UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS ODONTOLÓGICAS

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GENGIVITE PROXIMAL DIAGNOSTICADA PELO FIO DENTAL: AVALIAÇÃO CLÍNICA E HISTOLÓGICA

Santa Maria, RS
2017

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Odontológicas, Área de Concentração em Odontologia, ênfase em Periodontia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Ciências Odontológicas.**

Orientador: Prof. Dr. Fabricio Batistin Zanatta

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Aprovado em 11 de dezembro de 2017:

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Santa Maria, RS
2017

AGRADECIMENTOS

Ao **PPGCO** pela oportunidade em me proporcionar um curso de qualidade.

À **CAPES** pelo apoio financeiro, através de bolsa, durante todo o período do doutorado.

À minha família, **Alexandre, Janete** e **Camila**, pelo incentivo e apoio incondicional.

Ao meu orientador, professor **Fabricio Batistin Zanatta**, por ter disponibilizado seu tempo e permitido compartilhar seus conhecimentos e amizade, fundamental ao longo do doutorado.

À professora **Cristiane Cademartori Danesi**, pela disponibilidade, conhecimentos e contribuição neste trabalho.

Ao funcionário do LAPBIO, **Everton Pittaluga**, pelo envolvimento e disponibilidade para a realização deste trabalho.

À professora **Raquel Pippi Antoniazzi**, pela disponibilidade e contribuição neste trabalho.

Às colegas e amigas **Maisa Casarin** e **Stephanie Yuko Bessauer Tomitsuka**, pela disponibilidade, ajuda e contribuição neste trabalho.

Aos professores **Karla Zanini Kantoski** e **Carlos Heitor Cunha Moreira** pela ajuda e ensinamentos transmitidos.

Ao pessoal da Periodontia, pela amizade e momentos de risadas e descontração.

Aos coordenadores do PPGCO, professores **Thiago Machado Ardenghi** e **Luiz Felipe Valandro**, pela dedicação, ensinamentos e competência em prol do crescimento do nosso programa de pós-graduação.

A todos os professores do PPGCO pelos ensinamentos transmitidos.

À funcionária **Jéssica Dalcin da Silva** por todo o apoio que fornece aos pós-graduandos durante a nossa passagem pelo programa.

Aos **pacientes** que participaram pela disposição para contribuir em nossa pesquisa.

A todas as pessoas que de alguma maneira ajudaram e participaram na realização desta tese.

RESUMO

GENGIVITE PROXIMAL DIAGNOSTICADA PELO FIO DENTAL: AVALIAÇÃO CLÍNICA E HISTOLÓGICA

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ORIENTADOR: Fabricio Batistin Zanatta

Na presença do ponto de contato, o fio dental detecta mais sítios sangrantes que a sondagem, possivelmente pelo maior contato com a porção interna mais inflamada da papila. Entretanto, faltam evidências de validação. O objetivo desta tese foi validar o uso do fio dental para diagnóstico de gengivite proximal. Após diagnóstico clínico com fio dental contra a gengiva (FG) seguido pela sonda periodontal (ISG) após 10 minutos, três grupos de sujeitos foram identificados: FG+/ISG+ papilas sangrantes com ambos os métodos (n=26); FG+/ISG- sangrantes ao fio, mas não sangrantes à sondagem (n=26); FG-/ISG- não sangrantes com ambos os métodos (n=26). Posteriormente, uma papila de cada participante adulto, sem histórico de periodontite, foi biopsiada e analisada histologicamente por um examinador cego. Análise do infiltrado inflamatório no tecido conjuntivo gengival (escores 0-3) e porcentagem de fibras colágenas foram realizadas. Frequências significativamente maiores de inflamação moderada/severa foram observadas nos grupos FG+/ISG+ (100%) e FG+/ISG- (92,3%) em comparação ao FG-/ISG- (0%) e percentual de fibras colágenas significativamente diferente entre os três grupos [FG+/ISG+ (40,90$\pm$3,68) FG+/ISG- (45,78$\pm$4,55) e FG-/ISG- (60,01$\pm$3,66)] (P<0,001). Ainda, sítios proximais contralaterais não sangrantes com sondagem marginal (ISG-) e sangrantes (FG+) ou não (FG-) com fio dental foram identificados em 49 sujeitos. Após 24-48 horas, o volume de fluido crevicular gengival (VFCG) foi coletado com tiras de papel absorvente e comparado nos sítios teste (FG+/ISG-) e controle (FG-/ISG-). De um total de 172 sítios avaliados, sítios teste apresentaram um VFCG (unidades de Periotron) significativamente maior que sítios controle (FG+ 38 [26,5–68] *versus* FG- 25 [15,7–51,25]; P<0,001, teste Wilcoxon). Esta diferença se manteve tanto para sítios anteriores (FG+ 37 [23–66] *versus* FG- 21 [14–45]; P<0,001, teste Wilcoxon) como para sítios posteriores (FG+ 46 [28–92] *versus* FG- 34 [21–70]; P=0,04, teste Wilcoxon). Na ausência de sangramento após sondagem, sítios com sangramento ao fio dental apresentam inflamação significativamente maior que sítios sem sangramento ao fio. Nossos resultados sugerem a utilização do fio dental como método de diagnóstico de gengivite proximal em indivíduos sem histórico de periodontite.

Palavras-chave: Doenças Periodontais. Estudos de Validação. Inflamação. Periodontia.

ABSTRACT

PROXIMAL GINGIVITIS DIAGNOSED BY DENTAL FLOSS: CLINICAL AND HISTOLOGICAL EVALUATION

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In the presence of contact point, dental floss detects more bleeding sites than probing, possibly by greater contact with more inflamed internal part of papilla. However, there is a lack of validation evidences. The aim of this thesis was to validate the use of dental floss for the diagnosis of proximal gingivitis. After clinical diagnosis with dental floss against gingiva (BF) followed by periodontal probe (GBI) after 10 minutes, three subjects groups were identified: BF+/GBI+ bleeding papillae with both methods (n=26); BF+/GBI- bleeding with dental floss, but non-bleeding with probe (n=26); BF-/GBI- were non-bleeding with both methods (n=26). Subsequently, one papilla of each adult participant, with no history of periodontitis, was biopsied and histologically analyzed by a blind examiner. Inflammatory infiltrate analysis in gingival conjunctive tissue (scores 0-3) and percentage of collagen fibers were performed. Significantly higher frequencies of moderate/severe inflammation were observed in BF+/GBI+ (100%) and BF+/GBI- (92.3%) groups compared to BF-/GBI- (0%) and significantly different percentage of collagen fibers between three groups [BF+/GBI+ (40.90±3.68) BF+/GBI- (45.78±4.55) and BF-/GBI- (60.01±36.66)] (P<0.001). Also, non-bleeding contralateral proximal sites with marginal probing (GBI-) and bleeding (BF+) or not (BF-) with dental floss were identified in 49 subjects. After 24-48 hours, volume of gingival crevicular fluid (VGCF) was collected with absorbent paper strips and compared at test (BF+/GBI-) and control (BF-/GBI-) sites. From a total of 172 sites evaluated, test sites had a significantly higher VGCF (Periotron units) than control sites (BF+ 38 [26.5–68] *versus* BF- 25 [15.7–51.25]; P<0,001, Wilcoxon test). This difference was maintained for both anterior (BF+ 37 [23–66] *versus* BF- 21 [14–45], P<0.001, Wilcoxon test) and posterior sites (BF+ 46 [28–92] *versus* BF- 34 [21–70], P=0.04, Wilcoxon test). In absence of bleeding after probing, sites with flossing bleeding present significantly greater inflammation than sites with no flossing bleeding. Our results suggest flossing application as a diagnostic method for proximal gingivitis in subjects with no periodontitis history.

Keywords: Inflammation. Periodontal Diseases. Periodontics. Validation studies.

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1 INTRODUÇÃO

A gengivite induzida por placa bacteriana apresenta alta prevalência afetando quase 100% das pessoas (ALBANDAR; BRUNELLE; KINGMAN, 1999; GJERMO et al., 2002). Confirmando sua associação etiológica, altos níveis de biofilme supragengival na população também são observados, o que aponta falhas nos autocuidados de higiene bucal, especialmente nas regiões proximais (RAMBERG; AXELSSON; LINDHE, 1995; SILNESS; LÖE, 1964). Consequentemente, a gengivite é bastante frequente nestas áreas (HUGOSON; KOCH; RYLANDER, 1981).

A inflamação gengival pode ser diagnosticada pela presença de sangramento após estimulação mecânica com a sonda no sulco gengival (AINAMO; BAY, 1975). Quando comparada a sítios não sangrantes, a presença do sinal inflamatório se mostra associada com maior expressão inflamatória histológica (AMATO et al., 1986; APPELGREN; ROBINSON; KAMINSKI, 1979; CATON; POLSON, 1985; DAVENPORT; SIMPSON; HASSELL, 1982; ENGELBERGER et al., 1983; GREENSTEIN; CATON; POLSON, 1981; OLIVER; HOLM-PEDERSEN; LÖE, 1969) e maiores volumes de fluido crevicular gengival (FCG) (DANESHMAND; WADE, 1976; HATIPOGLU et. al., 2007).

Há evidências de que a inflamação gengival proximal parece iniciar na área central da papila (ABRAMS; CATON; POLSON, 1984; CATON; POLSON, 1985; THILO et al., 1986), porção esta que pode não ser estimulada corretamente pela sonda no momento do exame. Isso pode resultar em subestimação da presença de gengivite nas regiões proximais com ponto de contato estabelecido. Nesse sentido, o uso do fio parece trazer vantagens como método diagnóstico pelo contato do fio dental com uma maior extensão da papila dental internamente (CARTER; BARNES, 1974).

Tinoco & Gjermo (1992) demonstraram que o fio dental apresentou maior sensibilidade que o índice gengival (IG) (LÖE, 1967) e o índice de sangramento gengival (ISG) (AINAMO; BAY, 1975) para identificar reduções na inflamação gengival em crianças de 4 a 6 anos. Da mesma forma, Mariath et al. (2008) verificaram a acurácia do fio dental em crianças entre 3 e 6 anos em comparação ao

ISG (AINAMO; BAY, 1975) conduzindo estes índices em três momentos diferentes (intervalos de 3-4 dias). O fio dental apresentou concordância de 70% quando foi realizado após o ISG e de 76% quando realizado previamente ao ISG. Em outro estudo com adultos sem histórico de periodontite também foi comparado o fio dental ao ISG como método diagnóstico em sítios proximais com ponto de contato (GRELLMANN et al., 2016). Os achados demonstraram que tanto o fio dental friccionado contra o dente (FD) quanto o fio dental friccionado contra a gengiva (FG) detectaram mais sítios sangrantes comparados ao ISG, principalmente em regiões posteriores (pré-molares e molares). Dos 448 sítios em que o FG foi utilizado no primeiro exame, seguido pelo ISG após 10 minutos, 58,3% apresentaram sangramento ao fio (FG+) e ISG-. Além disso, comparando os dois métodos de fricção do fio dental, o FG detectou mais sítios sangrantes que o FD, o que demonstra sua maior capacidade de identificação do sinal inflamatório. Infelizmente, em nenhum dos estudos prévios a análise histológica e de volume de FCG foram conduzidas, o que seria importante para a validação do uso do fio contra a gengiva como método diagnóstico de gengivite proximal. Portanto, ainda não está claro se a presença de sangramento após fricção do fio dental contra a gengiva (FG+) está associada com maior infiltrado inflamatório e volume de FCG que sítios sem sangramento ao fio (FG-).

Assim, considerando os contextos expostos, no presente trabalho serão apresentados dois artigos. O primeiro deles, intitulado **“Flossing as a diagnostic method for proximal gingivitis”**, visou verificar a associação entre a presença e ausência de sangramento gengival marginal ao fio dental contra a gengival (FG) com aspectos histológicos e com o volume de FCG em adultos sem histórico de periodontite. O segundo artigo, uma revisão de literatura, intitula-se **“Diagnosis of gingivitis: state of the art”** e objetivou apresentar, comparar e discutir os principais métodos para o diagnóstico de gengivite e condições autoimunes associadas à inflamação gengival.

A validação de um método clínico de diagnóstico de gengivite proximal, supostamente mais sensível que o padrão ouro (sonda periodontal), permitirá o uso deste método em pesquisas clínicas que avaliem o impacto de estratégias

terapêuticas na inflamação gengival proximal em indivíduos sem periodontite. Ainda, este método poderá ser utilizado nas rotinas clínicas de diagnóstico/tratamento da inflamação gengival proximal. Nossa hipótese conceitual é de que papilas com FG+/ISG- estão associadas a infiltrados inflamatórios e volumes de FCG significativamente maiores, bem como percentuais de fibras colágenas significativamente menores que papilas com FG-/ISG-.

2 REVISÃO DE LITERATURA

2.1 ÍNDICES

A gengiva clinicamente saudável apresenta ausência de alterações na cor, edema e/ou sangramento marginal. Um dos sinais visuais da inflamação gengival é a vermelhidão da margem da gengiva, a qual se evidencia microscopicamente em decorrência da vasodilatação e do aumento no número de unidades vasculares no tecido conjuntivo subepitelial (EGELBERG, 1967). Já o edema e a textura lisa da gengiva livre refletem o extravasamento de células inflamatórias para a matriz extracelular e a perda de fibras colágenas. O sangramento após algum estímulo ocorre devido a micro-ulcerações no epitélio sulcular (GREENSTEIN, 1984). O sangramento é frequentemente utilizado como parâmetro na avaliação da gengiva devido a sua objetividade e facilidade (CHAVES et al., 1993; LANG; SCHATZLE; LOE, 2009).

Alguns índices gengivais são baseados em características clínicas da inflamação, contendo componentes avaliados de forma não invasiva por meio de exame visual (cor, textura, forma, sangramento espontâneo) e componentes inflamatórios que podem ser avaliados de forma invasiva após algum estímulo. Em alguns índices há uma mescla de aspectos visuais e a presença de sangramento marginal após estímulo mecânico (LOE, 1967; LOESCHE, 1979; MUHLEMANN; SON, 1971). Outros índices avaliam apenas aspectos visuais (APSE et al., 1991; LOBENE et al., 1986; SCHOUR; MASSLER, 1947) e há os que utilizam apenas o componente de sangramento de acordo com sua extensão (GARG; KAPOOR, 1985; MOMBELLI et al., 1987; MUHLEMANN, 1977), seu tempo de sangramento (BARNETT; CIANCIO; MATHER, 1980; NOWICKI et al., 1981) ou somente a presença ou ausência do sangramento (AINAMO; BAY, 1975; CARTER; BARNES, 1974; CATON; POLSON, 1985; EDWARDS, 1975; HOFER et al., 2011).

Diversos métodos para estimulação do sangramento marginal são utilizados: sonda periodontal (AINAMO; BAY, 1975; BARNETT et al., 1980; LOE, 1967; MOMBELLI et al., 1987; MUHLEMANN, 1977; MUHLEMANN; SON, 1971; NOWICKI

et al., 1981), madeira interdental (CATON; POLSON, 1985; LOESCHE, 1979), fio dental (CARTER; BARNES, 1974), fita dental (EDWARDS, 1975), escova dental (GARG; KAPOOR, 1985) e escova interdental (HOFER et al., 2011).

Um índice diagnóstico das condições gengivais deve idealmente ser simples e rápido de avaliar, com seus critérios claros e de fácil compreensão, bem como apresentar sensibilidade para identificar variações nos diferentes estágios da doença (LOBENE et al., 1986).

2.1.1 Alterações visuais X sangramento (ISG)

Muhlemann & Son (1971) relataram que um índice gengival deve ser capaz de detectar o sinal mais precoce da gengivite. Alguns autores demonstraram que, mesmo na ausência de alterações visuais, um expressivo percentual de sítios apresenta sangramento marginal (CATON et al., 1988; MEITNER et al., 1979; MUHLEMANN; SON, 1971), o que coloca a presença de sangramento como um sinal que antecede as alterações visuais. Já outros autores notaram que, em estágios iniciais da gengivite, mudanças na cor e edema precedem o sangramento marginal (APPELGREN; ROBINSON; KAMINSKI, 1979; BENAMGHAR et al., 1982; BRECX et al., 1987). Portanto, ainda não há um consenso na literatura sobre a cronologia de eventos visuais e inflamatórios no curso etiopatogênico da gengivite.

A utilização de critérios visuais (cor, edema, textura) dificulta a aplicação clínico-epidemiológica pelo tempo adicional de avaliação dispendido, pela dificuldade de visualização em regiões proximais, especialmente em dentes posteriores, pela subjetividade dos aspectos visuais e, ainda, por estes não serem determinados apenas por componentes inflamatórios, mas também por variações na intensidade da melaninogênese, grau de queratinização e vascularização (GREENSTEIN, 1984). Considerando limitações de aspectos visuais no diagnóstico de alterações gengivais, a presença ou ausência de sangramento após sondagem do sulco (AINAMO; BAY, 1975) é mais universalmente aplicável em estudos clínico-epidemiológicos, bem como pelos Cirurgiões-Dentistas por apresentar facilidade e rapidez (MUHLEMANN; SON, 1971).

2.2 ETIOPATOGENIA GENGIVITE – MARCADORES/MEDIADORES INFLAMATÓRIOS

A maioria das infecções em indivíduos sem alterações sistêmicas é de duração limitada e não deixam um dano permanente como resultado da capacidade do sistema imune do indivíduo para combater os agentes infecciosos. A imunidade inata atua como uma primeira linha de defesa contra infecções, e a maioria dos patógenos potenciais são eliminados antes de estabelecer uma infecção evidente (EBERSOLE et al., 2013). No entanto, durante esta fase, a ênfase na imunidade inata direcionada a atividades não específicas compreende um processo fisiológico natural chamado inflamação. A literatura focada na resposta inflamatória proporciona uma sequência ordenada de eventos coordenados concebidos para proteger o hospedeiro de infecções e minimizar danos aos tecidos do hospedeiro (EBERSOLE et al., 2013). Se essa primeira linha de defesa não tiver sucesso, uma resposta inflamatória crônica inicia, envolvendo células e biomoléculas do sistema imune adaptativo. Durante esta fase inflamatória crônica, a destruição tecidual aumenta, mesmo na presença de ativação de mais reações específicas para numerosas bactérias orais, incluindo os potenciais patógenos (EBERSOLE et al., 2013).

O processo inflamatório que se desenvolve nos tecidos periodontais em resposta à presença do biofilme bacteriano é protetor por intenção, mas resulta em dano tecidual. A resposta do hospedeiro é a principal responsável por este dano. Já que a maioria dos danos teciduais deriva da excessiva e desregulada produção de uma variedade de mediadores inflamatórios e enzimas destrutivas em resposta à presença do biofilme de placa bacteriana (CEKICI et al., 2014), é importante rever os principais tipos de marcadores que são responsáveis pela resposta do hospedeiro. Estes podem ser amplamente divididos em citocinas, metaloproteinases da matriz (MMPs) e prostaglandinas (PGs).

2.2.1 Citocinas

Citocinas são proteínas solúveis secretadas por células envolvidas na resposta do hospedeiro agindo como moléculas mensageiras que transmitem sinais para outras células. As citocinas ligam-se a receptores específicos em células-alvo e iniciam a sinalização por meio de cascatas intracelulares, resultando em alterações fenotípicas nas células através da regulação do gene alterado (BIRKEDAL-HANSEN, 1993b; TAYLOR; PRESHAW; DONALDSON, 2004). Estimulam a produção de proteínas pelas células-alvo que alteram o comportamento das células e podem levar ao aumento da secreção de mais citocinas, resultando em inflamação. Possuem numerosas ações que incluem iniciação e maturação das respostas imunes e inflamatórias e regulação da proliferação e diferenciação de células.

Citocinas são produzidas por vários tipos celulares incluindo células do infiltrado inflamatório (neutrófilos, macrófagos, linfócitos) e células residentes no periodonto (fibroblastos, células epiteliais) (TAKASHIBA; NARUISHI; MURAYAMA, 2003). As interleucinas (IL) são importantes membros do grupo das citocinas e estão envolvidas na comunicação entre leucócitos e outras células (epiteliais, endoteliais, fibroblastos) no processo inflamatório. Uma série de mais de 20 moléculas têm sido identificadas, as quais agem para recrutar células de defesa (polimorfonucleares, macrófagos, linfócitos) para áreas onde são requisitadas (LIU; LERNER; TENG, 2010).

As citocinas responsáveis pela resposta precoce à agressão microbiana incluem IL-1, IL-6 e fator de necrose tumoral alfa (TNF-α) (LAPPIN et al., 2001). A relevância das citocinas como mediadores biológicos da progressão das doenças periodontais já foi demonstrada, por exemplo, em resposta a terapia periodontal que resultou na diminuição dos níveis da citocina IL-1β (TOKER; POYRAZ; EREN, 2008) e TNF-α (IWAMOTO et al., 2003). Já as citocinas inflamatórias produzidas por células Th1 estão mais associadas a um processo de destruição tecidual ativa, enquanto citocinas com características anti-inflamatórias (IL-10, TGF-β), produzidas por células Th2, estão envolvidas na homeostasia tecidual e no subsequente processo de reparo tecidual (LAPPIN et al., 2001).

2.2.2 Metaloproteinases da matriz (MMPs)

As MMPs são uma família de enzimas proteolíticas responsáveis pela remodelação e degradação dos componentes da matriz extracelular (colágeno, proteoglicanas, elastina) (BIRKEDAL-HANSEN, 1993a; RYAN; RAMAMURTHY; GOLUB, 1996). Esses componentes estão constantemente no estado de *turnover*, razão pela qual existe intensa atividade enzimática da matriz na saúde, na doença e na reparação e remodelação tecidual (KINANE, 2000).

As MMPs são produzidas por uma variedade de células, incluindo neutrófilos, macrófagos, fibroblastos e células epiteliais. A MMP-8 e MMP-9 (derivadas dos neutrófilos) e MMP-13 (derivada de células ósseas ou epiteliais), destacam-se entre as predominantemente presentes no tecido gengival inflamado, as quais são encontradas em concentrações significativamente maiores em gengiva inflamada comparada a gengiva clinicamente saudável (OHLSSON; OLSSON; TYNELIUS-BRATTHALL, 1974). A presença aumentada dessas enzimas em sítios doentes, seu aumento durante a gengivite experimental (KOWASHI; JACCARD; CIMASONI, 1979) e sua diminuição após tratamento (HAERIAN et al., 1995, 1996) sugerem que a MMP-8, MMP-9 e MMP-13 estão envolvidas no colapso do tecido periodontal. A atividade de MMP e seus inibidores está associada com o *turnover* do tecido na gengivite e com a cicatrização após terapia (BUTLER; OVERALL, 2013).

2.2.3 Prostaglandinas (PGs)

As PGs são um grupo de compostos lipídicos derivados do ácido araquidônico, metabolizado para gerar uma série de compostos chamados de prostanoides que incluem as PGs. Macrófagos, assim como outras células, produzem prostaglandinas particularmente prostaglandina E2 (PGE2) que é um potente vasodilatador e indutor da produção de citocinas por várias outras células. A PGE2 é produzida por vários tipos de células (macrófagos, fibroblastos) e age sobre fibroblastos para induzir a produção de MMP, que são importantes para o *turnover* tecidual na gengivite (EBERSOLE et al., 2013).

2.3 ESTUDOS HISTOLÓGICOS

Histologicamente, a saúde gengival está associada a um tecido conjuntivo com feixes de fibras colágenas densas (GREENSTEIN; CATON; POLSON, 1981; PAGE; SCHROEDER, 1976), discretas áreas de infiltrado inflamatório e epitélio relativamente espesso (DANESHMAND; WADE, 1976; OLIVER; HOLM-PEDERSEN; LÖE, 1969). Já a presença de gengivite clinicamente detectável está associada a um tecido conjuntivo com baixo percentual de fibras colágenas (GREENSTEIN; CATON; POLSON, 1981; PAGE; SCHROEDER, 1976), presença de intenso infiltrado inflamatório e um epitélio relativamente delgado (ABRAMS; CATON; POLSON, 1984; EGELBERG, 1967).

Estudos encontraram uma área de tecido conjuntivo inflamado aproximadamente duas (DAVENPORT; SIMPSON; HASSELL, 1982; GREENSTEIN; CATON; POLSON, 1981; POLSON; GREENSTEIN; CATON, 1981) a três vezes maior em sítios sangrantes após estímulo comparado a sítios que não sangraram (ABRAMS; CATON; POLSON, 1984; AMATO et al., 1986). Sítios sangrantes apresentam um número significativamente maior de células inflamatórias comparado a sítios saudáveis (APPELGREN; ROBINSON; KAMINSKI, 1979; DAVENPORT; SIMPSON; HASSELL, 1982). Abrams, Caton & Polson (1984) compararam histologicamente papilas sangrantes e não sangrantes após estímulo com palito interdental removidas de sítios sem histórico de periodontite (profundidade de sondagem - PS e nível de inserção clínico - NIC ≤ 3mm). Os autores demonstraram que as regiões centrais das papilas sangrantes exibiram um percentual de infiltrado inflamatório aproximadamente três vezes maior que as não sangrantes. Ainda, estas regiões centrais papilares apresentavam o maior nível de inflamação nos sítios sangrantes (percentual de infiltrado inflamatório aproximadamente 3,5 vezes maior) em comparação que as porções vestibular/lingual.

É difícil determinar qual critério (alterações visuais ou sangramento gengival) expressa melhor o estado inflamatório da gengiva. Algumas evidências demonstraram correlações fracas entre critérios clínicos e status inflamatório observado histologicamente (APPELGREN; ROBINSON; KAMINSKI, 1979; OLIVER; HOLM-PEDERSEN; LÖE, 1969; PAYNE et al., 1975; ZACHRISSON, 1968). Sendo

assim, comparações entre os diferentes índices diagnósticos do processo saúde-doença gengival podem ser imprecisas.

2.4 FLUIDO CREVICULAR GENGIVAL (FCG)

O FCG é resultante da interação entre o biofilme bacteriano aderido ao dente e as células do tecido periodontal (CHAMPAGNE et al., 2003), e está constantemente sendo secretado (DEL FABBRO et al., 2001). Segundo Löe & Holm-Pedersen (1965), o fluxo de FCG é proporcional ao grau de severidade da inflamação. Volumes baixos de FCG são associados a tecidos saudáveis, e volumes maiores representariam o tecido inflamado (DANESHMAND; WADE, 1976; OLIVER; HOLM-PEDEREN; LÖE, 1969). Sinais visuais (alteração de cor e edema) e sangramento gengival são associados com o aumento do volume de FCG (BRIL; KRASSE, 1958; EGELBERG, 1964; ENGELBERGER et al., 1983; NOWICKI et al., 1981; OLIVER; HOLM-PEDEREN; LÖE, 1969; RUDIN; OVERDIEK; RATEITSCHAK, 1970; SHAPIRO; GOLDMAN; BLOOM, 1979). Ainda, evidências sugeriram que dentes multirradiculares apresentam maiores volumes de FCG que unirradiculares (HATIPOGLU et al., 2007; OZKAVAF et al., 2001). Isto possivelmente está relacionado com aspectos de higienização, a qual é mais difícil nos dentes posteriores e a anatomia de molares que apresentam uma maior área radicular interproximal (GOODSON, 2003; HATIPOGLU et al., 2007).

Inúmeros métodos foram desenvolvidos para a coleta do FCG: método de lavagem gengival (SKAPSKI; LEHNER, 1976), uso de túbulos microcapilares ou micropipetas (SUEDA; BANG; CIMASONI, 1969) e uso de tiras de papel filtro (LÖE; HOLM-PEDERSEN, 1965). O método com tiras de papel é o mais utilizado para a quantificação do volume de FCG devido a técnica de absorção ser rápida, de fácil utilização e minimamente invasiva (DEINZER; MOSSANEN; HERFORTH, 2000; GRIFFITHS, 2003).

Na utilização das técnicas de aferição do volume de FCG, as tiras de papel podem ser posicionadas na entrada do sulco (LOE; HOLM-PEDERSEN, 1965) ou dentro do sulco até que uma mínima resistência seja detectada (BRILL; KRASSE,

1958). Dentre os diferentes tipos de tiras absorvíveis, o Periopaper® tem sido o método de escolha para a coleta (DEINZER; MOSSANEN; HERFORTH, 2000; OZKAVAF et al., 2001). O Periotron® é um dispositivo que mede a capacitância da tira de papel filtro umedecida com o FCG via corrente elétrica (CIANTAR; CARUANA, 1998) e é utilizado para mensurar mais precisamente o volume de FCG (GRIFFITHS, 2003). Ao coletar o FCG no Periopaper®, a tira de papel deve ser imediatamente transferida para o Periotron® em até no máximo 2 segundos, a fim de evitar a evaporação do material (CHAPPLE et al., 1995; JIN; YU; CORBET, 2003). Outros aspectos como tempo de coleta, contaminação das amostras por sangue, saliva ou placa bacteriana, a temperatura e umidade do ar podem interferir no volume medido (BICKEL; CIMASONI, 1985; GOODSON, 2003; GRIFFITHS, 2003; GRIFFITHS; WILTON; CURTIS, 1992; TOZUM et al., 2004). Alguns autores determinaram um tempo de coleta de 30 segundos como seguro na identificação do grau de inflamação gengival (WASSALL; PRESHAW, 2016). Um tempo prolongado tem o risco de alterar a concentração de proteínas e influenciar no volume de FCG coletado (CURTIS et al., 1988). Uma das vantagens na determinação do volume de FCG é que este é um indicador dos estágios precoces de gengivite (CHAMPAGNE et al., 2003; GRIFFITHS, 2003).

Hancock, Cray & O'Leary (1979) e Oliver, Holm-Pedersen & Löe (1969) mensuraram o volume de fluido a partir do método da colorimetria através de solução de ninidrina e observaram que, em faces livres, sítios de escore 1 (alterações visuais de cor e edema e ausência de sangramento gengival) do índice de Löe (1967) apresentavam um maior volume de FCG quando comparados aos sítios de escore 0 (gengiva normal). Nowicki et al. (1981) avaliaram o volume de FCG através de tiras de papel absorvente inseridas no sulco gengival e em seguida colocadas no Periotron®. Sítios proximais com escore 1 também apresentaram maior volume de FCG comparado aos sítios proximais com escore 0. Entretanto, nesses estudos foi utilizado o método da colorimetria através de solução de ninidrina para aferição do FCG (HANCOCK; CRAY; O'LEARY, 1979; OLIVER; HOLM-PEDERSEN; LÖE, 1969), o qual não é tão preciso quanto o Periotron® (GRIFFITHS, 2003).

Posto isto, pode-se concluir que a medida do volume do FCG consiste em um método mais sensível para aferição dos sinais mais incipientes da inflamação gengival comparado a medidas subjetivas de cor, edema e sangramento.

3 ARTIGO 1 – FLOSSING AS A DIAGNOSTIC METHOD FOR PROXIMAL GINGIVITIS

Este artigo será submetido ao periódico *Journal of Clinical Periodontology*, ISSN: 1600-051X, Fator de impacto = 3.477; Qualis A1. As normas para publicação estão descritas no Anexo A.

Flossing as a diagnostic method for proximal gingivitis

Running Title: Gingivitis diagnosis using dental floss

KEYWORDS: histologic evaluation, histology, indexes, inflammation, periodontal diseases, periodontics, validation studies

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CONFLICT OF INTEREST: The authors report no conflicts of interest.

Funding information: This study was self-funded by the authors.

Clinical Relevance

Scientific rationale for the study: Probing is limited in making contact with the entire extension of interdental papilla at proximal sites. As proximal gingivitis seems to start in the central portion of papilla, flossing can be an advantageous method for early detection of gingivitis.

Principal findings: Sites that did not bleed on marginal probing, but bled with flossing had a higher volume of gingival crevicular fluid, greater inflammatory infiltrate and a lower percentage of collagen fibers compared to sites that did not bleed with either the application of a periodontal probe or dental floss.

Practical implications: Dental floss can be used as a diagnostic method for proximal gingivitis in adults without clinical attachment loss.

Abstract

Aim: To validate flossing for the diagnosis of proximal gingivitis.

Material and Methods: Dental floss was slid against the papilla. After ten minutes, a periodontal probe was applied. In the first study, three subjects groups were identified: bleeding (+) with both methods; bleeding (+) with dental floss, but not bleeding (-) with probing; and not bleeding (-) with both methods. One papilla from all 78 subjects was biopsied and histologically analyzed. Inflammatory infiltrate and percentage of collagen fibers were determined. In the second study, volume of gingival crevicular fluid (GCF) was collected with absorbent paper strips in 49 subjects exhibiting Flossing+/Probing- and Flossing-/Probing- at contralateral proximal sites. The GCF volume was compared between these sites (n=172).

Results: Higher frequencies of moderate/severe inflammation were found in the Flossing+/Probing+ (100%) and Flossing+/Probing- (92.3%) groups compared to the Flossing-/Probing- (0%) group. Significantly different percentages of collagen fibers were found among the three groups [Flossing+/Probing+ (40.90±3.68); Flossing+/Probing- (45.78±4.55); Flossing-/Probing- (60.01±36.66)] ($P<0.001$). Among the 172 sites evaluated, positive sites had more GCF volume [38 (26.5–68)] than negative sites [25 (15.7–51.25)] ($P<0.001$).

Conclusion: The findings suggest that flossing can be used as a diagnostic method for proximal gingivitis in subjects with no history of periodontitis.

1 INTRODUCTION

Gingivitis and periodontitis are caused by the buildup of bacterial biofilm at and below the gingival margin, respectively. These conditions are a continuum of the same inflammatory process (Kinane & Attström, 2005), with biofilm acting as a common primary factor for their development (Sanz et al., 2017). Although not all patients with gingivitis develop periodontitis, the management of gingivitis is considered both a primary prevention strategy for periodontitis and secondary prevention strategy for recurrent periodontitis (Chapple et al., 2015).

Marginal gingival bleeding following mechanical stimulus is a clinical parameter used to characterize tissue inflammation (Mariotti & Hefti, 2015). Bleeding after sulcus probing is related to increased inflammatory infiltrate in the adjacent tissue (Oliver, Holm-Pedersen, & Löe, 1969; Greenstein, Caton, & Polson, 1981; Caton & Polson, 1985), collagen depletion (Ejeil et al. 2003; Younes et al., 2009; Almeida et al., 2015) and a greater volume of gingival crevicular fluid (GCF) (Daneshmand & Wade, 1976; Hatipoglu, Yamalik, Berberoglu, & Eratalay, 2007) when compared to non-bleeding sites.

Previous studies have found that dental floss is a suitable tool for the diagnosis of proximal gingivitis in children (Tinoco & Gjermo, 1992; Mariath, Bressani, Haas, Araujo, & Rösing, 2008). Recently, Grellmann et al. (2016) compared the Gingival Bleeding Index (GBI) (Ainamo & Bay, 1975) with dental floss against teeth (DFT) and gums (DFG) in young adults using replication exams with a 10-minute interval. When probing was performed first, 41.7% and 50.7% of sites with negative results (Probing-) bled during second exam when DFT and DFG were used, respectively. When the exams were performed in the opposite order, 38.9% and 58.3% of sites that bled with DFT and DFG (first exam) did not bleed with probing (second exam).

The better performance of dental floss as a diagnostic method for gingival bleeding may be related to its better capacity in to reach the central area of the papilla, where gingival inflammation generally initiates (Abrams, Caton, & Polson, 1984; Caton & Polson, 1985; Thilo, Caton, Polson, & Espeland, 1986) and to the greater subgingival reach of dental floss (Waerhaug, 1981). Therefore, our findings (Grellmann et al., 2016) are inconclusive and require further histologic validation.

On basis of these considerations, we designed two validation studies to evaluate dental floss as a diagnostic method for proximal gingival inflammation by determining the association between the presence/absence of bleeding with histological outcomes (first study) and GCF volume (second study).

2 MATERIAL AND METHODS

2.1 Study design and eligibility criteria

Participants were recruited from the Dental School of *Universidade Federal de Santa Maria* (UFSM) and *Centro Universitário Franciscano* (UNIFRA). Each participant was enrolled in only one of the studies. Individuals were screened from March 2015 to March 2017 (histological study) and from May 2015 to March 2016 (GCF study) and submitted to an interview to determine the eligibility. The inclusion criteria were age ≥ 18 years and probing depth and/or clinical attachment loss ≤ 3mm at proximal sites with contact point (Eke et al., 2012). The exclusion criteria were relevant systemic condition (e.g. diabetes mellitus), the use of cyclosporine, phenytoin or nifedipine, current or past smoking habit, pregnant or lactating women, use of fixed orthodontic devices or fixed retainers and use of antibiotics/anti-inflammatory drugs in the three months prior to screening.

2.2 Evaluation methods (both studies)

The subjects were evaluated in dental chairs. Prior to the assessments, the teeth were air dried and isolated with cotton rolls. The following assessments were performed:

a. Application of dental floss (Figure 1a): method adapted from Carter & Barnes (1974); waxed floss (Sanifill, São Paulo, Brazil) was inserted with double gentle rub (one in mesial and one in distal site) and slid against the gingival tissue. Presence of bleeding within 10 seconds originating from buccal and/or lingual/palatal aspects of papilla indicated gingivitis;

b. Application of probe: method adapted from Ainamo & Bay (1975); a periodontal probe with a tip diameter of 0.5 mm (Williams, Neumar, São Paulo, Brazil) was positioned at the transition angle between free and proximal surfaces parallel to the long axis of the tooth. The probe was inserted into proximal gingival sulcus to approximately 2 mm and was extended as close as possible to the central region of the papilla. This procedure was performed once. Marginal bleeding was assessed within 10 seconds. Bleeding in buccal and/or lingual/palatal aspects of papilla indicated gingivitis.

2.2.1 Histological study

The clinical analysis (dental floss and periodontal probe) and surgical procedures were performed by three examiners (F.B.Z., M.C., R.P.A.). The histological and histomorphometric analyses were performed by one examiner (A.P.G.). All participants had to present a surgical need (e.g. exodontia, clinical crown lengthening) near the biopsied papilla. Moreover, no clinical situation that interferes with periodontal conditions (e.g. defective interproximal restorations, carious lesions and acute gingival condition) could be present at the biopsied site.

Eligible biopsied sites

- Flossing+/Probing+: bleeding within 10 seconds after flossing application; probing performed after 10 minutes (Grellmann et al., 2016) and bleeding within 10 seconds;
- Flossing-/Probing-: no bleeding within 10 seconds after flossing application; 10-minute interval; and no bleeding within 10 seconds after application of probe;
- Flossing+/Probing-: bleeding within 10 seconds after flossing application; 10-minute interval; and no bleeding within 10 seconds after application of probe.

The area was anesthetized and care was taken to not inject the anesthetic directly into the papilla. A split flap was performed in the buccal and lingual/palatal areas (Figure 1b). Gingivectomy was performed close to the alveolar ridge following

an intrasulcular incision with the aid of a custom Orban gingivectomy. Biopsied specimens were immediately placed in 10% neutral buffered formalin. Suturing was then performed (Figure 1c) and postoperative measurements were made. The minimum fixation period was 24 hours (Greenstein, Caton, & Polson, 1981). Prior to embedding, buccal and lingual tissues (Figure 1d) were separated to allow only the evaluation of the mid-interproximal tissue (0.4 cm) (Figure 1e), which was embedded in paraffin in the buccal-lingual direction.

Histochemical staining

Two serial histological sections (thickness: 4 μm) were obtained from each papilla [one stained with hematoxylin and eosin (H&E) and one stained with Masson's Trichrome] and mounted on glass slides (Exacta - Perfecta, São Paulo, Brazil). Histological (H&E) and histomorphometric (Masson's Trichrome) analyses were performed using a light microscope (Binocular Optical Microscope ZEISS, Axio Lab.A1, Germany) and the images were transferred to a monitor with a camera apparatus (AxioCam, ERc 5S, Germany). The examiner was blinded to the group allocation. The evaluation with H&E and Masson's Trichrome was in the upper connective tissue (directly under the epithelial basement membrane).

H&E staining followed the standardized methodology of the UFSM Pathology Laboratory. Two unmatched microscopic fields on each slide were selected and photographed (magnification: 100×) (Oliver, Holm-Pedersen, & Löe, 1969). The two fields enabled the analysis of the entire middle portion of the papilla. The first field was chosen and the second field was selected to the right of the first field. Histological analysis was performed using scores 0-3 (Oliver, Holm-Pedersen, & Löe, 1969). From the two fields, the scores mean was calculated per subject.

- Score 0 – No inflammatory cells in epithelium or connective tissue; non-significant inflammatory infiltrate (Figure 2a);
- Score 1 – Sparse distribution of inflammatory cells; discrete inflammatory infiltrate (Figure 2b);
- Score 2 – Moderate accumulation of inflammatory cells in isolated areas, sparse distribution in other areas (Figure 2c);

- Score 3 – Intense cellular inflammatory infiltration, mainly along subepithelial connective tissue (Figure 2d).

Masson's Trichrome staining (Easy Path, Erviegas - São Paulo, Brazil) was performed following the manufacturer's instructions and was used for the histomorphometric analysis of collagen fibers (color contrast between healthy and inflamed tissue). For each gingival sample, four mismatched fields were photographed on each slide (magnification: 200×) (Séguier, Godeau, & Brousse, 2000). The first field was chosen ramdomly and the others fields were selected to the right of the first field. The percentage area of collagen fibers was measured in each microscopic field (blue coloration) without considering color intensity. From the four fields, the mean percentage area of collagen fibers was calculated per subject. The Color Deconvolution tool of FIJI Image Analysis and Processing System for IMAGE J was used for the morphometric analysis.

2.2.2 GCF study

Eligible paired GCF collection sites

All clinical evaluations were performed by a single examiner (S.Y.B.T.). Participants needed to have at least one pair of contralateral teeth with the following characteristics:

- Flossing+/Probing-: bleeding within 10 seconds after flossing application; 10-minute interval; and no bleeding within 10 seconds after application of probe;
- Flossing-/Probing-: no bleeding within 10 seconds after flossing application; 10-minute interval; and no bleeding within 10 seconds after application of probe.

Between 24 and 48 hours after clinical evaluation with flossing and probing to select eligible paired sites, GCF volume was collected from the two contralateral sites in one of buccal proximal aspects. GCF volume collection was performed with relative isolation, drying of the tooth surfaces and adequate lighting. Absorbent paper strips (Periopaper, Oraflow Inc., New York, USA) were inserted into the gingival sulcus until light resistance (Brill & Krasse, 1958) and held for 30 seconds. Absorbent paper

strips were immediately transferred to a Periotron 8000 (Harco Electronics, New York, USA). Contamination with biofilm and saliva and injuries to the gingival tissue were avoided. Paper strips contaminated with blood were discarded and the site was excluded from the analysis. Exams were conducted in an air-conditioned room (approximately 20ºC) to minimize the influence of room temperature and humidity. One proximal site (mesial or distal) was evaluated when both sites bled. Proximal sites between 2nd and 3rd molars were not evaluated.

2.3 Training and calibration

2.3.1 Histological study

Training was conducted with an experienced examiner (F.B.Z.) to standardize the clinical evaluation of the gingival bleeding indexes (flossing and probing).

Training for the histological evaluation (H&E) was conducted with an experienced professional (C.C.D.). After the training period, intra-examiner agreement (Kappa coefficient: 0.96) was determined in 20 microscopic fields (magnification: 100×) not included in the study measured on two different days by the same examiner (A.P.G.) after a seven-day interval.

Using software (FIJI for ImageJ, version 1.47i, Wayne Rasband, National Institute of Health, USA), a superior and inferior value was calibrated in 20 microscopic fields not included in the study to calculate the percentage of collagen fiber area (Masson's Trichrome). These values were standardized for all microscopic fields.

2.3.2 GCF study

Training was conducted with an experienced examiner (F.B.Z.) to standardize the clinical evaluation of the gingival bleeding indexes (flossing and probing) and the method of inserting paper strips. Intra-examiner agreement was determined on 10 subjects not included in the study in duplicate exams with 10-minute intervals between flossing and probing. Kappa coefficients were 0.85 for flossing (n = 120 papillae) and 0.70 for probing (n = 105 papillae). Calibration of the Periotron 8000 was performed with a P2 model pipette (Pipetman, Gilson Inc., Middleton, WI, USA)

with operating range of 0.1 to 2 µl. Volumes of 0.2, 0.4, 0.8, 1, and 1.2 µl of saline were applied to the paper strips. The volume of 1.2 µl was used in triplicate. Between each sample, the upper and lower Periotron structures were cleaned with a cotton swab soaked in alcohol. A quadratic regression curve was constructed to determine the volume of GCF.

2.4 Sample size calculation

2.4.1 Histological study

Mean differences of 40% ± 10%, 30% ± 10% and 20% ± 10% of inflamed connective tissue (high leukocyte cell density and altered collagen) for papillae was considered for Flossing+/Probing+, Flossing+/Probing- and Flossing-/Probing-, respectively. These percentages were estimated based on Greenstein, Caton, & Polson (1981), who demonstrated a difference of approximately 10% in inflamed connective tissue between bleeding (28.7%) and non-bleeding (19.1%) sites after sulcus probing. Considering an 80% statistical power and $\alpha = 0.05$, the sample was determined to be 26 individuals per group (total: 78 subjects).

2.4.2 GCF study

A mean difference of 4 µl (Shapiro, Goldman, & Bloom, 1979) between Flossing+ and Flossing- papillae, a standard deviation of 7%, an 80% statistical power, $\alpha = 0.05$ and a paired design were considered, determining a sample size of 49 subjects.

2.5 Ethical considerations

Eligible participants were required to sign a consent form. These studies were conducted in accordance with the Declaration of Helsinki and were approved by Ethics Committee of *Universidade Federal de Santa Maria* – UFSM, RS, Brazil (CAAE: 39272314.0.0000.5346 and 15141013.5.0000.5346).

2.6 Statistical analysis

2.6.1 Histological study

The frequency distribution of the H&E scores in each subject group was described. H&E scores were dichotomized as absence (scores 0 and 1) or presence (scores 2 and 3) of clinically detected inflammation. Mean (standard deviation) percentages of collagen fibers (Masson's Trichrome staining) in each group were compared. The Shapiro-Wilk test was used to evaluate the normality of the collagen fiber percentage data. One-way ANOVA and Tukey's post hoc test were used for all comparisons of collagen fiber percentages among the three groups. A significance level of 5% was used for the statistical tests. The analyses were performed using the Statistical Package for the Social Sciences (SPSS version 23, Chicago, USA).

2.6.2 GCF study

The data were expressed as median and percentiles in Periotron units. Individual and site were used as units of analysis to evaluate the association between the volume of GCF and the presence/absence of bleeding with dental floss. Site was used as unit of analysis to determine differences in GCF volume between positive (Flossing+) and negative (Flossing-) sites in the anterior (incisor-incisor, incisor-canine and canine-pre-molar) and posterior (pre-molar-pre-molar, pre-molar-molar and molar-molar) regions. The Kolmogorov-Smirnov test was used to evaluate the normality of the GCF data. The Wilcoxon test was used for all GCF comparisons between Flossing+ and Flossing- sites. A significance level of 5% was used for statistical tests. The analyses were performed using the Statistical Package for the Social Sciences (SPSS version 23, Chicago, USA).

3 RESULTS

3.1 Histological study

One hundred thirteen subjects were screened and 35 were excluded (Figure 3). Among the 78 participants included, 40 (51.3%) were women, 38 (48.7%) were men, 59 (75.6%) were white, 19 (24.4%) were non-white, and mean age was 41 ± 13.64 years. Only one papilla was removed per subject. Table 1 displays the demographic characteristics of the three groups evaluated.

Frequencies of moderate/severe inflammation were significantly higher in the Flossing+/Probing+ (100%) and Flossing+/Probing- (92.3%) groups compared to the Flossing-/Probing- (0%) group. The percentage of collagen fibers differed significantly among the three groups [Flossing+/Probing+ (40.90 ± 3.68), Flossing+/Probing- (45.78 ± 4.55), and Flossing-/Probing- (60.01 ± 3.66)] ($P < 0.001$).

3.2 GCF study

Eighty subjects were screened and 31 were excluded (Figure 3). All 49 subjects included had 12 pairs of contralateral sites evaluated. Thirty-three (67.35%) subjects were women, 16 (32.65%) were men, 41 (83.67%) were white, 8 (16.33%) were non-white, and mean age was 23.23 ± 4.27 years.

Table 2 displays the results of the analysis by individual comparing Flossing+/Probing- and Flossing-/Probing- sites. Positive sites had a significantly larger volume of GCF [43 (29.25–68.75)] than negative sites [32 (16.75–47.75)] ($P < 0.001$, Wilcoxon test). This difference was maintained in the separate analyses for women [positive: 42.3 (28.7–87.3); negative: [32.5 (15.3–48.6)] ($P = 0.001$, Wilcoxon test) and men [positive: 57.5 (34–68.5)]; negative: [32 (18–44)] ($P = 0.003$, Wilcoxon test).

Figure 4 displays the results of the analysis by site comparing Flossing+ and Flossing- sites. Among the 172 sites evaluated, positive sites had a significantly larger volume of GCF [38 (26.5–68)] than negative sites [25 (15.7–51.25)] ($P < 0.001$, Wilcoxon test). This difference was maintained in the separate analyses of anterior [positive: 37 (23–66); negative: 21 (14–45)] ($P < 0.001$, Wilcoxon test) and posterior (positive: 46 (28–92); negative: 34 (21–70)] ($P = 0.04$, Wilcoxon test) regions.

4 DISCUSSION

The data confirmed our conceptual hypothesis of significantly greater inflammation at sites that bled after being rubbed with dental floss compared to non-bleeding sites. These findings are consistent with the different outcomes evaluated and complement previous data by Grellmann et al. (2016), reinforcing the hypothesis that dental floss is more sensitive than proximal sulcus probing for the detection of

proximal bleeding, probably due to the greater contact of dental floss with the inflamed connective tissue at proximal sites, which seems not to be sufficiently achieved when using a periodontal probe.

The findings revealed a clear difference (approximately 15%) regarding the loss of integrity of the connective tissue among Flossing+/Probing- and Flossing-/Probing- groups (when flossing detects proximal bleeding or not). Based on evidence that proximal gingival inflammation seems to initiate in the central area of the papilla (Abrams, Caton, & Polson, 1984; Caton & Polson, 1985; Thilo, Caton, Polson, & Espeland, 1986), the differences between the Flossing+/Probing- and Flossing-/Probing- groups indicate that the middle portion of the papilla is not stimulated by a periodontal probe. Therefore, we hypothesize that there is an initial inflammation focused in the central area of the papilla in the Flossing+/Probing- group.

A small difference (approximately 5%) was found between the Flossing+/Probing+ and Flossing+/Probing- groups. Although histologically both groups presented similar histological inflammation, only dental floss was able to clinically detect these changes. Therefore, if probing was a good method to diagnose proximal gingivitis, this difference (approximately 5%) should be greater.

A reduction in the total area occupied by collagen in inflamed gingival tissue has been described by other authors (Gogly et al., 1997; Séguier, Godeau, & Brousse, 2000; Younes et al., 2009; Almeida et al., 2015). Ejeil et al. (2003) found a 20% reduction in the gingival area occupied by collagen when comparing normal and severely inflamed gingival tissues in human patients. The authors also found that an area of approximately 60% is occupied by collagen under normal conditions, which is in agreement with our results. However, all gingival samples in the above studies were obtained from buccal marginal tissues rather than the middle of papilla. Therefore, probing is a good method to diagnose gingivitis in free surfaces.

In the study by Grellmann et al. (2016), when bleeding was absent during the first examination, the DFT-DFG sequence revealed bleeding in 19% of sites during the second exam. When the sequence was reversed, 43.6% of sites that bled during the first exam (DFG) did not bleed during the second exam (DFT). These results raise the hypothesis that DFG is more sensitive than DFT at detecting proximal gingival

bleeding. We hypothesized that DFG is possibly more sensitive and its execution is closer to the probing technique, in which the internal portion of the gingival sulcus is stimulated. Thus, we chose the DFG method for comparison to the use of a periodontal probe.

Grellmann et al. (2016) considered the possibility of gingival bleeding occurring due to mechanical trauma when exams are sequentially performed. The number of non-bleeding sites on a first examination and bleeding sites on second examination (after 10 minutes) was 19.5%, 9.4% and 12.5% for GBI-GBI, DFT-DFT, and DFG-DFG, respectively. The increase in bleeding sites shows that part of the bleeding during the second examination was due to trauma, with probing (GBI) having the greatest traumatic effect. Secondly, despite the greater probability of bleeding during the second examination due to the sequential mechanical traumatic effect, 38.9% and 58.3% of sites that bled after DFT and DFG during first examination did not bleed when probing was performed during the second examination. These data demonstrate the lower sensitivity of a periodontal probe compared to the flossing techniques (DFT and DFG). Based on these findings, we always performed probing as the second exam in both of the present studies (GCF and histological) in order to characterize the Flossing+/Probing- group. Even being a more traumatic method, probing did not detect bleeding in a second examination.

The GCF findings are in agreement with data described in previous studies, in which a positive association was found between a greater volume of GCF and the presence of gingival inflammation (Löe & Holm-Pedersen, 1965; Oliver, Holm-Pedersen, & Löe, 1969; Rüdin, Overdiek, & Rateitschak, 1970; Daneshmand & Wade, 1976; Shapiro, Goldman, & Bloom, 1979; Hatipoglu, Yamalik, Berberoglu, & Eratalay, 2007). Although no statistical analysis comparing anterior and posterior sites was performed in the present study, the mean volume of GCF was greater in posterior sites than anterior sites (Figure 4). Evidence suggests that multi-root teeth have a larger volume of GCF than single-root teeth (Ozkavaf et al., 2000; Hatipoglu, Yamalik, Berberoglu, & Eratalay, 2007), possibly due to hygienic aspects and anatomical characteristics, as posterior teeth are more difficult to clean and molars

have a greater interproximal root area (Goodson, 2003; Hatipoglu, Yamalik, Berberoglu, & Eratalay, 2007).

The differences in GCF volume and inflammation found between Flossing+/Probing- and Flossing-/Probing- groups demonstrate that flossing has ability to differentiate sites with different clinical and subclinical inflammatory expression and corroborates the hypothesis that flossing is more sensitive than proximal sulcus probing. Moreover, it helps to rule out the possibility of gingival trauma.

In the present investigation, strategies were used to reduce the possibility of bias. The selection of contralateral sites in the same subject (GCF study) decreases variability related to the individual inflammatory response (e.g. susceptibility and hormonal variations) (Lindhe & Attström, 1967; Liew et al., 1991; Wilton et al., 1992; Trombelli et al., 2004; Khosravisamani et al., 2014) and decreases the variability in the GCF volume collection site, enabling better standardization in the sulcular area of each pair of sites compared. Other strategies were the examiner blinding (histological study), sample size calculation and the choice of intracrevicular technique (Brill & Krasse, 1958) for the evaluation of GCF volume, which leads to less variability in the collection of gingival fluid samples (Egelberg & Attström, 1973). The limitations of present study were the analyzed papillary area (middle only), not allowing comparisons with the buccal and lingual tissues; and only the area just below the epithelium was evaluated, not being evaluated throughout height of papilla, only the entire extension of papilla.

The applicability of dental floss is in detect more incipient lesions. Moreover, flossing could be used more easily in epidemiological studies where proximal area is, in general, the region that presents greater chance of periodontal disease development (Eke et al., 2012).

5 CONCLUSION

Despite the presence of moderate/severe inflammatory infiltrate in 92.3% of cases in the Flossing+/Probing- group, and approximately 45% of collagen depletion in the gingiva portion immediately below the contact point, probing was not able to

clinically detect these histological changes. In contrast, flossing when used against gingiva detected bleeding in 100% of cases, being more sensitive to clinically identify inflammatory changes in this region immediately below the contact point.

ACKNOWLEDGMENTS

The authors would like to thank the staff at the UFSM Pathology Laboratory (LAPBIO) for their involvement and availability to support the histological study. The authors are also grateful to the Brazilian fostering agency *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (*CAPES).

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TABLE 1 Characteristics of subjects in three evaluation groups

		Flossing+ Probing+ (n = 26)	**Flossing+ Probing- (n = 26)**	**Flossing- Probing- (n = 26)**
Gender#	M	15 (57.69)	12 (46.15)	11 (42.31)
	F	11 (42.31)	14 (53.85)	15 (57.69)
Age*		39.88 (14.89)	37.26 (12.26)	45.84 (12.68)
Skin color#	White	18 (69.23)	21 (80.77)	20 (76.92)
	Non-White	8 (30.77)	5 (19.23)	6 (23.08)
Papilla location#	Anterior	7 (26.93)	8 (30.77)	6 (23.08)
	Posterior	19 (73.07)	18 (69.23)	20 (76.92)

All comparisons between groups: non-significant (P>0.05, chi-Square test for gender, skin color and papilla location, one-way ANOVA for age); *Mean (SD); #n (%); anterior (from incisor-incisor to canine-pre-molar) and posterior (from pre-molar-pre-molar to molar-molar)

TABLE 2 Comparison of volume of GCF (Periotron units) between positive and negative sites considering all subjects (n = 49) and separately for women (n = 33) and men (n = 16)

		Mean (SD)	**Min–Max**	**Median (P25%–P75%)**
Flossing-	Female[#]	37.16 (27.29)	2–112	32.5 (15.3–48.6)
	Male[&]	31.34 (17.70)	1–67	32 (18–44)
	Total[*]	35.38 (24.71)	1–112	32 (16.75–47.75)
Flossing+	Female[#]	55.50 (36.70)	10–145	42.3 (28.7–87.3)
	Male[&]	52.73 (22.95)	26–107	57.5 (34–68.5)
	Total[*]	54.71 (32.90)	10–145	43 (29.25–68.75)

SD: standard deviation
[#] Female flossing- versus flossing+ (n = 33), P=0.001, Wilcoxon's signed rank test
[&] Male flossing- versus flossing+ (n = 16), P = 0.003, Wilcoxon's signed rank test
* Total flossing- versus flossing+ (n = 49), P < 0.001, Wilcoxon's signed rank test

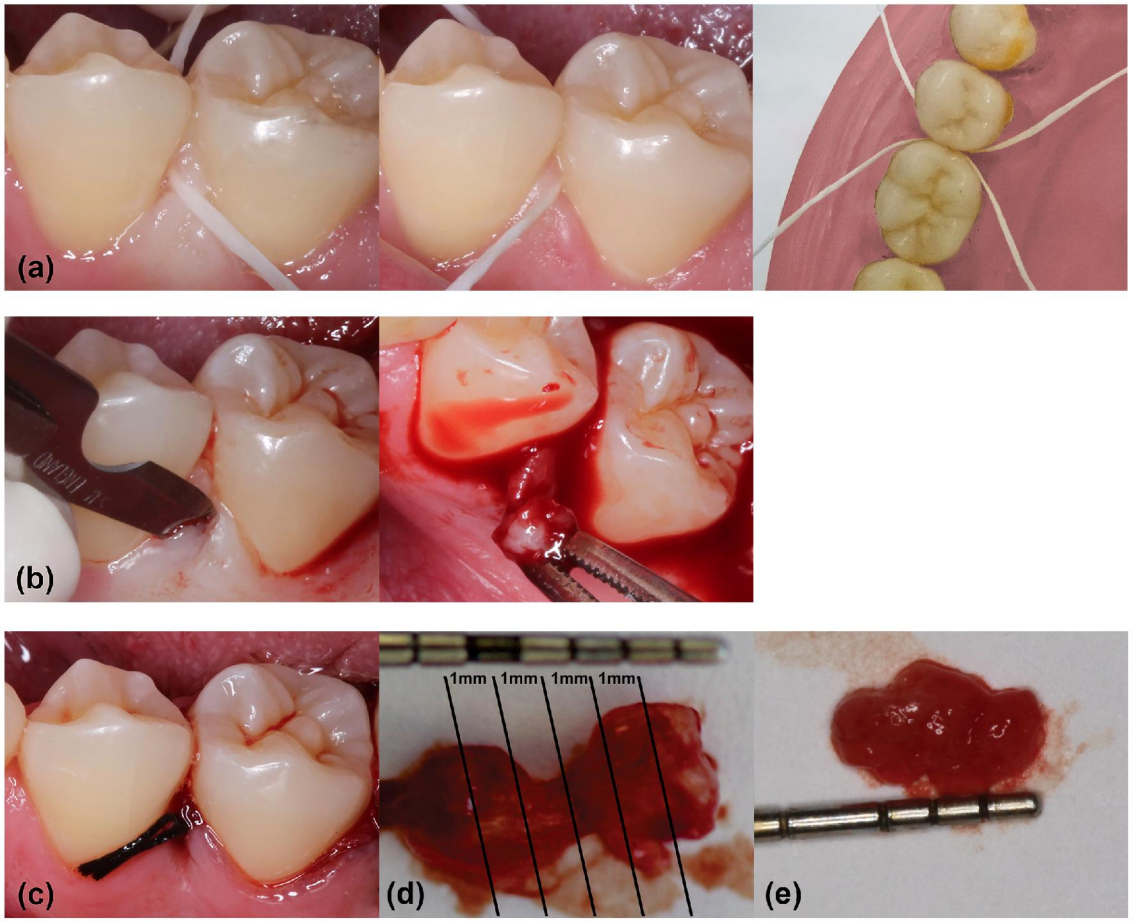
Figure Legends

FIGURE 1 (a) Evaluation method with dental floss. (b) Split flap by buccal and papilla removal. (c) Suture demonstrating no esthetic impairment. (d) Entire papilla. (e) Mid-interproximal tissue.

FIGURE 2 (a) score 0 – no inflammatory cells; (b) score 1 – sparse; (c) score 2 – moderate; (d) score 3 – intense. Magnification: 100×

FIGURE 3 Studies flowchart

FIGURE 4 Comparison of volume of GCF (Periotron units) between positive and negative sites considering all sites (n = 172) and separately for anterior (n = 102) and posterior (n = 70) regions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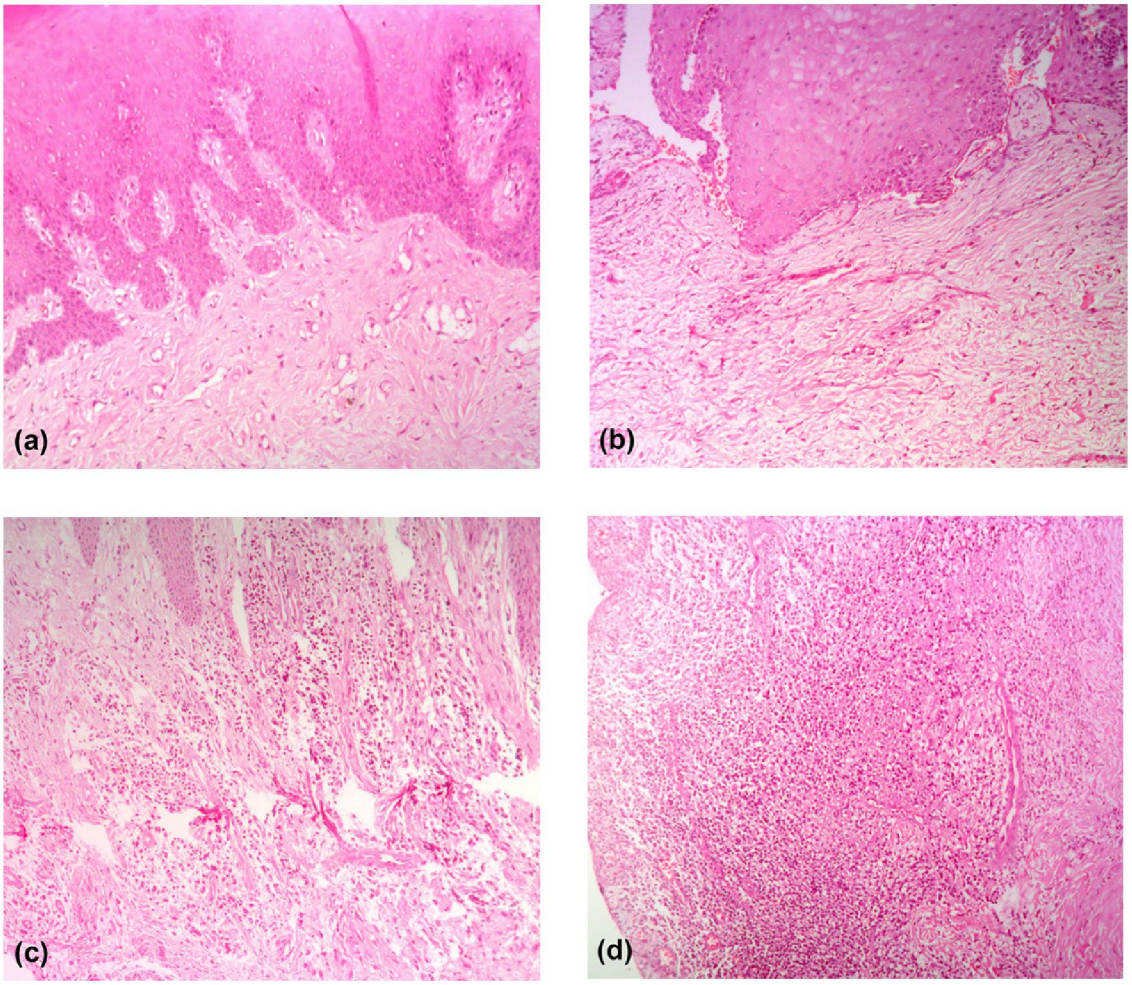
(a)
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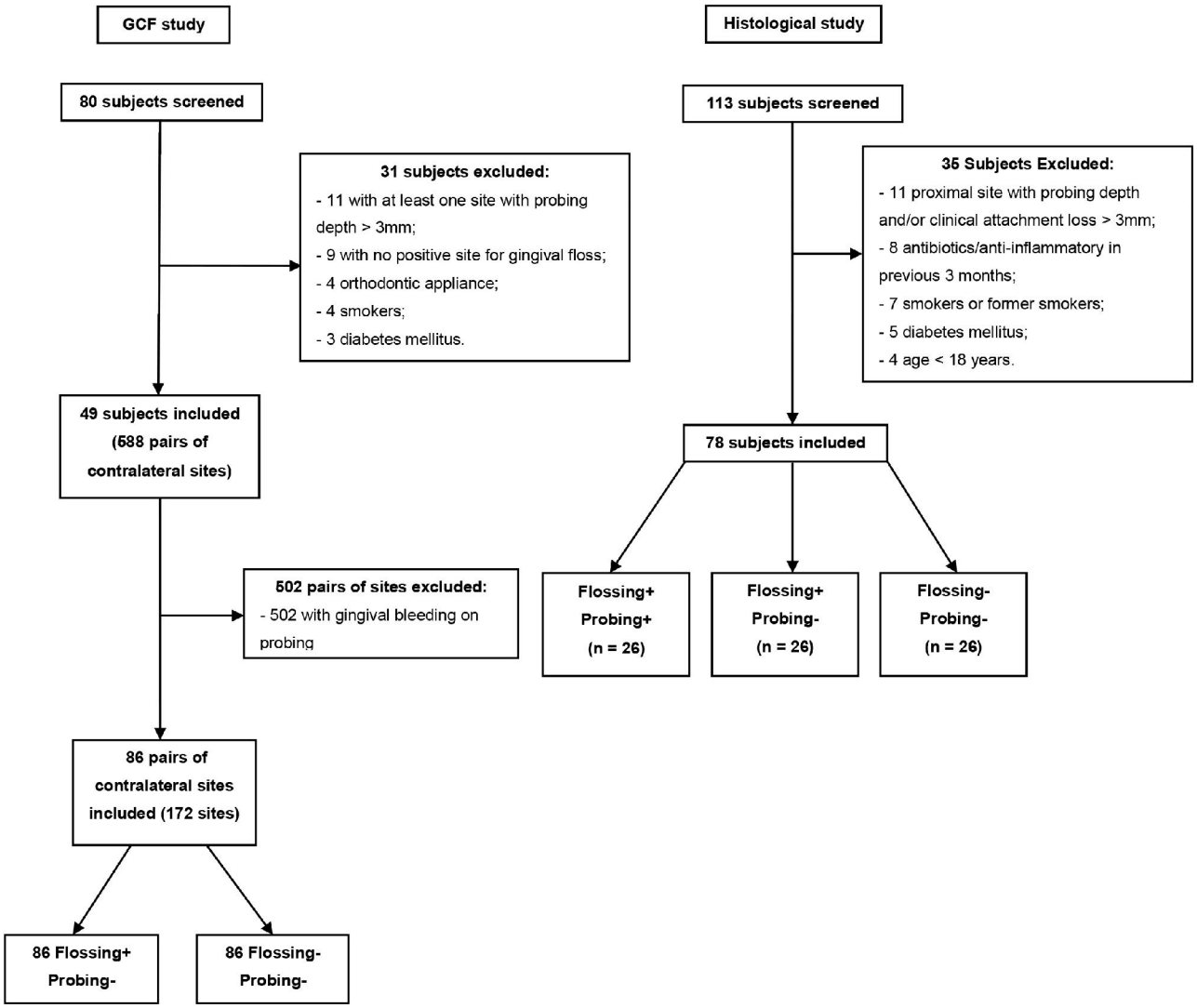
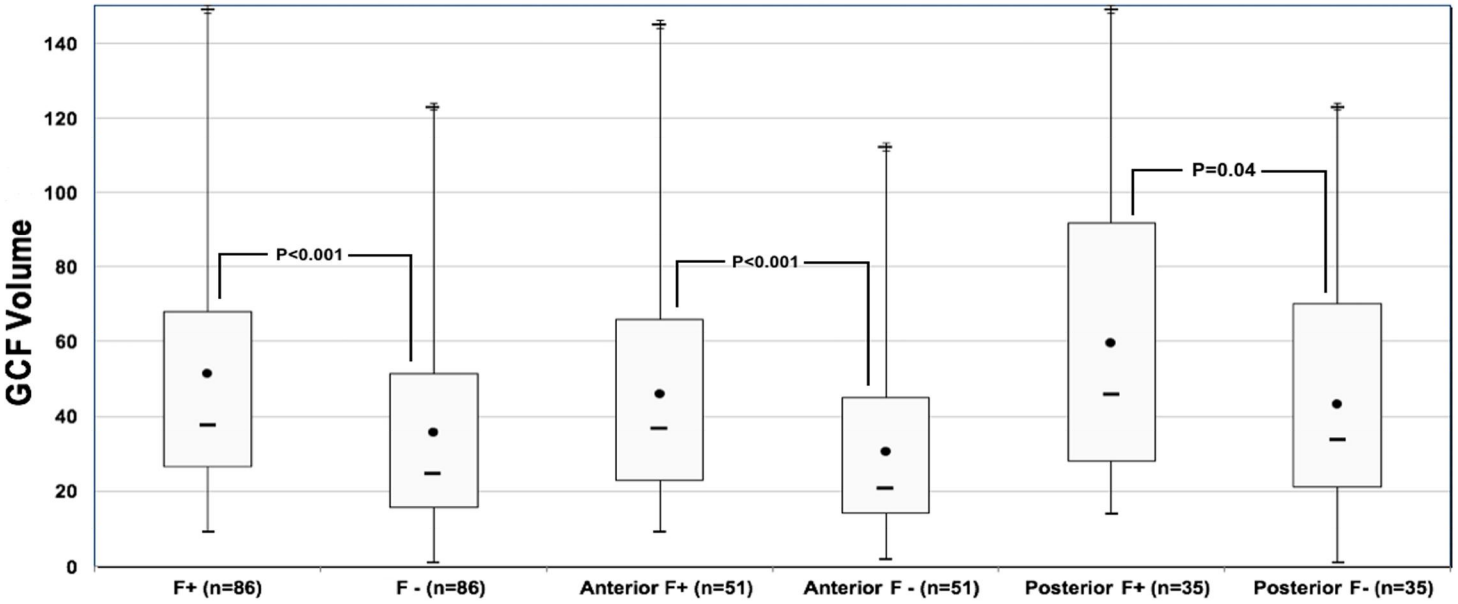

GCF study
80 subjects screened
31 subjects excluded:
- 11 with at least one site with probing depth > 3mm;
- 9 with no positive site for gingival floss;
- 4 orthodontic appliance;
- 4 smokers;
- 3 diabetes mellitus.
49 subjects included (688 pairs of contralateral sites)
502 pairs of sites excluded:
- 502 with gingival bleeding on probing
86 pairs of contralateral sites included (172 sites)
86 Flossing+ Probing-
86 Flossing- Probing-
Histological study
113 subjects screened
35 Subjects Excluded:
- 11 proximal site with probing depth and/or clinical attachment loss > 3mm;
- 8 antibiotics/anti-inflammatory in previous 3 months;
- 7 smokers or former smokers;
- 5 diabetes mellitus;
- 4 age < 18 years.
78 subjects included
Flossing+ Probing+ (n = 26)
Flossing+ Probing- (n = 26)
Flossing- Probing- (n = 26)



F Flossing; GCF Gingival Crevicular Fluid; • Mean; – Median; Wilcoxon's signed rank test

4 ARTIGO 2 – DIAGNOSIS OF GINGIVITIS: STATE OF THE ART

Este artigo foi publicado no periódico *Journal of Dentistry & Oral Disorders*, ISSN: 2572-7710. As normas para publicação estão descritas no Anexo B.

Diagnosis of gingivitis: State of the Art

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ABSTRACT

Gingivitis is a disease caused by accumulation of supragingival biofilm. Considering the fact that gingivitis always precedes periodontitis, the diagnosis of marginal inflammation allows monitoring the quality of at-home plaque control. Moreover, several inflammatory and/or autoimmune conditions are associated with oral mucosal manifestations. The aim of this review is to present, compare, and discuss the main methods for the diagnosis of gingivitis and autoimmune conditions associated with gingivitis. The autoimmune diseases may be diagnosed by various methods including histological examination, direct and indirect immunofluorescence microscopy, immunoblotting and quantitative immunoassay. Some gingival indices evaluate visual aspects and the presence of marginal bleeding after mechanical stimulation whereas other indices just evaluate visual aspects. In addition, some use only the extent, the length, or just the presence or absence of bleeding. Despite the fact that the collection and analysis of gingival crevicular fluid are suitable for scientific research, the diagnosis of gingivitis made by marginal bleeding is easier, faster, cheaper and, therefore, more widely applicable to routine clinical practice and epidemiological studies. The clinical diagnosis of gingivitis can thus be done by different methods. In the clinical setting, dichotomous scoring of bleeding seems to be simpler, faster, and less subjective. In the research setting, visual criteria associated with the presence of bleeding seem to more clearly detect small changes in gingival tissues, increasing the sensitivity of the selected method. The various gingival indices available share similarities and differences, but none of them is universally applied or accepted, and their selection depends on what will be evaluated.

Keywords: Inflammation; Diagnosis; Periodontics; Indices.

ABBREVIATION

Gingival crevicular fluid (GCF)

INTRODUCTION

Gingivitis is the first sign of imbalance in the periodontal health-disease process. Plaque-induced gingivitis is caused by the accumulation of supragingival plaque around the gingival margin and is triggered between 10 and 21 days according to interindividual differences [1]. Plaque control rebalances the health-disease process and promotes the restoration of gingival health between 7 and 10 days [1-4]. Gingivitis is confined to the tissues that protect the teeth and, while not causing irreversible damage, its presence is a prerequisite for the establishment of a subgingival biofilm, which eventually leads to periodontitis [5-8]. Advanced periodontitis and dental caries are the most common causes of tooth loss in adults [9, 10]. In addition, they are associated with greater impacts on quality of life for causing halitosis, pathologic tooth migration, gingival recession, bleeding, among others [11]. Besides the fact that gingivitis precedes periodontitis, the diagnosis of gingival inflammation helps the dentist to monitor sites where plaque control should be improved, i.e., the presence or absence of gingivitis is directly related to the frequency of appropriate at-home care [1]. Therefore, the diagnosis, prevention, and treatment of gingivitis are needed.

Oral lesions may be the first and occasionally the only manifestation for a number of immune-mediated diseases that affect the skin and mucosal surfaces. Autoantibodies directed against structural compounds of the skin and oral mucosa and/or inflammatory infiltrates cause tissue damage. An accurate diagnosis can be reached by utilizing a number of diagnostic tools such as direct immunofluorescence microscopy of a perilesional biopsy and serological testing for circulating autoantibodies in conjunction with histopathological analysis. An early and precise diagnosis of autoimmune and inflammatory diseases with oral involvement is a prerequisite for their effective treatment. That being considered, the present review aims to present and discuss the different methods for the diagnosis of gingivitis described in the literature.

LITERATURE REVIEW

Epidemiological studies have shown a high prevalence of gingivitis and periodontitis in the general population [12, 13]. Among periodontal diseases, gingivitis is the most prevalent one, affecting almost 100% of individuals [14]. High rates of supragingival biofilm accumulation have also been observed, denoting failure in oral hygiene self-care, especially in the cleaning of proximal surfaces [15, 16]. Consequently, gingivitis is quite frequent at these sites [17].

Visual signs (redness, swelling, change in texture) and/or presence of marginal bleeding have been included as components of different indices used for the diagnosis of gingivitis [18-31]. Muhlemann & Son (1971) reported that a gingival index should be able to detect the earliest sign of

gingivitis. However, there is still no consensus in the literature about the chronology of visual and inflammatory events in the pathophysiological course of gingivitis.

Diagnosis of gingivitis

Gingivitis can be diagnosed by various methods. Although histological evidence of inflammation is an accurate method to assess gingivitis, biopsies are impracticable. Therefore, a less invasive method is required [32]. The measurement of GCF has proven to play an important role in the assessment of gingivitis [33-36].

Categorical scores have been used by different indices. Such indices combine visual aspects and the presence of marginal bleeding after mechanical stimulus [26, 27, 29]. Other indices only evaluate visual aspects [25, 31]. Also, some use only the extent of bleeding [23, 28], bleeding time [19, 30], or just the presence or absence of bleeding [18, 20-22, 24]. It is difficult to determine which criteria (GCF volume, visual signs, or gingival bleeding) best indicate the inflammatory condition of the gingiva given that some evidence has shown weak correlations between clinical criteria/gingival fluid and inflammatory status observed histologically [35, 37-40]. Thus, comparisons between different diagnostic methods could be inaccurate.

According to Carter & Barnes (1974), a good index for evaluating gingivitis must have well-established validity in order to assess what actually needs to be assessed and enough sensitivity to detect slight changes. Moreover, its reproducibility by the same or different examiners is also crucial. Finally, an index should be simple to use, require few tools, and be as free as possible from subjective interpretation.

Several methods for stimulation of marginal bleeding have been used: periodontal probe [18, 19, 26, 28-30], wooden interdental cleaner [21, 27], dental floss [20], dental tape [22], toothbrush [23], and interdental brush [24]. Table 1 shows the main features of the indices used to date.

GCF

GCF results from the interaction between the bacterial biofilm attached to the tooth surface and periodontal tissue cells [41]. It is a complex mixture of substances derived from blood serum, leukocytes, structural cells of the periodontium, and oral microorganisms. Thus, GCF analysis is a noninvasive measure that assesses the pathophysiological state of the periodontium at a specific site [42].

GCF is constantly secreted [43]. Löe & Holm-Pedersen (1965) reported that GCF flow is proportional to the severity of inflammation, thereby highlighting its importance as an assessment tool. They concluded that, in order to obtain valid measurements of the fluid, paper strips should be positioned at the entrance (extrasulcular method) rather than within the gingival sulcus (intrasulcular method proposed by Brill & Krasse in 1958) until some resistance is felt. These methodological differences probably affect the results, since even a gentle insertion into the gingival sulcus causes sufficient damage, changing the permeability of the epithelium and, consequently, increasing the amount of gingival fluid [46].

A low GCF flow is associated with healthy tissue while a high GCF flow indicates inflamed tissues [33, 35]. Visual signs of inflammation have been associated with an increased GCF flow [35, 36, 45, 47], and so has gingival bleeding [30, 35, 36, 48-50]. A higher GCF flow is observed in multirooted teeth when compared to

single-rooted ones, probably due to the difficulty faced by individuals in performing oral hygiene on these teeth or to the anatomy of molars (greater interproximal root surface, possibility of larger root irregularities, and abundant vascularization) [34].

Several methods have been developed for GCF collection, such as the gingival washing method [51], the use of microcapillary tubules or micropipettes [52], and absorbent filter paper strip collection [44]. The downside of the washing method is that it does not provide information on the volume of collected fluid and, although the capillary tubing method measures different amounts of fluid, it requires a long time (around 30 minutes per site) for an accurate collection of small volumes [53]. Moreover, unlike the absorbent filter paper strip method, which is fast, easy to use, minimally invasive and has traditionally been the method of choice [53, 54], the use of capillary tubes can cause trauma and affect the measurement of the volume and components of the collected fluid.

Different types of absorbent strips are available: Durapore, Millipore [55], Whatman chromatography [56], and absorbent filter paper strip [57]; however, none of them have had their validity tested with Periopaper®. Periopaper® is a filter paper strip widely recognized as a method of choice for GCF collection via absorption [54, 58].

Due to the importance of quantifying GCF volume, a number of methods have been described for measuring it via absorption: colorimetry, weighing, and use of an electronic apparatus (Periotron®). Colorimetry is a valid method that uses ninhydrin or fluorescein to indicate areas of absorption; however, the stains obtained by this technique and by weighing do not allow the analysis of GCF components. More recently, the introduction of an electronic device known as Periotron® has allowed a more accurate determination of GCF volume, enabling subsequent laboratory research into sample composition [53]. The equipment measures the electrical capacitance of the filter paper strip [59]. There are three Periotron® models (600, 6000, and 8000) and all have shown accuracy in GCF volume measurement [53]. Periotron® 8000 (Ora Flow Inc., Amityville, NY, USA) quantifies the amount of GCF or saliva collected with filter paper strips and, by using a computer program, it converts the data into a unit of volume [60]. It is recommended that the GCF collected on Periopaper® strips be immediately transferred (within 0-2s) to Periotron® to prevent the material from evaporating [61, 62].

Other operational and technical aspects, such as collection time; contamination of GCF samples by blood, saliva, and plaque; and air temperature and humidity, can interfere with measurement accuracy [34, 53, 60, 63, 64]. Both knowledge and control of these aspects ensure that the observed results will actually reflect the condition of the investigated tissue. Previous studies on Periotron® have suggested that the filter paper strips should remain in place for 5s [53]. However, alternative approaches have been developed to increase the GCF volume available for subsequent laboratory analysis [53]. One of them consists in leaving the strip at the entrance of the gingival sulcus for 30s [65] or 3 min [36, 44]. A study with gingivitis patients compared these two collection times and found no difference in fluid volume proportional to the increase in measurement time [66]. Based on the results, the authors recommend restricting the collection time to 30s, thus safely determining the extent of gingival infection. Nevertheless, the problem with a long collection time is that the nature of the fluid samples may change, especially regarding protein concentration [67].

The volume and flow rate of GCF are indicators of vascular permeability changes at the early stages of inflammation [68]. Then, the standard clinical measurements used to determine gingival inflammation may be less sensitive than GCF results, showing better diagnostic accuracy of this method at earlier stages of gingivitis [41, 53]. However, although GCF collection and analysis are suitable for scientific research, the diagnosis of gingivitis made by marginal bleeding is easier, faster, cheaper and, therefore, more widely applicable to routine clinical practice and epidemiological studies.

Visual criteria *versus* marginal bleeding

Some gingival indices have been based on clinical features of inflammation, with some components evaluated noninvasively by visual examination (color, texture, shape, spontaneous bleeding), and inflammatory components measured invasively after some stimulus. The visual signs of gingival inflammation include redness of the gingival margin, which becomes evident from vasodilation, and increase in the number of vascular units in the subepithelial connective tissue [69], since edema and the smooth texture of the free gingiva indicate loss of fibrous connective tissue and extravasation of inflammatory cells into the extracellular matrix. Bleeding after a stimulus is due to microulcerations in the sulcular epithelium [70]. This parameter, for being objective and easy to use, has often been considered in the evaluation of the gum [71-73].

A diagnostic index for gingival conditions should be simple and quick to use, with clear and comprehensible criteria, and should also be sensitive to identify variations at different stages of the disease [25]. In this sense, visual criteria (color, swelling, texture) hinder clinical and epidemiological application as they are time-consuming, do not allow easy assessment of proximal regions (especially of posterior teeth), are subjective, and are not determined only by inflammatory components, but also by variations in the intensity of melanogenesis and in the degree of keratinization and vascularity [70].

Given the limitations of visual aspects in the diagnosis of gingival changes, the presence or absence of bleeding on probing [18] is more universally applicable in clinical and epidemiological studies and in clinical practice [20, 29]. Although gingival bleeding on probing is not a good diagnostic indicator of clinical attachment loss, its absence is an excellent negative predictive sign of future insertion loss [74].

Some authors have shown that, even in the absence of visual changes, a significant percentage of sites show marginal bleeding [29, 75, 76], which means that the presence of bleeding is a sign that precedes visual changes [18, 20, 29, 75-77]. Other authors have noted that changes in color and contour precede marginal bleeding at the early stages of gingivitis, [70, 78]. This discrepancy may be due to the subjectivity of visual inspection and to the differences in the techniques used to evaluate bleeding [70], possibly increasing the number of false positive results in consequence of trauma after mechanical stimulation.

Periodontal probe *versus* dental floss/tape

Variations in probing depth and angulation may interfere with the results by stimulating bleeding in deeper regions of the pocket or by causing injury, hindering the diagnostic value of marginal bleeding on probing [79, 80].

There is evidence that gingival inflammation in the proximal region likely arises in the center of the papilla [21, 81, 82], an area that is not often thoroughly assessed by the probe at sites without attachment loss and with an established point of contact. Thus, it appears that a marginal probe for the diagnosis of gingival conditions has a somewhat limited use in proximal regions. Therefore, the use of dental floss/tape as a diagnostic tool may be advantageous in the proximal region as it allows contact along the full length of the papilla.

Dental floss/tape *versus* wooden interdental cleaner *versus* interdental brush

Gingival indices that use wooden interdental cleaner for detecting proximal gingivitis [21, 27] can cause trauma to the tissue due to the shape and rigidity of these devices and should thus be used with caution. However, the index proposed by Hofer et al. (2011), which relies upon the insertion of an interdental brush into the vestibular region below the point of contact, cannot be used when the papilla fills the interproximal region. Among the four devices assessed, dental flosses and tapes seem to be the most suitable to detect proximal gingivitis, possibly because they do not cause trauma to the gingival tissue and can be inserted into the proximal sites with or without the presence of papillae.

Gingivitis associated with inflammatory and autoimmune diseases

A group of autoimmune diseases is characterised by autoantibodies against epithelial adhesion structures and/or tissue-tropic lymphocytes driving inflammatory processes resulting in specific pathology at the mucosal surfaces and the skin [83]. The most frequent site of mucosal involvement in autoimmune diseases is the oral cavity. Broadly, these diseases include conditions affecting the cell-cell adhesion causing intra-epithelial blistering and those where autoantibodies or infiltration lymphocytes cause a loss of cell-matrix adhesion or interface inflammation [84]. Several inflammatory and/or autoimmune conditions such as chronic ulcerative stomatitis, lichen planus, mucous membrane pemphigoid, pemphigus vulgaris, erythema multiforme, plasma cell gingivitis and graft-versus-host disease are associated with oral mucosal manifestations, including “desquamative gingivitis” [85]. This term was introduced to describe the presence of erythema, localized or generalized desquamation and /or erosion on the buccal aspect of attached gingiva mainly of the anterior teeth. In some cases, marginal gingiva may also be affected. Gingival desquamation has a subacute or chronic onset in the majority of cases, with variable degrees of extension and distribution [85].

Studies show that oral lichen planus is the most common immune-mediated disorder affecting the oral cavity, followed by pemphigus vulgaris and mucous membrane pemphigoid [86, 87]. Moreover, oral mucosa can be the first affected mucosal surface in many of these conditions, a fact that emphasizes the need for better understanding of clinical features and diagnostic tools for autoimmune diseases among practitioners. Precise and early diagnosis greatly facilitates timely, effective and specific treatment [86].

The definitive, accurate diagnosis of autoimmune diseases requires the detection of immunoreactant deposits in the tissues and the circulating autoantibodies by direct and indirect immunofluorescence microscopy, respectively. Direct immunofluorescence microscopy helps to detect molecules such as immunoglobulins and complement within biopsy specimens [88]. Selection of the site for the biopsy specimen is important. Direct IF microscopy is performed on non-bullous or non-eroded skin or mucosa (i.e. erythematous or normal appearing

tissue adjacent to blisters or erosions), because immune deposits may be degraded in the area where the dermal-epidermal separation occurs, leading to false negative results. False negative results may also occur as a result of improper handling or faulty preservation of the biopsy, which must be frozen immediately and stored at temperatures below −70 °C or placed in a saline or a special Michel's medium for transport for no longer than 48 hours for subsequent immunofluorescence testing [89].

Indirect immunofluorescence microscopy, is a test in which patient's serum is examined for the presence of circulating autoantibodies to a defined antigen. This test allows the differentiation between serum autoantibodies that bind to the roof and those that stain the floor of the artificial split reflecting the molecular difference in autoantibody specificity [89].

A number of other immunoassays, including enzyme-linked immunosorbent assay (ELISA), immunoblot or immunoprecipitation are available to facilitate the characterization of the molecular specificity of autoantibodies. Of these techniques, the ELISA is most commonly used. With the identification of target antigens and advancement of molecular biology and recombinant technology, antigens have been produced in bacteria and eukaryotic cells [88]. These recombinant, cell derived forms of the target antigens have been utilized in the development of sensitive and specific ELISA kits for detection of circulating autoantibodies. ELISA using recombinant antigens has several advantages over indirect immunofluorescence techniques on tissue sections. It provides information on the molecular specificity of autoantibodies, it is easy to perform and readily amenable to standardization, and, importantly gives quantitative results. Therefore, these are exquisite parameters for monitoring diseases, in which levels of serum autoantibodies have been shown to correlate with disease activity. Several commercially available ELISA kits are now used for the diagnosis and monitoring of immune-mediated diseases [90].

While the autoimmune disease may be suspected based on clinical manifestations, demonstration of tissue-bound and circulating autoantibodies, or lymphocytic infiltrates, by various methods including histological examination, direct and indirect immunofluorescence microscopy, immunoblotting and quantitative immunoassay is a prerequisite for definitive diagnosis.

CONCLUDING REMARKS

Gingivitis can be clinically diagnosed by different methods. In the clinical setting, dichotomous scoring of bleeding seems to be simpler, faster, and less subjective. Moreover, the absence of gingival bleeding on probing is desirable, indicating low risk of future clinical attachment loss. In the research setting, visual criteria associated with the presence of bleeding seem to more clearly detect small changes in gingival tissues, increasing the sensitivity of the selected method.

Moreover, given the frequency of oral involvement and the fact that oral mucosa is the initially affected site in many cases, the informed practitioner should be well acquainted with diagnostic and therapeutic aspects of autoimmune dermatosis with oral involvement.

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Table 1

Index	Author(s) (Year)	Instrument/Bleeding time (seconds)	Scores	Evaluated sites
Papillary Marginal Attachment (PMA)	Schour & Massler (1947)	Only visual/Not applicable	0: without gingivitis in any area of the mouth 1: mild gingivitis - inflammation located in the papilla in 1 to 3 of the 6 lower anterior teeth 2: moderate gingivitis - extension of inflammation to the gingival margin in more than 3 regions or teeth. Redness and glazing are increased in intensity 3: Severe gingivitis – extension of the inflammation to the attached gingiva. Redness, swelling, loss of dotted and tone. Spontaneous bleeding is usually present 4: very severe gingivitis - very severe generalized periodontitis	Buccal region of all teeth; papillary, marginal, and attached gingivae evaluated separately
Gingival Index (GI)	Löe (1967)	Probe*	0: normal gingiva 1: mild inflammation - slight color change and slight edema. No bleeding on probing 2: Moderate inflammation - redness, swelling, and glazing. Bleeding on probing 3: severe inflammation - marked redness and swelling. Ulceration. Tendency to spontaneous bleeding	Buccal, distobuccal, mesiobuccal, and lingual regions of all teeth
Sulcus Bleeding Index (SBI)	Muhlemann & Son (1971)	Probe (parallel to the long axis of the tooth)/30	0: healthy appearance of papillary and marginal gingiva, without bleeding from the sulcus 1: healthy appearance of papillary and marginal gingivae, no color change and no edema, but marginal bleeding on probing 2: bleeding on probing and color change due to inflammation. No edema 3: Bleeding on probing, color change, and slight edema 4: bleeding on probing, color change, and evident swelling or bleeding on probing and evident edema 5: bleeding on probing and spontaneous bleeding and color change, severe edema with or without ulceration	Buccal, distobuccal, mesiobuccal, and lingual regions of all teeth
Gingival Bleeding Index (GBI)	Carter & Barnes (1974)	Unwaxed dental floss (Twice)/Not reported; 30s is	Dichotomous (presence/absence of bleeding)	Interproximal region of all teeth except between 2nd and 3rd molars; areas cannot be evaluated when

		allowed for reinspection		the position of the tooth, diastema, or other factor has a desirable interproximal relationship
Bleeding Index (BI)	Edwards (1975)	Dental tape (Twice)/15	Dichotomous (presence/absence of bleeding)	Interproximal region of all teeth
Gingival Bleeding Index (GBI)	Ainamo & Bay (1975)	Probe (3 to 4 times)/10	Dichotomous (presence/absence of bleeding)	Buccal region of all teeth
Papillary Bleeding Index (PBI)	Muhlemann (1977)	Probe*	0: no bleeding 1: only one bleeding point 2: many isolated bleeding points or only a small area of bleeding 3: interdental triangle filled with blood after probing 4: profuse bleeding when probing, blood spreads towards the marginal gingiva	Interproximal region of all teeth
Papillary Bleeding Score (PBS)	Loesche (1979)	Wooden interdental cleaner*	0: Healthy gums, no bleeding 1: reddish gum with edema, no bleeding 2: bleeding without flow 3: bleeding with flow to marginal gingiva 4: profuse bleeding 5: severe inflammation; marked redness and edema, tendency to spontaneous bleeding	Interproximal region of all teeth
Modified Papillary Bleeding Index (MPBI)	Barnett et al. (1980)	Probe (Once)/0-30	0: no bleeding within 30s 1: bleeding between 3 and 30s 2: bleeding within 2s 3: Immediately bleeding upon probe placement	Mesial region of all teeth
Bleeding Time Index (BTI)	Nowicki et al. (1981)	Probe (Once or twice)/0-15	0: no bleeding within 15s of second probing 1: bleeding within 6 to 15s of second probing 2: bleeding within 11 to 15s of first probing or within 5s of second probing 3: bleeding within 10s after first probing 4: spontaneous bleeding	All teeth
Eastman Interdental Bleeding Index (EIBI)	Caton & Polson (1985)	Interdental wooden cleaner (4 times)/15	Dichotomous (presence/absence of bleeding)	Buccal in interproximal regions

Quantitative Gingival Bleeding Index (QGBI)	Garg & Kapoor (1985)	Dental brush*/30s is allowed for reinspection	0: no bleeding on brushing; bristles free of blood stains 1: slight bleeding on brushing; bristle tips stained with blood 2: moderate bleeding on brushing; about half of bristle length from tip downwards stained with blood 3: severe bleeding on brushing; entire bristle length of all bristles including brush head covered with blood	1 score for each 6 segments: canine to canine or premolars and molars, left or right, in the upper or lower arches
Modified Gingival Index (MGI)	Lobene et al. (1986)	Only visual/Not applicable	0: no inflammation 1: mild inflammation; slight color change, slight change in texture but not in all papillary or marginal gingivae 2: mild inflammation; same criterion as in score 1 but involving all papillary unit or marginal gingiva 3: moderate inflammation; glazing, redness, swelling and/or hypertrophy of the papilla or marginal gingiva 4: severe inflammation; marked redness, swelling and/or hypertrophy of the papilla or marginal gingiva, spontaneous bleeding or ulceration	Buccal, distobuccal, mesiobuccal, and lingual regions of all teeth
Bleeding on Interdental Brushing Index (BOIB)	Hofer et al. (2011)	Interdental brush (Once)/30	Dichotomous (presence/absence of bleeding)	Interproximal region of all teeth

5 DISCUSSÃO

Os dados confirmaram nossa hipótese conceitual de expressão inflamatória significativamente maior em sítios sangrantes ao fio dental em comparação com sítios não sangrantes ao fio dental. Esses achados foram consistentes em diferentes desfechos avaliados e complementam dados anteriores por Grellmann et al. (2016), reforçando a hipótese de que o fio dental é mais sensível do que a sondagem do sulco interproximal na detecção do sangramento interproximal. Estes resultados são provavelmente devido ao maior contato do fio dental com o tecido conjuntivo inflamado, o qual parece não ser alcançado suficientemente pela sonda periodontal em sítios proximais com ponto de contato.

A quantificação de fibras colágenas no grupo de sítios não sangrantes com fio dental e com sonda periodontal revelou que a fração de área ocupada por feixes de colágeno (AA%) foi de 60,01 ± 3,66. Uma diminuição significativamente maior de AA% foi observada no grupo de sítios sangrantes ao fio dental e não sangrantes a sonda periodontal (45,78 ± 4,55) quando comparado com o grupo de sítios não sangrantes. No grupo de sítios sangrantes com fio dental e sonda periodontal observou-se uma diminuição significativa de AA% (40,90 ± 3,68). Esses achados revelaram uma clara diferença em relação à perda de integridade do tecido conjuntivo entre os grupos do estudo.

A redução da área total ocupada por colágeno foi descrita por outros autores na gengiva inflamada (ALMEIDA et al., 2015; GOGLY et al., 1997; SÉGUIER; GODEAU; BROUSSE, 2000; YOUNES et al., 2009). De acordo com Ejeil et al. (2003), houve uma redução de 20% na área gengival ocupada por colágeno quando compara-se tecidos gengivais normais e severamente inflamados em pacientes humanos. Além disso, eles também descreveram que uma área de cerca de 60% é ocupada por colágeno em condições normais, em acordo com nossos resultados.

Informações de Grellmann et al. (2016) podem ser revistas quando considera-se a possibilidade de sangramento gengival ocorrendo devido ao trauma mecânico. Em primeiro lugar, o número de sítios não sangrantes em um primeiro exame e sangrantes em um segundo exame após 10 minutos foi de 19,5%, 9,4% e 12,5%

para ISG-ISG, FD-FD e FG-FG, respectivamente. Este aumento dos sítios sangrantes mostra que parte do sangramento do segundo exame é devido ao trauma, com a sondagem (ISG) tendo o maior efeito traumático. Em segundo lugar, embora a maior probabilidade de sangramento no segundo exame ser devido ao efeito traumático mecânico seqüencial, 38,9% e 58,3% dos sítios sangrantes após FD e FG no primeiro exame não sangraram após ISG realizado no segundo exame. Estes achados reforçam a menor sensibilidade da sonda periodontal comparadas às técnicas de uso do fio dental (FD e FG). Considerando estas informações, realizamos o ISG sempre como segundo exame para ambos os estudos (VFCG e histológico) para caracterizar o grupo de sítios sangrantes ao fio dental e não sangrantes com a sonda periodontal.

As diferenças de VFCG e inflamação observadas entre sítios sangrantes ao fio dental e não sangrantes à sonda periodontal comparados a sítios não sangrantes com fio dental e com a sonda mostram que o uso do fio dental possui capacidade para diferenciar sítios com diferentes expressões inflamatórias clínicas e subclínicas e corroboram a hipótese de que o fio dental é mais sensível do que a sondagem interproximal do sulco. Além disso, ajuda a descartar a hipótese de trauma gengival. Nossos achados do VFCG corroboram estudos prévios que encontraram associação positiva entre VFCG mais elevado e presença de inflamação gengival (DANESHMAND; WADE, 1976; HATIPOGLU et al., 2007; LÖE; HOLM-PEDERSEN, 1965; OLIVER; HOLM-PEDERSEN; LÖE, 1969; RÜDIN; OVERDIEK; RATEITSCHAK, 1970; SHAPIRO; GOLDMAN; BLOOM, 1979).

Outra observação de Grellmann et al. (2016) mostra que, na ausência de sangramento no primeiro exame, a sequência FD-FG revelou sangramento em 19% dos sítios no segundo exame. Quando a sequência foi invertida (FG-FD), 43,6% dos sítios que sangraram no primeiro exame com FG não sangraram na segunda avaliação com FD. Esses resultados levantam a hipótese de que FG é possivelmente mais sensível do que FD na detecção de sangramento gengival proximal. Nós hipotetizamos que FG é possivelmente mais sensível e sua característica de execução está mais próxima da técnica ISG, onde a porção interna do sulco gengival

é estimulada. Por esse motivo, escolhemos o método FG para ser comparado ao ISG.

Algumas estratégias foram desenhadas para reduzir a possibilidade de viés. Uma delas foi a seleção de sítios contralaterais no mesmo sujeito (estudo VFCG), o qual diminui a variabilidade relacionada à resposta inflamatória individual (por exemplo, susceptibilidade e variações hormonais) (KHOSRAVISAMANI et al., 2014; LIEW et al., 1991; LINDHE; ATTSTRÖM, 1967; TROMBELLI et al., 2004; WILTON et al., 1992) e diminui a variabilidade do sítio de coleta de VFCG, tentando padronizar a área sulcular de cada par de sítios comparados. Por exemplo, os dentes multirradiculares apresentam VGCF significativamente maior do que os dentes não unirradiculares (HATIPOGLU et al., 2007; OZKAVAF et al., 2000), possivelmente devido a características anatômicas, como maior área radicular interproximal (GOODSON, 2003; HATIPOGLU et al., 2007). Outra estratégia para reduzir o viés foi o cegamento do examinador nas análises histológicas, o cálculo do tamanho da amostra e a escolha da técnica intracrevicular (BRILL; KRASSE, 1958) para a avaliação do VFCG, o qual apresenta menor variabilidade na coleta de amostras de fluidos gengivais (EGELBERG; ATTSTRÖM, 1973). Por outro lado, as limitações para o presente estudo foram os critérios de elegibilidade rigorosos que limitam a validação externa dos resultados. Assim, estudos adicionais podem ser realizados em sujeitos com histórico de periodontite para esclarecer se o fio dental também pode ser utilizado neste tipo de paciente.

6 CONCLUSÃO

Os achados desta tese indicaram que o fio dental é um melhor método para se diagnosticar gengivite proximal em adultos quando comparado ao ISG. No entanto, esses resultados só podem ser inferidos para indivíduos adultos sem periodontite ou sem histórico de perda de inserção > 3mm em sítios proximais com ponto de contato.

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ANEXO A – NORMAS PARA PUBLICAÇÃO NO PERIÓDICO *JOURNAL OF CLINICAL PERIODONTOLOGY*

Author Guidelines

Content of Author Guidelines: 1. General, 2. Ethical Guidelines, 3. Manuscript Submission Procedure, 4. Manuscript Types Accepted, 5. Manuscript Format and Structure, 6. After Acceptance

Relevant Document: Sample Manuscript

Useful Websites: Submission Site, Articles published in *Journal of Clinical Periodontology*, Author Services, Wiley-Blackwell's Ethical Guidelines, Guidelines for Figures

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1. GENERAL

Journal of Clinical Periodontology publishes original contributions of high scientific merit in the fields of periodontology and implant dentistry. Its scope encompasses the physiology and pathology of the periodontium, the tissue integration of dental implants, the biology and the modulation of periodontal and alveolar bone healing and regeneration, diagnosis, epidemiology, prevention and therapy of periodontal disease, the clinical aspects of tooth replacement with dental implants, and the comprehensive rehabilitation of the periodontal patient. Review articles by experts on new developments in basic and applied periodontal science and associated dental disciplines, advances in periodontal or implant techniques and procedures, and case reports which illustrate important new information are also welcome.

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2. ETHICAL GUIDELINES

Journal of Clinical Periodontology adheres to the below ethical guidelines for publication and research.

2.1. Authorship and Acknowledgements

Authors submitting a paper do so on the understanding that the manuscript have been read and approved by all authors and that all authors agree to the submission of the manuscript to the

Journal.

Journal of Clinical Periodontology adheres to the definition of authorship set up by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE authorship criteria should be based on 1) substantial contributions to conception and design of, or acquisiation of data or analysis and interpretation of data, 2) drafting the article or revising it critically for important intellectual content and 3) final approval of the version to be published. Authors should meet conditions 1, 2 and 3.

It is a requirement that all authors have been accredited as appropriate upon submission of the manuscript. Contributors who do not qualify as authors should be mentioned under Acknowledgements.
Please note that it is a requirement to include email addresses for all co-authors at submission. If any of the email-addresses supplied are incorrect the corresponding author will be contacted by the journal administrator.

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

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

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

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

2.3 Clinical Trials
Clinical trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist should also be included in the submission material.

Journal of Clinical Periodontology encourages authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials/, http://isrctn.org/. The

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

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

2.5 Conflict of Interest and Source of Funding
Journal of Clinical Periodontology requires that all authors (both the corresponding author and co-authors) disclose any potential sources of conflict of interest. Any interest or relationship, financial or otherwise that might be perceived as influencing an author's objectivity is considered a potential source of conflict of interest. These must be disclosed when directly relevant or indirectly related to the work that the authors describe in their manuscript. Potential sources of conflict of interest include but are not limited to patent or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker's fees from a company. If authors are unsure whether a past or present affiliation or relationship should be disclosed in the manuscript, please contact the editorial office at cpeedoffice@wiley.com. The existence of a conflict of interest does not preclude publication in this journal.

The above policies are in accordance with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals produced by the International Committee of Medical Journal Editors (http://www.icmje.org/). It is the responsibility of the corresponding author to have all authors of a manuscript fill out a conflict of interest disclosure form, and to upload all forms together with the manuscript on submission. The disclosure statement should be included under Acknowledgements. Please find the form below:

Conflict of Interest Disclosure Form

2.6 Appeal of Decision
Under exception circumstances, authors may appeal the editorial decision. Authors who wish to appeal the decision on their submitted paper may do so by e-mailing the editorial office at cpeedoffice@wiley.com with a detailed explanation for why they find reasons to appeal the decision.

Please note that all revisions and resubmissions of papers should also include a separate rebuttal and a tracked changes document to assist in peer review.

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

3. MANUSCRIPT SUBMISSION PROCEDURE

Manuscripts should be submitted electronically via the online submission site http://mc.manuscriptcentral.com/jcpe. The use of an online submission and peer review site enables immediate distribution of manuscripts and consequentially speeds up the review process. It also allows authors to track the status of their own manuscripts. Complete instructions for submitting a paper is available on the submission site. Further assistance can be obtained from the Senior Editorial Office Assistant, Kim Harris, at cpeedoffice@wiley.com.

Please note that all revisions and resubmissions of papers should also include a separate rebuttal and a tracked changes document to assist in peer review.

3.1. Manuscript Files Accepted
Main manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rft) files (not write-protected). The text file must contain the entire manuscript including title page, abstract, clinical reference, main text, references, acknowledgement, statement of source of funding and any potential conflict of interest, tables, and figure legends, but no embedded figures. In the text, please reference any figures as for instance 'Figure 1', 'Figure 2' etc. to match the tag name you choose for the individual figure files uploaded.

Figure files should be uploaded separately to the main text. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing.

Manuscripts should be formatted as described in the Author Guidelines below.

Please ensure that ALL items (figures and tables) are cited in the main text.

3.2. Blinded Review
All manuscripts submitted to *Journal of Clinical Periodontology* will be reviewed by two or more experts in the field. Papers that do not conform to the general aims and scope of the journal will, however, be returned immediately without review. *Journal of Clinical Periodontology* uses single blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper.

3.3. Suggest a Reviewer
Journal of Clinical Periodontology attempts to keep the review process as short as possible to enable rapid publication of new scientific data. In order to facilitate this process, please suggest the name and current email address of one potential international reviewer whom you consider capable of reviewing your manuscript. In addition to your choice the editor will choose one or two reviewers as well.

3.4. Suspension of Submission Mid-way in the Submission Process
You may suspend a submission at any phase before clicking the 'Submit' button and save it to submit later. The manuscript can then be located under 'Unsubmitted Manuscripts' and you can click on 'Continue Submission' to continue your submission when you choose to.

3.5. E-mail Confirmation of Submission

After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may be caused by some sort of spam filtering on your e-mail server. Also, the e-mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their whitelist.

3.6 Resubmissions
If your manuscript was given the decision of reject and resubmit, you might choose to submit an amended version of your manuscript. This should be submitted as a new submission following the guidelines above under 3.2. In addition you should upload comments to the previous review as "supplementary files for review".

4. MANUSCRIPT TYPES ACCEPTED

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

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

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

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

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

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

Language: The language of publication is English. Authors for whom English is a second language may choose to have their manuscript professionally edited before submission to improve the English. It is preferred that manuscript is professionally edited. Please refer to English Language Editing Services offered by Wiley at http://wileyeditingservices.com/en/.

Japanese authors can also find a list of local English improvement services at http://www.wiley.co.jp/journals/editcontribute.html. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

Abbreviations, Symbols and Nomenclature: *Journal of Clinical Periodontology* adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. Abbreviations should be kept to a minimum, particularly those that are not standard. Non-standard abbreviations must be used three or more times and written out completely in the text when first used.

5.2. Structure

All articles submitted to Journal of Clinical Periodontology should include:

- Title Page
- Conflict of Interest and Source of Funding
- Clinical Relevance
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion
- References
- Tables (where appropriate)
- Figure Legends (where appropriate)
- Figures (where appropriate and uploaded as separate files)

All manuscripts should emphasize clarity and brevity. Authors should pay special attention to the presentation of their findings so that they may be communicated clearly. Technical jargon should be avoided as much as possible and be clearly explained where its use is unavoidable.

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

Conflict of Interest and Source of Funding: Authors are required to disclose all sources of institutional, private and corporate financial support for their study. Suppliers of materials (for free or at a discount from current rates) should be named in the source of funding and their location (town, state/county, country) included. Other suppliers will be identified in the text. If

no funding has been available other than that of the author's institution, this should be specified upon submission. Authors are also required to disclose any potential conflict of interest. These include financial interests (for example patent, ownership, stock ownership, consultancies, speaker's fee,) or provision of study materials by their manufacturer for free or at a discount from current rates. Author's conflict of interest (or information specifying the absence of conflicts of interest) and the sources of funding for the research will be published under a separate heading entitled "Conflict of Interest and Source of Funding Statement".

See Editor-in-Chief Maurizio Tonetti's Editorial on Conflict of Interest and Source of Funding and www.icmje.org/#conflicts for generally accepted definitions.

Abstract: is limited to 200 words in length and should not contain abbreviations or references. The abstract should be organized according to the content of the paper.

For Original Research Articles the abstract should be organized with **aim, materials and methods, results and conclusions**.

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

Clinical Relevance: This section is aimed at giving clinicians a reading light to put the present research in perspective. It should be no more than 100 words and should not be a repetition of the abstract. It should provide a clear and concise explanation of the rationale for the study, of what was known before and of how the present results advance knowledge of this field. If appropriate, it may also contain suggestions for clinical practice.

It should be structured with the following headings: **scientific rationale for study, principal findings, and practical implications**.

Authors should pay particular attention to this text as it will be published in a highlighted box within their manuscript; ideally, reading this section should leave clinicians wishing to learn more about the topic and encourage them to read the full article.

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

5.3. Original Research Articles

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

The word limit for original research articles is 3500 words, and up to 7 items (figures and tables) may be included. Additional items can be included as supplementary files online (please see 5.9 below).

Main Text of **Original Research Articles** should be organized with

- Introduction,
- Materials and Methods,
- Results and Discussion.
- References (Harvard, see section 5.7)

The background and hypotheses underlying the study, as well as its main conclusions, should be clearly explained. Please see Sample Manuscript.

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

Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced. As a condition of publication, authors are required to make materials and methods used freely available to academic researchers for their own use. This includes antibodies and the constructs used to make transgenic animals, although not the animals themselves.

(a) Clinical trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist should also be included in the submission material. If your study is a randomized clinical trial, you will need to fill in all sections of the CONSORT Checklist. If your study is not a randomized trial, not all sections of the checklist might apply to your manuscript, in which case you simply fill in N/A.

Journal of Clinical Periodontology encourages authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following free, public clinical trials registries: www.clinicaltrials.gov, http://clinicaltrials.ifpma.org/clinicaltrials/. The clinical trial registration number and name of the trial register will then be published with the paper.

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

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

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

an ethical board should also be included.

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

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

Results: should present the observations with minimal reference to earlier literature or to possible interpretations.

Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The discussion section should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.

The discussion may usefully be structured with the following points in mind (modified from the proposal by Richard Horton (2002), The Hidden Research Paper, The Journal of the American Medical Association, 287, 2775-2778). Not all points will apply to all studies and its use is optional, but we believe it will improve the discussion section to keep these points in mind.

Summary of key finding
* Primary outcome measure(s)
* Secondary outcome measure(s)
* Results as they relate to a prior hypothesis

Strengths and Limitations of the Study
* Study Question
* Study Design
* Data Collection
* Analysis
* Interpretation
* Possible effects of bias on outcomes

Interpretation and Implications in the Context of the Totality of Evidence
* Is there a systematic review to refer to?
* If not, could one be reasonably done here and now?
* What this study adds to the available evidence
* Effects on patient care and health policy

* Possible mechanisms
Controversies Raised by This Study Future Research Directions
* For this particular research collaboration
* Underlying mechanisms
* Clinical research

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

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

The main text of Clinical Innovation Reports should be organized with

- Introduction,
- Clinical Innovation Report,
- Discussion and Conclusion
- References (Harvard, see section 5.7)

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

The main text of Case Reports should be organized with

- Introduction,
- Case report,
- Discussion and Conclusion
- References (see section 5.7)

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

The word limit for reviews is 4000 words.

The main text of Reviews should be organized with

- Introduction,
- Review of Current Literature,
- Discussion and Conclusion
- References (Harvard, see section 5.7)

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

Reference style (Harvard):
References in the text should quote the last name(s) of the author(s) and the year of publication (Brown & Smith 1966). Three or more authors should always be referred to as, for example, Brown et al. 1966.

A list of references should be given at the end of the paper and should follow the recommendations in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors, (1975), p. 36. London: The Royal Society of Medicine.

a) The arrangement of the references should be alphabetical by first author's surname.
b) The order of the items in each reference should be:
(i) for journal references: name(s) of author(s), year, title of paper, title of journal, volume number, first and last page numbers.
(ii) for book references: name(s) of author(s), year, chapter title, title of book in italics, edition, volume, page number(s), town of publication, publisher.
c) Authors' names should be arranged thus: Smith, A. B., Jones, D. E. & Robinson, F. C. Note the use of the ampersand and omission of comma before it. Authors' names when repeated in the next reference are always spelled out in full.
d) The year of publication should be surrounded by parentheses: (1967).
e) The title of the paper should be included without quotation marks.
f) The journal title should be written in full, italicised (single underlining in typescript), and followed by volume number in bold type (double underlining on typescript) and page numbers.

Examples: Botticelli, D., Berglundh, T. & Lindhe, J. (2004) Hard-tissue alterations following immediate implant placement in extraction sites. *Journal of Clinical Periodontology* **10**, 820-828. doi:10.1111/j.1600-051X.2004.00565.x
Lindhe, J., Lang, N.P. & Karring, K. (2003) *Periodontology and Implant Dentistry*. 4th edition, p. 1014, Oxford. Blackwell Munksgaard.
Bodansky, O. (1960) Enzymes in tumour growth with special reference to serum enzymes in cancer. In *Enzymes in Health and Disease*, eds. Greenberg, D. & Harper, H. A., pp. 269-278.

Springfield: Thomas.

URL: Full reference details must be given along with the URL, i.e. authorship, year, title of document/report and URL. If this information is not available, the reference should be removed and only the web address cited in the text. Example: Smith A. (1999) Select Committee Report into Social Care in the Community [WWW document]. URL http://www.dhss.gov.uk/reports/report0394498.html [accessed on 7 November 2003]

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

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

5.8. Tables, Figures and Figure Legends

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

Figures: All figures should be planned to fit within either 1 column width (8.0 cm), 1.5 column widths (13.0 cm) or 2 column widths (17.0 cm), and must be suitable for photocopy reproduction from the printed version of the manuscript. Lettering on figures should be in a clear, sans serif typeface (e.g. Helvetica); if possible, the same typeface should be used for all figures in a paper. After reduction for publication, upper-case text and numbers should be at least 1.5-2.0 mm high (10 point Helvetica). After reduction symbols should be at least 2.0-3.0 mm high (10 point). All half-tone photographs should be submitted at final reproduction size. In general, multi-part figures should be arranged as they would appear in the final version. Each copy should be marked with the figure number and the corresponding author's name. Reduction to the scale that will be used on the page is not necessary, but any special requirements (such as the separation distance of stereo pairs) should be clearly specified.

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

Figures should be on a white background, and should avoid excessive boxing, unnecessary colour, shading and/or decorative effects (e.g. 3-dimensional skyscraper histograms) and highly pixelated computer drawings. The vertical axis of histograms should not be truncated to exaggerate small differences. The line spacing should be wide enough to remain clear on reduction to the minimum acceptable printed size. Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same typesize as used

elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field. Thousands should be separated by thin spaces (1 000). Unusual units or abbreviations should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general, visual cues (on the figures themselves) are preferred to verbal explanations in the legend (e.g. broken line, open red triangles etc.)

Preparation of Electronic Figures for Publication
Although low quality images are adequate for review purposes, print publication requires high quality images to prevent the final product being blurred or fuzzy. Submit EPS (lineart) or TIFF (halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Do not use pixel-oriented programmes. Scans (TIFF only) should have a resolution of 300 dpi (halftone) or 600 to 1200 dpi (line drawings) in relation to the reproduction size (see below). EPS files should be saved with fonts embedded (and with a TIFF preview if possible). For scanned images, the scanning resolution (at final image size) should be as follows to ensure good reproduction: lineart: >600 dpi; half-tones (including gel photographs): >300 dpi; figures containing both halftone and line images: >600 dpi.
Detailed information on our digital illustration standards can be found at
http://authorservices.wiley.com/bauthor/illustration.asp.
Check your electronic artwork before submitting it:
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Figure Legends: should be a separate section of the manuscript, and should begin with a brief title for the whole figure and continue with a short description of each panel and the symbols used; they should not contain any details of methods.

5.9. Supplementary Material
Supplementary material, such as data sets or additional figures or tables that will not be published in the print edition of the Journal but which will be viewable in the online edition, can be uploaded as 'Supporting information for review and online publication only'.

Please see **http://authorservices.wiley.com/bauthor/suppmat.asp** for further information on the submission of Supplementary Materials.

6. AFTER ACCEPTANCE

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

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

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

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

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

after acceptance. Given that copyright licensing is a condition of publication, a completed copyright form is required before a manuscript can be processed as an Accepted Article.

Accepted articles will be indexed by PubMed; therefore the submitting author must carefully check the names and affiliations of all authors provided in the cover page of the manuscript, as it will not be possible to alter these once a paper is made available online in Accepted Article format.

7. OnlineOpen
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Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper OnlineOpen if you do not wish to. All OnlineOpen articles are treated in the same way as any other article. They go through the journal's standard peer-review process and will be accepted or rejected based on their own merit.

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For authors signing the copyright transfer agreement
If the OnlineOpen option is not selected the corresponding author will be presented with the copyright transfer agreement (CTA) to sign. The terms and conditions of the CTA can be previewed in the samples associated with the Copyright FAQs below:

CTA Terms and Conditions: http://authorservices.wiley.com/bauthor/faqs_copyright.asp

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If the OnlineOpen option is selected the corresponding author will have a choice of the following Creative Commons License Open Access Agreements (OAA):

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Manuscript Sections

Manuscript Title
Abstract
Keywords
Abbreviations
Body Text
Equations and Formulas
Units
Supplementary
References
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Book References:

Gardner JG, Simmons MJ, Snustad PD. Principles of Genetics. 8th edn. New York: John Wiley & Sons. 2006.

Book Chapter:

Honn KV, Tang DG, Chen Y. Adhesion molecules and site-specific metastasis. Neri Serneri SS, Gensini GF, Abbate R, Prisco D, editors. In: Thrombosis: An Update. Scientific Press. 1992; 269-303.

Link/URL:

National Cancer Institute at National Institutes of Health. [hyperlinked with www.cancer.gov]

Proceedings of a Conference:

Gee JC, Joshi S, Pohl KM, Wells WM, Zollei L, editors. Information Processing in Medical Imaging. Proceedings of 23rd International Conference; 2013 June 28--July 3; CA, USA. New York: Springer, 2013.

PhD Theses/Dissertation:

Simonneau A. Gold-Catalyzed Cycloisomerization Reactions Through Activation of Alkynes [dissertation]. Springer Theses, 2014.

Datasets:

Zheng LY, Guo XS, He B, Sun LJ, Peng Y, Dong SS, et al. Genome data from sweet and grain sorghum (Sorghum bicolor). GigaScience. 2011.

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