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PERFIL LOCAL DE CITOCINAS E TRATAMENTO DE BOLSAS RESIDUAIS EM DIABÉTICOS TIPO 2 COM PERIODONTITE CRÔNICA

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RESUMO

Objetivo: Esse trabalho foi composto por dois estudos científicos cujos objetivos foram: *Estudo 1* – Comparar os níveis locais de citocinas (INF-γ, TNF-α, IL-17, IL-23 e IL-4), RANKL e OPG em indivíduos com periodontite crônica sistemicamente saudáveis e portadores de diabetes melito tipo 2 bem controlados e com controle glicêmico insatisfatório. *Estudo 2* – Avaliar os efeitos da terapia cirúrgica e não-cirúrgica associadas à administração de antibióticos sistêmicos nos parâmetros clínicos e níveis de citocinas (INF-γ, IL-17, IL-23 e IL-4) em bolsas residuais de diabéticos tipo 2. **Material e métodos:** No *Estudo 1*, os níveis de citocinas, RANKL e OPG no fluido gengival de bolsas periodontais de indivíduos sistemicamente saudáveis (n=20), diabéticos bem-controlados (n=17) e descompensados (n=20) foram avaliados por ELISA. No *Estudo 2*, 21 diabéticos com pelo menos 2 bolsas residuais/quadrante contralateral receberam raspagem cirúrgica ou não-cirúrgica por meio de um desenho experimental do tipo boca-dividida. Todos os sujeitos receberam metronidazol e amoxicilina por 10 dias. Parâmetros clínicos e níveis de citocinas foram avaliados no início e em 3 e 6 meses pós-terapia. **Resultados**: Em geral, os resultados do *Estudo 1* demonstraram que os diabéticos, (bem controlados e pobremente controlados), apresentaram níveis elevados de mediadores pró-inflamatórios, RANKL e OPG e reduzidos de IL-4, quando comparados aos não-diabéticos ($p<0,05$). Além disso, sujeitos bem-controlados apresentaram os maiores níveis de IFN-γ, enquanto os descompensados os maiores níveis de IL-17 ($p<0,05$). No *Estudo 2*, ambas as terapias promoveram melhoras clínicas em 3 e 6 meses ($p<0,05$); entretanto, não houve diferenças estatísticas entre elas ($p>0,05$). Os níveis de todas as citocinas estavam aumentadas após a terapia cirúrgica quando comparado à terapia não-cirúrgica ($p<0,05$). **Conclusões**: De acordo com o *Estudo 1*, diabéticos apresentaram uma resposta pró-inflamatória periodontal exacerbada quando comparados aos não-diabéticos e, o perfil periodontal de citocinas parece ser influenciado pelo controle glicêmico. De acordo com o *Estudo 2,* o tratamento cirúrgico e não-cirúrgico, associado a antibióticos sistêmicos, foram similarmente efetivos para tratamento de bolsas residuais em diabéticos tipo 2. O tratamento cirúrgico associado à antibioticoterapia, por sua vez, promoveu um aumento prolongado nos níveis de citocinas, principalmente aos 6 meses.

Palavras-chave: diabetes melito, periodontite crônica, citocinas, ligante RANK, osteoprotegerina, procedimentos cirúrgicos.

ABSTRACT

Aim: This work was composed by two studies, in which the aims were: *Study 1* - To compare the local levels of cytokines (IFN-γ, IL-17, IL-23 e IL-4), RANKL and OPG in systemically-healthy, better-controlled and poorly controlled type 2 diabetic subjects with chronic periodontitis. *Study 2*- To evaluate the effects of surgical and non-surgical debridement associated with systemic antibiotics, on clinical and cytokine outcomes of residual pockets in type 2 diabetic subjects. **Material and methods:** In the *Study 1*, the levels of cytokines, RANKL and OPG in the gingival crevicular fluid from periodontal pockets of systemically-healthy (n=20), better-controlled (n=17) and poorly controlled (n=20) diabetic subjects were evaluated by ELISA. In the *Study 2*, 21 diabetic subjects presenting at least two residual pockets/contralateral quadrant received surgical or non-surgical debridement using a split-mouth design. All subjects received metronidazole plus amoxicillin for 10 days. Clinical parameters and cytokine levels were assessed at baseline, 3 and 6 months post-therapies. **Results:** In general, the results from *Study 1* showed that diabetic subjects, (better-controlled and poorly-controlled), presented high levels of pro-inflammatory mediators, OPG and RANKL and, reduced levels of IL-4, when compared to non-diabetic subjects ($p<0.05$). In addition, better-controlled individuals exhibited the highest levels of IFN-γ, while poorly controlled subjects presented the highest levels of IL-17 ($p<0.05$). In *Study 2*, both therapies yielded clinical benefits at 3 and 6 months ($p<0.05$), without significant differences between them ($p>0.05$). Levels of all cytokines were increased after surgical therapy when compared to non-surgical therapy ($p>0.05$). **Conclusions:** According to *Study 1*, diabetic subjects presented a hyperproinflammatory periodontal response when compared to non-diabetic individuals and, the cytokine periodontal profile seems to be influenced by the glycemic control. According to *Study 2*, surgical and non-surgical debridements associated with systemic antibiotics were equally effective in treating residual pockets in type 2 diabetic subjects. Surgical treatment and systemic antibiotics produced a prolonged increase in the levels of cytokines, mainly at 6 months.

Key Words: diabetes melito, chronic periodontitis, cytokines, RANK ligand, osteoprotegerin, surgical procedures.

SUMÁRIO

1. INTRODUÇÃO

1.1. Diabetes melito

Diabetes melito (DM) é um distúrbio crônico do metabolismo dos carboidratos, lipídios e proteínas, caracterizado por níveis elevados de glicose (hiperglicemia) e subsequentes complicações sistêmicas e bucais (BROWNLEE, 1994). A hiperglicemia pode ocorrer como resultado da deficiência da secreção de insulina, causada por uma disfunção das células ß pancreáticas, da resistência à utilização da insulina no fígado e músculos ou da combinação de ambos fatores. Dentre as diversas desordens causadas pelo estado hiperglicêmico crônico podem ser destacados danos ao coração, olhos, rins, nervos e sistema vascular e algumas manifestações bucais, entre as quais se destaca a periodontite (MEALEY & OCAMPO, 2007).

O DM tipo 1 ocorre devido a destruição das células ß do pâncreas, geralmente levando a perda total da secreção de insulina. Embora seja mais comum em crianças e adolescentes, 15 - 30% dos casos são diagnosticados somente após 30 anos de idade (LAAKSO & PYORALA, 1985). Nesses indivíduos, a destruição das células ß acontece de forma mais lenta que nas crianças. A insulinopenia em pacientes com DM tipo 1 torna a insulina exógena indispensável pelo resto da vida. Isso explica porque antes o DM tipo 1 também era conhecida como DM insulinodependente (BINGLEY et al., 1997).

Pacientes com DM tipo 2, por sua vez, apresentam resistência insulínica, que consiste em uma alterada utilização da insulina endógena pelas células-alvo. Os mesmos podem ter também uma alteração na produção de insulina. Em muitos pacientes, principalmente no início da doença, a produção de insulina está aumentada, resultando em hiperinsulinemia. Com a progressão dessa condição, a produção de insulina geralmente diminui e os pacientes têm uma deficiência de insulina relativa associada à resistência insulínica periférica (RHODES, 2005). Esse tipo de DM foi inicialmente denominada DM não-insulino dependente. Isso porque, em alguns pacientes e principalmente nos estágios iniciais da doença, não há necessidade de tratamento com insulina para sobreviver, sendo necessário apenas o controle da dieta e/ou uso de hipoglicemiantes. Muitos indivíduos com DM tipo 2 podem não ser diagnosticados por muitos anos porque a hiperglicemia aparece gradualmente e, muitas vezes, sem sintomas (DEFRONZO & FERRANNINI, 1991).

Em 2000, a prevalência mundial de DM tipo 2 foi estimada em 150 milhões (ZIMMET et al., 2001). Em 2009, 285 milhões de pessoas no mundo eram portadoras de DM e cerca de 90%

destes indivíduos tinham DM tipo 2. No Brasil, um estudo transversal hipotético estimou que existem cerca de 6,48 milhões de brasileiros com DM tipo 2 (REZENDE et al., 2010). Um estudo multicêntrico demonstrou que entre 6671 brasileiros com DM, 979 (15%) tinham DM tipo 1 e 5692 (85%) apresentavam DM tipo 2 (MENDES et al., 2010). Além disso, a prevalência de pacientes com controle glicêmico inadequado [hemoglobina glicada (HbA1c) ≥7] foi de 90% em brasileiros com DM tipo 1 e 73% em brasileiros com DM tipo 2 (MENDES et al., 2010) enquanto apenas 57,1% de adultos com DM apresentavam um nível de HbA1c < 7% nos Estados Unidos (CHEUNG et al., 2009).

1.2. Relação bidirecional entre DM e periodontite

Periodontite é uma doença infecciosa que produz um processo inflamatório levando à destruição dos tecidos de proteção e suporte periodontal, culminando muitas vezes na perda do elemento dental (KINANE & MARK BARTOLD, 2007). Embora já esteja bem estabelecido que o biofilme bacteriano patogênico é o fator etiológico das doenças periodontais, alguns fatores sistêmicos e hábitos (ex. tabagismo, DM, estresse, osteoporose, etc) foram apontados como capazes de modular negativamente o estabelecimento, progressão e gravidade das periodontites. Neste contexto, vários estudos demonstraram um aumento na prevalência, gravidade e progressão de doenças periodontais em indivíduos diabéticos quando comparados aos não-diabéticos, suportando o DM como um verdadeiro fator de risco para as periodontites (EMRICH et al., 1991; LÖE, 1993; GROSSI et al., 1994; FIRATLI et al., 1996; SKREPCINSKI & NIENDORFF, 2000). Como uma via de mão dupla, as periodontites têm sido também sugeridas como importantes colaboradoras para o inadequado controle glicêmico nos indivíduos diabéticos (JANKET et al., 2005; DARRÉ et al., 2008).

Por mais de duas décadas, estudos epidemiológicos em populações com alta prevalência de DM demonstraram que a incidência e a gravidade da perda de inserção periodontal são maiores em diabéticos (LÖE, 1993). Emrich et al. (1991), estudando uma população de índios Pima no Arizona, demonstraram que o DM estava significativamente relacionado à prevalência e severidade das doenças periodontais, mesmo após ajuste estatístico para variáveis demográficas e índice de placa. Taylor et al. (1998) observaram que a progressão da periodontite, em dois anos, foi 4,23 vezes maior em indivíduos diabéticos quando comparados aos não-diabéticos. Novak et al. (2008) demonstraram que indivíduos hispano-americanos portadores de DM tipo 2 apresentaram maior gravidade e extensão de destruição periodontal. O DM foi associado também

à maior formação de cálculo e perda dentária. Kaur et al., em 2009, em um estudo realizado em indivíduos pomeranos, confirmaram a associação entre as DM tipo 1 e 2 e a periodontite e perda óssea. Susanto et al., em 2010, avaliando a população da Indonésia, também observaram que a prevalência e a severidade da periodontite foi significativamente maior em diabéticos tipo 2 comparado aos indivíduos sistemicamente saudáveis.

Tem sido sugerido que as bactérias, seus fatores de virulência e os mediadores inflamatórios presentes na periodontite podem desencadear não apenas seqüelas locais como também efeitos sistêmicos. Assim, alguns estudos sugeriram que as doenças periodontais podem induzir ou até mesmo perpetuar uma condição inflamatória crônica de baixa intensidade (D´AIUTO et al., 2004; LOOS et al., 2001). Dentro da linha de pesquisa sobre os efeitos sistêmicos da periodontite, estudos têm sugerido que indivíduos diabéticos com infecção periodontal apresentam mais dificuldades para controlar a glicemia em relação aos diabéticos que não possuem periodontite (TAYLOR et al., 1996). Muitos estudos testaram essa hipótese acompanhando os efeitos do tratamento periodontal sobre os níveis de HbA1c em diabéticos tipo 2 e os resultados ainda permanecem controversos (RODRIGUES et al., 2003; PROMSUDTHI et al., 2005; JANKET et al., 2005; FARIA-ALMEIDA et al., 2006; NAVARRO-SANCHEZ et al., 2007; DARRÉ et al., 2008; SANTOS et al., 2009). De maneira geral, recentes meta-análises demonstraram efeitos positivos das terapias periodontais no controle glicêmico de indivíduos diabéticos (DARRÉ et al., 2008; SIMPSON et al., 2010; TEEUW et al., 2010). Darré et al. (2008) sugeriram que o tratamento periodontal contribui para a melhora do controle glicêmico após avaliar uma série de estudos controlados intervencionais randomizados realizados em diabéticos tipo 1 e 2. Similarmente, Teew et al. (2010) e Simpson et al. (2010) propuseram que existe uma moderada, mas significante, melhora no controle glicêmico após o tratamento periodontal em indivíduos diabéticos tipo 2. Recentemente, um estudo clínico controlado e randomizado, realizado em 60 diabéticos tipo 2 com periodontite moderada-avançada, avaliou o efeito da terapia periodontal no controle glicêmico. Trinta diabéticos receberam raspagem e alisamento radicular não-cirúrgico, enquanto os demais não receberam tratamento por 6 meses. Parâmetros clínicos e nível de HbA1c foram mensurados em 1, 3 e 6 meses após a terapia. Todos os parâmetros clínicos melhoraram significativamente nos pacientes submetidos ao tratamento, o que contribuiu fortemente para melhorar o controle glicêmico nos diabéticos envolvidos no estudo (KOROMANTZOS et al. 2011).

1.3. Influência do controle glicêmico na condição periodontal

O grau de controle glicêmico tem sido determinado como uma variável muito importante na relação entre o DM e a periodontite. Embora algumas evidências demonstrem maior prevalência de inflamação gengival e destruição periodontal nos indivíduos que possuem controle ruim/insatisfatório da glicemia, outros não encontraram uma relação direta entre o controle glicêmico e a gravidade dos parâmetros periodontais. Um estudo realizado em diabéticos tipo 1 e 2 não demonstrou diferenças na gravidade da condição periodontal em relação ao nível de controle glicêmico (BACIC et al. 1988). Similarmente, Tervonen e Karjalainen (1997), avaliando uma população de diabéticos tipo 1, não encontraram nenhuma diferença no número de bolsas periodontais ≥ 4mm com sangramento à sondagem entre indivíduos com controle glicêmico insatisfatório, moderado ou bom. Recentemente, Kardeşler et al. (2010) demonstraram que a profundidade de sondagem, nível clínico de inserção e sangramento à sondagem foram semelhantes em diabéticos bem-controlados (HbA1c<7%) e não-controlados (HbA1c>7%) com periodontite crônica. Outros estudos, porém, demonstraram uma associação direta entre a gravidade da periodontite e o controle glicêmico insatisfatório. Tervonen e Knuuttila (1986) observaram que diabéticos bem controlados apresentaram uma melhor condição periodontal quando comparado aos indivíduos não-controlados. Seppala e Ainamo (1994) verificaram, por sua vez, que diabéticos não-controlados apresentaram maiores níveis de perda óssea alveolar quando comparado aos indivíduos controlados tipo 1, em um acompanhamento de 2 anos. Lim et al. (2007) , avaliando os diabéticos tipo 1 e 2 como um todo, encontraram correlações positivas entre os níveis de HbA1c e a porcentagem de sítios com profundidade ≥ 5 mm com sangramento à sondagem. Nesse et al. (2009) estabeleceram uma relação de dose-resposta entre a superfície periodontal inflamada e os níveis de HbA1c em diabéticos tipo 2. Recentemente, Chen et al. (2010) demonstraram que a média de profundidade de sondagem de boca-toda é uma variável preditora para níveis elevados de HbA1c, após ajustes para fatores de confundimento como tabagismo, idade, gênero e índice de massa corpórea. Bandyopadhyay et al. (2010) observaram que sítios periodontais de indivíduos não-controlados apresentaram maiores chances de progressão quando comparado aos indivíduos bem-controlados.

1.4. O papel das citocinas e fatores relacionados à osteoclastogênese na periodontite e na periodontite associada ao DM

Com o desenvolvimento e aprimoramento das técnicas imunológicas e de biologia molecular foi possível constatar que a produção local de mediadores inflamatórios regula a resposta do hospedeiro frente à agressão bacteriana periodontal, exercendo um papel protetor e/ou destrutivo no processo de doença e conseqüente destruição de tecidos periodontais (VAN DYKE & SERHAN, 2003). É importante destacar que os níveis locais dos mediadores imuno-inflamatórios podem ser avaliados, de acordo com a técnica laboratorial empregada, em nível de proteína ou RNA mensageiro (RNAm), tanto no fluido gengival como nos tecidos periodontais.

Os papéis de algumas citocinas que se destacam no processo de inflamação e na imunidade inata como a interleucina (IL)-1β e o fator de necrose tumoral (TNF)-α vêm sendo explorados por mais de duas décadas no campo da periodontia. As principais atividades biológicas destes mediadores são de caráter pró-inflamatório, incluindo o estímulo para a produção de moléculas de adesão, metaloproteinases, fatores relacionados à osteoclastogênese e outros mediadores pró-inflamatórios (GABAY et al., 2010; DINARELLO, 2009; BOYCE et al., 2005; APOSTOLAKI et al., 2010). Em geral, estudos científicos evidenciaram que os níveis locais de TNF-α são elevados em sítios com periodontite crônica e agressiva quando comparado aos sítios periodontalmente saudáveis (GÓRSKA et al., 2003; KURTIŞ et al., 2005; BASTOS et al., 2009). Embora muitos estudos tenham avaliado os níveis séricos de TNF-α em diabéticos com periodontite (IWAMOTO et al., 2001; GÓRSKA et al., 2003; CHEN et al., 2010; KOBAYASHI et al., 2010), poucos trabalhos focaram nos níveis locais deste mediador na periodontite. Salvi et al. (1998) sugeriram que indivíduos diabéticos com doença periodontal apresentam maior secreção de prostaglandina-E2, IL-1ß e TNF-α no fluido gengival quando comparados aos não-diabéticos. Gomes et al. (2006) observaram que a expressão de TNF-α em amostras de saliva é similar em diabéticos portadores de periodontite crônica moderada e avançada. Duarte et al., em 2007, não encontraram diferenças nos níveis de TNF-α entre indivíduos diabéticos e sistemicamente saudáveis. Recentemente, Venza et al. (2010) demonstraram que o tecido gengival de sítios com periodontite crônica em diabéticos tipo 2 descompensados apresentavam alta expressão de TNF-α. Aspriello et al. (2010) avaliaram os níveis de TNF-α no fluido gengival de diabéticos tipo 1 e 2 para verificar possíveis diferenças nos mecanismos inflamatórios da periodontite associada à ambos os tipos de DM. Os autores observaram que diabéticos tipo 1 apresentavam níveis mais elevados de TNF-α e IL-1β e que existia uma correlação negativa entre os níveis desses marcadores e a duração do DM tipo 1. Santos et al. (2010) compararam os níveis

de TNF-α, por meio de ELISA, no fluido gengival de diabéticos tipo 2 bem-controlados e descompensados com periodontite crônica antes e em 3 e 6 meses após terapia periodontal não-cirúrgica. Os autores não encontraram diferenças entre os níveis de TNF-α entre os grupos e em nenhum dos tempos experimentais.

No contexto da resposta imune adquirida, foi inicialmente sugerido que as sub-populações de células T helper (Th), denominadas Th1 e Th2, poderiam determinar uma resposta frente à agressão bacteriana periodontal pelo equilíbrio entre a liberação de mediadores pró- e anti-inflamatórios (OHLRICH et al., 2009). Por esse motivo, por vários anos uma série de estudos objetivaram delinear o perfil Th1 e Th2 na patogênese das doenças periodontais, estudando as principais citocinas produzidas pelas duas populações celulares em nível de proteína e RNAm. Interferon (INF)-γ é um mediador pró-inflamatório predominante na resposta Th1 e, assim, capaz de conduzir para uma resposta imune mediada por células. Em contraste, a IL-4 é uma citocina anti-inflamatória representativa da resposta Th2 que promove a imunidade humoral pela produção de fatores de crescimento e diferenciação para linfócitos B (FIETTA & DELSANTE, 2009). O INF-γ apresenta como principal função a ativação de macrófagos nas respostas imunes inata e mediada por células, que culmina na destruição tecidual. Por outro lado, a IL-4 é um potente inibidor de macrófagos que bloqueia a secreção de diversos mediadores pró-inflamatórios, incluindo o INF-γ (FIETTA & DELSANTE, 2009). Górska et al. (2003) encontraram altas concentrações e frequências de detecção de INF-γ em sítios com periodontite avançada. Honda et al. (2006) compararam o perfil de expressão gênica de diversos mediadores inflamatórios por meio de PCR quantitativo em gengivite e periodontite. Os autores verificaram que os níveis de INF-γ estavam mais elevados na periodontite quando comparado à gengivite. Dutzan et al., em 2009, correlacionaram o perfil de resposta imunológica (Th1, Th2 e Th17) em biópsias gengivais de lesões ativas e inativas em indivíduos com destruição tecidual periodontal. Houve maior expressão de INF-γ nas lesões ativas, quando comparado às inativas. A ausência localizada de IL-4 nos tecidos periodontais foi, por sua vez, associada à atividade e progressão de doença, como, por exemplo, à evolução da gengivite para a periodontite (SHAPIRA et al., 1992; TSAI et al., 2007). Pradeep et al. (2008) investigaram a relação entre condições clínicas periodontais e as concentrações de IL-4 no fluido gengival de sítios saudáveis, com gengivite, com periodontite crônica e com periodontite tratada. Os autores encontraram níveis de IL-4 mais elevados em indivíduos saudáveis, níveis intermediários nos tratados e com gengivite e os

menores níveis nos indivíduos com periodontite crônica, sugerindo que a presença dessa citocina está associada com a remissão ou melhora da periodontite.

Alguns estudos sugeriram ainda uma estreita relação entre o INF-γ e a IL-4, cujo equilíbrio seria capaz de regular a resposta imuno-inflamatória nas doenças periodontais. Ukai et al. (2001), por exemplo, verificaram que a razão IL-4/INF-γ em bolsas profundas e com sangramento é menor que em bolsas moderadas e rasas. Tsai et al. (2007) demonstraram que a terapia periodontal é capaz de reduzir a quantidade de INF-γ e aumentar a concentração de IL-4 no fluido gengival de indivíduos com periodontite crônica, sugerindo que a razão IL-4/INF-γ apresenta um papel importante na destruição dos tecidos periodontais. Até o presente momento, poucos estudos avaliaram os níveis de INF-γ e IL-4 em sítios com periodontite de pacientes diabéticos tipo 2. Duarte et al. (2007) avaliaram o mecanismo de interferência do DM na expressão gênica de citocinas e marcadores relacionados à osteoclastogênese em biópsias de indivíduos sistemicamente e periodontalmente saudáveis, sistemicamente saudáveis com periodontite e diabéticos tipo 1 com periodontite. Níveis mais elevados de INF-γ foram encontrados no grupo dos diabéticos quando comparado ao grupo controle. Shin et al. (2010) observaram e quantificaram a expressão de IL-4 e INF-γ em biópsias de indivíduos diabéticos tipo 2 com periodontite, sistemicamente saudáveis com periodontite crônica e sistemicamente e periodontalmente saudáveis. Independente do DM houve uma tendência para uma maior expressão de INF-γ nos grupos que apresentavam periodontite. Por outro lado, a maior expressão de IL-4 foi observada no grupo dos indivíduos sadios. Santos et al. (2010) compararam os níveis de IL-4 e INF-γ no fluido gengival de diabéticos tipo 2 com periodontite crônica bem-controlados e descompensados antes e em 3 e 6 meses após terapia. Níveis mais elevados de INF-γ foram encontrados em indivíduos bem-controlados quando comparado aos descompensados, em todos os tempos experimentais. Níveis mais baixos de IL-4 foram observados nos indivíduos bem-controlados no baseline.

Uma terceira população de células Th, antagônicas às células T regulatórias, foi mais recentemente identificada e denominada Th17. Essas células se caracterizam especialmente pela produção de IL-17 e são mantidas pela presença da IL-23 (TESMER et al., 2008). A IL-17 é uma citocina pró-inflamatória que compartilha as ações biológicas da IL-1β e do TNF-α (XU & CAO, 2010) e, por esse motivo, tem sido apontada como um mediador importante nas lesões periodontais. IL-23 é uma citocina pró-inflamatória envolvida na expansão da linhagem Th17,

que pertence a família da IL-12. É secretada como um heterodímero composto pela subunidade p40, idêntica a subunidade p40 da IL-12 e por uma única subunidade p19, similar a subunidade p35 da IL-12 (OPPMANN et al., 2000). Assim, os papéis da resposta Th17 e das citocinas IL-17 e IL-23 têm sido atualmente alvo de investigações no campo periodontal (TAKAHASHI et al., 2005; OHLRICH et al., 2009). Ohyama et al. (2009) observaram níveis mais elevados de IL-23 e IL-17 em lesões periodontais quando comparado aos tecidos sadios. Os autores sugeriram que essas citocinas são estimuladas em tecidos inflamados. Similarmente, um outro estudo comprovou que há uma expressão exacerbada de IL-17 em lesões periodontais ativas quando comparado às lesões inativas e, há uma correlação positiva entre a expressão de IL-17 e o ligante do receptor do ativador do fator nuclear kappa-ß (RANKL) (DUTZAN et al., 2009). Cardoso et al., em 2009, investigaram a presença de células Th17 na doença periodontal. Biópsias gengivais e de osso alveolar foram coletadas, tanto de pacientes saudáveis como portadores de periodontite. Dentre outras citocinas, os níveis de RNAm para a IL-17 e IL-23 em tecido gengival e de IL-17 e do RANKL no osso alveolar foram avaliadas por reação de polimerase em cadeia (PCR). Os dados demonstraram altos níveis de IL-17 e IL-23 nos tecidos doentes, assim como indicaram a presença de células Th17 nos tecidos gengivais dos pacientes com periodontite. Além disso, IL-17 e RANKL foram abundantemente expressos no osso alveolar dos pacientes doentes em comparação aos baixos níveis encontrados nos indivíduos sem periodontite. Até o presente momento, poucos trabalhos avaliaram os papéis de IL-17 e IL-23 em sítios periodontais de diabéticos. Santos et al. (2010), comparando diabéticos tipo 2 bem-controlados e descompensados com periodontite crônica, observaram que os indivíduos descompensados apresentavam níveis mais altos de IL-17 em relação aos bem-controlados, enquanto não havia diferenças entre os grupos para os níveis de IL-23. Entretanto, os autores não incluíram um grupo de não-diabéticos com periodontite. Duarte et al., em 2010, avaliaram os níveis de IL-17 em biópsias teciduais de sítios com periodontite crônica em fumantes, diabéticos tipo 2 e sem nenhum dos dois fatores de risco. Os níveis de IL-17 foram maiores no grupo dos diabéticos com periodontite quando comparado aos demais grupos.

O estudo das interações entre o sistema imunológico e o tecido ósseo, referido como osteoimunologia, tem ganhado espaço nos últimos anos movido pela busca de um melhor entendimento da fisiologia e patologias que acometem o tecido ósseo. Neste contexto, a sinalização de algumas citocinas tem sido apontada como importante para a regulação da

formação e função de células ósseas (LEE & LORENZO, 2006). A interação entre as moléculas osteoprotegerina (OPG), RANKL e o receptor do ativador do fator nuclear kappa-ß (RANK) no é um dos mecanismos moleculares mais importantes no processo de reabsorção óssea. Resumidamente, a formação (osteoclastogênese) e atividade dos osteoclastos e, consequentemente, a reabsorção do tecido ósseo ocorre a partir da ligação RANK/RANKL. A OPG, por sua vez, é capaz de regular este processo de reabsorção pela sua capacidade de união ao RANKL, evitando assim a interação RANKL/RANK e por consequência, a reabsorção óssea (TANAKA et al., 2005). O sistema RANK/RANKL/OPG é um exemplo importante da conexão entre osso e células imunológicas (LEE & LORENZO, 2006), pois alguns estudos já demonstraram que citocinas pró-inflamatórias como TNF-α (BOYCE et al., 2005) e IL-17 (SATO et al., 2006; NAKASHIMA & TAKAYANAGI, 2009) são capazes de induzir a expressão de RANKL. Na doença periodontal, níveis aumentados de RANKL e reduzidos de OPG têm sido encontrados (VERNAL et al., 2004; LU et al., 2006; BOSTANCI et al., 2007). Vernal et al. (2004) observaram maiores níveis de RANKL no fluido gengival de indivíduos com periodontite quando comparado aos indivíduos periodontalmente sadios, sustentando a hipótese de que RANKL apresenta uma correlação positiva com a perda óssea alveolar da doença periodontal. Bostanci et al., em 2007, compararam os níveis de RANKL, OPG e sua proporção (RANKL/OPG) no fluido gengival entre pacientes saudáveis e com doenças periodontais. Os níveis de RANKL estavam baixos nos grupos saudáveis e com gengivite e altos nos grupos com periodontite crônica e agressiva. Os níveis de OPG foram mais altos no grupo saudável comparado aos demais grupos com qualquer tipo de doença periodontal. A proporção RANKL/OPG estava mais elevada nos grupos com periodontites comparado à saúde e gengivite e apresentou uma correlação positiva com parâmetros clínicos importantes como profundidade de sondagem e nível clínico de inserção. Altos níveis de RNAm para RANKL e baixos níveis para OPG foram encontrados em indivíduos diabéticos tipo 1 com periodontite (DUARTE et al. 2007). Segundo Santos et al. (2010), concentrações mais elevadas de RANKL e RANKL/OPG são observadas em diabéticos tipo 2 descompensados, sugerindo que o controle glicêmico pode interferir negativamente na proporção RANKL/OPG nesses indivíduos.

Embora esteja clinicamente bem definido que indivíduos diabéticos apresentam maior risco para a periodontite, os mecanismos inflamatórios e imunológicos que explicam esse dado clínico ainda não estão claros. Uma vez que diabéticos e não-diabéticos com periodontite apresentam poucas

diferenças microbiológicas (HINTAO et al., 2007), a possível explicação para a exacerbada destruição periodontal em diabéticos é uma resposta do hospedeiro alterada. Em geral, a hiperglicemia induz alterações microvasculares relacionadas à espessura da camada basal, remodelação tecidual, adesão celular, inflamação, nível de citocinas e fatores de transcrição, que são responsáveis pelas complicações oculares, renais, neuronais e periodontais nos diabéticos (ROY et al., 2010). Um mecanismo molecular plausível para explicar a gravidade de periodontite em diabéticos consiste no fato da hiperglicemia crônica gerar glicolisação progressiva das proteínas corpóreas, formando os produtos finais avançados da glicolisação denominados AGEs (*advanced glycation end products*) (MÉNDEZ et al., 2010). Estes compostos, que estão presentes nos tecidos periodontais (KATZ et al., 2005), se ligam a um receptor específico (RAGE) e podem causar a exacerbação das respostas inflamatórias sistêmicas e periodontais (LALLA et al., 1998; LALLA et al., 2001; TAKEDA et al., 2006).

1.5. Tratamento periodontal e bolsas periodontais residuais em diabéticos e não-diabéticos

Atualmente, como regra para a prática clínica, o tratamento periodontal básico compreende uma fase inicial não-cirúrgica para controle do agente etiológico, na qual é realizada a remoção mecânica do biofilme supra e subgengival pelo profissional por meio de diferentes dispositivos (curetas manuais, instrumentos sônicos e ultrassônicos, lasers, etc), na ausência de procedimentos cirúrgicos, associado ao controle do biofilme supragengival pelo paciente. Em geral, essas medidas visam a eliminação de biofilme e cálculo dental para a redução dos patógenos periodontais e recolonização dental por uma microbiota compatível com o hospedeiro, cujos principais reflexos clínicos são o ganho de inserção e as reduções da profundidade de sondagem e inflamação tecidual (CUGINI et al., 2000; PETERSILKA et al., 2002; COBB, 2002; COLOMBO et al., 2005; IOANNOU et al., 2009). Diversos estudos demonstraram que esse protocolo de terapia básica quando aplicado aos diabéticos também promove benefícios clínicos, semelhantes aos observados em indivíduos não-diabéticos. Faria-Almeida et al. (2006) compararam a resposta clínica e glicêmica ao tratamento periodontal convencional entre indivíduos com e sem DM tipo 2, em 3 e 6 meses. Houve melhoras em todos os parâmetros clínicos sem diferenças estatísticas entre os grupos, exceto para profundidade de sondagem que apresentou uma melhora superior para os sujeitos não-diabéticos. A melhora observada no nível de HbA1c confirmou a resposta positiva metabólica ao tratamento periodontal. Navarro-Sanchez et al. (2007) compararam, em 3 e 6 meses, os efeitos clínicos da terapia não-cirúrgica em diabéticos tipo 2 e não-diabéticos e seu

efeito no controle glicêmico dos diabéticos. Ambos os grupos responderam positivamente à terapia proposta e apresentaram melhoras significativas nos parâmetros clínicos. Melhoras clínicas e imunológicas foram ainda acompanhadas por uma redução significativa nos valores de HbA1c nos diabéticos. Da Cruz et al. (2008) avaliaram as alterações clínicas, microbiológicas e glicêmicas em 3 meses após a terapia periodontal convencional em indivíduos com e sem DM tipo 2. Houve melhoras nos parâmetros clínicos para ambos os grupos após 3 meses, mas não foram observadas alterações na taxa de HbA1c. Apesar de algumas reduções bacterianas, não houve diferenças entre os grupos. Kardesler et al. (2010) avaliaram os efeitos da terapia periodontal nos parâmetros clínicos, no controle glicêmico e parâmetros imunológicos séricos de indivíduos com DM tipo 2 portadores de periodontite crônica. Os indivíduos foram divididos em diabéticos compensados (HbA1c <7%), diabéticos descompensados (HbA1c ≥ 7%) e sistemicamente saudáveis e, submetidos à raspagem e alisamento radicular. Todos os grupos apresentaram melhoras nos parâmetros clínicos em 1 e 3 meses pós-tratamento. Os níveis de HbA1c dos diabéticos descompensados diminuíram significativamente após 3 meses, enquanto não houve alterações para o grupo dos compensados.

Com objetivo de ampliar os benefícios clínicos da raspagem e alisamento radicular durante a terapia básica, alguns estudos propuseram a inserção de terapias adicionais na fase inicial do tratamento periodontal, incluindo a administração de antibióticos sistêmicos, aplicação de antimicrobianos locais subgengivais e/ou técnicas para um controle mais efetivo do biofilme supragengival, anti-inflamatórios e terapia fotodinâmica (KRAYER et al., 2010). Tais agentes adjuntos também foram propostos para o tratamento periodontal de indivíduos diabéticos com o objetivo de melhorar os efeitos da raspagem e alisamento radicular aplicados neste grupo de risco para as doenças periodontais (JANKET et al., 2005; PROMSUDTHI et al., 2005; SINGH et al., 2008; AL-ZAHRANI et al., 2009; DEO et al., 2010).

Embora a terapia periodontal inicial forneça resultados clínicos e microbiológicos satisfatórios que possam ser mantidos com a instituição de uma terapia de manutenção sistemática, tratamentos adicionais são algumas vezes necessários para bolsas residuais, que não apresentaram boa resposta à terapia básica e permanecem profundas (≥ 5mm) e com sangramento à sondagem. Alguns estudos demonstraram que esses sítios residuais não-responsivos estão localizados predominantemente em regiões de difícil acesso (SERINO et al., 2001) e áreas de bi/trifurcação (NORDLAND et al., 1987; EGELBERG & CLAFFEY, 1994). Entretanto, outros fatores

relacionados aos hábitos do paciente (consumo de cigarros, controle de placa inadequado, etc) também podem contribuir para a presença de sítios não-responsivos (TONETTI et al., 1998; MATULIENE et al., 2008). A necessidade de instituição de tratamentos adicionais para bolsas residuais está embasada em estudos que demonstraram que as mesmas apresentam maior risco para perda futura de inserção. Matuliene et al. (2008) investigaram a influência de bolsas residuais ≥ 5mm e sangramento a sondagem após terapia ativa periodontal na progressão da doença e perda dentária. Os autores observaram que uma profundidade de sondagem = 5mm representa um risco para perda dentária com *odds ratio* de 5,8 e 7,7 em nível de sítio e dente, respectivamente. Os correspondentes *odds ratio* para bolsas = 6mm foram 9,3 e 11 e para bolsas ≥ 7mm foram 37,9 e 64,2. Ao nível de paciente, fumantes pesados, duração da terapia de suporte e bolsas ≥ 6mm e índice de sangramento à sondagem ≥ 30% representou risco para perda dentária.

Diversas terapias foram propostas para tratamento de sítios residuais após terapia básica e/ou durante a fase de manutenção periodontal, incluindo a repetição da raspagem e alisamento radicular isoladamente ou em associação com a aplicação de lasers, dispositivos de liberação antimicrobiana local, antibióticos sistêmicos e acesso cirúrgico (SERINO et al., 2001; SALVI et al., 2002; EICKHOLZ et al., 2005; McCOLL et al., 2006; ZEE et al., 2006; KÖNIG et al., 2008; LULIC et al., 2009). Surpreendentemente, não existem na literatura estudos em que o foco foi o tratamento de bolsas residuais em diabéticos. O presente trabalho tem como um dos objetivos avaliar efeito da raspagem radicular repetida associada ao uso de antibióticos sistêmicos, especificamente amoxicilina e metronidazol, e acesso cirúrgico para tratamento de bolsas residuais. Por esse motivo, uma breve descrição da contribuição destas terapias para o tratamento da periodontite crônica como um todo e de sítios não-responsivos se faz necessária.

1.5.1. Tratamento cirúrgico periodontal antinfeccioso

Estudos clínicos longitudinais desenvolvidos nas últimas décadas que compararam diferentes tratamentos cirúrgicos periodontais anti-infecciosos entre si e com a terapia não-cirúrgica, representam bases científicas de grande importância em periodontia. Os estudos clássicos de um grupo de pesquisadores de Michigan compararam os efeitos clínicos da curetagem periodontal, eliminação cirúrgica de bolsa por meio da gengivectomia, retalho com ou sem recontorno ósseo, retalho de Widman modificado e raspagem e alisamento radicular não-cirúrgico, em 2 ou 5 anos (RAMFJORD et al., 1975; RAMFJORD et al., 1987). Em geral, os resultados demonstraram que

a curetagem, raspagem e alisamento radicular e retalho de Widman modificado promoveram melhores resultados em relação ao nível de inserção, enquanto que a eliminação de bolsa resultou em maior redução de profundidade de sondagem. Outras observações relevantes do grupo foram que os molares apresentaram uma resposta menos favorável que os demais dentes, sítios rasos perderam inserção após qualquer um dos tratamentos e, os sítios profundos apresentaram ganho de inserção.

A comparação entre a raspagem e alisamento radicular não-cirúrgico e retalho de Widman modificado, desenvolvida por Lindhe et al. (1982) forneceu uma importante informação sobre a "profundidade crítica de sondagem" para se empregar um procedimento cirúrgico ou não-cirúrgico. Os autores estabeleceram as profundidades de sondagem acima das quais ocorreria ganho de inserção clínica e abaixo das quais haveria uma tendência à perda de inserção, sendo estas 2,9mm para raspagem e alisamento radicular não-cirúrgico e 4,2mm para acesso cirúrgico. Em 1996, Kaldahl et al. (1996) demonstraram que, em 3 anos, não havia diferenças entre a raspagem radicular não-cirúrgica e o retalho de Widman modificado para a profundidade de sondagem em sítios com bolsas iniciais de 5 à 6mm. Ao final de 5 anos, não havia diferenças para a profundidade de sondagem entre os mesmos grupos de tratamento para as bolsas maiores que 7 mm. Mais tarde, um estudo clínico desenvolvido por Serino et al. (2001) comparou o efeito clínico do tratamento inicial não-cirúrgico e cirúrgico em indivíduos com periodontite avançada e, a incidência de recorrência da doença durante 12 anos de terapia de manutenção. Um grupo recebeu acesso cirúrgico e raspagem e o outro apenas raspagem não-cirúrgica. Os autores concluíram que a terapia cirúrgica foi mais efetiva para a redução da profundidade de sondagem e eliminação de bolsas profundas. Além disso, os pacientes que receberam a terapia não-cirúrgica exibiram sinais mais evidentes de progressão da doença em 1-3 anos após a terapia ativa, comparado ao grupo que recebeu cirurgia.

Uma revisão sistemática de Hung & Douglass (2002) demonstrou que a terapia cirúrgica apresentou melhores resultados que o tratamento não-cirúrgico para a redução de profundidade de sondagem para bolsas inicialmente profundas. Entretanto, essas diferenças apresentaram uma tendência a se igualar após 3 anos de acompanhamento. Heitz-Mayfield et al. (2002) sugeriram, em uma revisão sistemática, que tanto a terapia não-cirúrgica como a terapia cirúrgica são efetivas para tratamento de periodontite crônica em relação ao ganho de inserção e redução da

inflamação. Entretanto, o tratamento cirúrgico promove redução de profundidade de sondagem e ganho de inserção mais favoráveis para bolsas profundas.

Alguns estudos avaliaram os efeitos da terapia cirúrgica especificamente em bolsas residuais e periodontite recorrente. Em 1994, Sigurdsson et al. compararam os efeitos clínicos e microbiológicos da terapia cirúrgica e não-cirúrgica em pacientes com sítios residuais apresentando sinais de progressão de periodontite. Em 1 ano, a profundidade de sondagem, o nível clínico de inserção e o perfil bacteriano melhoraram em relação ao tempo inicial, mas sem diferenças evidentes entre os grupos. Os autores concluíram que ambas as terapias foram efetivas para o tratamento de lesões recorrentes em pacientes susceptíveis à periodontite. Rawlinson et al. (1995) avaliaram o efeito microbiológico do retalho de Widman modificado para tratamento de bolsas residuais profundas. Os resultados demonstraram uma redução mais significativa de patógenos após o emprego da terapia cirúrgica quando comparado à raspagem não-cirúrgica. König et al. (2008) desenvolveram um estudo prospectivo para comparar os efeitos da raspagem e alisamento radicular repetida e da terapia cirúrgica em bolsas residuais de pacientes com periodontite crônica generalizada. Os autores demonstraram que ambos os tratamentos reduziram a profundidade de sondagem e melhoraram o nível clínico de inserção. Foi sugerido que a raspagem subgengival adicional em bolsas não-responsivas é capaz de promover ganho de inserção clínica e reduzir a necessidade de tratamento cirúrgico periodontal.

Existem poucas evidências na literatura do impacto do tratamento cirúrgico em diabéticos. Westfelt et al. (1996) compararam o efeito clínico do tratamento cirúrgico de bolsas residuais (> 5mm com sangramento) em 6 meses após raspagem não-cirúrgica entre diabéticos (tipo 1 e 2) e não-diabéticos. Os indivíduos foram engajados em uma terapia de manutenção e re-avaliados em 12, 24 e 60 meses. Diabéticos e não-diabéticos responderam de forma similar ao protocolo de tratamento proposto.

1.5.2. Terapia antibiótica sistêmica

Como descrito anteriormente, embora a raspagem e alisamento radicular promova resultados positivos nos parâmetros clínicos, em alguns sítios, a mesma não é capaz de diminuir a infecção de forma satisfatória (HAFFAJJE et al., 1997; CUGINI et al., 2000; CARVALHO et al., 2005; MATARAZZO et al., 2008). Desta forma, o uso de antibióticos sistêmicos tem sido proposto como coadjuvante ao tratamento de infecção periodontal tanto na fase inicial como para bolsas residuais (HERRERA et al., 2002; HERRERA et al., 2008; KRAYER et al., 2010). Até o

presente momento, os efeitos de diversos grupos de antibióticos foram avaliados no tratamento de periodontite crônica e agressiva, em destaque para os antibióticos tetraciclíneos (tetraciclinas, minociclina, doxiciclina), macrolídeos (eritromicina, azitromicina), penicilinas (amoxicilina), nitroimidazol (metronidazol), quinolonas (ciprofloxacinas) e lincomicinas (clindamicinas) (KRAYER et al., 2010).

Em indivíduos diabéticos, a doxiciclina tem sido o antibiótico mais estudado como coadjuvante ao tratamento periodontal. Estudos sugeriram o uso de doxiciclina não apenas como antibiótico, mas em doses subantimicrobianas para utilizar o efeito inibitório deste medicamento sobre as metaloproteinases. Promsudthi et al. (2005) examinaram o efeito da terapia periodontal associada à administração de doxiciclina 100mg/dia por 14 dias no controle glicêmico de diabéticos tipo 2. Alguns indivíduos (controle) não receberam nem o tratamento periodontal nem o antibiótico. Parâmetros clínicos, glicemia em jejum e HbA1c foram mensurados no baseline e em 3 meses. Após a terapia, a condição periodontal melhorou, porém não houve diferenças para as taxas de glicemia em jejum e HbA1c. Singh et al. (2008) avaliaram diabéticos tipo 2 com periodontite generalizada não-tratados, que receberam apenas raspagem ou raspagem associada à doxiciclina sistêmica, em relação aos parâmetros periodontais e controle glicêmico. Após 3 meses, ambas terapias foram efetivas para melhoras dos parâmetros clínicos e metabólicos, demonstrando reduções significativas quando comparado ao grupo não-tratado. Entretanto, um resultado mais favorável foi encontrado para o grupo que recebeu o antibiótico. Deo et al. (2010) avaliaram, em 6 meses, o efeito clínico da administração de doses baixas de doxiciclina (20mg 2xdia/6meses) como terapia adjunta à raspagem e alisamento radicular em pacientes diabéticos com periodontite crônica. Houve uma maior redução da profundidade de sondagem e ganho de inserção clínica no grupo teste comparado ao grupo controle, que recebeu raspagem e placebo. Os autores sugeriram que a doxiciclina pode potencializar o efeito benéfico da raspagem em diabéticos com periodontite avançada. O´Connel et al. (2008) observaram significante melhora clínica, glicêmica e imunológica em indivíduos diabéticos portadores de periodontite que receberam terapia mecânica associada à doxiciclina (100mg/dia por 2 semanas) quando comparado ao grupo que recebeu apenas a raspagem.

O metronidazol é um nitromidazol sintético de característica bactericida, ativo contra bactérias Gram negativas, especialmente *Porphyromonas gingivalis* e *Prevotella intermedia*. Pahkla et al., em 2005, observaram a concentração no plasma, saliva e fluido gengival após administração

sistêmica de metronidazol (500mg, 2 ou 3x ao dia, por pelo menos 2 dias) em indivíduos com periodontite crônica generalizada. Os autores verificaram que o metronidazol possui boa capacidade para atingir o fluido gengival e a saliva, sendo portanto indicado para tratamento da periodontite. Trabalhos científicos avaliaram o efeito do metronidazol nos parâmetros clínicos periodontais e, em muitos deles, os resultados clínicos foram melhores quando o metronidazol foi associado à raspagem, comparado a terapia mecânica isolada (JOYSTON-BECHAL et al., 1986; LOESCHE et al., 1992; WINKEL et al., 1997; ELTER et al., 1997; FERES et al., 2001; HAFFAJJE et al., 2003 e 2007; CARVALHO et al., 2004; MATARAZZO et al., 2008). Alguns estudos sugeriram ainda que o metronidazol apresenta melhor ação em bolsas profundas (LOESCHE et al., 1981; HAFFAJEE et al., 2007). Haffajee et al., em 2008, observaram, ao longo de um ano, uma mudança positiva no perfil microbiológico dos pacientes com periodontite crônica que receberam metronidazol (250mg, 3x ao dia/14 dias) ou azitromicina (500mg, 1x ao dia/3 dias) sistêmicos, quando comparado ao grupo que recebeu apenas a raspagem.

A amoxicilina, por sua vez, é um antibiótico de amplo espectro, pertencente ao grupo das penicilinas, que atua sobre espécies anaeróbias estritas e facultativas, cocos e bacilos Gram negativos e positivos (KULIK et al., 2008). Ainda assim, os resultados dos estudos que avaliaram o efeito da amoxicilina associada à raspagem demonstraram que esse antibiótico oferece apenas uma modesta contribuição nos parâmetros clínicos periodontais quando comparado à terapia mecânica isolada (HELOVUO & PAUNIO, 1989; FERES et al., 2001). Feres et al., em 2001, associaram a raspagem à administração de metronidazol (250mg 3x ao dia) ou de amoxicilina (500mg 3x ao dia) por 14 dias para tratamento de indivíduos portadores de periodontite crônica. Os indivíduos que receberam metronidazol obtiveram uma redução significativa dos patógenos pertencentes ao complexo vermelho (*Tannerella forsythia, P. gingivalis e Treponema denticola*) em 1 ano pós-tratamento. Os indivíduos que receberam amoxicilina apresentaram recolonização desses patógenos após 1 ano. Além disso, esse antibiótico reduziu a proporção de espécies compatíveis com o hospedeiro, como Actinomyces. Um único estudo em diabéticos tipo 2 administrou amoxicilina como coadjuvante à raspagem e alisamento radicular (RODRIGUES et al., 2003). Os autores avaliaram, em 3 meses, os efeitos da raspagem e alisamento radicular de boca-total em sessão única associada ou não à administração de amoxicilina/ácido clavulânico 875 mg no controle glicêmico e parâmetros clínicos de diabéticos. Ambos os grupos demonstraram melhoras clínicas pós-terapias. O valor de HbA1c reduziu significativamente para

o grupo que recebeu apenas a raspagem e a glicemia em jejum não apresentou diferença significativa para nenhum grupo.

Há cerca de 20 anos, a combinação de amoxicilina e metronidazol tem sido sugerida como a mais promissora para tratamento da periodontite crônica e agressiva (HERRERA et al., 2002; MOMBELLI et al., 2005; MESTNIK et al., 2010). van Winkelholff et al., em 1992, associaram raspagem e alisamento radicular ao metronidazol (250mg 3x ao dia) e amoxicilina (375mg, 3x ao dia) por 7 dias. Os autores observaram melhora significativa em parâmetros clínicos importantes (ex. profundidade de sondagem e nível clínico de inserção) e também na redução de *Aggregatibacter actinomycetemcomitans.* Os benefícios da raspagem e alisamento radicular associada ao metronidazol e amoxicilina no tratamento de periodontite recorrente foram demonstrados por van Winkelhoff et al. (1992) e Pavicić et al. (1994). Winkel et al. (2001) demonstraram que a administração sistêmica de amoxicilina e metronidazol, em 6 semanas após raspagem e alisamento radicular, produziu efeitos clínicos e microbiológicos superiores à raspagem e alisamento radicular sozinha, em 3 meses. López et al. (2006) avaliaram o efeito do metronidazol associado à amoxicilina como monoterapia sobre a microbiota subgengival da periodontite crônica. Os autores submeteram um grupo à terapia antibiótica por 7 dias e outro grupo a raspagem e placebos. Após 12 meses, ambas terapias apresentaram resultados semelhantes, com melhoras clínicas e reduções bacterianas significativas. Um estudo placebo controlado de Moeintaghavi et al. (2007) avaliou os efeitos clínicos e microbiológicos da administração sistêmica de metronidazol (250mg) e amoxicilina (500mg), ambos de 8/8h por 7 dias, como terapia adjunta à raspagem e alisamento radicular em pacientes com periodontite crônica moderada/avançada. O grupo teste apresentou melhoras mais significativas em todos os parâmetros clínicos e microbiológicos quando comparado ao controle em 8 semanas, reforçando o benefício que a terapia antibiótica pode fornecer para o tratamento da periodontite crônica. Recentemente, Cionca et al. (2009) demonstraram que a combinação de amoxicilina (375mg) e metronidazol (500mg), 3x dia por 7 dias, associada ao debridamento de boca-total em 48 horas resultou em efeitos clínicos mais favoráveis que o debridamento sozinho. Nos indivíduos tratados com antibióticos, houve maior redução de bolsas e sítios com sangramento, diminuindo a necessidade de terapias adicionais em 6 meses após a terapia inicial. Um estudo desenvolvido por Serino et al. (2001) utilizou por 14 dias a associação de amoxicilina (750mg 2X/dia) e metronidazol (400mg 3X/dia) como coadjuvante à raspagem não cirúrgica para o tratamento de

bolsas residuais. O tratamento proposto foi efetivo para aumentar o número de bolsas rasas (<4mm) e, após 1 ano, apenas 5% dos sítios ainda permaneceram com bolsas profundas (>6mm).

Até o momento, não existem estudos na literatura que utilizaram a associação metronidazol e amoxicilina como adjunto à raspagem e alisamento radicular para tratamento de periodontite em diabéticos na fase inicial ou para bolsas residuais.

2. PROPOSIÇÃO

Esse trabalho foi dividido em dois estudos, cujos objetivos foram:

ESTUDO 1 – Comparar os níveis de citocinas (INF-γ, TNF-α, IL-17, IL-23 e IL-4) e fatores relacionados a osteoclastogênese (RANKL e OPG) no fluido gengival de indivíduos com periodontite crônica sistemicamente saudáveis ou portadores de DM tipo 2 bem controlados ou com controle glicêmico insatisfatório.

ESTUDO 2 – Avaliar, em 3 e 6 meses, os efeitos da terapia cirúrgica e não-cirúrgica associadas à administração de antibióticos sistêmicos (metronidazol e amoxicilina) nos parâmetros clínicos e níveis de citocinas (INF-γ, IL-17, IL-23 e IL-4) em bolsas residuais de DM tipo 2.

3. ESTUDO 1

Cytokines and bone-related factors in systemically-healthy and type 2 diabetic subjects with chronic periodontitis (aceito no Journal of Periodontology).

Abstract

Background: This study compared the levels of cytokines and bone-related factors in the gingival crevicular fluid (GCF) of systemically-healthy, better-controlled and poorly controlled type 2 diabetic subjects with chronic periodontitis. **Methods:** Thirty-seven type 2 diabetic subjects and 20 systemically-healthy subjects with chronic periodontitis were enrolled in this study. The subjects with diabetes mellitus (DM) were categorized as better-controlled (n=17; HbA1c levels ≤ 8%) or poorly controlled subjects (n=20; HbA1c > 8%). Levels of tumor necrosis factor (TNF)-α, interleukin (IL)-4, interferon (IFN)-γ, IL-23, IL-17, soluble receptor activator of nuclear factor-κβ ligand (sRANKL) and osteoprotegerin (OPG) in GCF of diseased sites were analyzed by ELISA. **Results**: Type 2 DM, as a whole, upregulates the levels of OPG, RANKL, IFN-γ, IL-17 and IL-23 and downregulates the production of IL-4 in sites with chronic periodontitis ($p<0.05$). Better-controlled individuals exhibited the highest levels of IFN-γ, while poorly controlled subjects presented the highest levels of IL-17 ($p<0.05$). There were no differences in the levels of TNF-α, OPG and IL-23 among systemically-healthy, better- and poorly controlled diabetic subjects ($p>0.05$). **Conclusion**: Increased levels of proinflammatory cytokines and RANKL were observed in the GCF of type 2 diabetic subjects with chronic periodontitis, when compared to non-diabetic ones. In addition, poor or good glycemic status seems to modulate osteo-immuno-inflammatory mediators in a different manner.

Key-words: Diabetes Mellitus; Chronic Periodontitis; Cytokines, RANK Ligand; Osteoprotegerin.

Sentence Summary: Type 2 diabetic subjects as a whole had increased levels of proinflammatory cytokines and RANKL when compared to non-diabetic subjects. Moreover, a poor or good glycemic status may interfere in the pattern of osteo-immuno-inflammatory periodontal response against pathogens in type 2 diabetic subjects.

Introduction

Periodontal diseases are mediated by the interaction between pathogens and host immune-inflammatory responses[1]. Since inflammatory mediators can profoundly affect the protective or destructive nature of the host response, there has been an increasing interest in identifying the role played by cellular and molecular factors in the pathogenesis of periodontal diseases. Several inflammatory mediators [i.e. interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-17, IL-23, interferon (INF)-γ and tumor necrosis factor (TNF)-α] and bone-related factors [osteoprotegerin (OPG), receptor activator of NF-kB (RANK) and RANK ligand (RANKL)] have been identified around periodontal tissues at both mRNA and protein levels[2-9]. TNF-α is a major mediator of inflammation and, when overexpressed, can stimulate the establishment of chronic inflammatory and autoimmune diseases[10]. In periodontitis, TNF-α exerts pro-inflammatory activities that are related to the loss of attachment and bone[11,12]. IL-17 is a pro-inflammatory cytokine that shares biological functions with TNF-α and IL-1β. This cytokine is implicated in bone destruction via RANKL and in the production of other inflammatory markers such as IL-6, IL-8 and prostaglandins[13-16]. IL-23 promotes the expansion of T helper (Th)- 17 cells, which are characterized by the production of IL-17[17]. Both IL-17 and IL-23 have been currently related to periodontitis[18-20]. IL-4 is an anti-inflammatory cytokine that has been related to healthy periodontal tissues[21] and, is considered the counterpart of IFN-γ, a pro-inflammatory mediator, which has been associated with the progression of periodontitis[7, 21, 22].

Cytokines, signaling molecules, transcription factors and membrane receptors appear to mediate the interactions of the immune system with bone. The RANK/RANKL/OPG system, which regulates bone metabolism and osteoclastogenesis, is an important example of the communication between bone and immune cells[16, 23]. RANK binds to RANKL to induce osteoclastogenesis, while OPG acts as a decoy receptor by binding to RANKL and preventing RANK signaling and osteoclast activation[24]. Increased levels of RANKL and decreased levels of OPG have been found in periodontitis[25-27].

Diabetes mellitus (DM) is a well-recognized risk factor for the severity and progression of periodontal diseases[28]. Although the role of some biomarkers including cytokines, chemokines and bone related factors have been investigated in subjects with DM and periodontitis[29-34], the

immuno-inflammatory system involved in the development of periodontal diseases in diabetic subjects has not been thoroughly comprehend. Since only minor periodontal microbiological differences have been found between diabetic and non-diabetic subjects[35], it is important to understand the immuno-inflammatory mechanisms that determine the higher susceptibility to periodontitis in diabetic subjects, when compared to the non-diabetic individuals. Therefore, this study compared the levels of cytokines (TNF-α, IL-4, IFN-γ, IL-23 and IL-17) and bone-related factors (RANKL and OPG) in the gingival crevicular fluid (GCF) of systemically-healthy, better-controlled and poorly controlled type 2 diabetic subjects with chronic periodontitis. We hypothesized that better-controlled, poorly controlled diabetic subjects and systemically-healthy individuals with chronic periodontitis exhibit distinct dysregulations of cytokines and bone related factors, which could affect their susceptibility to periodontal breakdown.

Material and Methods

Subjects

Thirty-seven type 2 diabetic subjects and 20 systemically-healthy individuals (aged 41 to 63 years), all with chronic periodontitis, were selected from the population referred to the Periodontal Clinic of Guarulhos University, from January 2009 until July 2010. Detailed medical and dental records were obtained. Subjects who fulfilled the following described inclusion/exclusion criteria were invited to participate in the study. All eligible subjects were informed of the nature, potential risks and benefits of their participation in the study and signed their informed consent. This study protocol was previously approved by the Guarulhos University's Ethics Committee in Clinical Research.

Inclusion and exclusion criteria

Data concerning the duration of DM and medications were retrieved from the medical records of the subjects. Diabetic subjects should have presented type 2 DM diagnosis by a physician for at least the past 5 years. These subjects were on insulin supplementation, diet regimen and/or oral hypoglycemic agents. All subjects were diagnosed with generalized chronic periodontitis, based on the clinical and radiographic criteria proposed by the 1999 World Workshop for Classification of Periodontal Diseases and Conditions[36]. The subjects should be > 30 years old

(range: 30 to 65 years), present at least 15 teeth (excluding third molars and teeth with advanced decay indicated by exodontias) and more than 30% of the sites with probing depth (PD) and clinical attachment levels (CAL) ≥ 4 mm.

Exclusion criteria were pregnancy, lactation, current smoking and smoking within the past 5 years, periodontal or/and antibiotic therapies in the previous 6 months, use of mouthrinses containing antimicrobials in the preceding 2 months, any systemic condition that could affect the progression of periodontal disease (e.g. immunological disorders) and long-term administration of anti-inflammatory and immunosuppressive medications. Subjects with periapical pathology, orthodontic appliances and multiple systemic complications of DM were also excluded from the study.

Fasting plasma glucose and glycated hemoglobin monitoring

A single laboratory performed the blood analyses in the diabetic subjects (Clinical Analysis Laboratory, Guarulhos University) including fasting plasma glucose (FPG) and glycated hemoglobin (HbA1c). FPG was measured using the glucose oxidase method and expressed in milligrams per deciliter (mg/dl). HbA1c was measured by high-performance liquid chromatography and expressed as a percentage. Diabetic subjects who had HbA1c values > 8% were categorized as poorly controlled (n=20), whereas subjects who presented HbA1c levels ≤ 8% were categorized as better-controlled (n=17).

Clinical examination

All clinical examinations were performed by one examiner (VRS) who was calibrated, as previously described[32]. The intra-examiner variability was 0.20 mm for PD and 0.23 mm for CAL. This trained examiner was able to provide reproducible measurements of under 0.5 mm. The clinical parameters, registered dichotomously, i.e. bleeding on probing (BoP) and suppuration (SUP), were calculated by the Kappa-Light test and the intra-examiner agreement was > 0.85. The examiner was unaware of the systemic status of the subjects.

The following parameters were assessed at six sites of all teeth, excluding third molars (mesio-buccal, medio-buccal, disto-buccal, mesio-lingual, medio-lingual, disto-lingual), using a manual

periodontal probe[†]: plaque index (PI – presence or absence)[37], BoP (presence or absence), SUP (presence or absence), PD (mm) and CAL (mm).

GCF sampling

GCF was collected one week after clinical examination, in order not to modify the GCF composition. Two non-contiguous sites per subject distributed in different quadrants and presenting PD and CAL ≥ 5mm, BoP and no furcation involvement were chosen for sampling. After removal of the supragingival biofilm with cotton pellets, the sites were isolated with cotton rolls and gently dried with an air syringe to eliminate the possibility of contamination with saliva. GCF was collected by inserting standard paper strips[‡] approximately 2 mm into the sulcus/pocket for 30s. Strips visually contaminated with blood were discarded. The GCF sample volume was measured in a calibrated Periotron 8000[§] and the readings were then converted to an actual volume (µl) by reference to the standard curve. The strips from the two selected sites were immediately placed into separate microcentrifuge tubes containing 250 µl phosphate-buffered saline and protease inhibitor cocktail[‖]. The samples were stored at -20ºC for subsequent assays.

Enzyme linked immunosorbent assay (ELISA)

GCF samples were analyzed by ELISA for TNF-α[¶], IL-4[¶], IFN-γ[¶], IL-17[¶], IL-23[¶], RANKL[#] and OPG[#] using commercially available kits. The tubes were homogenized for 30s and centrifuged for 5min at 1,500xg in order to elute. Assays were carried out according to the manufacturer's recommendations. The negative controls for the assays were the PBS plus protease inhibitor cocktail without GCF sample. The minimum detectable doses (sensitivity) for TNF-α, IL-4 and

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[†] UNC15, Hu-Friedy, Chicago, IL, USA

[‡] Periopaper, Oraflow Inc., Smithtown, NY, USA

[§] Periotron 8000, Proflow Inc., Amityville, NY, USA

[‖] Sigma-Aldrich, Saint Louis, MO, USA

[¶] Quantikine; R&D Systems Inc., Minneapolis, MN, USA

[#] Biomedica Medizinprodukte GmbH & Co KG, Wien, Austria

IL-23 ranged from 0.038 – 0.191 pg/ml, 0.03 - 0.22 pg/ml and 2.7 - 16.3 pg/ml, respectively. The minimum detectable doses for IFN-γ and IL-17 were < 8.0 pg/ml and < 15 pg/ml, respectively. The limit of detection for sRANKL and OPG were 1.6 pg/ml and 2.8 pg/ml, respectively. In relation to specificity, the manufacturers reported no significant cross reactivity or interference for the ELISA kits used in this study. Results were reported as total amount (pg) of each protein per site in 30s of sampling. Calculations of the protein concentrations in each site (pg/µl) were established by dividing the total amount of the each mediator by the GCF volume.

Statistical Analysis

The number of subjects and sites for GCF sampling included in this study was based on previous studies from our and other laboratories that found differences in the levels of the cytokines studied, when comparing different clinical periodontal status [2,21,32,33]. The statistical analysis was performed using a software program[**]. Data were first examined for normality by the Kolmogorov-Smirnov test. Since the data achieved normality, parametric methods were used for the comparisons. The percentages of sites with visible plaque accumulation, BoP, SUP and the means PD and CAL were computed for each subject. Clinical parameters were averaged across subjects. The levels of each cytokine and bone related factor from the two selected sites were also averaged across subjects. Subsequently, clinical parameters and the levels of cytokine and bone-related factors were averaged into following two groups: systemically healthy and type 2 diabetic subjects. The significance of differences in clinical parameters, age and total amounts and concentrations of the biomarkers between both groups were compared using the Student t test. Subsequently, the data were also organized into systemically-healthy, poorly and better-controlled groups and compared by One-Way ANOVA. When there were significant differences by ANOVA, a pair-wise comparison was performed by the Tukey test. The differences in the duration of DM, HbA1c and FPG levels between poorly and better-controlled diabetic subjects were compared by the Student t test. The χ^2 test was used to detect differences in the frequencies of gender. The level of significance was set at 5%.

[**] BioEstat 3.0, Sociedade Civil Mamirauá, CNPq, Tefé, AM, Brazil

Results

Clinical results

No significant differences were observed between groups for any clinical and demographic parameters, except for the mean levels of plaque accumulation that were higher for poorly controlled subjects than better-controlled and non-diabetic subjects ($p<0.05$). As expected, better-controlled diabetic subjects demonstrated lower levels of HbA1c and FPG than poorly controlled ones ($p<0.05$) (Table 1).

Levels of cytokines and bone-related factors

Systemically-healthy X type 2 diabetic subjects

The total amounts (pg/site) and concentrations (pg/µl) of cytokines in systemically-healthy and type 2 diabetic groups are presented in Figure 1. The total amount of IL-4 was lower in the diabetic group, when compared to the systemically-healthy group ($p<0.05$). On the other hand, total amount and concentrations of IL-17 and concentrations of IL-23 and IFN-γ were increased in the diabetic group, when compared to systemically-healthy controls ($p<0.05$). The total amounts (pg/site) and concentrations (pg/µl) of bone-related factors in systemically-healthy and type 2 diabetic groups are shown in Figure 2. The levels of sRANKL and the concentration of OPG were higher in the diabetic than in the systemically-healthy group ($p<0.05$).

Systemically-healthy subjects x poorly controlled x better-controlled diabetic subjects

The levels of cytokines and bone-related factors of systemically-healthy, poorly and better-controlled type 2 diabetic groups are presented in Table 2. Levels of IL-4 were higher in systemically-healthy than better-controlled diabetic subjects ($p<0.05$). Better-controlled subjects presented the highest levels of IFN-γ ($p<0.05$), while poorly controlled subjects demonstrated the highest levels of IL-17 and sRANKL ($p<0.05$). The RANKL/OPG ratio was increased in poorly controlled when compared to better-controlled diabetic subjects ($p<0.05$). There were no differences among groups in the levels of TNF-α, OPG and IL-23 ($p>0.05$).

Discussion

It has been proposed that individuals with type 2 DM display certain features of inflammation and immunity that can alter the pathogenesis of several diseases. Therefore, this study hypothesized that better-controlled and poorly controlled diabetic subjects with chronic periodontitis exhibit distinct profile of cytokines and bone related factors, when compared to systemically-healthy individuals, which could explain their distinct susceptibility to periodontal breakdown. The results demonstrated that the levels of sRANKL, OPG, IL-17, IL-23, and IFN-γ were higher in the GCF of subjects with DM as a whole, when compared to systemically-healthy controls. Conversely, the total amount of IL-4 was lower in type 2 diabetic than non-diabetic subjects. Collectively, these findings suggest that the balance between pro- and anti-inflammatory mediators in periodontal tissues of diabetic subjects is shifted towards hyper-proinflammation, that could potentially exacerbate the host response against pathogens and periodontal destruction. Additionally, it was also observed that the glycemic control of the diabetic subjects may modulate the levels of the majority of these biomarkers differently, as previously demonstrated by our research group[32,33]. In order to overcome the possible impact of periodontitis severity on the levels of biomarkers studied, the sampling sites from diabetic and non-diabetic subjects were matched for clinical parameters including PD, CAL (Table 1) and BoP.

The RANK/RANKL/OPG system regulates bone resorption in various inflammatory bone diseases, including periodontitis[24]. In general, a high local ratio of RANKL/OPG, which characterizes an increased osteoclastogenesis, has been reported in periodontitis[24-27]. OPG has been shown to play a role not only in bone but also in the vasculature and immune system. In type 2 diabetic subjects, serum levels of OPG, but not RANKL, appear to be higher when compared to non-diabetic controls. However, it is not clear whether this finding is due to the hyperglycemic state or a marker of DM-related vascular complications[38]. The results of the present study showed higher levels of sRANKL in the GCF of diabetic individuals when compared to systemically-healthy ones, suggesting an increased risk of osteoclast production and loss of alveolar bone in subjects with DM. A increased local concentration of OPG was also observed in diabetic subjects compared to non-diabetic ones, probably as a result of a regulatory circuit of negative feedback in an attempt to slow down the bone resorption process in these subjects (Figure 2). Additionally, in general, poorly controlled subjects presented the highest

levels of sRANKL and RANKL/OPG ratio (Table 2). This further confirms our previous findings in which hyperglycemia in type 2 diabetic subjects is strongly related to enhanced RANKL/OPG ratio[32] and, consequently, may be critical to alveolar bone loss. To date, few studies have assessed the role of RANKL and OPG in periodontitis under the challenge of DM[31,32,39]. In relation to type 1 DM, Mahamed et al.[39] demonstrated that diabetic mice had enhanced alveolar bone loss, associated with the increased RANKL expressions during infection by *Actinomyces actinomycetemcomitans*, when compared to non-diabetic mice. Similarly, Duarte et al.[31] also indicated a trend towards a higher mRNA RANKL/OPG ratio in gingival tissues from type 1 diabetic subjects, compared to systemically-healthy controls.

Increased osteoclast activity due to an imbalance in the RANKL/OPG system can be a result of dysregulated immune responses. In the context of osteoimmunology, signal transductions via cytokines are important pathways in osteoclastic cells, linking the immune and bone systems in many inflammatory diseases[16,23]. Evidence suggests that periodontitis is one of the inflammatory diseases that is caused by the dysregulation of T cell–derived or their related cytokines. Briefly, TNF-α is a pro-inflammatory cytokine that stimulates not only the production of chemokine, cytokines, collagenases and prostaglandins but also bone resorption-related factors[12,14]. IFN-γ is the signature pro-inflammatory cytokine of Th1 cells while the anti-inflammatory IL-4 is mainly produced by Th2 cells[40]. Recently, a lineage of IL-17-producing effector $CD4^+$ T lymphocytes, termed Th17 cells, has been also described and seems to be maintained by IL-23[41]. In general, high levels of IL-4 have been related to healthy periodontal tissues while increased levels of IFN-γ, TNF-α, IL-17 and IL-23 have been related to periodontitis progression [5,6,11,14,18-22,42]. In this study, type 2 DM, as a whole, upregulates the levels of pro-inflammatory cytokines including IFN-γ, IL-17 and IL-23 and downregulates the production of IL-4 in diseased periodontal sites. In addition, although there was a tendency towards high levels of TNF-α in diabetic subjects, no differences between groups were detected in the levels of this cytokine (Figure 1). Interestingly, better-controlled individuals exhibited the highest levels of IFN-γ, while poorly controlled subjects presented the highest levels of IL-17. In addition, both profiles of immune responses were different from those of non-diabetic subjects. These findings suggest a polarization for an overproduction of Th1- or Th17-cytokines, modulated by the glycemic status of the diabetic subjects (Table 2). Since the Th1 cytokine, IFN-γ, suppresses the differentiation of Th17 cells[40], it could be speculated that the antagonism between the Th1 and Th17 subsets in better-controlled

diabetic subjects resulted in minimal production of IL-17 in these subjects. On the other hand, the high expression of IL-17 may be responsible for the exacerbated periodontal destruction[13,14,20] and alveolar bone resorption[13,43] in poorly controlled subjects, as observed in previous clinical investigations[44,45]. Worthy of note is that our results did not show the same immunological response to periodontal pathogens for well-controlled diabetic and non-diabetic subjects. However, it is important to consider that, in this study, subjects were classified as better-controlled subjects based on a single measurement of HbA1c, which reflects glycemic control over the preceding 1 - 3 months. Possibly, hyperglycemia induces changes in gene expression and biological reactions such as advanced glycation-end product (AGE) in long-lived molecules that may not be reversed quickly when glycemia returns to normal. Thus, episodes of poor control are progressively imprinted on the cells, resulting in long-term effects on cellular immune activity.

Very few studies have compared the periodontal levels of the cytokines evaluated in the current investigation in diabetic and non-diabetic subjects. In agreement with the present study, a recent investigation showed that the levels of IL-4 were significantly decreased in diseased gingival tissues from subjects with type 2 DM compared to non-diabetic controls[46]. On the other hand, contrary to our findings, the authors did not demonstrate differences in the levels of IFN-γ in sites with chronic periodontitis between subjects with or without DM. However, the aforementioned study did not elucidate the glycemic status of the study population, which may have been formed predominantly by poorly controlled subjects. In contrast to our findings, Venza et al.[34] showed increased expression of TNF-α in poorly controlled type 2 diabetic individuals at mRNA levels, when compared to well-controlled and non-diabetic subjects. However, it is important to note that mRNA expression does not estimate biologically-active molecules and not necessarily imply the synthesis of the protein.

Various cellular and molecular disturbances could contribute to the hyper-proinflammation observed in the type 2 diabetic subjects when compared to non-diabetic individuals. Among several biological consequences, hyperglycemia induces AGE formation. AGE, when linked to its receptor (RAGE), increases the secretion of pro-inflammatory mediators. For this reason, systemic levels of several pro-inflammatory markers (ex. IL-17, IFN-γ, soluble cell adhesion molecules, C-reactive protein) are increased in type 2 diabetic subjects when compared to healthy

controls[47,48], confirming that DM is a proinflammatory state[49]. In addition, a higher expression of RAGE was observed in periodontal tissues of type 2 diabetic than non-diabetic subjects, which indicates a possible local involvement of this receptor in the periodontal destruction associated with type 2 DM[50]. Finally, it seems that inflammatory mediators act on DM and periodontitis in the same direction and, the synergism between the inflammatory cytokines produced in DM and periodontitis may aggravate periodontal destruction[44,45].

In conclusion, high levels of proinflammatory cytokines and RANKL were observed in the GCF of type 2 diabetic subjects in the GCF of type 2 diabetic subjects with chronic periodontitis, when compared to non-diabetic ones. In addition, poor or good glycemic status seems to modulate osteo-immuno-inflammatory mediators in a different manner.

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Table 1 – Clinical and demographic parameters of the study population.

		Type 2 diabetic subjects		
Parameter (Mean, ± SD)	Systemically-healthy (n=20)	All (n=37)	Poorly controlled (n=20)	Better-controlled (n=17)
Age (years)	51.5 ± 8.3	52.5 ± 8.7	51.4 ± 9.6	53.4 ± 8.0
Gender (n; M/F)	8/12	16/21	9/11	7/10
Duration of DM	_____	6.4 ± 1.1	6.3 ± 1.1	6.5 ± 1.2
FPG (mg/dl)	_____	169.6 ± 60.5	199.4 ± 56.9 #	131.8 ± 41.4
HbA1c (%)	_____	9.0 ± 2.2	10.6 ± 1.3 #	7.0 ± 1.1
GCF (µl)	0.40 ± 0.10	0.40 ± 0.03	0.39 ± 0.03	0.40 ± 0.03
Full-mouth PI (%)	63.8 ± 38.7	73.2 ± 29.3	83.1 ± 23.0*	61.4 ± 32.3
Full-mouth BoP (%)	63.4 ± 23.9	55.1 ± 29.7	60.1 ± 29.5	49.1 ± 29.7
Full-mouth SUP (%)	5.4 ± 7.1	3.0 ± 5.2	3.0 ± 4.7	3.0 ± 6.0
Full-mouth PD (mm)	3.8 ± 1.0	3.5 ± 0.7	3.5 ± 0.6	3.5 ± 0.8
Full-mouth CAL (mm)	4.3 ± 1.2	4.1 ± 0.9	4.1 ± 0.8	4.1 ± 1.0
Sampled site PD (mm)	6.3 ± 1.2	5.9 ± 1.0	6.0 ± 1.0	5.9 ± 1.1
Sampled site CAL (mm)	6.8 ± 1.7	6.3 ± 1.5	6.3 ± 1.4	6.2 ± 1.1

PI: plaque index; BoP: bleeding on probing; SUP: suppuration; PD: probing depth; CAL: clinical attachment level; GCF: gingival crevicular fluid; HbA1c: glycated hemoglobin; FPG: fasting plasma glucose; M: male; F: female. # Differences between poorly and better-controlled diabetic subjects by Student t test ($p<0.05$). * Significantly higher than better-controlled and non-diabetic subjects by ANOVA and Tukey test ($p<0.05$).

Table 2 – Mean (± SD) of the total amounts and concentrations of the biomarkers.

		Type 2 diabetic subjects	
Biomarkers	Systemically-Healthy	Poorly controlled	Better-controlled
IL-4 (pg/site)	0.05 (± 0.06) a	0.02 (± 0.03) ab	0.001 (± 0.003) b
IL-4 (pg/µl)	0.09 (± 0.11) a	0.08 (± 0.12) ab	0.004 (± 0.02) b
IFN-γ (pg/site)	4.2 (± 4.2) ab	3.4 (± 3.6) b	6.8 (± 2.4) a
IFN-γ (pg/µl)	9.2 (± 8.9) b	17.8 (± 22.0) b	39.9 (± 26.7) a
IL-17 (pg/site)	8.6 (± 10.1) b	23.9 (± 13.2) a	7.3 (± 7.6) b
IL-17 (pg/µl)	19.0 (± 21.7) b	160.6 (± 131.1) a	47.1 (± 64.2) b
IL-23 (pg/site)	2.2 (± 4.2)	4.6 (± 10.4)	2.5 (± 3.5)
IL-23 (pg/µl)	4.9 (± 7.9)	39.9 (± 96.8)	13.3 (± 23.5)
TNF-α (pg/site)	0.04 (± 0.07)	0.05 (± 0.07)	0.07 (± 0.18)
TNF-α (pg/µl)	0.09 (± 0.20)	0.33 (± 0.59)	0.40 (± 1.22)
sRANKL (pg/site)	34.7 (± 9.7) b	66.6 (± 26.5) a	24.6 (± 5.6) b
sRANKL (pg/µl)	86.2 (± 39.9) b	402.5 (± 286.5) a	222.8 (± 294.2) ab
OPG (pg/site)	5.8 (± 4.7)	5.6 (± 4.3)	5.6 (± 2.6)
OPG (pg/µl)	13.4 (± 12.5)	45.0 (± 66.6)	50.6 (± 61.6)
RANKL/OPG	12.3 (± 12.7) ab	24.7 (± 26.6) a	5.3 (± 61.6) b

Different letters indicate statistically significant differences by ANOVA and Tukey test ($p<0.05$).

Figure 1. Distribution of the total amount (pg/site) and concentration (pg/µl) of cytokines in the GCF from diseased sites of systemically-healthy and type 2 diabetic subjects. The horizontal bars show the mean values. The individual symbols represent the cytokine level at each site. * Differences between groups (Student t test; $p<0.05$).

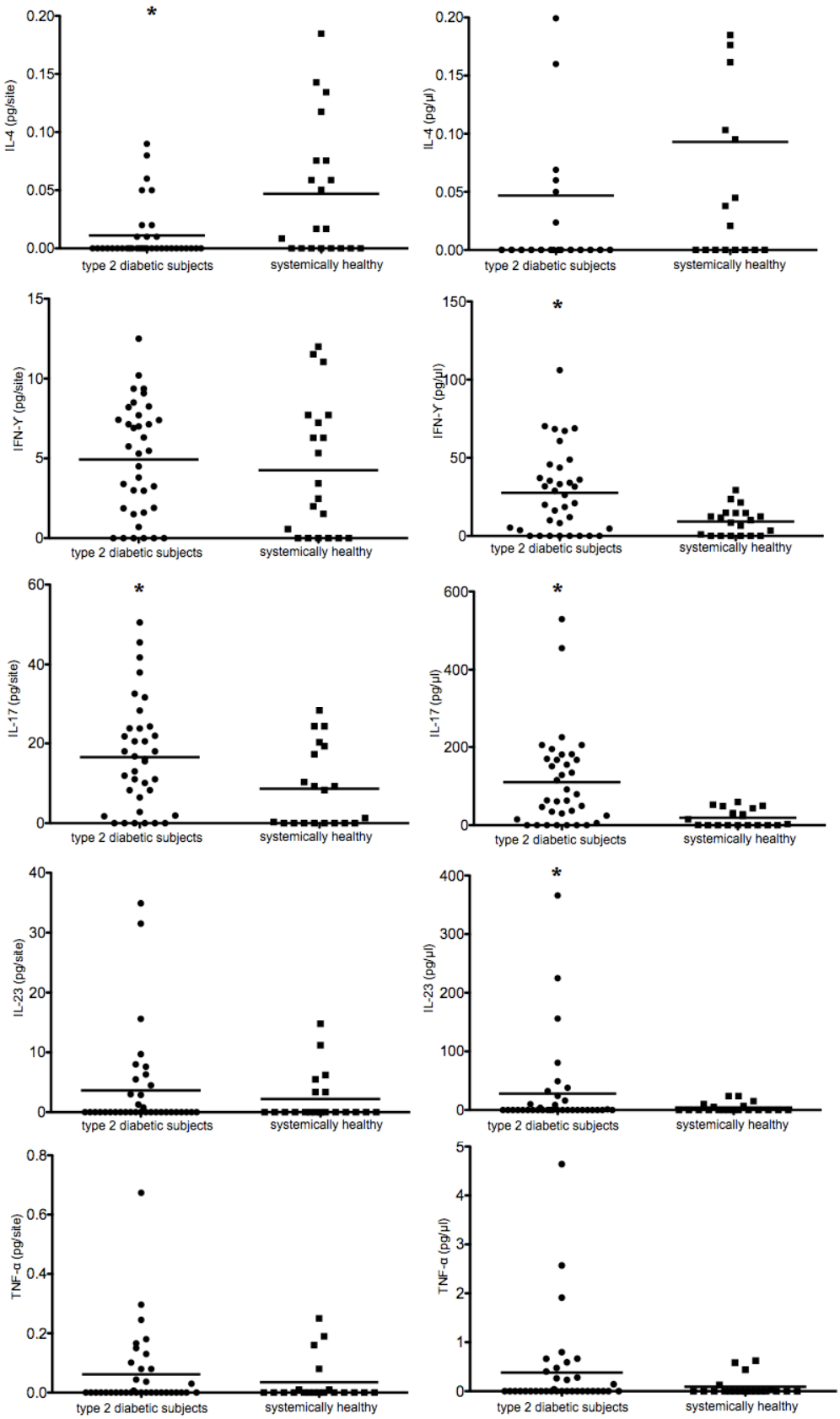
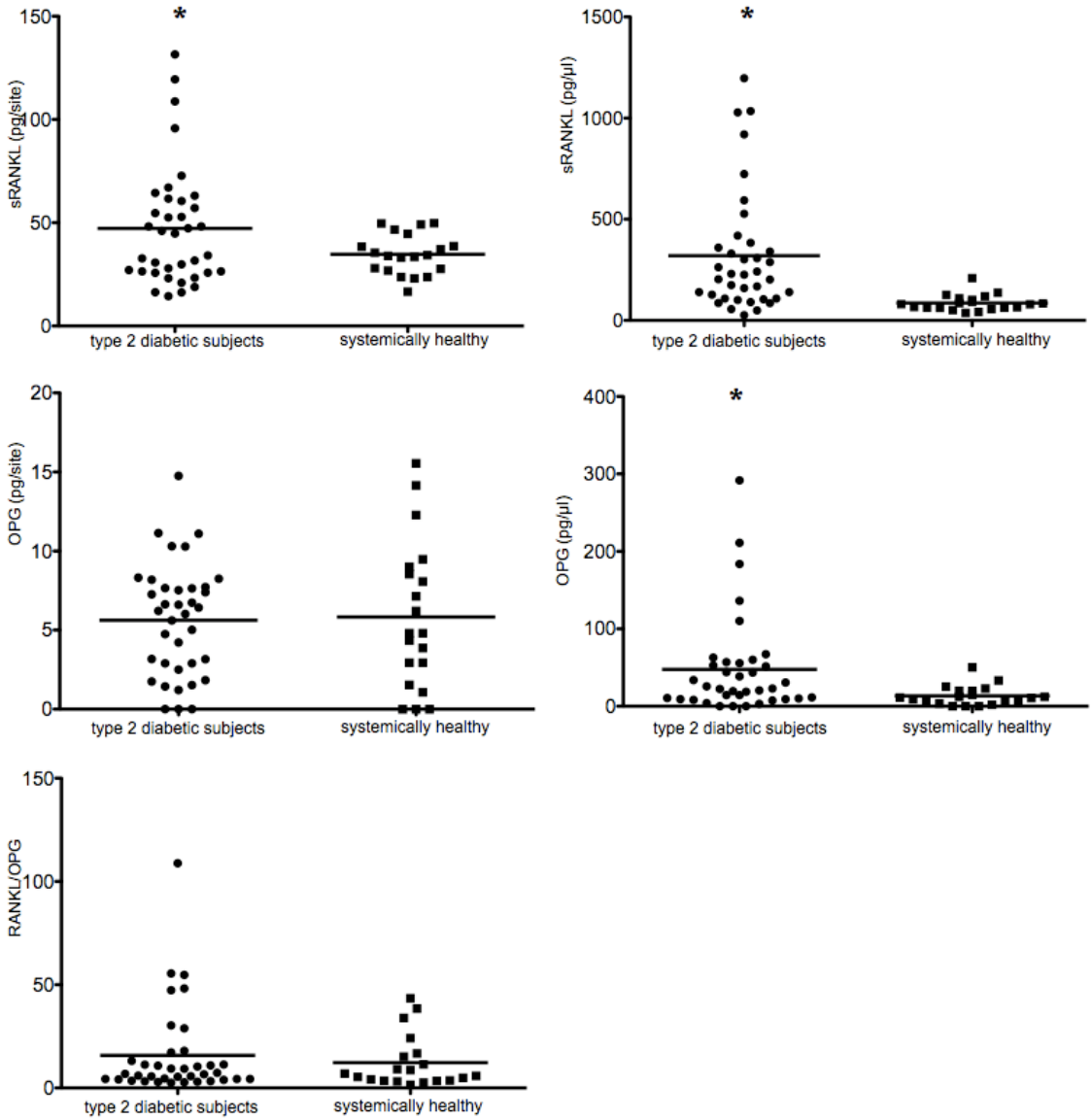

IL-4 (pg/site)
IL-4 (pg/µl)
IFN-Y (pg/site)
IFN-Y (pg/µl)
IL-17 (pg/site)
IL-17 (pg/µl)
IL-23 (pg/site)
IL-23 (pg/µl)
TNF-α (pg/site)
TNF-α (pg/µl)
type 2 diabetic subjects
systemically healthy
*

Figure 2. Distribution of the total amount (pg/site) and concentration (pg/µl) of bone-related factors in the GCF from diseased sites of systemically-healthy and type 2 diabetic subjects. The horizontal bars show the mean values. The individual symbols represent the protein level at each site. * Differences between groups (Student t test; $p<0.05$).



4. ESTUDO 2

Surgical and non-surgical therapy with systemic antibiotics for residual pockets in type 2 diabetics with chronic periodontitis (submetido ao Journal of Clinical Periodontology).

Abstract

Aim: This study evaluated the effects of surgical (SD) and non-surgical (NSD) debridements, associated with systemic antibiotics, on clinical and cytokine outcomes of residual pockets (RP) in type 2 diabetics. **Material and Methods:** A blind, split-mouth, controlled, randomized clinical trial was conducted in 21 subjects presenting at least two RP per contralateral quadrant. All subjects received metronidazole plus amoxicillin for 10 days and the selected quadrants were assigned to receive SD or NSD. Clinical parameters, the number of RP and local levels of interferon (IFN)-γ, interleukin (IL)-17, IL-23 and IL-4 were assessed at baseline, 3 and 6 months post-therapies. **Results:** Overall, clinical parameters improved significantly after both therapies ($p<0.05$), without differences between groups at any time-point ($p>0.05$). There was a trend towards a greater reduction in the number of RP following SD ($p>0.05$). Levels of all cytokines were increased after SD when compared to NSD ($p<0.05$). **Conclusion:** SD and NSD associated with antibiotic therapy provide similar 6-month clinical benefits for RP in diabetic subjects. Increased levels of cytokines were found in the RP treated by surgery, probably as a consequence of the wound healing following surgical trauma.

Clinical Relevance

Scientific rationale for study*:** Residual pockets (RP) exhibit an increased risk for disease progression. Therefore, the establishment of ideal therapies for RP is necessary, especially in groups at risk of periodontitis, such as diabetic subjects. ***Principal findings: Surgical (SD) and non-surgical debridement (NSD) associated with antibiotics, yielded similar clinical improvements in RP. Additionally, the levels of all cytokines were increased following SD compared to NSD probably as a result of tissue trauma. ***Practical implication:*** A conservative approach including NSD, associated with metronidazole and amoxicillin, could be successfully recommended for the treatment of RP in type 2 diabetic subjects.

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INTRODUCTION

Studies have demonstrated that initial periodontal therapy, including scaling and root planing (SRP) alone or in conjunction with adjunctive approaches such as antimicrobials, may improve clinical parameters in non-diabetic (Carvalho et al. 2004, Haffajee et al. 2007) and diabetic subjects (Rodrigues et al. 2003, Navarro-Sanchez et al. 2007, da Cruz et al. 2008, Santos et al. 2009) due to the reduction of pathogens and the establishment of a local host-compatible microbiota (Cugini et al. 2000, da Cruz et al. 2008, Feres et al. 2009). Subsequently, the majority of the clinical benefits achieved in this initial phase can be successfully maintained by the institution of a systematic supportive periodontal program (Lindhe & Nyman 1984, Tonetti et al. 1998, Rosling et al. 2001).

In this maintenance phase, repeated SRP for recurrent/refractory pockets may produce some additional reduction in probing depth (PD), bleeding on probing (BoP) and gain in clinical attachment levels (CAL) (Badersten et al. 1984). However, repeated SRP alone with the same technique seems to be ineffective for persistent residual sites [PD ≥ 5mm with BoP], especially in areas of deep pockets, angular defects and furcation involvements (Anderson et al. 1996, Jenkins et al. 2000). Since residual pockets (RP) represent a risk factor for further periodontal breakdown (Tonetti et al. 1998, Renvert & Persson 2002, Matuliene et al. 2008), surgical approaches and local and systemic antimicrobials have been proposed for the retreatment of such unresponsive sites, when SRP alone fails to arrest disease progression (Winkel et al. 1998, Serino et al. 2001a, König et al. 2008).

Although some studies have focused on the establishment of therapeutic protocols for the treatment of unresponsive pockets in non-diabetic subjects (Serino et al. 2001a, König et al. 2008), to date, no study has evaluated the clinical and/or immunological effects of the retreatment of RP in type 2 diabetic subjects, which is recognized as a group at risk of periodontitis progression. Therefore, the aims of this study were to evaluate: 1- the clinical effects of surgical (SD) and non-surgical (NSD) debridements, associated with systemic antibiotics, for the treatment of RP in type 2 diabetic subjects at 3 and 6 months; 2- the effects of both therapeutic protocols on the local levels of pro- (IL-17, IL-23, IFN-γ) and anti- (IL-4) inflammatory cytokines. We hypothesized that SD associated with systemic

antibiotics would promote the best clinical and cytokine profiles for the treatment of RP in type 2 diabetic subjects.

MATERIALS AND METHODS

Sample size calculation

The sample size was calculated using α=0.05 and the power (1-β)=80%. For the variability (σ=SD), the value of 1mm was used considering PD change in the initial RP (PD $\geq$ 5mm with BoP), as a variable outcome. The minimum clinically significant value (δ) considered was 1mm. Based on these data, the minimum number of subjects required was calculated to be 18. However, based on anticipated attrition about 15%, the number of subjects enrolled in this study was 21.

Experimental design and subject population

In this prospective, split-mouth, blinded, randomized and controlled clinical study, 21 type 2 diabetic subjects (42-67 years old) with RP were treated with NSD and SD, associated with systemic amoxicillin and metronidazole. In the pre-study phase, 61 subjects diagnosed with type 2 DM and generalized chronic periodontitis (Armitage 1999) were selected from the population referred to Guarulhos University, from December 2007 until January 2009. All subjects should present diagnosis of type 2 DM for at least 6 years. Subjects were > 40 years old and had at least 15 teeth, excluding third molars, more than 30% of the sites with PD and CAL $\geq$ 4mm. Exclusion criteria were pregnancy, lactation, current smoking and smoking within the past 6 years, antibiotic therapies in the previous 6 months, use of mouthrinses containing antimicrobials in the preceding 2 months, systemic conditions (except DM) that could affect the progression of periodontitis, orthodontic appliances, multiple systemic complications of DM and long-term administration of anti-inflammatory and immunosuppressive medications. These subjects underwent an initial periodontal treatment and were engaged in a supportive periodontal program *(see Pre-study phase)*.

After this pre-study phase (Fig. 1), 21 subjects who presented at least two non-contiguous RP per contralateral quadrant distributed in posterior teeth, had a full-mouth plaque index score $\leq$ 25% (Ainamo & Bay 1975), were non-allergic to amoxicillin or metronidazole and, did not take any antibiotic during the pre-study phase entered the present study. The contralateral quadrants presenting the RP were randomly assigned, by a computer-

generated table, to receive SD (test group) or NS (control group) *(see Treatment protocol)*. All eligible subjects were thoroughly informed of the nature, potential risks and benefits of their participation in the study and signed their informed consent. This study protocol was previously approved by the Guarulhos University's Ethics Committee in Clinical Research.

Treatment protocol

Pre-study phase: 61 subjects were submitted to calculus removal, exodontia, provisional restorations and supragingival plaque control. SRP was performed in two to four appointments using curettes and ultrasonic device in a maximum of 21 days without the use of systemic or local antimicrobials. All subjects received supportive therapy at 3, 6 and 9 months. Clinical examination was performed at 12 months to select eligible subjects presenting RP.

SD (test group): On one eligible quadrant, a full-thickness flap was reflected, the granulation tissue was removed and SRP was performed with curettes and ultrasonic device. Chlorhexidine gluconate mouthwash (0.12%, 2x/day for 2 weeks) was prescribed and the sutures were removed at 6 days post-surgery. Surgical therapy, without osteoplasty and regeneration, was performed at all teeth presenting RP and at the respective adjacent teeth.

NSD (control group): On the other side, SRP was performed using periodontal curettes and ultrasonic device without a flap at six days after surgery. Non-surgical therapy was performed at all teeth with RP and at the neighboring teeth.

Metronidazole (400mg/3x/day) plus amoxicillin (500mg/3x/day) were prescribed for 10 days, starting on the day of the SD. All volunteers received the same brand of toothpaste to use during the course of the study (Colgate Total®, Colgate-Palmolive Ind. e Com. Ltda., São Paulo, SP, Brazil). Supragingival plaque control was performed at 3 months after therapies.

Compliance and monitoring of adverse events

The subjects received packs containing 30 capsules of metronidazole/ amoxicillin. An assistant monitored the compliance to antibiotic therapy by calling the patients 3x during the 10 days of antibiotic intake. The subjects were asked to bring the packs back at the end of the 10 days with any remaining capsule. All subjects also answered a questionnaire

(administered by a study assistant) about any self-perceived side effects of the medications on the 3rd and 10th day of antibiotic therapy.

Examiner calibration

Clinical examinations were performed by one examiner (ACM), calibrated according to the method described by Araujo et al. (2003). The intra-examiner variability was 0.19mm for PD and 0.21mm for CAL. The clinical parameters registered dichotomously were calculated by the Kappa-Light test and the intra-examiner agreement was > 0.85. The examiner was unaware of the treatment allocation of the quadrant.

Clinical monitoring

Glycated hemoglobin (HbA1c) was measured by a single laboratory only at baseline. The following parameters were assessed at six sites of all teeth, excluding third molars, using a manual periodontal probe (North Carolina, Hu-Friedy, Chicago, IL, USA): plaque index (PI) (Ainamo & Bay 1975), marginal bleeding (MB, presence/absence), BoP (presence/absence), suppuration (SUP, presence/absence), PD (mm) and CAL (mm).

Gingival crevicular fluid (GCF) sampling and enzyme linked immunosorbent assay (ELISA)

Two non-contiguous RP from each eligible quadrant, presenting no furcation involvement, were chosen for GCF sampling at baseline and at 3 and 6 months post-therapies. GCF samples were collected and stored as previously described by Santos et al. (2010). GCF was collected by paper strips (Periopaper, Oraflow Inc., Smithtown, NY, USA) and fluid volume was measured in a calibrated Periotron 8000 (Periotron 8000, Proflow Inc., Amityville, NY, USA). Samples were analyzed by ELISA for IL-4, IFN-γ, IL-17 and IL-23 (Quantikine; R&D Systems Inc. R&D Systems Inc., Minneapolis, MN, USA) using commercially available ELISA kits, according to the manufacturer's recommendations. Results were reported as total amount (pg) of cytokine per site per 30s of sampling. Cytokine concentrations per site (pg/µl) were established by dividing the total amount of the cytokines by the GCF volume.

Statistical Analysis

The primary outcome variable was the mean changes of PD in sites initially presenting RP. Secondary outcomes included the additional clinical parameters and the cytokine levels. Quadrant data consisted of the values from the teeth presenting RP and their adjacent teeth. Site data comprised the values from the RP. Data were examined for normality by the Kolmogorov-Smirnov test and were analyzed using parametric methods. The percentage of sites with visible plaque accumulation, BoP, SUP, the mean PD, and CAL were computed for control and test quadrants, separately. The number and the mean PD of RP were computed for each eligible quadrant. RP were also classified according to PD (<5, 5-6, 7-8 and ≥9mm) in each time-point. Clinical parameters were averaged across quadrants. Clinical and cytokine results were averaged according to the therapeutic groups. The changes in the number of pockets with PD ≥5mm without BoP and in the number of RP (from baseline to 3 and 6 months) were examined for both groups. Clinical and cytokine differences between groups were compared using the paired Student's t-test. Repeated measures ANOVA and Tukey test were employed to detect differences within each group among the three time-points. The level of significance was set at 5%. The biostatician was unaware of the treatment allocation of the quadrants.

RESULTS

The study population comprised 13 males and 8 females (mean age: 53.2 ± 9.1 years). The mean HbA1c levels and duration of DM were 11.3 ± 2.3% and 7.1 ± 0.9 years, respectively. There were no subject or site dropouts during the study (Fig.1).

The subjects did not report adverse effects such as fever and indisposition after treatment. Six subjects reported one or two adverse events to the medication including diarrhea, vomiting, headache, metallic taste or irritability. All subjects informed full adherence to the antibiotic therapy.

Quadrant data

No significant differences were observed between groups for any clinical parameter at any time-point ($p>0.05$). Both therapies led to a significant decrease in the mean percentage of BoP and in the mean PD ($p<0.05$). Only SD produced significant reductions in the mean CAL at 3 and 6 months post-therapy when compared to baseline ($p<0.05$) (Table 1).

Most of the subjects presented attachment gain, independently of the treatment applied. Twelve quadrants treated with SD presented better results for CAL than those receiving NSD. Six quadrants treated with NSD showed more attachment gain than those treated by surgery (Fig. 2).

Site data

141 and 178 RP were treated by NSD and SD, respectively. The mean number and PD of RP reduced significantly over time for both therapies ($p<0.05$), without differences between groups ($p>0.05$) (Table 2).

There were no differences between SD and NSD regarding the reductions in the number of pockets with PD ≥ 5mm without BoP ($p>0.05$). There was a trend towards a greater reduction in the number of RP following SD at 3 ($p=0.07$) and 6 months ($p=0.06$) (Fig.3).

Overall, independently of the therapy, there were reductions in the mean PD of the RP. Twelve of the 21 quadrants from the SD group showed a greater reduction in the mean PD of the RP when compared to the contralateral quadrant submitted to the NSD (Fig.2).

The percentage of pockets < 5mm without BoP increased significantly at 3 and 6 months for both therapies ($p<0.05$; Fig. 4). There were no RP ≥ 9mm at 3 and 6 months after both treatments. Also, no significant differences were observed between groups for the percentage of any PD categories at any time-point ($p>0.05$; Fig. 4).

The mean PD, CAL and GCF volume of the sites selected for cytokine evaluations reduced significantly at 3 and 6 months for both groups ($p<0.05$), without differences between groups at any time-point ($p>0.05$; Table 3).

Cytokines

There were no significant differences between groups regarding total amounts and concentrations of any cytokine at baseline ($p>0.05$; Table 3). The concentrations of all cytokines and the total amounts of IFN-γ, IL-17 and IL-4 were higher for the SD group at 6 months post-therapy ($p<0.05$). There was a significant increase in the levels of all cytokines at 6 months post-surgery when compared to baseline ($p<0.05$; Table 3).

DISCUSSION

Previous investigations have demonstrated that RP have an increased risk for disease progression (Tonetti et al. 1998, Renvert & Persson 2002, Matuliene et al. 2008). Therefore, persisting pockets ≥ 5mm with BoP are recognized as needing supplementary therapies, especially in groups at risk of periodontitis, such as diabetic subjects. This study evaluated, for the first time, the clinical and immunological effects of NSD and SD, associated with metronidazole and amoxicillin, for the re-treatment of RP in type 2 diabetic subjects. Overall, both therapeutic modalities displayed similar positive clinical effects in almost all clinical parameters, including the primary variable (Table 2). Also, no changes in cytokine levels were observed after NSD, while there were increases in levels of all cytokines evaluated at 3 and/or 6 months following SD, probably as a reflection of the tissue trauma induced by surgery. Therefore, the hypothesis that SD associated with systemic antibiotics would promote the best clinical and cytokines outcomes for the treatment of RP of type 2 diabetic subjects was rejected.

Previous studies have demonstrated that NSD and SD are useful methods for the treatment of chronic periodontitis, resulting in reductions in PD and gingival inflammation and gain in CAL. However, SD seems to be more effective than NSD in reducing the PD at sites with initially deep pockets (Isidor & Karring 1986, Lindhe et al. 1984, Becker et al. 2001, Hung & Douglass 2002, Serino et al. 2001b, Heitz-Mayfield 2005). In this study, at the quadrant level, there were no significant clinical differences between NSD and SD at any time-point. In addition, both therapies were effective in reducing the mean PD and the sites presenting BoP (Table 1) and promoting some attachment gain (Fig. 2) when the whole quadrant was analyzed, although a significant reduction in the mean CAL was observed exclusively for SD group. At site level, both therapies promoted a similar reduction in the mean PD and in the number of RP (Table 2), with a slight advantage of the SD over the NSD in relation to the reduction in the number of RP (Fig. 3). Furthermore, SD and NSD were equally effective in increasing the percentage of pockets with PD < 5mm without BoP (Fig. 4). In agreement with our results, König et al. (2008) demonstrated that repeated SRP and SD similarly reduced PD and CAL in RP in non-diabetic subjects with generalized advanced periodontitis. However, in the aforementioned study, the re-treatment of unresponsive sites was performed at 5 months after the initial therapy. Worthy of note is

that the subjects included in this study were on supportive therapy 3 times/year in the pre-study phase (Fig. 1) and therefore, had already received repeated non-surgical subgingival instrumentation (without antibiotics) in their RP. Indeed, the value of performing repeated subgingival scaling in persistent pockets at 3-month intervals has already been questioned (Jenkins et al. 2000). Thus, in the present study, it was observed that the administration of metronidazole and amoxicillin significantly enhanced the effects of the non-surgical SRP in the residual sites, in which the mechanical re-instrumentations alone failed to improve periodontal health in the maintenance phase. Therefore, it may be suggested that the clinical benefits observed for both therapies could be attributed, to some extent, to the beneficial effects of the systemic antibiotic therapy. In fact, the use of systemic antibiotics, in combination with mechanical debridement, has been shown to be effective in initial periodontal therapy (Herrera et al. 2002, Haffajee et al. 2007, Herrera et al. 2008, Matarazzo et al. 2008, Cionca et al. 2009) and in the re-treatment of recurrent periodontitis (van Winkelhoff et al. 1992, Pavicić et al. 1994, Winkel et al. 2001, Serino et al. 2001a). Winkel et al. (2001) showed that systemic metronidazole and amoxicillin, administered at 6 weeks after an initial SRP, produced significantly better clinical and microbiological periodontal results than SRP alone in 3 months. The benefits of SRP, associated with a one-week administration of metronidazole and amoxicillin, in the treatment of recurrent periodontitis were also demonstrated by van Winkelhoff et al. (1992) and Pavicić et al. (1994). Similarly to the antibiotic protocol used in the present study, Serino et al. (2001a) employed metronidazole and amoxicillin as an adjunct to the mechanical re-treatment of unresponsive sites in non-diabetic subjects. The percentage of shallow pockets (<4mm) increased and there were few sites (<5%) with remaining deep pockets (>6mm) at 12 months post-therapy. Therefore, since NSD and SD associated with systemic antibiotics showed only minor clinical differences, a more conservative approach could be successfully recommended in the management of RP in type 2 diabetic subjects. The present findings should also take into consideration the cost-benefit of these procedures, since SD may cause morbidity, increased time and cost of treatment, increased anxiety for patients and possibility of root sensitivity and esthetic problems (Patel et al. 2006).

Periodontitis has been related to low levels of anti-inflammatory mediators such as IL-4 and elevated levels of pro-inflammatory cytokines, such as IFN-γ, IL-17 and IL-23 (Ukai et

al. 2001, Garlet et al. 2004, Vernal et al. 2005, Lester et al. 2007, Pradeep et al. 2008, Dutzan et al. 2009). In the present study, even though the clinical parameters improved after both treatments, the local levels of all pro-inflammatory cytokines evaluated were higher at 3 and/or 6 months for SD, when compared to NSD (Table 3). In addition, high levels of IL-4 were also observed at 6 months post-surgery, probably as a result of a regulatory circuit of negative feedback, in an attempt to slow down the local production of pro-inflammatory mediators (Table 3). These findings suggest the presence of an apparent inflammatory process that persists for a prolonged period at the sites treated by SD. One could argue that these findings were somewhat unexpected since both therapies yielded clinical health, and high levels of pro-inflammatory cytokines in GCF have been suggested as tools to indicate diseased periodontal tissues. It is recognized that a better clinical healing and more favorable clinical effects of the periodontal therapies depend on the reduction of pathogens. Although a further study from our research group will focus on the microbiological effects of the therapies evaluated, based on the present clinical results, it is expected that both therapies positively affected the composition of the subgingival microbiota. Therefore, it is speculated that the cytokine "storm" observed after surgery in this study is not attributed to the host response against pathogens, but a consequence of the reparative process after the injury induced by the surgery, due to greater manipulation of the soft tissues and bone exposure. The long-term stability of the clinical benefits achieved after SD will be essential to detect the consequences of these cytokine findings in the diabetic subjects. Furthermore, since cytokines may arise from surgical trauma, pathogen-induced inflammation or a combination of both stimuli, future studies including multiple profiles of clinical, microbiological and host response measurements will be important to assure the actual impact of the therapies proposed.

Periodontal and bone wound healing involve complex biological events including the secretion of growth factors and cytokines that are also released during periodontitis development (Polimeni et al. 2006, Tsiridis et al. 2007). In support of our findings, previous investigations in non-diabetic subjects have indicated that the healing process is prolonged until 6 months following SD, causing continuous production of healing involving molecules (Reinhardt et al. 1993, Needleman et al. 2000, Keles et al. 2007). Reinhardt et al. (1993) showed an increase in the concentration of IL-1β in GCF at 6

months following SD when compared to NSD, along with the improvement in clinical parameters in the surgically treated sites. Needleman et al. (2000) demonstrated an increase in the levels of prostaglandin E_2 at 6 months after surgery with and without local application of 25% metronidazole gel. Keles et al. (2007) found a significant increase in angiogenesis (number of blood vessels) in the periodontal tissues at 6 months after guided tissue regeneration and open debridement surgeries, which indicate a still uncompleted healing activity at this time-point post-surgery. Since a group of non-diabetic subjects was not included in this study, it is not possible to discern whether and, to what extent, the high levels of cytokines observed in the diabetic subjects after surgery were aggravated by a delayed wound healing and exacerbated inflammation due to DM (Guo and Dipietro 2010) or were a normal consequence of the repair activity of the periodontal tissues after surgical trauma.

In conclusion, SD and NSD associated with antibiotic therapy provide similar 6-month clinical benefits for RP in type 2 diabetic subjects. Furthermore, there were increased local levels of cytokines in RP treated with SD, which most probably reflects the wound healing following surgical trauma.

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Table 1. Mean (± SD) values of clinical parameters for both therapeutic groups over time (Quadrant data).

	Non-surgical debridement n=21			**Surgical debridement n=21**		
Parameters	Baseline	3 months	6 months	Baseline	3 months	6 months
PI (%)	26.4 ± 28.9	22.2 ± 26.2	27.5 ± 22.4	28.3 ± 28.9	20.2 ± 21.1	23.7 ± 16.6
MB (%)	6.0 ± 12.5	2.3 ± 5.1	1.6 ± 4.6	4.4 ± 9.5	1.9 ± 5.2	2.5 ± 5.8
PD (mm)	3.8 ± 0.5[a]	3.1 ± 0.8[b]	3.4 ± 0.4[ab]	4.3 ± 1.0[a]	3.3 ± 0.3[b]	3.3 ± 0.3[b]
CAL (mm)	4.5 ± 0.9	4.3 ± 1.5	4.4 ± 1.6	5.0 ± 1.0[a]	4.3 ± 1.2[b]	4.4 ± 1.5[b]
BoP (%)	34.0 ± 16.9[a]	15.9 ± 15.4[b]	14.7 ± 13.9[b]	43.5 ± 24.9[a]	22.3 ± 21.9[b]	22.8 ± 14.8[b]
SUP (%)	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 1.3	1.2 ± 3.5	0.08 ± 3.7	0.3 ± 1.7

Different letters indicate significant differences over time by Repeated measures ANOVA and Tukey tests ($p<0.05$). There were no differences between treatment groups at any time point by paired Student's t-test ($p>0.05$).

Table 2. Mean (± SD) number and PD of the RP for both therapeutic groups over time (Site data).

	Non-surgical debridement			**Surgical debridement**		
	Baseline	3 months	6 months	Baseline	3 months	6 months
Number	6.7 ± 3.4[a]	2.8 ± 2.6[b]	2.7 ± 2.1[b]	8.5 ± 5.7[a]	3.4 ± 3.5[b]	3.0 ± 3.4[b]
PD (mm)	6.0 ± 0.9[a]	4.3 ± 0.7[b]	4.3 ± 0.8[b]	6.1 ± 1.0[a]	4.0 ± 0.9[b]	4.0 ± 0.9[b]

Different letters indicate significant differences over time by Repeated measures ANOVA and Tukey tests ($p<0.05$). There were no differences between treatment groups at any time point by paired Student's t-test ($p>0.05$).

Table 3. Mean (±SD) of total amounts (pg/site), concentrations (pg/µl) of cytokines and clinical parameters of sampled sites for non-surgical and surgical debridement groups overtime.

	Non-surgical debridement			**Surgical debridement**		
Cytokines	Baseline	3 months	6 months	Baseline	3 months	6 months
IFN-γ (pg/site)	11.1 ± 12.1	12.5 ± 13.2*	14.0 ± 11.4*	12.4 ± 13.6 [a]	16.2 ± 14.3 [ab]	21.8 ± 7.9 [b]
IFN-γ (pg/µl)	55.3 ± 94.1	116.2 ± 386.4	41.0 ± 38.4 *	41.4 ± 51.0 [a]	57.2 ± 60.9 [ab]	100.5 ± 83.8 [b]
IL-4 (pg/site)	0.05 ± 0.1	0.03 ± 0.07	0.06 ± 0.06*	0.03 ± 0.05 [a]	0.07 ± 0.12 [b]	0.14 ± 0.14 [b]
IL-4 (pg/µl)	0.2 ± 0.5	0.08 ± 0.15	0.20 ± 0.24*	0.12 ± 0.18 [a]	0.30 ± 0.55 [a]	0.71 ± 0.75 [b]
IL-17 (pg/site)	2.9 ± 6.1	3.2 ± 6.3*	7.9 ± 8.3*	6.7 ± 8.6 [a]	6.7 ± 7.2 [a]	15.6 ± 14.3 [b]
IL-17 (pg/µl)	14.0 ± 35.3	7.9 ± 14.1*	29.3 ± 40.6*	21.5 ± 32.5 [a]	32.5 ± 39.2 [a]	120.3 ± 241.4 [b]
IL-23 (pg/site)	4.7 ± 10.6	10.7 ± 15.2	12.7 ± 12.7	8.9 ± 11.7	12.0 ± 14.3	17.2 ± 11.2
IL-23 (pg/µl)	24.2 ± 58.9	28.1 ± 35.4*	35.6 ± 54.7*	21.5 ± 39.6 [a]	49.9 ± 64.5 [a]	101.4 ± 149.2 [b]
GCF (µl)	0.39 ± 0.20 [a]	0.20 ± 0.10 [b]	0.17 ± 0.14 [b]	0.36 ± 0.17 [a]	0.28 ± 0.16 [b]	0.18 ± 0.12 [b]
Sampled sites PD (mm)	6.7 ± 1.3 [a]	4.6 ± 1.2 [b]	4.6 ± 1.2 [b]	6.9 ± 1.8 [a]	4.4 ± 1.4 [b]	4.2 ± 1.2 [b]
Sampled sites CAL (mm)	7.4 ± 1.5 [a]	5.7 ± 1.8 [b]	5.9 ± 1.8 [b]	7.5 ± 1.9 [a]	5.6 ± 1.7 [b]	5.7 ± 2.2 [b]

Different letters indicate significant differences over time by Repeated measures ANOVA and Tukey tests ($p<0.05$). * Differences between treatment groups at each time point by paired Student's t-test ($p<0.05$).

Figure 1. Flow chart of the study design.

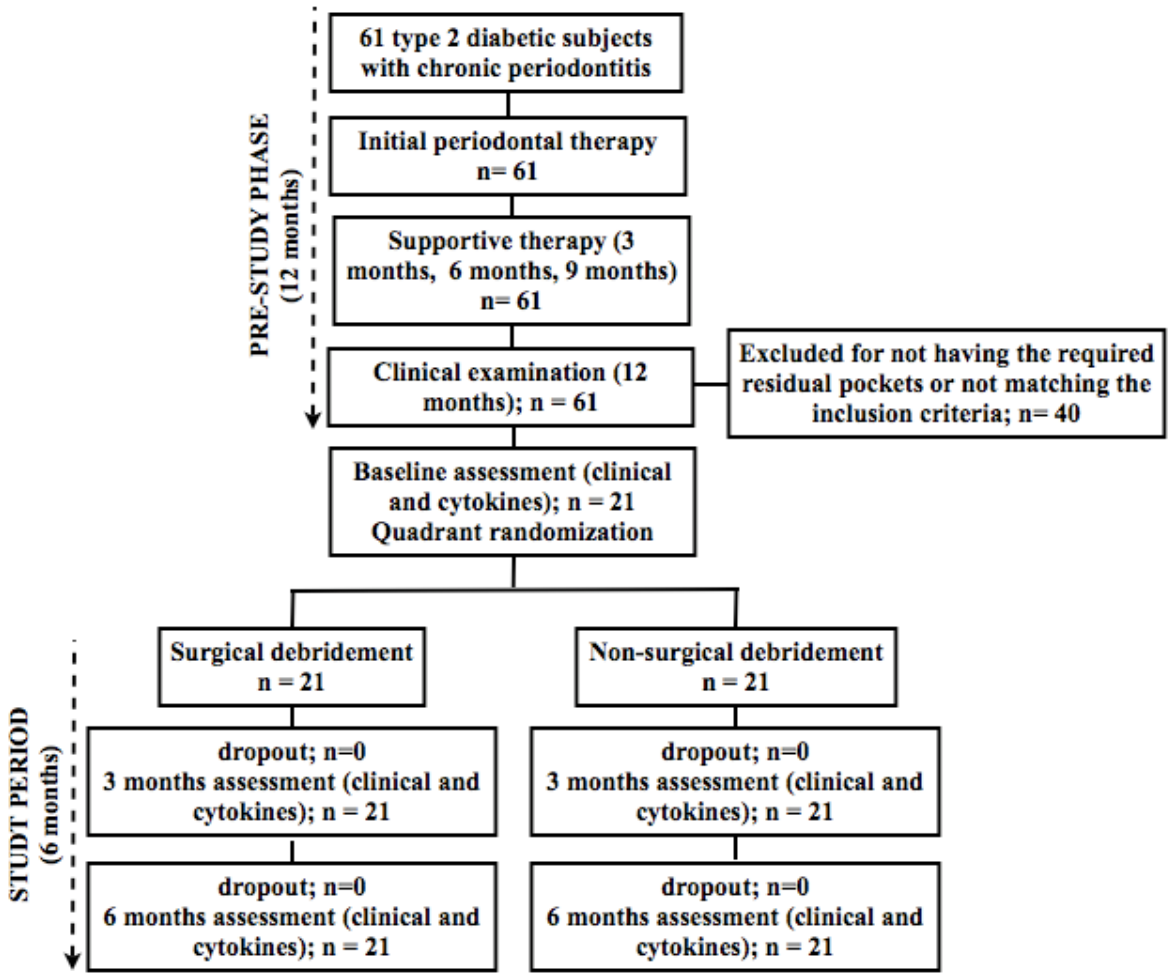


Figure 2. A - Changes in mean CAL from baseline to 6 months post-therapies for both quadrants of each subject (Quadrant data). **B -** Individual changes in the mean PD of the RP from baseline to 6 months (Site data).

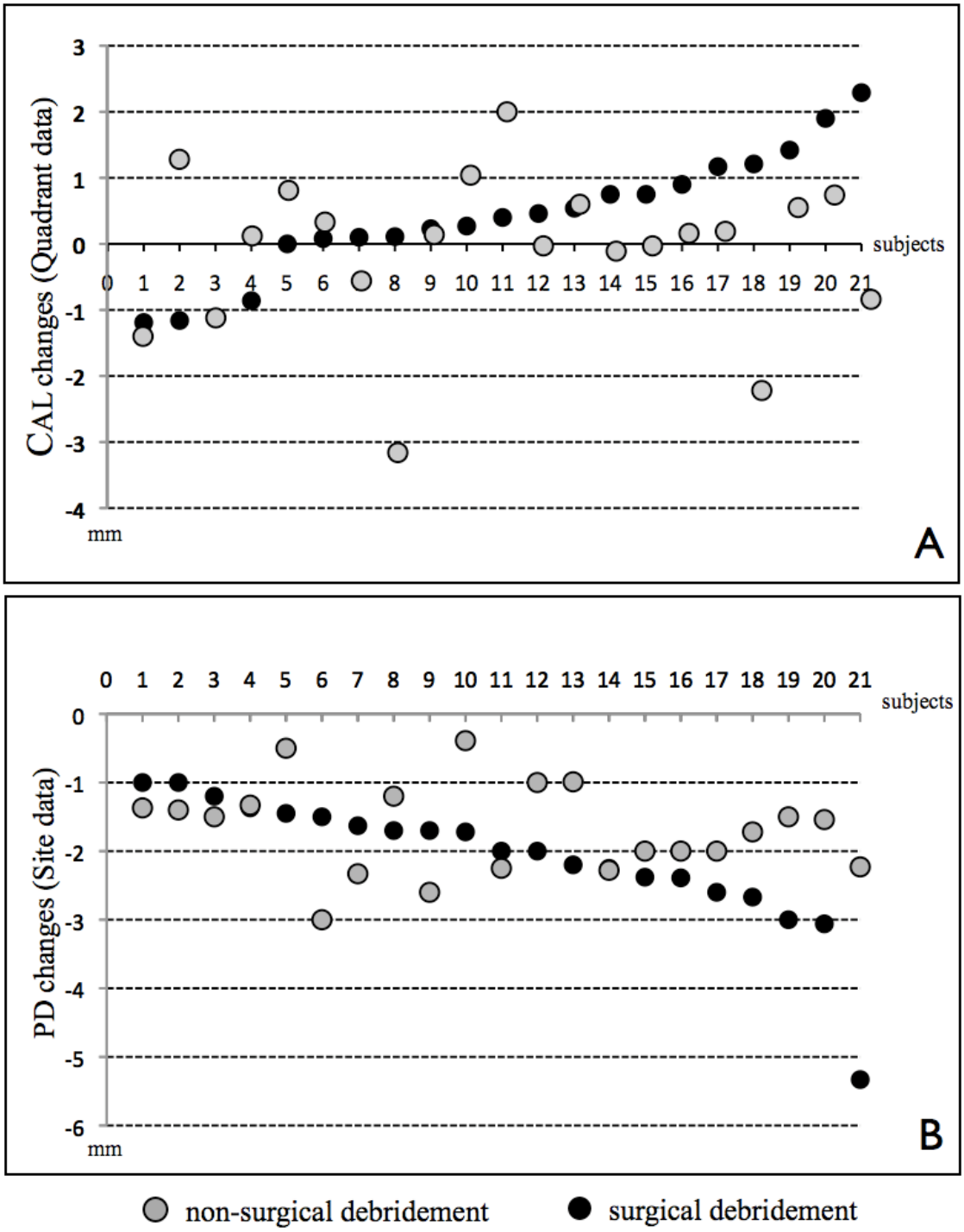


Figure 3. Bar charts of the mean changes (± SD) in the number of pockets with PD ≥ 5mm without BoP and in the number of RP (PD ≥ 5mm with BoP) from baseline to 3 and to 6 months. There were no differences between treatment groups at any time point by paired Student's t-test (p>0.05).

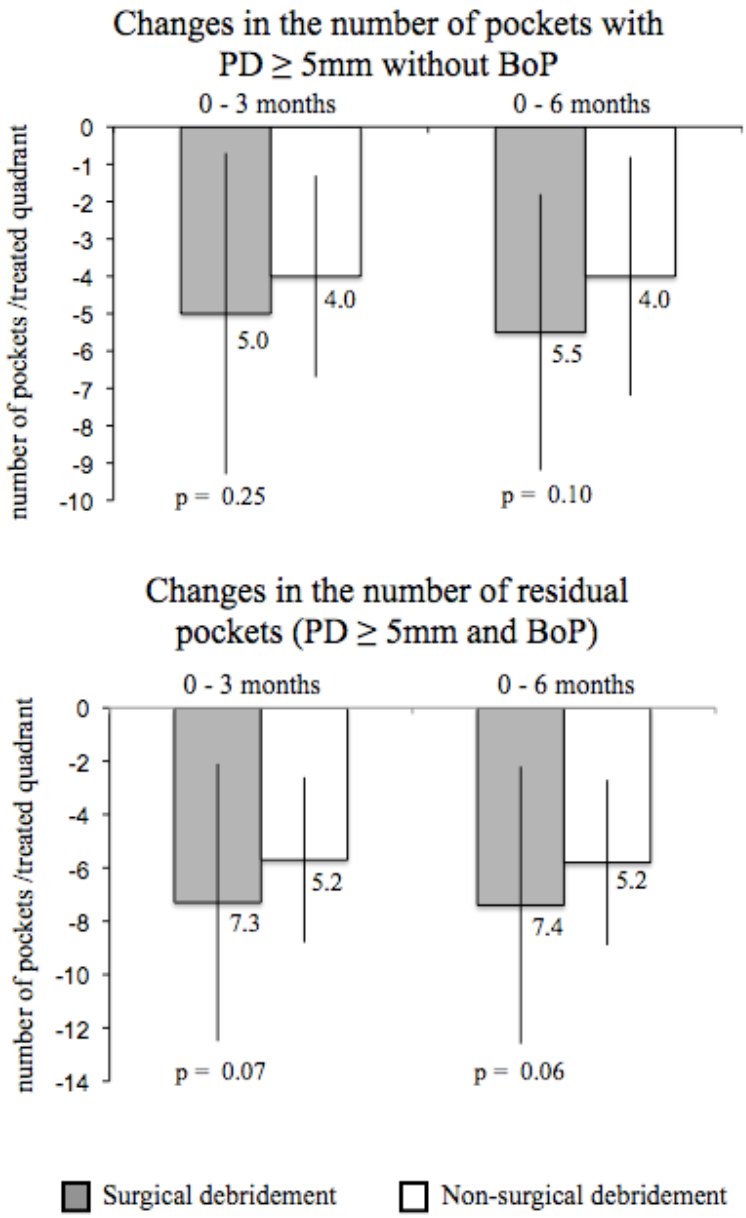
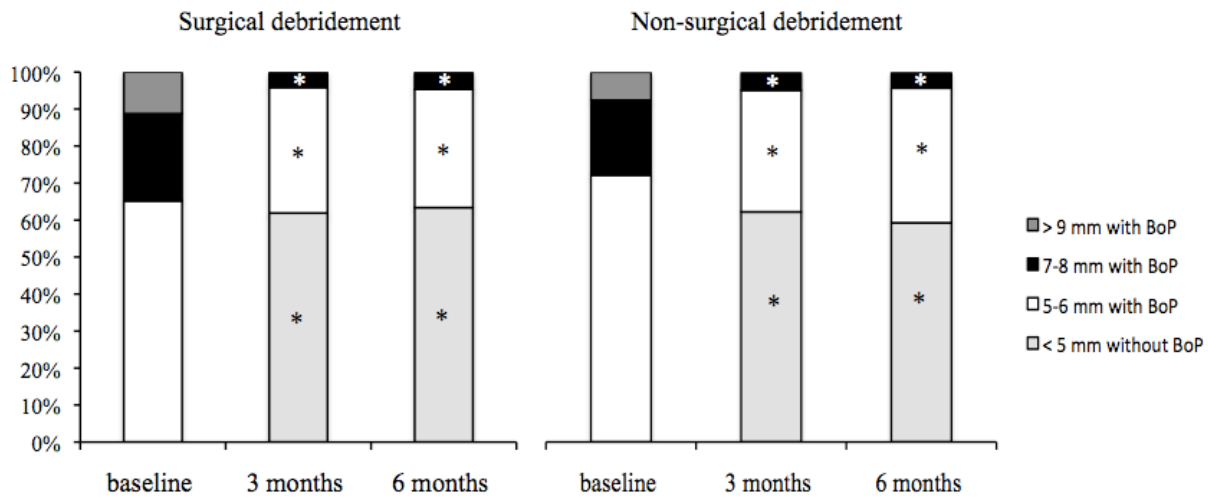


Figure 4. Distribution for the clusters of PD at baseline, 3 and 6 months for all RP. There were no differences between treatment groups at any time point by paired Student's t-test ($p>0.05$). * Significant differences when compared to baseline (Repeated measures ANOVA and Tukey tests, $p<0.05$). There were no RP $\geq$ 9 mm at 3 and 6 months after both treatments.



5. CONSIDERAÇÕES FINAIS

ESTUDO 1 – Existe uma escassez de estudos científicos que avaliaram o perfil imuno-inflamatório de indivíduos diabéticos tipo 2 com periodontite crônica, principalmente, levando em consideração o controle glicêmico. O delineamento do aspecto imuno-inflamatório periodontal em diabéticos é importante para um melhor entendimento da exacerbada destruição clínica nestes indivíduos e sugestões de terapias específicas para esse grupo de risco. Esse estudo contribuiu com a importante informação de que diabéticos tipo 2, avaliados como um todo, independente do controle glicêmico, apresentam níveis locais aumentados de RANKL e citocinas pró-inflamatórias. Além disso, o controle glicêmico parece modular o perfil de citocinas produzido localmente contra patógenos periodontais, polarizando para a hiperprodução de citocina da resposta Th1 (INF-γ) em indivíduos bem-controlados ou Th17 (IL-17) em diabéticos com controle glicêmico insatisfatório. A implicação clínica deste dado molecular ainda necessita ser futuramente investigada.

ESTUDO 2 – Bolsas residuais apresentam risco cientificamente comprovado para futura perda de inserção, o que pode representar um prejuízo ainda maior para pacientes de risco para periodontite como os diabéticos. Entretanto, a literatura não apresenta estudos sobre terapias voltadas para bolsas residuais em diabéticos. Neste estudo, a raspagem e alisamento radicular com ou sem acesso cirúrgico, associada à administração sistêmica de amoxicilina e metronidazol foram propostas para tratamento de sítios não-responsivos em diabéticos tipo 2 engajados em uma terapia de manutenção durante 1 ano. Os resultados demonstraram que ambos protocolos terapêuticos produziram benefícios clínicos similares para as bolsas residuais, com uma modesta vantagem para redução de um maior número de bolsas residuais após terapia cirúrgica. Entretanto, a terapia cirúrgica resultou em níveis elevados de citocinas pró- e anti-inflamatórias em 3 e 6 meses, provavelmente devido ao trauma cirúrgico. Dessa forma, considerando o custo benefício das terapias propostas, uma abordagem mais conservadora, incluindo a re-raspagem e alisamento radicular em campo aberto associada à terapia antibiótica poderia ser seguramente recomendada para tratar bolsas residuais em diabéticos tipo 2.

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ANEXOS

Guarulhos, 14 de abril de 2009.

Exma. Sra.
Poliana M. Duarte

PARECER Nº65/2009

Referência: **Aprovação de Projeto**
SISNEP/426 - "Raspagem e alisamento radicular por meio de acesso cirúrgico em sítios residuais de indivíduos diabéticos tipo 2: estudo clínico, microbiológico e imunológico"

O Comitê de Ética em Pesquisa da Universidade Guarulhos analisou o Projeto de Pesquisa de sua autoria "Raspagem e alisamento radicular por meio de acesso cirúrgico em sítios residuais de indivíduos diabéticos tipo 2: estudo clínico, microbiológico e imunológico" - SISNEP/426, na reunião de 07.04.2009, e no uso das competências definidas na Res. CNS 196/96, considerou o Projeto acima **aprovado.**

As orientações abaixo devem ser consideradas pelo Pesquisador Responsável durante a realização da pesquisa, visando que a mesma se desenvolva respeitando os padrões éticos:

- O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado.
- O pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou, aguardando seu parecer, exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade de regime oferecido a um dos grupos da pesquisa que requeiram ação imediata.
- Eventuais modificações ou emendas e eventos adversos ao protocolo, devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas.
- Esclarecemos a necessidade da apresentação de relatório de andamento até **31.03.10** e relatório final até **31.03.11.**

Jumara Silvia Van De Velde Vieira
Coordenadora do Comitê de Ética em Pesquisa

Guarulhos, 08 de agosto de 2007.

Exma. Sra.
Profa. Poliana Mendes Duarte

PARECER Nº 100/2007

Referência: **Aprovação de Projeto**
SISNEP/277 - "Raspagem a alisamento radicular em até 24 horas ou por quadrantes em 4 semanas em pacientes com periodontite crônica portadores de diabetes mellitus: avaliação clínica, microbiológica e do controle metabólico"

O Comitê de Ética em Pesquisa da Universidade Guarulhos analisou o Projeto de Pesquisa de sua autoria "Raspagem a alisamento radicular em até 24 horas ou por quadrantes em 4 semanas em pacientes com periodontite crônica portadores de diabetes mellitus: avaliação clínica, microbiológica e do controle metabólico" - SISNEP/277, na reunião de 08.08.2007, e no uso das competências definidas na Res. CNS 196/96, considerou o Projeto acima **aprovado**.

As orientações abaixo devem ser consideradas pelo Pesquisador Responsável durante a realização da pesquisa, visando que a mesma se desenvolva respeitando os padrões éticos:

- O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na Integra, por ele assinado.
- O pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou, aguardando seu parecer, exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade de regime oferecido a um dos grupos da pesquisa que requeiram ação imediata.
- Eventuais modificações ou emendas e eventos adversos ao protocolo, devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas.
- Esclarecemos a necessidade da apresentação de relatório de andamento I até **15.08.08,** relatório de andamento II até **15.08.09,** e final até **15.09.10.**

Luciene Cristina de Figueiredo
Coordenadora do Comitê de Ética em Pesquisa

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Subject: Journal of Periodontology - Manuscript JOP-10-0643.R1

13-Jan-2011

Dear Professor Duarte,

I am pleased to inform you that your manuscript, Cytokines and bone-related factors in systemically-healthy and type 2 diabetic subjects with chronic periodontitis (JOP-10-0643.R1), is accepted for publication in the Journal of Periodontology, with an acceptance date of 14-Dec-2010.

Within the next few weeks, your manuscript will be published online in the "Ahead of Print" section at http://www.joponline.org/toc/jop/0/0. More information on "Ahead of Print" is available at this link.

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Sincerely,

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02-Feb-2011

Dear author of "Surgical and non-surgical therapy with systemic antibiotics for residual pockets in type 2 diabetics with chronic periodontitis",

The manuscript entitled "Surgical and non-surgical therapy with systemic antibiotics for residual pockets in type 2 diabetics with chronic periodontitis" has been submitted by Prof. Poliana Duarte to Journal of Clinical Periodontology and will shortly be checked for its suitability for the journal and then forwarded for review.

You have been listed as (co-)author for the manuscript. If this is not the case, please reply to this email.

Sincerely,
Rosie Ledger
Journal of Clinical Periodontology Editorial Office

[Universidade Guarulhos]