investigation provided valuable information about the presence of several biomarkers in different peri-implant sites. Our data reinforced the relationship of deeper sites (PD > 4mm) associated with BoP and/or SUP with higher levels in IL-1β, IL-17a, TNF-α, sCD-40L, MDC, FGF-2, GM-CSF and PDGF-BB. A significant relation between PICF biomarkers and clinical inflammatory signs in peri-implant sites was present, even though few studies evaluating this significant amount of inflammatory mediators are available.

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Tables

Table 1: Clinical Parameters for the Clinical Groups (mean per site)

Clinical Parameters	Group 0 n=27	Group 1 n=10	Group 2 n=52	Group 3 n=61	p value
PI (%)	7.4	10.0	11.5	13.1	0.88*
GBI (%)	96.3	100.0	88.5	91.8	0.35*
PD (mm)	2.9 ± 0.9	5.1 ± 0.3	3.3±0.8	5.8±1.2	< 0.001**
AL (mm)	3.3 ± 1.5	4.8 ± 1.4	3.9±0.9	5.8±1.3	< 0.001**
BoP (%)	0	0	100	98.4	< 0.001*
SUP (%)	0	0	17.3	41	< 0.001*

* Pearson Chi-Square Test

** ANOVA

Table 2: Biomarkers' profiles in each group

	Biomarkers (pg/30s)	Geometric means (per group) 0	1	2	3	Unadjusted *p* value
Pro – Inflammatory Cytokines (a)	IL-1β	11.13	60.79	49.26	63.62	0.003*
	IL-2	0.74	1.22	2.16	2.21	0.028*
	IL-6	2.76	4.44	5.10	7.25	0.112
	IL-12p40	5.60	9.71	12.45	12.09	0.238
	IL-12p70	1.06	2.30	2.76	3.01	0.095
	IL-15	0.92	1.90	1.97	2.75	0.157
	IL-17a	0.38	1.84	1.61	1.51	0.010*
	TNF-α	1.72	3.55	3.64	4.04	0.011*
	sCD-40L	22.03	54.03	51.18	72.73	< 0.001*
Anti-Inflammatory Cytokines (b)	IL-10	6.65	16.06	10.14	9.46	0.467
	IL-13	1.80	4.13	4.00	4.16	0.064
	IL-1ra	1,246.76	2,257.02	1,928.89	1,710.09	0.025*
Chemokines (c)	Eotaxin	10.11	16.90	20.33	21.34	0.055
	MCP-3	6.34	14.38	14.14	14.37	0.119
	MDC	30.83	45.25	46.52	50.01	0.003*
Growth-Factors (d)	FGF-2	14.76	49.61	43.58	48.34	0.012*
	Flt-3L	4.49	7.07	8.13	8.92	0.070
	GM-CSF	0.37	1.63	1.48	1.98	0.013*
	PDGF-BB	15.86	70.68	32.87	42.28	< 0.001*
	VEGF	59.69	99.11	74.62	80.64	0.038*

Table 3: Mean Ratio comparisons among groups

		1 x 0			2 x 0			3 x 0		
	Biomarker	Mean Ratio	95%CI	Adjusted p value	Mean Ratio	95%CI	Adjusted p value	Mean Ratio	95%CI	Adjusted p value
a	IL-1β	5.46	0.35-84.15	0.477	4.43	1.15-17.03	0.022*	5.72	1.56-20.97	0.002*
a	IL-2	1.64	0.21-12.78	0.989	2.92	0.97-8.80	0.063	2.97	0.93-9.50	0.079
a	IL-6	1.61	0.28-9.28	0.979	1.85	0.62-5.55	0.600	2.63	0.93-7.43	0.084
a	IL-12p40	1.73	0.28-10.85	0.966	2.22	0.71-6.99	0.339	2.16	0.75-6.21	0.290
a	IL-12p70	2.16	0.36-13.11	0.836	2.60	0.83-8.12	0.156	2.83	0.88-9.06	0.107
a	IL-15	2.07	0.32-13.57	0.891	2.14	0.64-7.16	0.458	2.99	0.85-10.57	0.128
a	IL-17a	4.82	0.72-32.39	0.166	4.23	1.29-13.93	0.009*	3.97	1.12-14.11	0.025*
a	TNF-α	2.07	0.69-6.20	0.403	2.11	0.98-4.57	0.062	2.35	1.12-4.94	0.015*
a	sCD-40L	2.45	0.61-9.84	0.430	2.32	1.17-4.61	0.007*	3.30	1.58-6.90	< 0.001*
b	IL-10	2.41	0.38-15.30	0.756	1.52	0.56-4.12	0.842	1.42	0.57-3.54	0.893
b	IL-13	2.30	0.44-12.01	0.710	2.22	0.88-5.61	0.133	2.31	0.99-5.38	0.054
b	IL-1ra	1.81	0.83-3.95	0.243	1.55	1.02-2.35	0.034*	1.37	0.85-2.22	0.410
c	Eotaxin	1.67	0.62-6.69	0.910	2.01	0.92-4.41	0.111	2.11	0.98-4.53	0.059
c	MCP-3	2.27	0.74-6.93	0.283	2.23	0.89-5.60	0.126	2.27	0.91-5.67	0.108
c	MDC	1.47	0.66-3.26	0.750	1.51	1.02-2.24	0.037*	1.62	1.12-2.34	0.003*
d	FGF-2	3.36	0.81-13.86	0.138	2.95	1.07-8.13	0.029*	3.28	1.20-8.97	0.012*
d	Flt-3L	1.58	0.52-4.76	0.859	1.81	0.92-3.57	0.119	1.99	0.99-3.98	0.054
d	GM-CSF	4.45	0.50-39.46	0.360	4.06	1.06-15.53	0.036*	5.43	1.29-22.80	0.011*
d	PDGF-BB	4.46	1.74-11.40	< 0.001*	2.07	0.83-5.16	0.195	2.67	1.27-5.58	0.003*
d	VEGF	1.66	0.63-4.36	0.665	1.25	0.80-1.96	0.715	1.35	1.00-1.83	0.053

a- Pro-inflammatory Cytokines

b- Anti-inflammatory Cytokines

c- Chemokines

d- Growth-Factors

*Statistically significant difference

3. CAPÍTULO II

Artigo 2

Effects of anti-infective surgical therapy on PICF biomarkers in patients with peri-implantitis

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Effects of anti-infective surgical therapy on PICF biomarkers in patients with peri-implantitis

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Abstract:

Aim: The objective of the present longitudinal clinical study was to evaluate the levels of 20 peri-implant crevicular fluid (PICF) biomarkers over time (6 months and 12 months) after anti-infective surgical therapy in patients with peri-implantitis.

Material & Methods: Fifty-two patients presenting implants affected by peri-implantitis had their clinical parameters measured and PICF samples collected from the mesio-buccal site of every implant. These subjects were submitted to anti-infective surgical therapy. Clinical parameters and PICF biomarkers were re-measured at 6 and 12 months after treatment. PICF samples were analyzed using multiplex bead immunoassay for 20 biomarkers. Differences over time were analyzed using generalized estimating equations. The significance level established for all analyses was 5% ($p < 0.05$).

Results: Significant reductions in clinical parameters were detected after surgical therapy. However, significant increases of IL-1β, IL-6, IL-12p40, IL-12p70, IL-13, IL-17a and sCD-40L levels in PICF were demonstrated after therapy.

Conclusion: The anti-infective surgical treatment improved the clinical parameters and also the IL-13, Flt-3-L and GM-CSF levels in PICF, but was accompanied by an increase in levels of several PICF biomarkers such as the pro-inflammatory cytokines.

Key words: peri-implantitis; dental implants; biological markers; cytokines; therapy

Introduction

Dental implant therapy constitutes a well-established approach of replacing missing teeth. However, some treatment complications may occur and one of the most common is peri-implantitis (Renvert et al. 2012). It has been said to be expected up to one of every five patients (Klinge & Meyle 2012).

Peri-implantitis is an infectious disease resulting in an inflammatory process that affects alveolar bone and soft tissues around an implant under functional load (Esposito et al. 1999). There are some factors that have been shown to affect peri-implantitis prevalence including smoking, poor oral hygiene and a history of periodontitis (Esposito et al. 1998). If undiagnosed, peri-implantitis may lead to complete loss of osseointegration and, consequently, implant failure (Heitz-Mayfield 2008).

Bacterial products from periodontal pathogens stimulate the production of inflammatory mediators that may lead to tissue destruction (Gemmel et al. 1997). Clinical and radiographic parameters provide limited information about the dynamic pathophysiological mechanisms during the installation and progression of the disease. Therefore, both researchers and clinicians have proposed that the measurement of molecular markers in the peri-implant crevicular fluid (PICF) could serve as a diagnostic parameter, indicating the presence and/or progression of peri-implantitis (Ozçakir-Tomruk et al., 2012).

Based on the clinical and microbial similarities between peri-implant and periodontal diseases, similar therapies have been proposed for the peri-implantitis treatment (Duarte et al. 2009). Attempts have been performed to evaluate useful treatment protocols that might contribute to the achievement of clinical resolution of peri-implantitis (Claffey et al. 2008). Local antimicrobials, systemic antibiotics, mechanical debridement and/or regenerative techniques have been proposed to the treatment of this infection (Roos-Jansaker et al. 2003).

Studies have investigated the clinical and microbiologic profiles of peri-implantitis before and after different treatment approaches (Roos-Jansaker et al. 2003, Klinge & Meyle 2012). However, there are few data regarding clinical and immunologic effects of anti-infective surgical therapy for peri-implantitis. Therefore, the main objective of the present study was to examine the levels of 20 PICF biomarkers over time (6 months and 12 months) after surgical therapy, and also to correlate them to the clinical parameters.

Materials and Methods

Study Design

The present longitudinal clinical study evaluated the levels of PICF biomarkers in implants affected by peri-implantitis by means of multiplexed bead immunoassay (Luminex). PICF samples from mesio-buccal sites were collected at baseline and 6 and 12 months after completion of a peri-implantitis therapy, which involved access flap surgery and surface decontamination.

Subject Population and Clinical Examination

Fifty-two patients who had been rehabilitated with implants were recruited at the Guarulhos University Oral Implantology Clinic (Guarulhos, SP, Brazil). The study population presented 150 implants affected by peri-implantitis, including 41 women and 11 men with mean age of 55.7 years, 80% were Caucasian and 21% were smokers.

The Guarulhos University's Ethics Committee in Clinical Research approved the present study protocol. All the participants were informed about the purposes of the study and confirmed their agreement by signing an Informed Consent Form. Also, to be included in the present investigation, all subjects should present a loaded implant-supported restoration for at least 12 months and should not have medical history of any chronic diseases, recent use of mouthwashes and history of antibiotic therapy and/or steroid or non-steroid anti-inflammatory medications in the 6 months preceding the study. Individuals who were either pregnant or nursing were also excluded. The subjects with untreated advanced periodontitis, previous peri-

implantitis treatment and abusive use of alcohol or drugs were also excluded from the study.

Clinical parameters were measured in each patient at 6 specific sites around implants and natural teeth. The diagnosis of peri-implantitis was determined by the presence of probing depth ≥ 5mm associated with the presence of bleeding and/or suppuration after probing and bone loss > 4 mm (Shibli et al. 2008).

The clinical parameters including visible plaque index - PI (0 or 1), gingival bleeding index – GBI (0 or 1), probing depth - PD (mm), attachment loss - AL (mm), bleeding on probing - BoP (0 or 1) and suppuration - SUP (0 or 1) were measured at six sites per implant or tooth (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual and disto-lingual) by a calibrated examiner. PD and AL were recorded in millimeters using a North Carolina periodontal probe (PCPUNC 15 Hu-friedy Mfg Co, Chicago, IL).

The mesio-buccal implant sites of the patients were distributed into 4 groups at baseline according with PD, BoP and SUP parameters at mesio-buccal site, due to the PICF samples had been collected from the mesio-buccal site. Group 0 had PD ≤ 4mm and no bleeding on probing and no suppuration, group 1 had PD > 4 mm and BoP and SUP=0, group 2 had PD ≤ 4mm and BoP or SUP=1 and group 3 had PD > 4 mm and BoP or SUP=1.

Anti-infective Surgical Therapy

All patients were submitted to the surgical treatment of peri-implantitis because this condition was present in at least one implant of each patient. First, oral hygiene instructions were provided and supragingival biofilm removal were performed, and the surgery was executed ninety days later. Following surgical exposure of the contaminated dental implant surface, mechanical debridement was performed with metallic mini-five Gracey curettes (Hu-friedy, Chicago, IL), in order to remove the granulation tissue. After mechanical debridement with curettes, the implant surfaces were decontaminated using a sodium bicarbonate air-powder system (ProfiNeo, Dabi Atlante, Ribeirão Preto, SP, Brazil). The flap was positioned at its original position and sutured in place (Nylon 5-0, Ethicon Inc. Johnson &

Johnson Company, Somerville, NJ, EUA). Paracetamol or dipyrone and chlorhexidine gluconate (0.12%) mouthwash were prescribed. The suture was removed 10 days after the procedure.

Peri-implant Crevicular Fluid Collection

The samples were collected before the clinical measurements (to avoid the interference of bleeding on probing) at baseline, 6 and 12 months after surgical mechanical treatment of peri-implantitis. PICF samples were obtained from the mesio-buccal site of all implants. Following the removal of supramucosal biofilm, the sites were isolated with cotton rolls, gently dried with an air syringe to eliminate the possibility of contamination with saliva and a 30-s PICF sample was collected on filter strips (Periopaper®, Interstate Drug Exchange, Amityville, NY, USA). The strips were inserted 1-2mm into the peri-implant sulcus or pocket. Samples were immediately placed in Eppendorf tubes on ice, transported to the laboratory and stored at -80ºC. Samples visually contaminated with blood were discarded. All PICF samples were then shipped to the laboratory (The Forsyth Institute) for analysis.

Quantification of cytokines using multiplexed bead immunoassay (Luminex)

Biomarkers levels were determined using the human cytokine 20-plex (magnetic bead panel) by Millipore (Millipore Corporation, Billerica, MA, USA). Before the assay, PICF samples were eluted using 60µl of the assay buffer provided in the Millipore kit by vortexing for 30 min. and then centrifuged for 10 min. at 10,000 rpm. Twenty biomarkers, including: interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-12 p40 (IL-12p40), interleukin-12 p70 (IL-12p70), interleukin-15 (IL-15), interleukin-17 (IL-17a), tumor necrosis factor alpha (TNF-α), soluble human CD40 ligand (sCD-40L), interleukin-10 (IL-10), interleukin-13 (IL-13), interleukin-1 receptor antagonist (IL-1ra), eotaxin, monocyte chemoattractant protein-3 (MCP-3), macrophage-derived chemoattractant (MDC), fibroblast growth factor-2 (FGF-2), fms-like tyrosine kinase-3 ligand (Flt-3L), granulocyte-macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor BB (PDGF-BB) and vascular endothelial growth factor (VEGF) were measured. The assays were

performed in 96-well solid plate following the manufacturer's instructions. Briefly, the plate was washed with wash buffer and controls and standards (ranging from 3.2 to 10,000 pg/ml) were added into the wells in duplicate. The samples were placed in respective wells and the microsphere beads coated with monoclonal antibodies against the twenty different target analytes were added to all wells. The plate was wrapped with aluminum foil and incubated with agitation on a plate shaker overnight at 4ºC. The well contents were removed using a hand-held magnetic plate and the plate was washed twice. The Detection Antibodies were added into each well and the plate was incubated with agitation on a plate shaker for one hour at room temperature. After incubation, the Streptavidin-Phycoerythrin solution was added and incubated with agitation on a plate shaker for 30 minutes at room temperature. After washing two times to remove the unbound reagents using the hand held magnetic plate, sheath fluid (Luminex Corporation, Austin, TX, USA) was added to the wells and the beads (minimum of 50 per analyte) were immediately analyzed in the Luminex 200™ instrument (Luminex Corporation, Austin, TX, USA). The Luminex 200™ monitors the spectral properties of the beads to distinguish the different analytes, while simultaneously measuring the amount of fluorescence associated with R-phycoerythrin, reported as median fluorescence intensity. The concentrations of the unknown samples (antigens in PICF samples) were estimated from a standard curve using the Bio-Plex® software (Bio-Rad Laboratories, Hercules, CA, USA) and expressed as picograms/30seconds (pg/30s). Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification of the standard curves were assigned the highest value of the curve.

Data analysis

Clinical parameters and the profiles of 20-PICF biomarkers after surgical therapy were compared over time (6 months and 12 months) using generalized estimating equations. Log-transformation was applied to the measurements of biomarkers (Olivier et al. 2008). Generalized estimating equations were used to model this association while accounting for the clustering of implant sites within subjects (Wu et al. 2014). The mesio-buccal site was used as the unit of analysis. Generalized estimating equations with an exchangeable working correlation, normal link and semirobust standard errors were used to estimate mean ratios. Statistical

analysis was performed using statistical software (SPSS version 22.0). The significance level established for all analyses was 5% ($p<0.05$).

Results

Subject retention

The study was conducted between June 2011 and May 2014. The study population consisted of 52 patients. A hundred and fifty implants affected by peri-implantitis were evaluated over time, however 6 patients dropped out during the study.

Clinical findings

Table 1 presents the mean clinical parameters of the patients over time and Table 2 presents the mean clinical parameters for each analyzed group at mesio-buccal site at baseline, 6 months and 12 months. According with Table 2, PI parameter had no statistically significant difference over time for all groups. However, GBI parameter had statistically significant difference over time for all groups. All groups, except group 0, had a significant reduction at PD mean post-surgical therapy. Group 0 and group 2 had a significant increase in AL mean after therapy. Group 2 and 3 had a significant reduction in percentage of BOP and SUP parameters over time.

Table 1: Clinical Parameters over time (mean per patient/full mouth)

Clinical Parameters	Baseline	6 M	12 M	*p* value*
PI (%)	40	38	40	0.90
GBI (%)	88	6	25	< 0.001
PD (mm)	2.73	2.16	2.00	< 0.001
AL (mm)	4.07	3.96	4.02	0.23
BOP (%)	25	7	6	< 0.001
SUP (%)	5	1	1	< 0.001

*Generalized Estimating Equations

Table 2: Clinical Parameters for the clinical groups over time (mean per mesio-buccal site)

Clinical Parameters	Baseline n=150	6 M n=126	12 M n=124	*p* value*
PI (%)	G0 7	G0 8	G0 0	0.959
	G1 11	G1 1	G1 1	0.297
	G2 12	G2 1	G2 1	0.040
	G3 13	G3 12	G3 0	0.946
GBI (%)	G0 96	G0 10	G0 32	0.001
	G1 99	G1 1	G1 41	0.001
	G2 91	G2 7	G2 40	0.001
	G3 90	G3 10	G3 30	0.001
PD (mm)	G0 2.83	G0 2.43	G0 2.25	0.156
	G1 5.05	G1 3.05	G1 2.85	< 0.001
	G2 3.44	G2 2.76	G2 2.59	< 0.001
	G3 5.59	G3 3.26	G3 3.45	< 0.001
AL (mm)	G0 3.27	G0 4.31	G0 4.35	0.006
	G1 5.04	G1 4.64	G1 4.94	0.835
	G2 3.97	G2 4.61	G2 4.45	0.029
	G3 5.61	G3 4.47	G3 4.98	0.059
BOP (%)	G0 0	G0 0	G0 0	-
	G1 0	G1 0	G1 19	0.239
	G2 100	G2 8	G2 8	< 0.001
	G3 99	G3 12	G3 9	< 0.001
SUP (%)	G0 0	G0 0	G0 0	-
	G1 0	G1 20	G1 0	0.239
	G2 17	G2 2	G2 2	0.002
	G3 41	G3 2	G3 1	< 0.001

*Generalized Estimating Equations

G0= group 0

G1= group 1

G2= group 2

G3= group 3

Peri-implant Crevicular Fluid Biomarkers

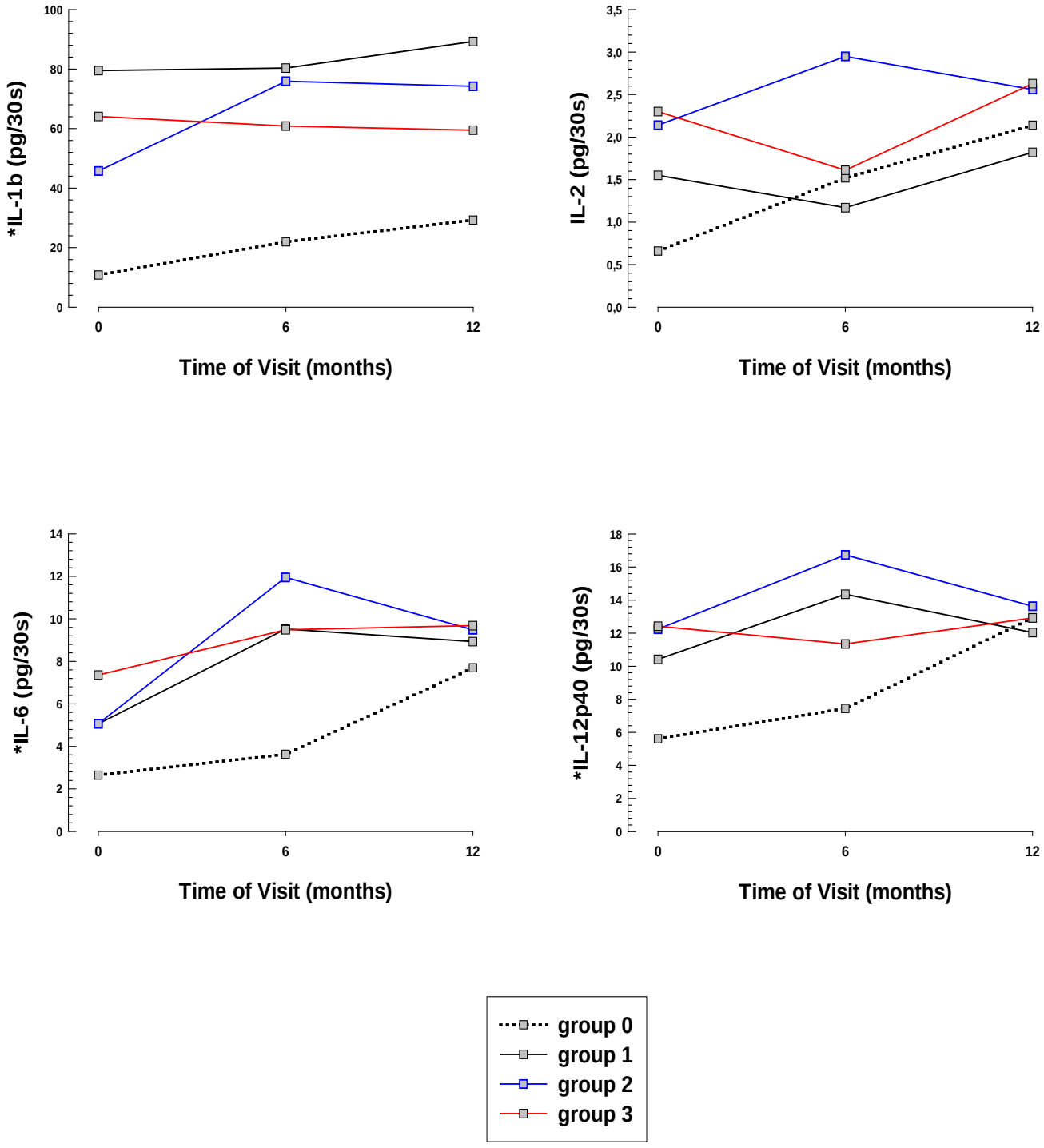
A total of 400 PICF samples were processed as part of the study (150 PICF samples at baseline, 126 PICF samples at 6 months and 124 PICF samples at 12 months). Table 3 and Figure 1 present the geometric mean of each biomarker (pg/30s) for all groups at baseline, 6 months and 12 months post-therapy. Table 4 presents the p-values for the comparisons between groups over time. The pro-inflammatory biomarkers, IL-1β, IL-6, IL-12p40, IL-12p70, IL-17a and sCD-40L had a statistically significant difference at 6 months post-therapy when comparing group 0 with group 2. sCD-40L was the only biomarker that had a statistically significant difference at 6 months after treatment when comparing group 0 with group 3. These biomarkers presented significant increase in mean PICF levels at 6 months post-therapy. The anti-inflammatory cytokine, IL-13, had a statistically significant difference when comparing group 0 with group 1 at 6 months post-therapy. IL-13 presented increases in mean PICF levels. Eotaxin and MCP-3 levels had a statistically significant difference when comparing group 0 with group 2 and also, MCP-3 levels presented a statistically significant difference when group 0 was compared to group 3 at 6 months post-therapy. These chemokines had a significant increase in mean PICF levels at 6 months after treatment. The growth factors showed more significant differences between groups than other biomarkers. FGF-2 and Flt-3L levels had a statistically significant difference when comparing group 0 with groups 1, 2 and 3 at 6 months post-therapy. GM-CSF levels presented significant difference only in comparison between group 0 and group 2 at 6 months post-therapy. PDGF-BB levels showed a statistically significant difference when group 0 was compared to groups 2 and 3 at 6 months post-therapy. FGF-2 and PDGF-BB levels had reductions in mean PICF levels at 6 months post-therapy. Comparisons between groups had no statistically significant differences at 12 months post-therapy for any biomarker.

Table 3: Biomarker geometric means over time

	Biomarkers (pg/30s)	Group	Geometric Means /(time) 0m	6m	12m
Pro-Inflammatory Cytokines	IL-1b	0	10.79	21.93	29.24
		1	79.48	80.35	89.29
		2	45.74	75.91	74.25
		3	64.10	60.84	59.49
	IL-2	0	0.66	1.52	2.14
		1	1.55	1.17	1.82
		2	2.14	2.95	2.56
		3	2.30	1.61	2.63
	IL-6	0	2.65	3.63	7.70
		1	5.06	9.52	8.93
		2	5.07	11.95	9.49
		3	7.36	9.49	9.68
	IL-12p40	0	5.61	7.45	12.94
		1	10.42	14.35	12.04
		2	12.24	16.73	13.63
		3	12.42	11.35	12.92
	IL-12p70	0	0.96	1.79	3.59
		1	2.92	4.11	5.72
		2	2.68	5.26	4.85
		3	3.16	3.28	4.47
	IL-15	0	0.92	2.10	6.72
		1	2.53	5.54	4.82
		2	1.83	5.96	6.62
		3	2.94	4.11	6.20
	IL-17a	0	0.33	1.08	3.34
		1	2.35	2.37	3.72
		2	1.55	3.79	3.36
		3	1.60	1.69	3.49
	TNF-a	0	1.53	4.99	9.30
		1	4.32	7.88	6.67
		2	3.42	11.76	7.71
		3	4.69	4.56	7.90
	sCD-40L	0	21.07	14.55	14.07
		1	52.10	32.68	18.25
		2	52.49	26.77	19.64
		3	71.21	28.92	18.54

Anti-Inflammatory Cytokines	IL-10	0	6.33	11.19	14.61
		1	16.29	21.80	15.10
		2	9.73	14.93	13.68
		3	10.10	12.92	10.75
	IL-13	0	1.82	4.26	10.27
		1	5.30	11.98	8.64
		2	3.74	6.79	5.70
		3	4.49	6.32	6.71
	IL-1ra	0	1,304.40	1,405.29	1,197.27
		1	2,158.57	1,622.79	1,399.96
		2	1,783.26	1,547.51	1,316.33
		3	1,752.33	1,894.67	1,336.89
Chemokines	Eotaxin	0	9.67	11.42	28.27
		1	20.76	25.81	32.23
		2	20.59	28.92	25.32
		3	21.84	19.59	27.97
	MCP-3	0	6.38	7.83	11.02
		1	15.55	12.69	11.99
		2	13.55	13.04	11.71
		3	14.80	12.50	9.97
	MDC	0	31.18	26.16	22.41
		1	45.39	38.77	28.23
		2	43.66	33.47	25.88
		3	51.02	35.35	25.48
Growth Factors	FGF2	0	15.10	14.42	21.19
		1	51.40	36.59	27.34
		2	43.77	29.06	21.33
		3	48.58	33.52	21.19
	Flt-3L	0	4.53	5.17	10.06
		1	7.80	12.00	10.59
		2	7.95	10.91	10.30
		3	9.16	9.17	9.19
	GM-CSF	0	0.31	0.90	2.41
		1	2.01	2.43	5.01
		2	1.43	3.82	2.74
		3	2.10	1.77	3.57
	PDGF-BB	0	17.64	15.18	24.41
		1	79.25	30.57	36.83
		2	30.10	30.85	27.12
		3	42.93	31.50	23.06
	VEGF	0	61.74	52.81	42.08
		1	102.28	98.25	47.65
		2	71.06	70.06	50.30
		3	80.44	76.71	46.16

Figure 1: Biomarkers over time post surgical therapy



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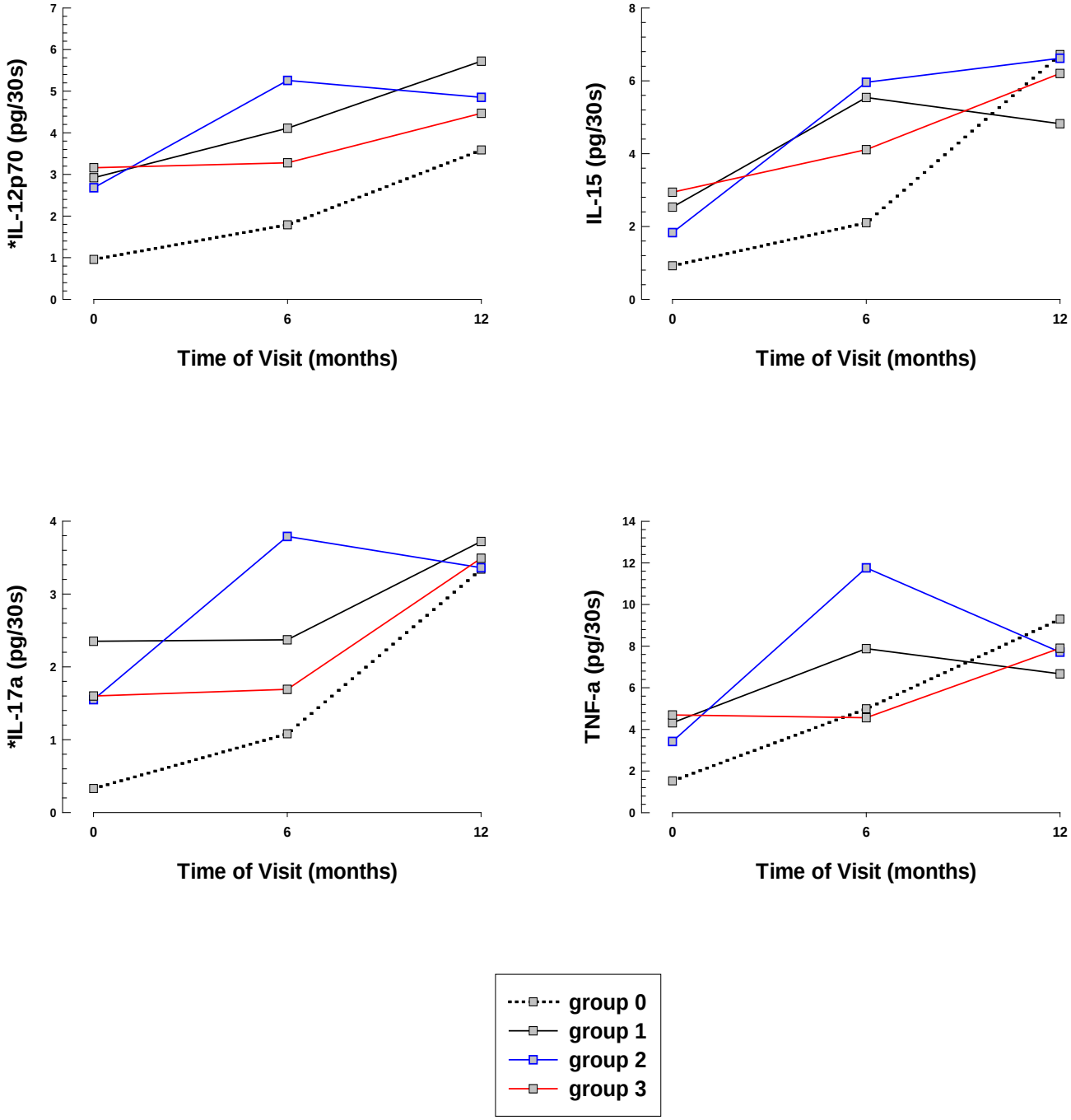

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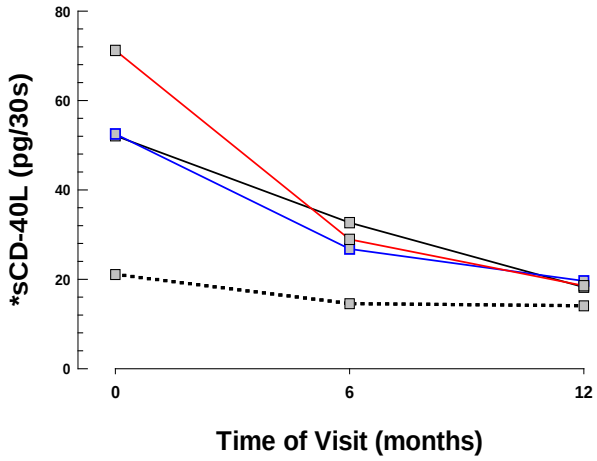

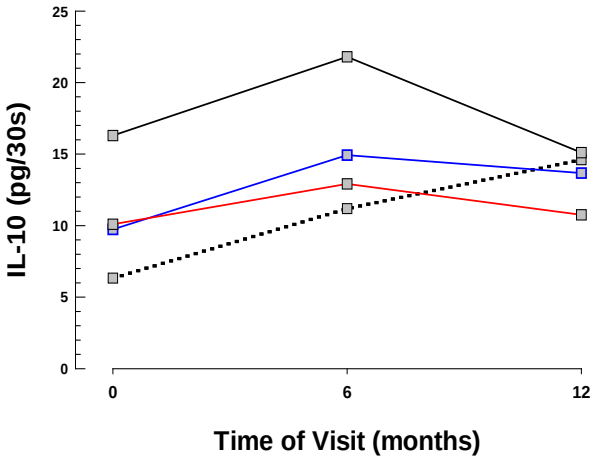

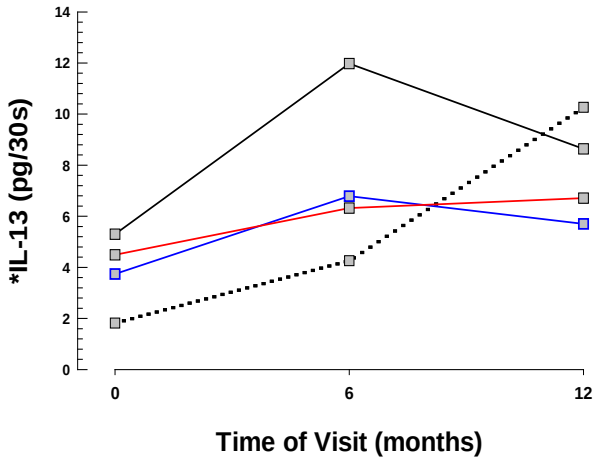

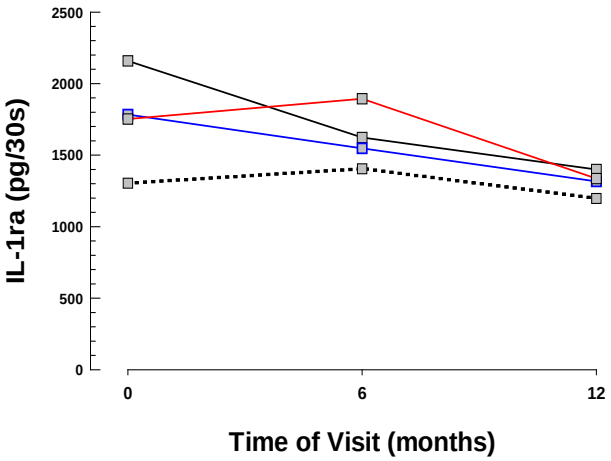

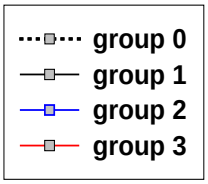

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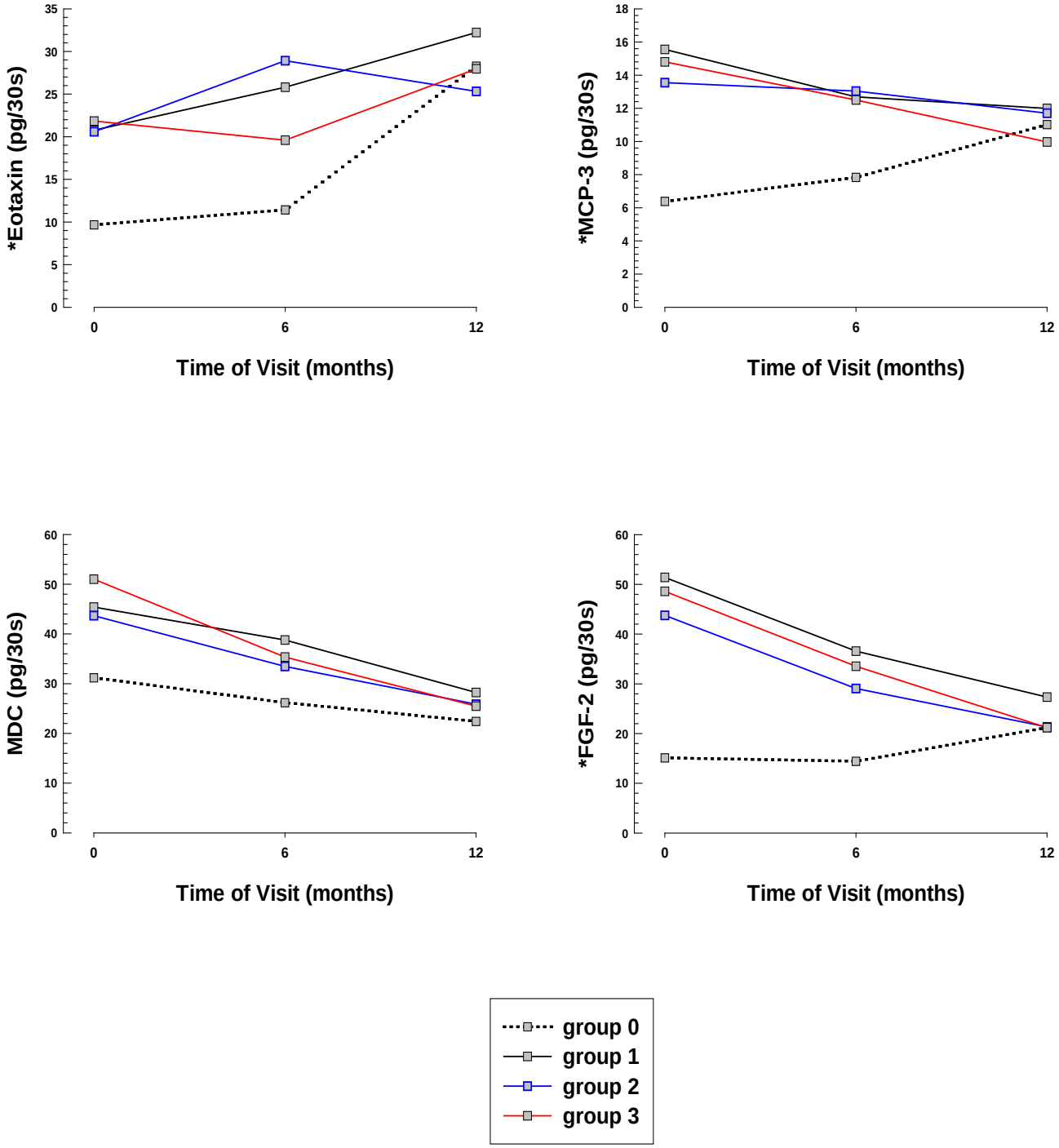

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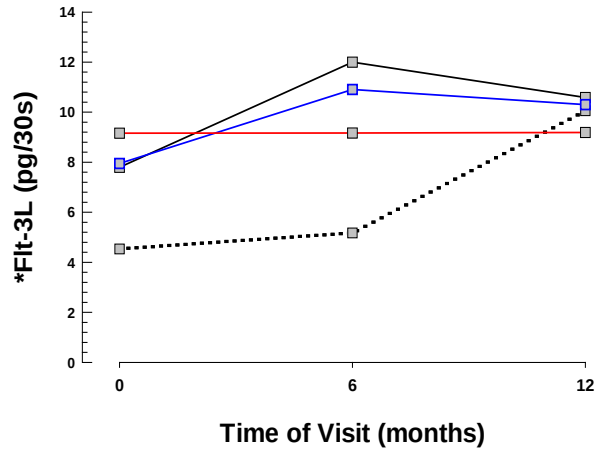

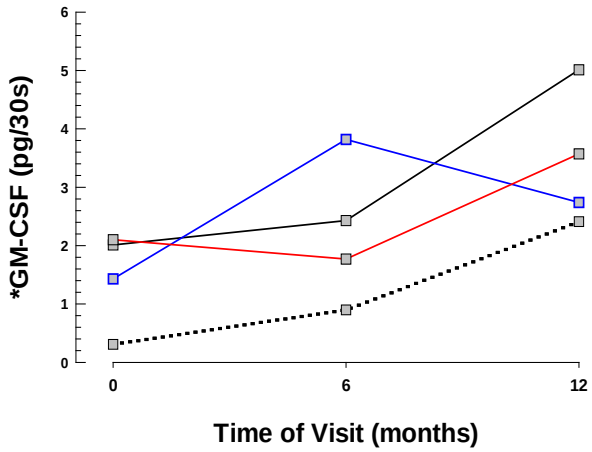

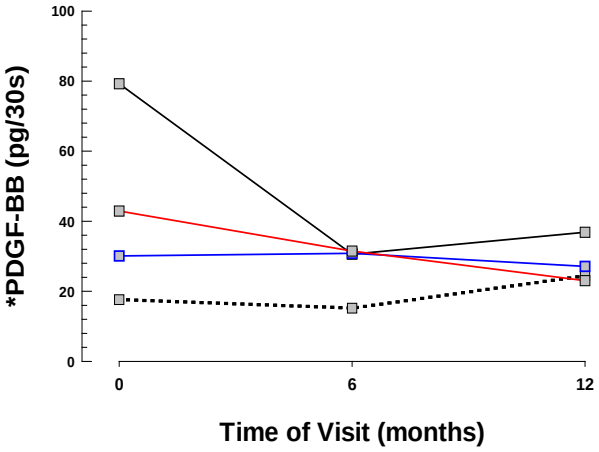

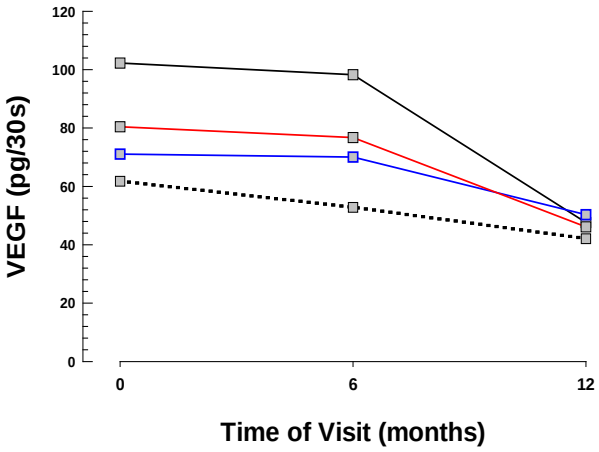

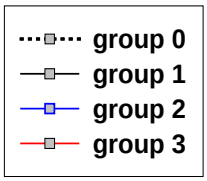

* Statistically significant difference

Table 4: *P*-values for the comparisons among groups over time

			Time (visit)	
			6 m	12 m
	Biomarker	Group	0	0
Pro–Inflammatory Cytokines	IL-1b	1	0.195	0.337
		2	0.043*	0.172
		3	0.077	0.289
	IL-2	1	0.741	0.847
		2	0.122	0.787
		3	0.899	0.757
	IL-6	1	0.172	0.829
		2	0.035*	0.731
		3	0.069	0.688
	IL-12p40	1	0.176	0.872
		2	0.028*	0.880
		3	0.259	0.996
	IL-12p70	1	0.276	0.448
		2	0.011*	0.532
		3	0.199	0.651
	IL-15	1	0.116	0.617
		2	0.052	0.977
		3	0.211	0.868
	IL-17a	1	0.383	0.889
		2	0.040*	0.992
		3	0.462	0.940
	TNF-a	1	0.517	0.657
		2	0.070	0.756
		3	0.848	0.781
	sCD-40L	1	0.063	0.557
		2	0.001*	0.164
		3	0.002*	0.290
Anti-inflammatory Cytokines	IL-10	1	0.169	0.944
		2	0.473	0.881
		3	0.709	0.462
	IL-13	1	0.012*	0.725
		2	0.278	0.188
		3	0.342	0.292
	IL-1ra	1	0.609	0.612
		2	0.712	0.775
		3	0.253	0.725

Table 4 – (Continued).

			Time (visit)	
			6 m	12 m
	Biomarker	Group	0	0
Chemokines	Eotaxin	1	0.087	0.784
		2	0.005*	0.761
		3	0.117	0.975
	MCP-3	1	0.095	0.750
		2	0.030*	0.754
		3	0.018*	0.630
	MDC	1	0.066	0.498
		2	0.124	0.507
		3	0.069	0.431
Growth Factors	FGF-2	1	0.012*	0.438
		2	0.012*	0.979
		3	0.003*	> 0.99
	Flt-3L	1	0.010*	0.850
		2	0.007*	0.917
		3	0.043*	0.673
	GM-CSF	1	0.338	0.390
		2	0.032*	0.870
		3	0.299	0.593
	PDGF-BB	1	0.108	0.319
		2	0.026*	0.759
		3	0.024*	0.850
	VEGF	1	0.058	0.766
		2	0.315	0.209
		3	0.120	0.596

* Statistically significant difference

Discussion

The main goal of the present study was to examine the impact of therapy on PICF biomarker levels longitudinally in 4 different groups of implant sites in patients presenting clinically assessed signs of peri-implantitis. The clinical parameters were also evaluated after surgical treatment.

The anti-infective surgical therapy resulted in decrease of GBI percentage and reduction of probing depth mean. Groups presenting PD ≤ 4 mm had an increase in mean attachment loss and groups presenting BOP=1 and/or SUP=1 at baseline had a great reduction of these parameters over time. The principal objectives for treatment of peri-implantitis are resolution of inflammation and preservation of supporting bone (De Wall et al. 2013). Access flap surgery, removal of granulation tissue and implant surface decontamination has been demonstrated to reduce BOP, suppuration and probing depths of implant sites over 5 years (Renvert et al 2012).

Our current findings demonstrate that anti-infective surgical therapy in patients with peri-implantitis significantly increased PICF levels of some pro-inflammatory biomarkers, meaning IL-1β, IL-6, IL-12p40, IL-12p70, IL-17a and sCD-40L. In contrast to these findings, the literature has shown decreases in pro-inflammatory biomarkers post-therapy. Other reports demonstrated significantly reduction of TNF-α levels in PICF at 3 months post-mechanical therapy in subjects with peri-implant diseases. However, the assessed decreases of PD and BOP means were comparable to our study (De Mendonça et al. 2009, Duarte et al. 2009). Those studies measured the cytokines profiles using enzyme-linked immunosorbent assay (ELISA) and it was reported that there were interassay disparities and interlaboratory variations, suggesting that results from studies using a given assay could not be directly extrapolated and/or compared with others evaluated by a different method (Fichorova et al. 2008).

The inflammatory response that occurs in peri-implantitis sites is similar to the ones observed in periodontally affect patients (Hultin et al. 2002). Other reports confirmed that periodontal therapy effectively reduces pro-inflammatory cytokines and chemokines in diseased patients, showing significant reductions in gingival crevicular fluid (GCF) levels of GM-CSF, IL-1β and increases in GCF levels of IL-6 after generalized aggressive periodontitis therapy (Thunell et al. 2010, Oliveira et al.

2012), similarly to the present investigation, where higher levels of IL-6 were verified in PICF post-surgical therapy. These studies used the same multiplex bead immunoassay that has been used in the present investigation, which is a high-throughput technique to determine the PICF biomarker expression, allowing the simultaneous quantification of several targets in a large number of samples at the same assay (Fonseca et al. 2014).

Duarte et al. (2009) stated that IL-10 (anti-inflammatory) levels in PICF were not different between healthy and diseased implants. However, our data showed that IL-13 was the only anti-inflammatory cytokine presenting increased levels post-therapy.

The chemokines (Eotaxin and MCP-3) had an increase in their levels post-therapy. Dezerega et al. (2010) showed that MCP-3 presented highly expressed levels in patients with chronic periodontitis, mainly in those with progressive periodontal lesions. The growth factors (Flt-3L and GM-CSF) had an increase in their levels post-therapy. Growth factors have been believed to modulate the tissue healing process, promoting bone and soft tissue regeneration (Kaigler et al. 2011). In the present study, FGF-2 and PDGF-BB had a decrease in their profiles post therapy. In contrast, Kitamura et al. 2011, suggested that FGF-2 might have induced new alveolar bone formation in vertical defects at 36 weeks post-periodontal surgery, exhibiting potent angiogenic activity during periodontal regeneration. There is a lack of evidence in the literature about the PICF levels of these biomarkers after peri-implantitis treatment.

Our conflicting data about the increased levels of some pro-inflammatory cytokines post-therapy could be explained by the executed treatment approach (access flap surgery), which might have exacerbated the inflammatory process. According with Li & Wang (2014), cytokine levels usually increase as a result of implant placement, but gradually decrease for 8 months after surgery. However, Meyle (2012) showed that non-surgical therapy of implants with peri-implantitis might present an insufficient treatment outcome, corroborating the surgical approach performed here. Furthermore, the currently available evidence does not recommend a specific protocol for the surgical therapy of peri-implantitis. The following elements of therapy seem to be beneficial concerning surgical therapy: access by a full-

thickness flap, thorough cleaning of the contaminated implant surfaces, antibiotic systemic administration and oral chlorhexidine rinse. The stabilization of the defect with a bone substitute might also be advantageous (Mombelli et al. 2012). Laser decontamination, use of abrasive devices or implantoplasty on the exposed part of the implant surface as adjuncts to surgical regenerative therapies might lead to improved clinical results compared to conventional treatment alone (Renvert et al. 2012), although not fully supported by the present literature.

Metallic curettes and ultrasonic tips, although more effective than non-metallic alternatives for tissue debridement, have been said to damage the titanium implant surface (Mann et al. 2012), but this type of curettes has been used to remove the granulation tissue during the surgical treatment in the present investigation. Air-powder abrasive systems have a potential for inducing surgical emphysema (Duarte et al. 2009), nonetheless this complication was not observed in the present investigation.

In summary, results of the present investigation suggest that the applied anti-infective surgical therapy might have positively influenced the assessed clinical parameters. And also, it might have increased some of pro-inflammatory cytokines levels and the anti-inflammatory cytokine IL-13 levels in PICF. There is a lack of evidence regarding the presence and levels of biomarkers in peri-implant crevicular fluid after the treatment of peri-implantitis, as well as their role in the treatment outcome. Further investigations are needed for a better understanding of the host immune response modulation, in order to develop other therapeutic strategies generating better tissue healing responses for peri-implant disease.

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Clinical Relevance:

Scientific rationale for the study: Few studies have been reported about PICF biomarkers levels in subjects with peri-implantitis after surgical therapy. The present investigation evaluated the levels of 20 PICF biomarkers after surgical treatment of peri-implantitis.

Principal findings: Clinical parameters have improved post-surgical therapy. The levels of IL-13, Flt-3L and GM-CSF in PICF were significantly higher after surgical treatment, however were accompanied by a significant increase in the levels of several PICF biomarkers such as the pro-inflammatory cytokines.

Practical implications: Better understanding of the biomarkers' levels in peri-implant crevicular fluid after the treatment of peri-implantitis, may improve the strategies to solve and to monitor this disease.

4. DISCUSSÃO

A importância do conhecimento da resposta imunológica do hospedeiro frente à agressão bacteriana na peri-implantite é crucial para o entendimento da patogênese e desenvolvimento de terapias efetivas para a resolução de doenças peri-implantares. A resposta imune em relação à doença peri-implantar tem sido relatada na literatura, porém os estudos ainda são muito escassos, com isso o presente estudo avaliou os biomarcadores imunológicos em sítios peri-implantares e investigou o efeito da terapia cirúrgica em relação a esses marcadores para a melhor compreensão dessa resposta do hospedeiro.

A colonização bacteriana ao redor dos implantes, pelos periodonto-patógenos, ocorre em poucas semanas após a sua instalação. Os produtos bacterianos desses microrganismos estimulam a produção de mediadores inflamatórios. O desequilíbrio entre os marcadores pró- e anti-inflamatórios na resposta imune, resultará na destruição tecidual nas doenças peri-implantares (VAN DYKE & SERHAN, 2003; MELO *et al.*, 2012).

A peri-implantite é bastante prevalente, acometendo um a cada cinco pacientes reabilitados com implantes osseointegrados. Porém, diferenças na definição dessa doença têm resultado numa variação muito grande nos valores referentes à prevalência da mesma. Essas diferenças incluem a utilização de diferentes limites em relação aos parâmetros radiográficos, para se determinar a perda óssea, e aos parâmetros clínicos (KLINGE & MEYLE, 2012).

A detecção precoce e confiável de qualquer reação adversa do tecido peri-implantar é um requisito para a instituição do tratamento dos implantes dentários. Os mediadores inflamatórios associados à peri-implantite são secretados no fluido crevicular peri-implantar, o qual se torna um valioso método diagnóstico não

invasivo, para a avaliação da resposta imunológica, potencialmente indicando precocemente o risco de doença peri-implantar (GRANT *et al.*, 2010). Portanto, a análise dos níveis de biomarcadores, no fluido crevicular peri-implantar, pode auxiliar na detecção de lesões inflamatórias em um estágio precoce, o qual poderia estar clinicamente latente.

A determinação da quantidade dos níveis de biomarcadores no fluido crevicular peri-implantar pode ser determinada por diversas técnicas. Nessa investigação foi utilizado o ensaio imunológico Luminex (multi-análise com micro-esferas), o qual possui um alto-rendimento, permitindo a quantificação de diversos biomarcadores em um grande número de amostra simultaneamente, melhorando assim a qualidade e o espectro de análise (FONSECA *et al.*, 2014). Outras técnicas têm sido relatadas na literatura para a avaliação dos níveis de biomarcadores no fluido crevicular, tais como o ensaio de ELISA (ensaio de imunoabsorção acoplado a enzimas), citometria de fluxo, *western blotting e PCR* (reação em cadeia da polimerase). O ensaio de ELISA, por ser um método de coloração imunohistoquímica, pode não ser sensível o suficiente para a detecção de determinados biomarcadores, como quimiocinas e seus receptores (GEMMEL & SEYMOUR, 2001; DEZEREGA *et al.,* 2010).

No primeiro capítulo desse trabalho, observou-se uma forte correlação positiva entre os parâmetros clínicos e os níveis de biomarcadores presentes nos sítios peri-implantares. Sítios com bolsas mais profundas (profundidade de sondagem > 4 mm) associadas a sangramento à sondagem e/ou supuração mostraram níveis mais elevados de citocinas pró-inflamatórias como a IL-1β (interleucina-1 beta), IL-17a (interleucina-17), TNF-α (fator de necrose tumoral-alfa) e sCD-40L (ligante CD40 solúvel).

Esses achados concordam com os de LUO et al. (2011), que demonstraram que sítios com peri-implantite apresentaram níveis mais elevados de IL-1β e TNF-α no fluido crevicular peri-implantar. Corroborando também com os estudos de DARABI et al. (2013), o qual observou o aumento dos níveis de IL-17. O presente estudo também mostrou que a presença de inflamação nos sítios peri-implantares (presença de sangramento à sondagem e/ou supuração) resultou no aumento dos níveis de sCD-40L (ligante CD40), MDC (quimiotático derivado de macrófago), FGF-2 (fator de crescimento de fibroblasto – básico) e GM-CSF (fator estimulador de colônias de macrófagos e granulócitos), o que para o conhecimento dos autores ainda não foi relatado na literatura até o momento.

Ao analisar o capítulo 2 desse trabalho, pode-se perceber que os parâmetros clínicos reduziram significativamente após a terapia cirúrgica anti-infecciosa. O tratamento cirúrgico da peri-implantite adotado nesse estudo, sob o ponto de vista clínico, foi efetivo na resolução da inflamação. É consenso na literatura que a terapia não cirúrgica não é efetiva para as lesões de peri-implantite (RENVERT; ROOS-JANSAKER & CLAFFEY, 2008). Diversas modalidades de tratamento cirúrgico são relatadas, com o objetivo de descontaminar a superfície do implante, para alcançar a resolução da lesão inflamatória (LINDHE & MEYLE, 2008). No presente trabalho, foram associados agentes mecânicos e químicos, para a descontaminação da superfície dos implantes, como debridamento mecânico com curetas metálicas e jato de bicarbonato com irrigação de solução salina respectivamente. De acordo com a literatura, a combinação de técnicas de descontaminação mecânica e química deve ser aplicada para melhores resultados (WISMEIJER & SUBRAMANI, 2012).

No segundo capítulo foi observado que a terapia cirúrgica anti-infecciosa aumentou significativamente os níveis de IL-13 (interleucina-13) nos sítios peri-

implantares. Esse resultado está de acordo com os estudos descritos na literatura, os quais mostram que o acúmulo de IL-13 contribui para a resolução de diversas inflamações não-orais, devido à inibição da síntese de citocinas pró-inflamatórias (JOHNSON & SERIO, *et al.,* 2007). Além disso, na presente pesquisa foi observado o aumento significativo dos níveis de Flt-3L (ligante relacionado ao receptor de classe III da tirosina cinase) e GM-CSF (fator estimulador de colônias de macrófagos e granulócitos), o que está de acordo com a literatura, a qual mostra que os fatores de crescimento aceleram o processo de cicatrização, promovendo a regeneração óssea e tecidual (KAIGLER et al. 2011).

O presente trabalho também demonstrou um aumento significativo dos níveis dos biomarcadores pró-inflamatórios como IL-1β (interleucina-1beta), IL-6 (interleucina-6), IL-12p40 (interleucina-12 p40), IL-12p70 (interleucina-12 p70), IL-17a (interleucina-17a) e sCD-40L (ligante CD40 solúvel) no fluido crevicular após a terapia cirúrgica. Entretanto, esses resultados não estão de acordo com a evidência atual, que mostra a diminuição dos biomarcadores anti-inflamatórios após a terapia cirúrgica anti-infecciosa (DE MENDONÇA *et al.,* 2009; DUARTE *et al.,* 2009). Esses estudos descritos na literatura avaliaram os níveis de citocinas através do método de ELISA. Porém, a literatura sugere que investigações, que usaram métodos de avaliação imunológica diferentes, não podem ser comparadas, pois existem disparidades e variações entre os laboratórios (FICHOROVA *et al.,* 2008).

Esse resultado conflitante na presente pesquisa poderia ser explicado pelo tipo de abordagem realizada no tratamento da peri-implantite, que foi uma modalidade cirúrgica com rebatimento de retalho, exacerbando assim o processo inflamatório. Entretanto, o estudo de MEYLE (2012) mostrou que a terapia não

cirúrgica em implantes com peri-implantite, não é efetiva na resolução dessa doença.

É importante destacar que os achados do presente estudo, referentes aos níveis de biomarcadores no fluido crevicular em sítios peri-implantares antes e após a terapia cirúrgica anti-infecciosa, necessitam ser confirmados por pesquisas adicionais para o melhor entendimento da resposta inflamatória na peri-implantite, com o objetivo de determinar outras estratégias terapêuticas, para se obter um melhor efeito na cicatrização tecidual das doenças peri-implantares.

5. CONCLUSÕES

De acordo com o trabalho apresentado, pode-se concluir que:

- os parâmetros clínicos mostraram uma forte correlação positiva com as concentrações de biomarcadores presentes nos sítios peri-implantares;

-os parâmetros clínicos reduziram significativamente após a terapia cirúrgica anti-infecciosa;

- a terapia cirúrgica anti-infecciosa aumentou significativamente os níveis de IL-13 (citocina anti-inflamatória) no fluido crevicular;

- a terapia cirúrgica aumentou significativamente os níveis dos biomarcadores pró-inflamatórios como IL-1β, IL-6, IL-12p40, IL-12p70, IL-17a e sCD-40L no fluido crevicular dos sítios peri-implantares.

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7. APÊNDICE

Biomarkers table: type and main function

Biomarkers	Type	Main Function
IL-1β: interleukin 1 beta	Pro-inflammatory cytokine	Stimulating the production and release of other inflammatory mediators such as IL-6, Matrix Metalloproteinases (MMPs), Prostaglandin E2 (PGE2). They're released by macrophages.
IL-2: interleukin 2	Pro-inflammatory cytokine	Proliferation of T lymphocytes and B cells. It also induces the production of other cytokines, such as IFN-γ and TNF-β resulting in the activation of monocytes, neutrophils and natural killer cells.
IL-6: interleukin 6	Pro-inflammatory cytokine	Progenitor cell stimulation, platelet production, immunoglobulin production in B cells.
IL-12p40: subunit of IL-12	Pro-inflammatory cytokine	Blocking IL-23 functions by competing for the IL-23 receptor. It's chemotactic for macrophages.
IL-12p70: subunit of IL-12	Pro-inflammatory cytokine	Stimulating the production of IFN-γ by lymphocytes and natural killer cells.
IL-15: interleukin 15	Pro-inflammatory cytokine	Activation T lymphocytes, in order to produce IFN-γ.
IL-17: interleukin 17	Pro-inflammatory cytokine	Induction of cytokine production and cellular infiltration. It is produced by Th17 cells. It stimulates the production of IL-1β, IL-6, IL-8 and TNF-α.
TNF-α: tumor necrosis factor alpha	Pro-inflammatory cytokine	Stimulating the production and release of other inflammatory mediators such as IL-6, MMPs, PGE2. And it's an inducer of bone resorption.
sCD40L: soluble human CD40 ligand	Receptor for pro-inflammatory signaling	Playing a key role in immune activation: co-stimulation of T-cell proliferation . CD40L is expressed on activated T helper cells (CD4+), basophils and mast cells.
IL-10: interleukin 10	Anti-inflammatory cytokine	This immunoregulatory cytokine can inhibit a series of pro-inflammatory signals (TNF,IL-1 and IL-6) and has also been involved in the suppression of MMPs and in stimulation of osteoprotegerin (OPG), an inhibitor of bone resorption.
IL-13: interleukin 13	Anti-inflammatory cytokine	Stimulating the growth and differentiation of B-cells.

IL-1ra: interleukin 1 receptor antagonist	Receptor for IL-1	It binds to IL-1. It inhibits the activity of IL-1α, IL-1β, attenuating its response.
Eotaxin	Chemokine	Recruitment of eosinophils.
MCP-3: monocyte chemoattractant protein-3	Chemokine	Chemotaxis' promotion. It induces IL-1β, TNF-α and IFN-ɣ.
MDC: macrophage-derived chemoattractant/chemokine	Chemokine	It is a potent chemoattractant for dendritic cells, natural killer cells, monocytes and the T-lymphocytes (Th2 cells).
FGF-2: Basic fibroblast growth factor	Growth factor	Promoting endothelial cell proliferation (stimulating the angiogenesis).
Flt-3L:fms-like tyrosine kinase-3 ligand	Growth factor	Small molecule that act as a growth factor (fms). Flt-3L promotes expansion and differentiation of B cells in the presence of IL-7 or in combination of IL-7 and IL-13.
GM-CSF: granulocyte-macrophage colony stimulating factor	Growth factor	Stimulating the proliferation and differentiation of precursor of neutrophils, eosinophils and monocytes.
PDGF-BB: platelet-derived growth factor BB	Growth factor	Potent mitogen for a wide range of cells types, including fibroblasts and connective tissue.
VEGF:vascular endothelial growth factor	Growth factor	Influencing vascular permeability. It's a potent chemoattractant for monocytes.

8. ANEXO

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA

OF.CEP-410/12 Porto Alegre, 16 de abril de 2012.

Senhor Pesquisador,

O Comitê de Ética em Pesquisa da PUCRS informa que, de acordo com o Parecer CONEP nº. 180/2012 (Protocolo aprovado com recomendação), cabe ao CEP verificar o cumprimento das questões expostas pela CONEP no referido parecer. Em vista disso, o CEP-PUCRS apreciou e aprovou sua resposta ao parecer citado acima, referente ao seu protocolo de pesquisa intitulado **"Análise microbiológica e imunológica associada à saúde e doença Peri-implantar"**, bem como o novo Termo de Consentimento Livre e Esclarecido.

Ressaltamos que seu estudo pode ser iniciado a partir desta data.

Os relatórios parciais e final deverão ser encaminhados a este CEP.

Atenciosamente,

Prof. Dr. Rodolfo Herberto Schneider
Coordenador do CEP-PUCRS

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