ALINE CRISTIANE PLANELLO

ANÁLISE DE POLIMORFISMOS NO PROMOTOR DOS GENES *MMP1, MMP3 e MMP9* NA DESORDEM DA ARTICULAÇÃO TEMPOROMANDIBULAR.

Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas - UNICAMP como parte dos requisitos para obtenção do Título de Mestre em Biologia Buco-Dental, área de concentração Histologia e Embriologia.

Orientadora: Profa. Dra. Ana Paula de Souza Pardo

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"Tenha em mente que tudo que você aprende na escola é trabalho de muitas gerações. Receba essa herança, honre-a, acrescente a ela e, um dia, fielmente, deposite-a nas mãos de seus filhos."

Albert Einstein

RESUMO

Objetivo: As Metaloproteinases da Matriz (MMPs) são enzimas que degradam a matriz extracelular (MEC) e tem sido associadas às desordens temporomandibulares (DTM). Nós investigamos a freqüência dos -1607 1G/2G *MMP1* polimorfismo (rs1799750), -1171 6A/5A *MMP3* polimorfismo (rs3025058) e -1562 C/T *MMP9* polimorfismo (rs3918242) em indivíduos com sinais de degeneração da ATM, diagnosticados por exame de imagem, a fim de analisar a associação desses polimorfismos e a DTM. Métodos: A população estudada foi composta por 115 indivíduos diagnosticados por exame de imagem (grupo DTM) e 117 controles. Os polimorfismos genéticos foram determinados por PCR/RFLP. Resultados: A freqüência do genótipo 2G/2G no gene *MMP1* foi significantemente mais alta no grupo DTM do que no grupo Controle ($p = 0.008$). O genótipo 2G/2G no grupo DTM mostrou um risco aumentado para a DTM com um OR = 2.25 (95% IC = 1.26 – 3.99) quando comparado com os genótipo 1G/2G e 1G/1G. A freqüência dos alelos do gene *MMP1* não mostrou diferença significativa entre os grupos ($p > 0.05$). A distribuição dos genótipos e alelos dos genes *MMP3* e *MMP9* não mostrou diferença significativa ($p > 0.05$). Conclusão: Nossos resultados mostram a associação entre o polimorfismo -1607 *MMP1* e a suscetibilidade à DTM.

Palavras-Chave: MMP, Polimorfismos genéticos, Desordem Temporomandibular, ATM

ABSTRACT

Objective. Matrix metalloproteinases (MMPs) degrade extracellular matrix components and have been implicated to play an important role in temporomandibular joint disorder (TMD). We investigated the frequency of -1607 1G/2G *MMP1* polymorphism (rs1799750), -1171 6A/5A *MMP3* polymorphism (rs3025058) and -1562 C/T *MMP9* polymorphism (rs3918242) in individuals with TMJ degeneration diagnosed by image exam in order to analyze the association of these MMPs polymorphisms and TMD.

Methods. The studied population comprised 115 TMD individuals diagnosed by image exam and 117 healthy controls. Genotypes were determined using polymerase chain reaction/Restriction fragment length polymorphism PCR/RFLP.

Results. The *MMP1* 2G/2G genotype was significantly higher in the TMD group than in the Control group (p = 0.008). The genotype 2G/2G in the TMD group showed an increased risk to TMD with an OR = 2.25 (95% CI = 1.26 – 3.99) when compared with 1G/2G and 1G/1G genotypes. Analysis of *MMP1* allele frequencies showed no significant difference (p > 0.05). The MMP3 and MMP9 genotypes distribution and alleles frequency did not differ between the groups (p > 0.05).

Conclusion. Our results report the association of –1607 *MMP1* gene polymorphism and increased risk to TMD.

Key Terms: MMP, Polymorphism, SNP, Temporomandibular joint, TMJ

SUMÁRIO

INTRODUÇÃO

Desordem Temporomandibular (DTM) é um termo que engloba um número de problemas que envolvem a musculatura mastigatória e a articulação temporomandibular, representando freqüentemente a causa de dor orofacial e alteração da função (McNeill *et al*., 1990). Os Critérios de Diagnóstico para Pesquisa das Desordens Temporomandibulares (RDC-TMD), divide a DTM em dois Eixos: o Eixo I envolve as condições clínicas da ATM como, as desordens musculares, os deslocamentos de disco e as osteoartrites. O Eixo II compreende o grau de função da ATM e o status psicossocial do individuo (Dworkin & LeResche, 1992). A Academia Americana de Dor Orofacial classifica a DTM em: DTM relacionada às alterações musculares e DTM relacionada às alterações da articulação (McNeill *et al*., 1990). Em se tratando de alterações articulares, o revestimento de tecido conjuntivo denso, a cartilagem e osso subcondral são os tecidos mais acometidos (Stegenga *et al.*,1991).

O côndilo de um indivíduo jovem é estruturalmente diferente do côndilo de um indivíduo adulto. No indivíduo jovem o côndilo é revestido por tecido conjuntivo denso havendo logo abaixo deste uma camada de células indiferenciadas, uma região de ossificação endocondral e finalmente tecido ósseo. Já o côndilo de um indivíduo adulto é revestido por tecido conjuntivo denso que possui abaixo uma camada de células indiferenciadas, fibrocartilagem e tecido ósseo.

A ATM difere das demais articulações sinoviais. A superfície articular da ATM é recoberta por cartilagem fibrosa e tecido conjuntivo, os quais são compostos predominantemente por colágeno tipo I, enquanto que, as outras articulações sinoviais são recobertas por cartilagem hialina com predominância de colágeno tipo II (Wadhwa & Kapila, 2008).

Quando as alterações degenerativas acometem a cartilagem articular que recobre o côndilo mandibular e a eminência articular, suas propriedades físicas são alteradas afetando sua capacidade de suportar o stress imposto à articulação. Uma vez que a cartilagem é danificada ocorre um aumento na fricção entre as

superfícies articulares que irá atrapalhar o movimento fisiológico levando a uma resposta compensatória ou patogênica da cartilagem e seus tecidos adjacentes como o osso subcondral, cápsula, ligamentos, membrana sinovial e musculatura associada. A resposta patogênica leva a alterações dos tecidos que se assemelham a osteoartrite de outras articulações e podem levar a degradação irreversível da cartilagem articular e a uma remodelação óssea alterada (Stegenga, 2001).

Essas alterações degenerativas são resultado de um desequilíbrio entre os processos de catabolismo e anabolismo e são caracterizadas pela progressiva degradação da matriz extracelular (MEC) (Dijkgraaf *et al*., 1995).

As metaloproteinases da matriz (MMPs) são proteases que clivam multiplos componentes da MEC. As MMPs estão divididas em classes: as colagenases, as gelatinases, as estromelisinas, as MMPs trans-membrana e as matrilisinas, de acordo com a estrutura proteica e a especificidade pelo substrato. Sob condições fisiológicas a atividade das MMPs é precisamente regulada. Elas são secretadas sob forma de zimógeno e a atividade é zinco dependente. A atividade é controlada na razão 1:1 por inibidores específicos encontrados na MEC, chamados inibidores teciduais das metaloproteinases da matriz (TIMPs) (Visse & Nagase, 2003). A regulação da transcrição é uma importante via de controle uma vez que a maioria dos genes das MMPs é expressa quando requerida pela célula. Muitos estudos já demonstraram que a natural ocorrência de variações na seqüência da região promotora dos genes das MMPs pode resultar em diferença na expressão dessas enzimas (Ye, 2000). Essas variações são conhecidas como polimorfismos genéticos, os quais apresentam mais de uma variante (alelo) com uma freqüência de pelos menos 1% na população humana (Sherry *et al*., 1999).

Um polimorfismo de nucleotídeo único (SNP) na região promotora do gene *MMP1* foi identificado sendo resultado da inserção/deleção de uma guanina na posição -1607 (rs1799750). Dois alelos foram criados, um com uma única guanina (1G) e o outro com duas guaninas (2G). Ensaios *in vitro* e *in vivo* mostraram que o

alelo 2G aumenta a expressão do gene *MMP1*, uma vez que a as duas guaninas estão adjacentes a uma adenina (5´GGA 3´), criando um sitio de ligação para fatores de transcrição da família Ets (Rutter *et al*., 1998; Coon *et al*., 2009).

Um SNP na posição -1612 do gene *MMP3* (rs3025058) foi identificado como uma variação na seqüência de adeninas, sendo um alelo com 5 adeninas (5A) e outro com 6 adeninas (6A). Análises funcionais *in vitro* mostraram que o alelo 5A tem maior atividade quando comparado com o 6A. Isto porque fator de transcrição repressor da atividade tem maior afinidade pelo alelo 6A, diminuindo sua atividade e fazendo com que a expressão do gene *MMP3* esteja aumentada na presença do alelo 5A (Ye *et al*., 1995; Ye *et al*., 1996).

Inúmeros polimorfismos no gene *MMP9* foram identificados e um deles na região promotora se mostrou funcionalmente importante. O SNP na posição -1562 (rs3918242) é caracterizado pela substituição de uma citosina por uma timina, resultando em um alelo C e outro alelo T. O alelo T mostrou maior atividade de transcrição *in vitro*, isso porque o alelo C tem maior afinidade com fatores repressores de transcrição (Zhang *et al*., 1999).

Todos esses SNPs já foram associados com algumas doenças como: arteriosclerose, câncer, periodontite, infarto do miocárdio, aneurisma da aorta, e osteoartrite de joelho (Ye *et al*., 1996; de Souza *et al*., 2003; Astolfi *et al*., 2006; Deguara *et al*., 2007; Barlas *et al*., 2009; Peng *et al*., 2010). A MMP-1, -3 e -9 parecem exercer um importante papel nas doenças degenerativas da DTM (Kubota *et al*., 1998; Song *et al*., 2006) e os SNPs acima descritos modificam a expressão gênica destas enzimas, estando relacionados com várias doenças. Assim, o presente estudo teve como objetivo investigar a possível associação destes SNPs na região promotora dos genes *MMP1, MMP3 e MMP9* com a DTM.

CAPÍTULO 1

Association of Matrix Metalloproteinase promoter gene polymorphisms with susceptibility to temporomandibular joint disorder

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Short running footnote: MMP SNP and

Abstract

Objective. Matrix metalloproteinases (MMPs) degrade extracellular matrix components and play an important role in temporomandibular joint disorder (TMD). We investigated the frequency of -1607 1G/2G *MMP1* polymorphism (rs1799750), -1171 6A/5A *MMP3* polymorphism (rs3025058) and -1562 C/T *MMP9* polymorphism (rs3918242) in individuals with TMJ degeneration in order to analyze the association of these MMPs polymorphism with TMD.

Subjects and Methods. The studied population comprised 115 TMD individuals diagnosed by image exam and 117 healthy controls. Genotypes were determined using polymerase chain reaction/Restriction fragment length polymorphism PCR/RFLP.

Results. The *MMP1* 2G/2G genotype frequency was significantly higher in the TMD group than in Control group (p = 0.008). The individuals with 2G/2G genotype showed an increased risk to TMD with an OR = 2.25 (95% CI = 1.26 – 3.99) when compared with 1G/2G and 1G/1G genotypes. Analysis of *MMP1* alleles frequency showed no significant difference ($p > 0.05$). The *MMP3* and *MMP9* genotypes distribution and alleles frequency did not differ between the groups ($p > 0.05$).

Conclusion. Our results report the association of −1607 *MMP1* gene polymorphism with increased risk to TMD.

Key index term: MMP, genetic polymorphism, SNP, temporomandibular joint

Introduction

Temporomandibular disorder (TMD) is a term that embraces a number of clinical problems involving masticatory musculature, the temporomandibulat joint (TMJ) and associated structures. This disorder is a frequent cause of orofacial pain and physical impairment (Dworkin & LeResche, 1992, McNeill et al., 1990) and generally promotes slowly and progressive degeneration of articular components such as articular cartilage, bone, capsule, ligaments and synovial membrane. These degenerative changes of tissues alter physical characteristics of TMJ and its functional properties, leading first to the reversible changes and finally to the irreversible effects in joint (Stegenga, 2001).

An imbalance between synthesis and destruction of different types of extracellular matrix (ECM) collagens and aggrecan, in favor of proteolysis, is characteristic of degenerative changes in TMJ (Dijkgraaf et al., 1995). Some evidences have indicated that these events are associated to inflammatory process of TMJ, since increased levels of inflammatory modulators like cytokines, nitric oxide, cartilage matrix catabolites and proteinases such as matrix metalloptoteinases (MMPs) were found in the synovial fluid (Srinivas et al., 2001, Kubota et al., 1998, Kanyama et al., 2000, Ishimaru et al., 2000, Yoshida et al., 2006). In fact, MMPs derived from fibrocartilage and cartilage cells have been considered the key enzymes responsible for TMJ extracellular matrix breakdown (Song et al., 2006).

MMPs represent a family of metal-dependent enzymes that are capable of degrading all extracellular matrix (ECM) components, including all types of collagen, fibronectin and proteoglycans. All MMPs are secreted from cells as inactive pro-enzymes, becoming activated in the ECM after proteolytic cleavage of the N-terminal pro-peptide domain.

After activation, the function of MMPs in the ECM is still controlled by tissue inhibitors of MMPs (TIMPs), natural inhibitors of MMPs. The balance between MMP activity and TIMP inhibition plays a crucial role in ECM homeostasis and the disequilibrium between them may result in destruction of ECM proteins (Visse & Nagase, 2003).

Transcriptional regulation is also implicated in the control of MMPs function. Several functional genetic polymorphisms have been described in the promoter region of MMPs genes and these variances may modify basal and inducible levels of MMPs genes expression. Single-nucleotide polymorphisms (SNPs) described in the *MMP1* (Rutter et al., 1998), *MMP3* (Ye et al., 1996) and *MMP9* (Zhang et al., 1999) gene promoters have been associated with degenerative disease such as arthritis, atherosclerosis and periodontal disease (Ye et al., 1995, Deguara et al., 2007, Barlas et al., 2009, Astolfi et al., 2006, de Souza et al., 2003).

MMP1, MMP3 and *MMP9* are constitutively or transiently transcripted by fibroblasts and chondrocytes, implying that these enzymes could play a role in the TMJ degeneration. In this way, the present study investigated the frequency of some *MMPs* SNPs in individuals with TMJ degeneration diagnosed by image exam in order to analyze the association of those SNPs with TMD.

Material and Methods

This study was carried out including a total of 232 individuals, with the approval of the FOP/UNICAMP Ethic Committee (058/2008) and informed consent was obtained from all subjects. All participants were unrelated Brazilians from Northeastern region. The TMD group included 115 individuals, both gender, which underwent magnetic resonance imaging

(MRI) and/or computed tomography (CT), between the years of 2000 to 2005 at a private clinic. In order to be included in the TMD group, the individuals had to present at least one sign of degenerative change on MRI and/or CT exams, in one or both mandibular condyles. The degenerative changes considered were osteophyte, erosion, avascular necrosis, subcondral cyst and intra-articular loose bodies. The scans were performed using a Signa Horizon system model scanner (General Electric, Milwaukee, WI, USA), at a magnetic field magnitude of 1.5 T, using a bilateral radiofrequency surface coil of 6.5 x 6.5 cm in size. For the acquisition of final scans, an axial scout was performed. Based on that, the condyle was located and the right orientation of parasagittal and paracoronal slice sequences was established. The scans were interpreted by two experienced Head and Neck/Maxillofacial radiologists. Any disagreements were discussed until consensus was reached. In case of doubtful diagnoses, the scan was thrown aside. As a rule, before the image exams all the subjects were submitted to interview toward the chief complaint, including pain on mandibular movements and TMJ sounds. Presence of joint was recorded as "Yes" or "No", according to the patient's declaration and without palpation. There was no record of severity and period of joint pain. The radiologists that interpreted the images were not aware of the interview. The Control group comprised 117 gender-and-age-matched healthy individuals without any symptoms on TMJ. Ethnic status was not matched due to high genetic miscegenation of the Brazilian population (Parra et al., 2003).

DNA purification and PCR reaction

The genomic DNA was isolated from epithelial buccal cells as previously described (Aidar & Line, 2007). MMP1 PCR reaction was performed in a total volume of 15 µl containing

7.5 µl of Taq DNA polymerase (GoTaq green master mix, Promega corporation USA), 0.1 µM of each primer, 200 ng genomic DNA. The solution was incubated for 5 min at 95ºC, followed by 40 cycles of 1 min at 95ºC, 1 min at 55ºC and 1 min at 72ºC, with a final extension of 72ºC for 7 min MMP3 PCR reaction was carried out in a total volume of 10 µl containing 5.0 µl of Taq DNA polymerase (GoTaq green master mix, Promega corporation USA), 0.1 µM of each primer, 200 ng genomic DNA. Then the solution was incubated for 5 min at 94ºC followed by 35 cycles of 30 s at 94ºC, 30 s at 65ºC and 30 s at 72ºC, with a final step at 72ºC for 7min. MMP9 PCR was performed in a total volume of 10 µl cointainig 5.0 µl of Taq DNA polymerase (GoTaq green master mix, Promega corporation USA), 0.1 µM of each primer, 200 ng genomic DNA. Then, the solution was incubated for 3 min at 95ºC followed by 35 cycles of 1min at 95 ºC, 45 s at 65ºC and 45 s at 72ºC, with a final step at 72ºC for 7 min. Primer sequences are listed in the Table 1.

Genotype analysis

The MMPs genotypes were identified through RFLP. An amount of 10 µl of MMP1 PCR product was digested with 6 U of *Xmn*I (New England Biolabs, Inc., USA) for 16 hours at 37ºC overnight. A 2 µl aliquot of MMP3 PCR product was digested with 4 U of *Tth111*I enzyme (New England Biolabs Inc., USA) for 4 hours at 65ºC. To MMP9, 2 µl aliquot of PCR product was digested with 3 U of *Sph*I enzyme (New England Biolabs Inc., USA) for 16 hours at 37ºC. The MMPs digested products were electrophoresed on a 10% poliacrylamide gel at 20 mA and then the gel was stained with silver nitrate according to previously described (Sanguinetti et al. 1994). The *MMP1* 1G allele was identified as bands with sizes at 89 bp and 29 bp. The 2G allele was not cleaved by XmnI, keeping the original

PCR product size of 118 bp. The *MMP3* 5A allele resulted in 97 bp and 32 bp and the 6A allele was not cleaved by *Tth111*I and the PCR product size of 130 bp was kept. *MMP9* C allele was not recognized by *SphI* enzyme and only showed the PCR product, one band of 435 bp. The T allele was cleaved by *Sph*I and had two bands with sizes of 247 bp and 188 bp.

Statistical analyses

Statistical analyses of differences in the genotypes distribution observed between groups were performed in the the BioEstat 5.0 software using χ^2 test. Odds ratio (OR) and Hardy-Weinberg equilibrium (HWE) test were also performed in BioEstat 5.0 software. A *p*-value of less than 5% was taken as statistically significant. The haplotypes frequencies were estimated using Arlequin ver.3.0 software for population genetics data analyses.

RESULTS

Demographic characteristics of the subjects are shown in table 2. The groups were matched regarding age and gender distribution (p = 0.59 and 0.98 respectively). Clinical characteristics are listed in table 3.

Genotypes distribution and alleles frequency of *MMP1*, *MMP3* and *MMP9* SNPs were successfully performed in all subjects and then compared between the groups. The results of genetic analyses are presented in table 4. There were significant differences regarding the genotypes distribution of *MMP1* polymorphism. The *MMP1* 2G/2G genotype was significantly higher in the TMD group than in the Control group (p=0.008). The

individuals with genotype 2G/2G showed an increased risk of TMD with an OR 2.25 (95% CI = 1.26 – 3.99) when compared with 1G/2G and 1G/1G genotypes. Analysis of alleles frequency of *MMP1* gene did not show significant difference ($p > 0.05$). The *MMP3* and *MMP9* genotypes distribution and alleles frequency did not differ between the groups ($p > 0.05$). In order to verify statistical power of our sample, we used G*POWER software (Faul et al., 2007). The input parameters were: moderate effect size=0.3, based on genotype distribution data; statistical significance level $\alpha=0.05$; one and two degrees of freedom for allelic and genotype analysis, respectively. Statistical power for our sample was higher than 90% for association detection. The genotypes distribution was in Hardy-Weinberg equilibrium in the total sample.

Since the *MMP1* and *MMP3* genes are in the same chromosome cluster (11q22.3), the haplotype analysis was undertaken. The estimated haplotype frequencies are shown in Table 5. Significant differences were not observed between TMD group and controls regarding haplotype frequencies ($p > 0.05$).

DISCUSSION

Previous study reported that −1607 *MMP1* polymorphism affects gene transcription, increasing MMP-1 mRNA synthesis (Rutter et al., 1998). The 2G allele located at an adjacent adenosine creates a consensus binding site (5'-GGA-3') for Ets family transcription factors, which are the downstream targets of several growth factors. This binding site is closely to an AP-1 site, promoting a 37-fold increase in transcription activity *in vitro* (Rutter et al., 1998). A recent study of the same group, using transgenic mice, validated the increased expression of *MMP1* 2G allele *in vivo* (Coon et al., 2009).

We observed differences in genotype distribution between TMD individuals and control individuals for the *MMP1* −1607 1G/2G polymorphism. The probability of developing TMD in the homozygous 2G/2G Brazilian subjects is 2.25 times higher than in the 1G/2G and 1G/1G individuals. Our results are in accordance with several clinical studies that observed the presence of 2G allele associated with increased development risk of certain types of cancer (Peng et al., 2010) and degenerative diseases, such as osteomyelitis (Montes et al., 2009) and periodontitis (de Souza et al., 2003, Astolfi et al., 2006). The association of *MMP1* gene polymorphism with TMD is reinforced by other studies reporting increased levels of MMP1 protein in the synovial fluid of TMJ (Srinivas et al., 2001, Kanyama et al., 2000). However, we are not in agreement with the results found in knee osteoarthritis and in rheumatoid arthritis. In the former, the authors observed 1 G high frequency allele associated with knee osteoarthritis disease (Barlas et al., 2009) and, in the latter, the authors found no association between *MMP1* polymorphism and radiographic damages or its progression (Constantin et al., 2002a).

The different results could be explained by differences between TMJ and systemic joints. In contrast to other synovial joints in the body, where the articular surfaces are covered by hyaline cartilage, the TMJ surface is covered by fibrocartilage. This contains type II collagen and great amount of type I collagen, which is degraded by MMP1, while hyaline cartilage only contains type II collagen (Wadhwa & Kapila, 2008).

The −1171 *MMP3* 5A allele showed in vitro a greater promoter activity when compared with 6A (Ye et al., 1996). This polymorphism has been associated with atherosclerosis susceptibility in a number of genetic epidemiological studies. The frequency of 5A allele is significantly higher in affected individuals than in control subjects,

increasing the risk of acute myocardial infarction in individuals carrying one or two copies of the 5A allele. This risk was estimated to be 2.25-fold (Terashima et al., 1999, Ye, 2000). The 5A allele was associated with progressive radiographic damage in rheumatoid arthritis (RA) and the homozygous 6A/6A was observed as a protective factor against development of erosive RA in individuals from Czech Republic (Nemec et al., 2007). On the other hand, the 6A/6A genotype was associated with radiographic severity of RA and with the highest progression of joint in a French population (Constantin et al., 2002b).

MMP3 activity has been detected during initial phase of TMD, indicating the occurrence of changes in the TMJ (Fujita et al., 2009). MMP3 degrades cartilage proteoglycan, fibronectin type IV and IX collagen and laminin *in vitro.* It is capable to activate pro-MMP1, pro-MMP-8 and pro-MMP13, increasing the rate of tissue degradation by other MMPs (Fujita et al., 2009). In spite of it, we did not observe *MMP3* SNP association with our sample. The TMJ histology could explain our results as the articular surface is not covered by hyaline cartilage. Another possibility is that the *MMP3* gene polymorphism promotes a limited influence on the disease, requiring a larger sample than 232 individuals to detect the association.

A C-to-T exchange at position −1562 of *MMP9* gene promoter alters the binding of an inhibitory nuclear protein to this region, leading to increased transcriptional activity of the gene. MMP9 represents the most complex member of MMP family in terms of protein structure and activity regulation (Opdenakker et al., 2001). It is secreted by limited cell types and is not constitutively produced. Its activity is mainly controlled at the transcriptional level since MMP9 promoter responds to several cytokines and growth factors signals (Huhtala et al., 1991, Kondapaka et al., 1997). We did not observe a

relationship between *MMP9* gene polymorphism and TMD; however, MMP9 can be detected at high levels in the synovial fluid of TMD individuals. This fact may indicate the presence of inflammatory cells during the active phase of TMJ destruction (Srinivas et al., 2001).

The TMD diagnosis was made using image exam. The criterion of this diagnosis was mainly dependent on morphological changes in the TMJ region (Campos et al., 2008). So, the majority of our sample individuals were classified as TMJ osteoarthritis. MMPs play an important role in degenerative diseases like osteoarthritis, where the control of MMPs activity is essential to limit the tissue breakdown. At least three important regulatory mechanisms may control MMPs activity and part of extracellular matrix homeostasis: the regulation of transcription levels, the activation of zymogens into the extracellular matrix (including plasmin-dependent or MMP-dependent pathway) and the inhibition by TIMPs. Evidences have indicated that regulation of transcription is the most important step in the regulation of MMPs since these genes are expressed only when active physiological or pathological tissue remodeling takes place (Ye, 2000). On the other hand, it is not possible to discard the importance of the protein activity control. Besides MMPs activation by endogenous enzymes and inhibition by TIMPs, MMP activity is influenced by cell surface transportation and secretion, intracellular degradation and clearance (Sternlicht & Werb, 2001).

Concluding, we are reporting for the first time the relationship between −1607 *MMP1* gene polymorphism and increased risk to TMD. Probably, the mechanism of TMJ destruction is polygenic and other functional genetic polymorphisms as important as -1607 1G/2G *MMP1* must be involved with joint components destruction. Further studies are

required to improve additional knowledge about genetics of TMD and to enhance our understanding of the molecular mechanisms of this complex disorder.

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Table 1. Primers and restriction enzymes for genotyping *MMP1, MMP3, MMP9* SNPs by PCR-RFLP

SNP	Forward Primer	Reverse Primer	Restriction Enzyme
MMP1	5'-CGTGAGAATGTCTT CCCATT-3'	5'-CTTGGATTGATTTGAGA TAAGTGAAATC- 3'	XmnI
MMP3	5'-GTTCTCCATTCCTT TGATGGGGGGAAAgA-3'	5'-TTCCTGGAATTCACATC ACTGCCACCACT-3''	Tth111I
MMP9	5´-GCCTGGCACATAGTA GGCCC-3	5´-CTTCCTAGCCAGC CGGCATC -3´	SphI

Table 2. Demographic characteristics of TMD group and Control group

	TMD	Control
Age		
Age (mean ±SD)	42.82 ±14.96	38.04 ±14.17
Gender		
Female (%)	87.82	87.93
Male (%)	12.17	12.06
Ethinic group		
Caucasian (%)	95 (82.06)	78 (67.24)
Afro-american (%)	8 (6.95)	22 (18.96)
Mestizo (%)	12 (10.43)	16 (13.79)

Table 3. Clinical characteristics of TMD group

Clinical features	%
Interview	
Joint Pain	73.91
Joint sounds	90.43
Pain on systemic joints	37.90
Familiar history of TMD	43.15
Image exam	
Osteophyte	71.57
Erosion	38.94
Avascular necrosis	7.38
Subcondral cyst	2.10
Articular loose bodies	6.3

Most individuals presented more than one sign of degenerative changes on image exam.

Table 4. Allele and genotype frequencies of matrix metalloproteinase (*MMP*) gene polymorphisms in individuals in TMD group an Control group

Alleles and genotypes	TMD n (%)	Controls n (%)	P value	OR (95% CI)
MMP1 -1607				
Alleles				
1G	96 (41,73%)	116 (49.57%)	0.10	
2G	134 (58,26%)	118 (50.42%)		
Genotypes				
1G/1G	26 (22.60%)	25 (21.26%)	0.008*	2.25 (1.26-3.99)*
1G/2G	44 (38.26%)	66 (56.41%)		p=0.008
2G/2G[a]	45 (39.13%)	26 (22.22%)		
MMP3 -1171				
Alleles				
5A	82 (35.65%)	80 (34.18%)	0.81	
6A	148 (64.34%)	154 (65.81%)		
Genotypes				
5A/5A	15 (13.04%)	15 (12.82%)	0.91	
5A/6A	52 (45.21%)	50 (42.73%)		
6A/6A	48 (41.73%)	52 (44.44%)		
MMP9 -1562				
Alleles				
C	199 (86.52%)	212 (90.59%)	0.21	
T	31 (13.47%)	22 (9.40%)		
Genotypes				
C/C	85 (73.91%)	95 (81.19%)	0.23	
C/T	29 (25.21%)	22 (18.80%)		
T/T	1 (0.86%)	0 (0 %)		

[a] reference genotype for OR

Table 5. Haplotype distribution of MMP1 e *MMP3* gene polymorphism in TMD groups and control group

Haplotype	TMD n (%)	Controls n (%)	P value
1G-5A	51 (22.17)	63 (26.92)	0.30
1G-6A	45 (19.56)	54 (23.07)	0.42
2G-5A	30 (13.04)	19 (8.11)	0.15
2G-6A	104 (45.21)	98 (41.88)	0.72

CONCLUSÃO

Nossos resultados mostraram:

- A relação entre o polimorfismo -1607 1G/2G *MMP1* e a suscetibilidade à DTM;

- Nenhuma associação significante foi encontrada para os polimorfismos -1171 6A/5A *MMP3* e -1562 C/T *MMP9* e a DTM.

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ANEXO 1

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Análise de polimorfismos genéticos no promotor do gene da MMP-1, MMP-3 e MMP-9 em pacientes com desordens temporomandibulares**", protocolo nº **058/2008**, dos pesquisadores **ANA PAULA DE SOUZA PARDO e ALINE CRISTIANE PLANELLO**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 25/07/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Analysis of genetic polymorphisms in the gene promoter of MMP-1, MMP-3 and MMP-9 in patients with tempomandibular joint disease**", register number **058/2008**, of **ANA PAULA DE SOUZA PARDO and ALINE CRISTIANE PLANELLO**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 25/07/2008.

Prof. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 2

From: odiedoffice@wiley.com

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Subject: Oral Diseases - Manuscript ODI-02-10-OM-1560

Body: 11-Feb-2010

Dear Mrs. Aline Planello,

Your manuscript entitled "Association of matrix metalloproteinase gene polymorphism with temporomandibular joint disorder." has been successfully submitted online and is presently being given full consideration for publication in Oral Diseases.

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Yours sincerely,

Rosie Ledger
Editorial Assistant
Oral Diseases

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