FLAVIA MAGAZONI GONÇALVES

"EFEITOS DE POLIMORFISMOS GENÉTICOS SOBRE AS CONCENTRAÇÕES CIRCULANTES DE METALOPROTEINASES DA MATRIZ EXTRACELULAR EM MULHERES COM MIGRÂNEA"

"EFFECTS OF GENETIC POLYMORPHISMS ON THE CIRCULATING CONCENTRATIONS OF EXTRACELLULAR MATRIX METALLOPROTEINASES IN WOMEN WITH MIGRAINE"

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Orientador: Prof. Dr. José Eduardo Tanus dos Santos

"EFFECTS OF GENETIC POLYMORPHISMS ON THE CIRCULATING CONCENTRATIONS OF EXTRACELLULAR MATRIX METALLOPROTEINASES IN WOMEN WITH MIGRAINE"

Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas - UNICAMP para obtenção de título de Doutora em Farmacologia.

Doctorate thesis presented to the Pharmacology Postgraduation Programme of the School of Medical Sciences of the University of Campinas to obtain the Ph.D grade in Pharmacology.

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DEDICATÓRIA

Aos meus queridos pais

Luiz e Fátima

pelo amor incondicional

em todos os momentos de minha vida

e pelos exemplos de conduta.

À minha irmã Patrícia pelo carinho

e companheirismo.

Ao meu noivo Reinaldo por todo amor,

apoio e compreensão.

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"Tudo posso naquele que me fortalece"

(Filipenses 4:13)

RESUMO

A migrânea é uma cefaleia primária comum e altamente incapacitante que atinge cerca de 10% da população mundial, especialmente as mulheres. Apesar dos esforços, a fisiopatologia da migrânea não está completamente elucidada. No entanto, acredita-se que as metaloproteinases da matriz (MMPs) estejam envolvidas no rompimento da barreira-hematoencefálica durante uma crise de migrânea. O objetivo do presente trabalho é avaliar se polimorfismos funcionais nos genes da MMP-2 ($C^{-1306}T$ e $C^{-735}T$) e da MMP-9 ($C^{-1562}T$, $-90(CA)_n$ e R(279)Q) e haplótipos estão associados com a migrânea e se eles podem modificar os níveis circulantes de MMPs na migrânea. Para avaliar o efeito de polimorfismos da MMP-9, foram estudadas 102 mulheres sem migrânea (grupo controle) e 187 mulheres com migrânea (141 com migrânea sem aura; MSA e 46 com migrânea com aura; MA). As genotipagens para os polimorfismos $C^{-1562}T$, $-90(CA)_n$ e R(279)Q da MMP-9 foram realizadas por PCR-RFLP, PCR convencional seguida de eletroforese em gel de poliacrilamida e PCR em Tempo Real, utilizando-se o sistema Taqman® para discriminação de alelo, respectivamente. As concentrações plasmáticas de MMP-9 e TIMP-1 foram determinadas por ELISA. Os genótipos para os polimorfismos da MMP-2 foram determinados por PCR em Tempo Real, utilizando-se o sistema Taqman® para discriminação de alelo em 148 mulheres sem migrânea e em 204 mulheres com migrânea (153 MSA e 51 MA). As concentrações plasmáticas da MMP-2 e do TIMP-2 foram avaliadas, respectivamente, por zimografia e por ELISA. Os haplótipos foram inferidos utilizando o programa PHASE. Este estudo é o primeiro a mostrar que

polimorfismos funcionais e haplótipos nos genes da MMP-2 e da MMP-9 podem afetar os níveis circulantes das MMPs em pacientes com migrânea. Enquanto o haplótipo H6 (CLQ) da MMP-9 foi associado aos maiores níveis plasmáticos de MMP-9 nas pacientes com migrânea (359,8±69,53ng/ml *versus* 195,8±9,70ng/ml para o CLR; 201,5±18,67ng/ml para o CHR e 200,2±17,02ng/ml para o CHQ), as maiores concentrações de MMP-2 foram encontradas nas pacientes com migrânea com aura com o genótipo CC para o polimorfismo $C^{-735}T$ (1,29±0,07U.A. *versus* 0,96±0,06U.A. para os genótipos CT ou TT) e com o haplótipo H1 (CC) (1,24±0,05U.A. *versus* 0,94±0,05U.A. para o haplótipo CT) no gene da MMP-2. Apesar de não investigarmos os mecanismos moleculares que explicam esses resultados, podemos sugerir que o aumento dos níveis das MMPs associados a genótipos e haplótipos específicos podem predispor essas pacientes a um aumento da permeabilidade vascular da barreira hematoencefálica, promovendo assim o desenvolvimento de um ambiente neuroinflamatório no sistema nervoso central, contribuindo para uma crise de migrânea.

Palavras-chave: Migrânea, Metaloproteinases da matriz extracelular (MMPs), Inibidores tecidual das metaloproteinases da matriz extracelular (TIMPs), Polimorfismos e Haplótipos.

ABSTRACT

Migraine is a common primary headache and highly disabling, affecting approximately 10% of the population worldwide, especially women. Despite the efforts, the pathophysiology of migraine is not completely understood. However, it is believed that the matrix metalloproteinases (MMPs) are involved in the disruption of blood-brain barrier (BBB) during migraine attacks. The aim of this study was to evaluate whether functional polymorphisms in *MMP-2* ($C^{-1306}T$ and $C^{-735}T$) and *MMP-9* ($C^{-1562}T$, $-90(CA)_n$ and R(279)Q) genes and haplotypes are associated with migraine and whether they modify circulating MMPs levels in migraine. To evaluate the effect of MMP-9 polymorphisms, we studied 102 healthy women (controls) and 187 women with migraine (141 migraine without aura; MWA, and 46 migraine with aura; MA). Genotypes for $C^{-1562}T$, $-90(CA)_n$ e R(279)Q MMP-9 polymorphisms were determined by PCR-RFLP, by conventional PCR followed by electrophoresis in polyacrylamide gels and by real time-PCR using Taqman® allele discrimination assays, respectively. The plasma MMP-9 and TIMP-1 concentrations were measured by ELISA. Genotypes for MMP-2 polymorphisms were determined by real time-PCR using Taqman® allele discrimination assays in 148 healthy women without history of migraine and in 204 women with migraine (153 MWA and 51 MA). The plasma concentrations of MMP-2 and TIMP-2 were evaluated by gelatin zymography and ELISA, respectively. Haplotypes were inferred using the PHASE program. This is the first study to show that functional MMP-2 and MMP-9 polymorphisms and haplotypes can affect the circulating MMPs levels in patients with migraine. While the MMP-9 H6 (CLQ) haplotyple is associated with high

MMP-9 concentrations in patients with migraine (359,8±69,53ng/ml *versus* 195,8±9,70ng/ml for CLR; 201,5±18,67ng/ml for CHR and 200,2±17,02ng/ml for CHQ) , the highest concentrations of MMP-2 were found in patients with migraine with aura carrying the CC genotype for $C^{-735}T$ polymorphism (1,29±0,07A.U. *versus* 0,96±0,06A.U. for CT or TT genotypes) and the H1 (CC) haplotype (1,24±0,05A.U. *versus* 0,94±0,05A.U. for CT haplotype) in the *MMP-2* gene. Although we have not investigated the molecular mechanisms explaining these results, we can suggest that an increase of the MMPs levels associated with these genotypes and haplotypes may predispose these patients to increased vascular BBB permeability, thus promoting the development of an inflammatory environment in their central nervous systems, which contributes to migraine attacks.

Key words: Migraine, Extracellular matrix metalloproteinases (MMPs), Tissue inhibitors of extracellular matrix metalloproteinases (TIMPs), Polymorphisms and Haplotypes.

% - Porcentagem

AVC – Acidente vascular cerebral

BHE – Barreira hematoencefálica

CGRP – Peptídeos relacionados ao gene da calcitonina

DCA – Depressão cortical alastrante

ELISA - *Enzyme-linked immunosorbent assays*

H - *high* – alto; conjunto de alelos do microssatélite -90 $CA_{(14-24)}$ da MMP-9 englobando todos os alelos acima de 21 repetições do dinucleotídeo

L - *low* – baixo; alelo do microssatélite -90 $CA_{(14-24)}$ da MMP-9 englobando todos os alelos abaixo de 21 repetições do dinucleotídeo

MA – Migrânea com aura

MMPs – Metaloproteinases da matriz extracelular

MSA – Migrânea sem aura

MT-MMP - Metaloproteinase da matriz extracelular do tipo membrana

RFLP - *Restriction Fragment Length Polymorphisms*

SNP – Polimorfismo de base única *(Single Nucleotide Polymorphism)*

STGV – Sistema trigeminovascular

TIMPs – Inibidores endógenos teciduais das MMPs

SUMÁRIO

Pág.

RESUMO ---------- x

ABSTRACT ---------- xiii

1. INTRODUÇÃO ---------- 18

1.1 Migrânea - Epidemiologia, Classificação e Fisiopatologia ---------- 19

1.2 Metaloproteinases da Matrix Extracelular (MMPs) ---------- 25

1.3 Polimorfismos Genéticos da MMP-2 e da MMP-9 ---------- 29

2. OBJETIVOS ---------- 31

3. CAPÍTULOS ---------- 33

3.1 Capítulo 1 ---------- 34

3.2 Capítulo 2 ---------- 41

4. DISCUSSÃO ---------- 50

5. CONCLUSÕES ---------- 65

6. REFERÊNCIAS BIBLIOGRÁFICAS ---------- 67

7. ANEXOS ---------- 77

7.1 Critérios Diagnóstico para a Migrânea ---------- 78

7.2 Aprovação do Comitê de Ética em Pesquisa ---------- 80

7.3 Autorização para a inclusão do artigo 1 na tese ---------- 81

7.4 Autorização para a inclusão do artigo 2 na tese ---------- 86

1 - INTRODUÇÃO

1.1 Migrânea - Epidemiologia, Classificação e Fisiopatologia.

A migrânea, conhecida popularmente como enxaqueca, é uma cefaleia primária comum com grandes impactos sociais e econômicos. Atualmente, dentre as doenças mais incapacitantes, é classificada como a 19ª no *ranking* mundial, segundo a Organização Mundial da Saúde (OMS) [1].

Segundo critérios de diagnóstico da Sociedade Internacional de Cefaleias, a migrânea é clinicamente caracterizada por episódios de cefaleia que duram de 4 a 72 horas, com localização unilateral, caráter latejante ou pulsátil, de intensidade moderada ou grave, exacerbada por atividades físicas corriqueiras e que podem estar associada com náuseas e/ou fotofobia e fonofobia [1]. Pode ser dividida em dois subtipos principais, a migrânea sem aura (MSA) e a migrânea com aura (MA) (ANEXO). A MSA é o subtipo mais comum e geralmente mais incapacitante, enquanto que a MA é o menos comum e primariamente caracterizada por alterações neurológicas, frequentemente visuais, que antecedem e às vezes acompanham a cefaleia [1].

Estudos epidemiológicos indicam que a migrânea atinge cerca de 10% da população mundial, sendo mais prevalente na Europa e na América do Norte [2]. Nos Estados Unidos cerca de 6% dos homens e 18% das mulheres têm migrânea [3]. No Brasil, um estudo epidemiológico considerando os 27 estados brasileiros, mostrou que essa condição clínica atinge aproximadamente 15% da população, com as maiores incidências nas regiões Sudeste e Sul [4].

A prevalência da migrânea é dependente do gênero e da idade [2]. Antes da puberdade praticamente não há diferenças na incidência da migrânea entre homens e mulheres. No entanto, após a adolescência há um aumento significativo de sua ocorrência que é mais acentuado nas mulheres, em detrimento dos homens, atingindo uma proporção de três mulheres para cada homem na quarta década de vida. Após alcançar o pico na quarta década de vida, a prevalência da doença tende a cair em ambos os sexos [2, 5]. Evidências clínicas indicam que diferenças hormonais podem contribuir para essa discrepância [6, 7]. Independente do sexo, a idade mais comum para o aparecimento da migrânea é em torno da segunda ou terceira década de vida, que coincide exatamente com a faixa etária de maior produtividade no âmbito profissional do indivíduo.

De fato, alguns estudos têm relatado grandes prejuízos sociais e econômicos na vida dos indivíduos com migrânea [2]. Nos Estados Unidos, estima-se um gasto de aproximadamente 14,4 bilhões de dólares com os 22 milhões de indivíduos entre 20 a 65 anos de idade que apresentam a doença [8]. Dos custos relatados, apenas 1 bilhão é considerado como gastos diretos, com hospitalização e aquisição de medicamentos, enquanto que a maior parte desses gastos são considerados gastos indiretos, relacionados com a queda da produtividade em função de perda de dias de trabalho ou eficácia reduzida ao trabalhar com cefaleia. Cerca de 80% desses custos são com as mulheres [8].

Além dos prejuízos causados, tanto na vida social quanto no rendimento profissional do portador, atenção especial tem sido dedicada a essa condição clínica, já que vários estudos epidemiológicos têm apontado uma forte associação da migrânea, especialmente àquela com aura, com a incidência de doenças cardiovasculares e AVC isquêmico [9, 10]. Interessantemente, o risco de desenvolver alguma lesão cerebral isquêmica tem-se mostrado ainda maior em mulheres mais jovens com idade inferior a 45 anos, que são tabagistas ou que fazem uso de anticoncepcional oral [11]. Apesar da associação positiva entre migrânea e um risco cardiovascular aumentado não se sabe qual é o mecanismo exato responsável por essa associação.

De um modo geral uma crise de migrânea pode ser representada por algumas fases clínicas que são bem caracterizadas [12]. O pródromo ou período premonitório está presente em aproximadamente 20 a 60% dos casos. Ocorre de horas a dias antes do início da cefaleia e é caracterizado por sintomas bem variados como fadiga, alterações de humor e de apetite, entre outros [12, 13]. A aura migranosa atinge cerca de 20 a 30% dos pacientes, desenvolve-se ao longo de 5 a 20 minutos antes da cefaleia e consiste em sintomas neurológicos focais e reversíveis que podem ser visuais, sensoriais e motores [12-14]. Dentre as alterações, as visuais são as mais comuns e envolvem fenômenos positivos (presença de pontos cintilantes ou manchas) ou negativos (escotomas ou perda da acuidade visual) [1]. A fase da cefaleia propriamente dita apresenta as mesmas características clínicas já citadas acima, tanto para a MSA quanto para MA [1].

Tem um início gradual, atingindo uma intensidade moderada ou grave até sua completa resolução [12]. Apesar de os fatores desencadeantes de uma crise de migrânea não serem bem conhecidos, estresse, privação de sono, dieta, estímulos luminosos ou odores, mudanças de temperatura, têm sido relacionados com um aumento da predisposição às crises [15].

A fisiopatologia da migrânea ainda não está completamente elucidada**.** Atualmente a teoria mais aceita é a teoria neurovascular, fundamentada na ocorrência de um importante fenômeno conhecido como inflamação neurogênica [16, 17]. A depressão cortical alastrante (DCA) tem sido sugerida como o evento primário na patogênese da migrânea, especialmente naquela com aura, já que existe uma clara correlação temporal entre esses dois fenômenos [18]. A DCA é conhecida também como depressão alastrante de Leão, pois foi descrita pela primeira vez em 1944 por Aristides Leão, um pesquisador brasileiro da Universidade Federal do Rio de Janeiro que estava em Harvard estudando a eletrofisiologia em córtex de coelhos. Trata-se de um fenômeno neuroelétrico caracterizado por uma onda de despolarização que se propaga na superfície cortical a uma velocidade de 3-5 mm/min, no sentido póstero-anterior, e é seguida de uma supressão da atividade neuronal que é mais demorada. Durante a fase de despolarização há um aumento do fluxo sanguíneo cerebral, enquanto que a fase de supressão da atividade neuronal é associada a uma redução desse fluxo sanguíneo [18, 19].

Após a DCA**,** ocorre no córtex cerebral uma liberação de íons, como hidrogênio e potássio, e de outras moléculas, como óxido nítrico, que se difundem através dos vasos sanguíneos locais, despolarizam terminais perivasculares e causam a ativação da porção caudal do núcleo trigeminal no tronco cerebral [20].

A ativação de fibras sensoriais aferentes do sistema trigeminovascular (STGV) que inervam os vasos cerebrais, leva à liberação de neuropeptídeos importantes, nas terminações nervosas sensoriais, tais como a substância P, a neurocinina A e peptídeos relacionados ao gene da calcitonina (CGRP). Essas substâncias, uma vez liberadas, podem causar um aumento do fluxo sanguíneo intra e extracraniano, dilatação dos vasos meníngeos, extravasamento de proteínas plasmáticas e degranulação de mastócitos, culminando com o quadro da inflamação neurogênica [21, 22]. Além disso, a liberação desses peptídeos vasoativos é capaz de modular a dor durante uma crise de migrânea, à medida que podem sensibilizar e ativar nociceptores periféricos e centrais, além de inibir vias endógenas antinociceptivas responsáveis pelo controle da dor [16].

A migrânea é considerada uma doença complexa e multifatorial. Alguns estudos com gêmeos monozigóticos ou dizigóticos têm sido realizados para avaliar o envolvimento de fatores ambientais e genéticos e tem revelado um forte componente genético, já que as chances do indivíduo apresentar migrânea aumentam em gêmeos monozigóticos, quando comparados aos dizigóticos [23]. Recentemente muitos estudos genéticos têm sido conduzidos para a migrânea

hemiplégica familiar, um subtipo raro de migrânea com aura que apresenta herança autossômica dominante, e têm identificado importantes mutações em canais iônicos de cálcio, sódio e na bomba sódio/potássio que poderiam alterar o transporte e a liberação de alguns íons e de glutamato no cérebro e, dessa forma, aumentar a excitabilidade da região cortical [24, 25].

Em relação aos subtipos mais comuns, a MSA e a MA, alguns estudos genéticos de associação com genes canditatos têm sido conduzidos sem muito sucesso quanto à replicação dos achados. Dentre os principais genes candidatos, podemos citar alguns genes relacionados ao metabolismo dos sistemas serotoninérgicos e dopaminérgicos, genes realacionados à disfunção endotelial e alterações da permeabilidade vascular, entre outros [26]. Nos últimos anos alguns GWAS (*Genome wide association study)* têm sido conduzidos na tentativa de se entender melhor os subtipos mais comuns de migrânea [27-29]. Os resultados identificam alguns *SNPs* que estão localizados entre genes que possivelmente estejam envolvidos na homesostase do glutamato (genes *MTDH/AEG-1* e *PGCP*) [27] e em mecanismos relacionados à dor (gene *TRPM8*) [28]. No entanto, de forma similar ao que se observa com estudos de associações, os resultados gerados pelos *GWAS* são controversos e na maioria das vezes não podem ser replicados [24, 26].

Apesar de vários esforços, muitas questões acerca dos mecanismos fisiopatológicos e genéticos envolvidos na migrânea não estão claras. Alguns

estudos recentes têm sugerido o papel das metaloproteinases da matriz extracelular, as MMPs, na fisiopatologia da migrânea [30-33], no entanto, até o presente momento nenhum estudo de associação avaliou a influência de polimorfismos das MMPs na migrânea.

1.2 Metaloproteinases da Matrix Extracelular (MMPs)

As MMPs compreendem uma grande família de endopeptidases zinco e cálcio dependentes envolvidas na degradação de vários componentes da matriz extracelular como colágeno, elastina, proteoglicanas, fibronectina, entre outros [34, 35]. Essas enzimas exercem papéis importantes tanto em processos fisiológicos (proliferação, migração e diferenciação celular, embriogênese e morfogênese), quanto nos patológicos que envolvam atividade excessiva dessas MMPs [34, 36]. Atualmente mais de 20 subtipos diferentes de MMPs foram descritos e classificados de acordo com a afinidade pelo substrato que são capazes de degradar [35, 37], os quais incluem:

1. As colagenases (MMP-1, MMP-8, MMP-13 e MMP-18) responsáveis por clivar colágeno fibrilar (colágeno do tipo I, II e III);

2. As gelatinases (MMP-2 ou gelatinase A e MMP-9 ou gelatinase B): degradam principalmente o colágeno desnaturado (gelatina), sendo a

MMP-2 uma enzima que é constitutivamente expressa em diversos tecidos e a MMP-9 induzida;

3. As estromelisinas (MMP-3, MMP-10 e MMP-11)
4. As Matrilisinas (MMP-7 e MMP-26), as quais não possuem domínio hemopexina;
5. As MMPs do tipo membrana (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24 e MMP-25);
6. Outras MMPs (as metaloelastases ou MMP-12; MMP-9, MMP-20, MMP-22, entre outras).

As MMPs possuem similaridade estrutural, independentemente do substrato que são capazes de degradar. O domínio pró-peptídeo das MMPs apresenta uma porção N-terminal que permite que a enzima seja transportada para o meio extracelular e uma cisteína responsável por proteger o domínio catalítico da enzima. O sítio catalítico das MMPs apresenta um íon zinco, cuja ligação com o resíduo de cisteína do pró-domínio mantém a enzima em sua forma inativa e impede sua ligação ao seu substrato. O domínio catalítico das gelatinases (MMP-2 e MMP-9) é exclusivo, já que é o único que contém três fibronectinas do tipo 2, que formam um domínio de ligação com o colágeno [38, 39].

A atividade das MMPs pode ser regulada de várias maneiras, tanto em nível transcripcional quanto pós-traducional [34, 39]. Primariamente elas são secretadas na sua forma inativa ou zimogênios, cuja latência é mantida pela ligação da cisteina do pró-peptídeo com o íon zinco presente no sítio catalíco. A clivagem dessa ligação, e a consequente ativação dessas enzimas podem ocorrer por ação não proteolítica (estresse oxidativo) ou por meio da ação de proteases e de outras MMPs (ex: MT-MMPs) [37]. Além disso, a interação das MMPs com seus inibidores endógenos teciduais, conhecidos como TIMPs, é um passo muito importante na regulação das metaloproteinases [34]. Os TIMPs compreendem pequenas proteínas classificadas em quatro subtipos principais, que formam com as MMPs um complexo na proporção de 1:1 e impedem a ligação da enzima ao seu substrato. Apesar de não haver uma seletividade entre TIMPs e MMPs, sabe-se que o TIMP-1 é o inibidor preferencial da MMP-9 e o TIMP-2, o inibidor preferencial da MMP-2 [40].

O equilíbrio tecidual entre as MMPs e seus inibidores é essencial para a manutenção da homeostase tecidual e o aumento exagerado na atividade das MMPs contribui para importantes condições patológicas [41, 42]. Dentre as MMPs, a MMP-2 e a MMP-9 têm sido intensamente estudadas, pois níveis aumentados dessas gelatinases foram associados com aumento do risco de doenças cardiovasculares [43, 44]. Estudos clínicos e experimentais têm mostrado um aumento significativo na atividade e/ou expressão da MMP-2 e da MMP-9 em

diversas patologias envolvendo o sistema cardiovascular, como aterosclerose, hipertensão arterial, pré-eclâmpsia e AVC isquêmico, entre outras [45-50].

No sistema nervoso central, as MMPs contribuem para importantes danos teciduais à medida que degradam importantes componentes da barreira hematoencefálica (BHE). A BHE é composta por uma camada contínua de células endoteliais conectadas por junções intercelulares, astrócitos, pericitos e pela lâmina basal, uma estrutura muito importante que contém diversos componentes da matriz extracelular que são substratos para as gelatinases. Dessa forma, a degradação proteolítica intensa desses componentes da lâmina basal e das junções intercelulares leva a um aumento da permeabilidade da BHE que pode culminar com seu rompimento [51, 52].

Em relação à migrânea, *Gursoy-Ozdemir et al.* demostraram pela vez que a DCA é capaz de causar um aumento significativo na expressão e atividade da MMP-9 que, por sua vez, promove rompimento da BHE, formação de edema e extravasamento de proteínas plasmáticas durante uma crise de migrânea. Esses danos foram completamente revertidos com o uso de inibidores das MMPs, sugerindo, por conseguinte, que as MMPs poderiam constituir importantes alvos terapêuticos em algumas patologias envolvendo o sistema nervoso central [32, 53]. Alguns trabalhos clínicos vêm apontando para alterações nas concentrações plasmática da MMP-9 em pacientes com migrânea. *Leira et al.* mostrou pela primeira vez um aumento significativo nos níveis plasmáticos de MMP-9 na

migrânea em relação aos controles e ainda mostrou que as concentrações plasmáticas da MMP-9 eram maiores nos pacientes que estavam em período de crise, em relação aos estavam assintomáticos. Nenhuma diferença estatisticamente relevante foi encontrada quando comparados os subtipos de migrânea (com e sem aura) [54]. Posteriormente, nosso grupo mostrou pela primeira vez que havia uma importante alteração nos níveis plasmáticos de MMP-2 em mulheres com migrânea, tanto nas mulheres sem aura quanto nas com aura, quando comparadas às mulheres do grupo controle [33]. Desta forma, as gelatinases vêm sendo indicadas como importantes moléculas candidatas envolvidas em mecanismos fisiopatológicos da migrânea.

1.3 Polimorfismos Genéticos da MMP-2 e da MMP-9

Nos últimos anos, vários polimorfismos genéticos nos genes que codificam as gelatinases e que podem alterar a sua expressão e atividade têm sido estudados. Em relação ao gene da MMP-9 que está localizado no cromossomo 20, na região 20q11.2-q13.1, relevantes polimorfismos funcionais vêm sendo analisados, incluindo o SNP (S*ingle Nucleotide Polymorphism*) $C^{-1562}T$ *(rs3918242)* no promotor do gene que leva à substituição de uma citosina por uma timina [55], um microssatélite $-90(CA)_n$ *(rs2234681)* também na região promotora do gene, constituindo-se de repetições do dinucleotídeo CA entre 14 e 24 vezes [56] e, por último, um SNP Q279R *(rs17576)* no exon 6 do gene, constituindo-se de uma

troca de adenina para guanina na posição 855 que gera a substituição de um aminoácido glutamina por arginina na posição 279 da proteína [57]. Esses polimorfismos podem predispor os indivíduos a riscos aumentados de desenvolverem doenças cardiovasculares [43, 58].

O gene da MMP-2 está localizado no cromossomo 16, na região 16q13-q21. Até o presente momento, dois SNPs na região promotora do gene da MMP-2, correspondentes à troca de uma citosina por uma timina, são capazes de alterar a expressão e atividade da MMP-2 e têm sido amplamente estudados: o polimorfismo $C^{-1306}T$ *(rs243865)* [59] e o polimorfismo $C^{-735}T$ *(rs2285053)* [60]. Ambos podem predispor os indivíduos a condições patológicas, principalmente na presença do alelo polimórfico que é responsável por um aumento nas concentrações de MMP-2 [61-63].

Dessa forma, para um melhor entendimento acerca do envolvimento das MMPs na fisiopatologia da migrânea, torna-se pertinente avaliar as concentrações circulantes das gelatinases, bem como a influência de seus polimorfismos genéticos sob suas concentrações plasmáticas na migrânea.

2 - OBJETIVOS

Com a hipótese de que variações genéticas nos genes da MMP-2 e da MMP-9 poderiam estar associadas à migrânea e exercer importantes efeitos modulatórios sob as concentrações plasmáticas circulantes dessas MMPs, os objetivos do presente trabalho foram:

1. Avaliar a associação de genótipos e haplótipos de polimorfismos funcionais nos genes da MMP-9 ($C^{-1562}T$, $(CA)_{14-25}$ e Q279R) e da MMP-2 ($C^{-1306}T$ e $C^{-735}T$) com a migrânea;

2. Avaliar os efeitos dos diferentes polimorfismos da MMP-9 e da MMP-2, separadamente ou associados em haplótipos, sob as concentrações circulantes dessas MMPs nas mulheres com migrânea e controles

3 - CAPÍTULOS

3.1 - CAPÍTULO 1

SPECIFIC MATRIX METALLOPROTEINASE 9 (MMP-9) HAPLOTYPE AFFECT THE CIRCULATING MMP-9 LEVELS IN WOMEN WITH MIGRAINE

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Specific matrix metalloproteinase 9 (MMP-9) haplotype affect the circulating MMP-9 levels in women with migraine

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ABSTRACT

We investigated whether three relevant polymorphisms (C-1562T, microsatellite −90(CA)$_{14-24}$, and Q279R) in the MMP-9 gene, or MMP-9 haplotypes, are associated with migraine and affect MMP-9 and tissue inhibitor of MMPs (TIMP)-1 levels in patients with migraine. We studied 102 healthy women (controls) and 187 women with migraine (141 without aura — MWA, and 46 with aura — MA). Patients with MWA had higher plasma MMP-9 concentrations than patients with MA. Patients with MA had the highest TIMP-1 and lowest MMP-9/TIMP-1 ratios. The MMP-9 "C L Q" haplotype was associated with higher plasma MMP-9 concentrations in migraine patients.

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1. Introduction

Migraine is a chronic, intermittent, neurological disorder associated with a complex combination of cerebral and vascular events (Moskowitz, 2007; Goadsby et al., 2009; Markus Schürks et al., 2009). In this context, a neurovascular hypothesis has been proposed to explain the pathophysiology of migraine (Schurks et al., 2009), although the precise event responsible for migraine attacks still remains incompletely understood (Pietrobon and Striessnig, 2003). In this respect, the disruption of the blood–brain barrier (BBB) has been implicated during a migraine attack (Leira et al., 2007; Imamura et al., 2008). Despite no clear evidence of BBB proteolysis during migraine attacks (Edvinsson and Tfelt-Hansen, 2008), experimental induction of cortical spreading depression showed that breakdown of BBB may result of matrix metalloproteinases (MMP)-dependent mechanisms (Gursoy-Ozdemir et al., 2004).

MMPs are a large family of zinc-dependent enzymes involved in the degradation of a wide spectrum of extracellular matrix proteins. Their activation and activity is modulated by endogenous inhibitors, the tissue inhibitors of MMP (TIMPs), and imbalanced MMP activities have been suggested as relevant pharmacological targets in many disease conditions (Castro et al., 2011; Fontana et al., 2012; Marson et al., 2012; Romi et al., 2012). Importantly, MMP-9 has been implicated as a major mediator of BBB disruption in neuroinflammatory conditions that may promote migraine (Montaner et al., 2001; Castellanos et al., 2003; Gursoy-Ozdemir et al., 2004; Gurney et al., 2006). Indeed, experimental studies with MMP-9 knockout mice showed reduced BBB leakage and edema formation (Asahi et al., 2001), thus strongly indicating that MMP-9 is very important for this process. Moreover, previous studies showed increased circulating MMP-9 levels in migraine patients (Leira et al., 2007; Imamura et al., 2008), and it is possible that circulating MMP-9 levels may reflect migraine attacks (Leira et al., 2007; Imamura et al., 2008; Martins-Oliveira et al., 2009). However, it is not known whether genetic variations in the MMP-9 gene may affect the susceptibility to migraine and whether there are subgroups of patients that are genetically exposed to increased MMP-9 levels as a result of genetic differences. Such patients would potentially benefit from MMPs inhibitors.

Mounting evidence suggests that MMP-9 gene polymorphisms may affect MMP-9 levels, the progression of disease conditions and therapeutic responses (Demacq et al., 2009; Jacob-Ferreira et al., 2010b; Lacchini et al., 2010; Belo et al., 2012; Palei et al., in press). Relevant functional MMP-9 polymorphisms include the C(−1562)T polymorphism (Zhang et al., 1999), a microsatellite −90(CA)$_n$ (13–25 repeats) in promoter region (Shimajiri et al., 1999), and the Q279R in exon 6 (Allan et al., 1995). Therefore, in the present study, we examined

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whether these polymorphisms and their combinations (haplotypes) are associated with migraine or with altered MMP-9 concentrations in migraine patients. We have also studied the TIMP-1 concentrations and MMP-9/TIMP-1 ratio, which may provide a better index of net MMP-9 activity.

2. Materials and methods

2.1. Subjects

This study was approved by the Ethics Committee at Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. After complete description of nature of the study, each participant gave written informed consent.

We enrolled a total of 187 women with migraine and 102 healthy women without history of migraine. Among them, 141 women were diagnosed with migraine without aura (MWA) and 46 with aura (MA). Migraine patients were enrolled at the Headache Clinic of the Neurology Department, University Hospital of the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo. Diagnosis of migraine was made according to the International Classification of Headache Disorders criteria Anon (2004).

All study subjects were of Brazilian origin and underwent a complete medical history and physical examination. Furthermore, we excluded patients with preexistent diseases, pregnant as well as other kinds of headache. The control group included healthy women without headache were randomly selected from the local population visiting our University for non-medical reasons and unrelated to the patients.

2.2. Biochemical measurements

After written, informed consent was obtained, venous blood samples were collected into vacutainer plastic tubes (Becton-Dickinson, Brazil) containing sodium/potassium EDTA. We collected blood samples from patients either in the interictal phase or under migraine attack because previous results showed no differences in MMPs, TIMPs, or MMPs/TIMPs ratios when MA or MWA patients with headache attack were compared with asymptomatic MA or MWA patients, respectively (Martins-Oliveira et al., 2009). The blood samples were centrifuged at 1000×g for 10 min. The plasma samples were separated and immediately stored at −70 °C until used to measure plasma MMP-9 and TIMP-1 concentrations. In addition, aliquots of whole blood were separated and stored at −20 °C for genomic DNA extraction.

2.3. DNA isolation and genotype analyses

Genomic DNA was obtained from the cellular component of 1 mL of whole blood by a salting-out method and stored at −20 °C until analysis. To determine the genotypes for the −90(CA)14–24 (rs2234681) polymorphism, a PCR was carried out using the primers: 5′-GAC TTG GCA GTG GAG ACT GCG GGC A-3′ (sense) e 5′-GAC CCC ACC CCT CCT TGA CAG GCA A-3′ (antisense) (Demacq et al., 2008). The PCR conditions were performed as previously described (Demacq et al., 2008) and the amplified products were separated in a 7% polyacrylamide-urea gel and visualized by silver staining. Differences in molecular weight (or number of bases), from 146 bp (CA 14 repeats) to 166 bp (CA 24 repeats) were determined by comparison with migration of a 10 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) and with some samples from homozygotes that were sequenced. The alleles for the microsatellite −90(CA) 14–24 polymorphism were classified as "low" (L) count when the number of CA repeats was less than 21, and as "high" (H) when the number of CA repeats was 21 or more (Demacq et al., 2009).

Genotypes for the (C-1562T (rs3918242)) polymorphism of MMP-9 were determined by polymerase chain reaction (PCR) amplification using the primers 5′-GCC TGG CAC ATA GTA GGC CC-3′ (sense) and 5′-CTT CCT AGC CAG CCG GCA TC-3′ (antisense) and PCR conditions as previously described (Demacq et al., 2006, 2008). The amplified products were digested with *Sph I* (New England Biolabs, Ipswich, MA, USA) overnight at 37 °C, producing fragments of 247 bp and 188 bp in the case of a polymorphic variant (allele T), or an undigested 435 bp band in the case of a wild type allele (allele C) (Jacob-Ferreira et al., 2010b). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized by silver staining.

Genotypes for Q279R (rs17576) were determined using TaqMan® Allele Discrimination assay (Applied Biosystems, Foster City, CA). The PCR conditions were performed according to appointed by manufacturer's instructions.

2.4. Determination of plasma MMP-9 and TIMP-1 levels

To investigate the effects of MMP-9 polymorphisms or haplotypes on the circulating levels of MMP-9, we measured the plasma MMP-9 concentrations using a commercially available enzyme-linked immunosorbent assay (R&D Systems, Inc., Minneapolis MN) according to manufacturer's instructions. Moreover, we measured the plasma concentrations of TIMP-1 using commercially available enzyme-linked immunosorbent assays (R&D Systems, Inc., Minneapolis MN) and calculated the MMP-9/TIMP-1 ratio because this ratio may be a better index of net MMP-9 activity (Belo et al., 2009).

2.5. Haplotype inference

Haplotypes were inferred using the Bayesian statistical based program PHASE version 2.1 (http://www.stat.washington.edu/stephens/software.html) (Stephens et al., 2001) to estimate the haplotype frequencies in the population and the most likely pairs of haplotypes for each individual (Table 4). The possible haplotypes including genetic variants for three MMP-9 polymorphisms studied (H or L variants for −90(CA) 14–24, C or T variants for the C-1562T and Q or R variants for Q279R) were: H1 (CLR), H2 (CHR), H3 (CHQ), H4 (THQ), H5 (TLR), H6 (CLQ), H7 (THR) and H8 (TLQ). Therefore, we evaluated whether MMP-9 haplotypes modulate circulating MMP-9, TIMP-1 and MMP-9/TIMP-1 ratio among studied groups. However, due to low frequency of the H7 and H8 haplotypes, we excluded them of analysis. To assess differences in haplotype frequency distributions was used chi-square test, and to compare haplotype frequencies in controls and migraine a value of $p<0.00625$ (0.05/number of haplotypes) was considered significant to correct for the number of comparisons made.

2.6. Statistical analysis

The clinical data of study participants were compared by Kruskall-Wallis test followed, by Dunn's multiple comparison test for continuous variables and were expressed as mean ± standard deviation. Categorical variables were compared by Fisher's exact test or χ^2 test and expressed as frequencies and percentages (StatView, Cary, NC, USA).

The distribution of genotypes for each polymorphism was examined for deviation from the Hardy-Weinberg equilibrium by using chi-squared tests (StatView, Cary, NC, USA). Differences in the genotype and allele frequencies of each polymorphism among the groups were analyzed using chi-squared tests. In function of the relatively low frequency of the TT genotype, we combined both TT and CT genotypes (CT + TT group) to compare the effect of the genotypes on MMP-9 plasma levels. A value of $p<0.05$ was considered the minimum level of statistical significance.

3. Results

The data regarding clinical characteristics of the individuals enrolled in the present study population are summarizes in Table 1. There were no significant differences in the clinical parameters among groups ($p>0.05$).

We found higher plasma MMP-9 concentrations in MWA patients compared with MA patients ($p<0.05$; Table 1). In addition, we found higher plasma TIMP-1 concentrations in the MA group of women with MA compared with those found in MWA and the control group ($p<0.05$; Table 1). Interestingly, we found that women with MA had lower MMP-9/TIMP-1 ratios when compared with MWA and with the control group ($p<0.05$; Table 1).

Table 2 shows the genotype and allele distributions in migraine and control groups. The distribution of genotypes for the three polymorphisms evaluated in this study showed no deviation from Hardy-Weinberg equilibrium in both case and control groups. We found no significant difference in the genotype and allelic distribution for the three MMP-9 polymorphisms when patients and controls were compared ($p>0.05$).

We examined the effects of MMP-9 polymorphisms on plasma MMP-9 concentrations in the three study grups (Table 3). While we found no major effects of genotypes on plasma MMP-9 levels in the three study groups ($p>0.05$; Table 3), we found lower MMP-9 levels in patients with the CT or TT genotypes for the C-1562T polymorphism in the MA group than in controls or in MWA patients ($p<0.05$; Table 3).

We estimated haplotype frequencies in the three groups (Table 4). We found no significant differences in the distribution of MMP-9 haplotype frequencies when the three groups were compared ($p>0.05$; Table 4).

Interestingly, the analysis of how MMP-9 haplotypes affect MMP-9 levels in plasma showed the slightly higher MMP-9 levels in healthy subjects carrying the H4 (T H Q) haplotype compared with the other haplotypes ($p<0.05$; Fig. 1A). While no significant effects were found for MMP-9 haplotypes on plasma MMP-9 in the MWA and in the MA groups ($p>0.05$; Fig. 1C and D, respectively), the H6 haplotype was associated with the highest MMP-9 levels when all migraine patients were combined ($p<0.05$; Fig. 1C).

Finally, the analysis of TIMP-1 levels and MMP-9/TIMP-1 ratios showed no effects of MMP-9 haplotypes in the three groups ($p>0.05$; Figs. 2 and 3, respectively).

Table 1
Clinical characteristics of study participants.

Variables	Controls	MWA	MA	p value
N	102	141	46	–
Age (years)	36.3 ± 11.2	38.4 ± 11.3	39.2 ± 10.2	0.188
BMI (kg/m^2)	26.4 ± 5.3	27.2 ± 5.8	26.2 ± 5.7	0.617
Smoking (%)	20.4	24.0	17.3	0.620
Family history (%)	–	68.2	82.6	0.084
Frequency (%)				
1 to 3 per month	–	10.8	15.2	0.436
3 to 5 per month	–	18.6	8.7	0.159
5 to 10 per month	–	11.6	19.6	0.212
10 to 15 per month	–	24.1	19.6	0.683
> 15 days per month	–	34.9	36.9	0.858
Intensity of attacks (%)				
Mild	–	–	4.3	0.068
Moderate	–	32.6	26.1	0.461
Severe	–	67.4	69.6	0.855
Pain free (%)	–	48.8	45.6	0.734
Under migraine attack (%)	–	51.2	54.4	
Ethnicity (%)				
Whites	69.3	62.5	58.7	0.444
Non-whites	30.7	37.5	41.3	
MMP-9 (ng/ml)	213.5 ± 168.0	219.2 ± 157.0*	159.3 ± 118.1	<0.05
TIMP-1 (ng/ml)	394.2 ± 158.7	374.8 ± 145.9	515.1 ± 155.7**	<0.05
MMP-9/TIMP-1 ratio	0.641 ± 0.595	0.665 ± 0.501	0.374 ± 0.399**	<0.05

BMI, body mass index; MWA, migraine without aura; MA, migraine with aura. Values are the mean ± S.D. or % number of subjects.
* $p<0.05$ vs. MA.
** $p<0.05$ vs. controls and MWA.

Table 2
MMP-9 genotype and allele distributions.

Polymorphism	Genotypes	Controls (n = 102)	Migraine		
			All (n = 187)	MWA (n = 141)	MA (n = 46)
−90(CA)$_n$	LL	0.196 (20)	0.246 (46)	0.248 (35)	0.239 (11)
	HL	0.510 (52)	0.487 (91)	0.482 (68)	0.500 (23)
	HH	0.294 (30)	0.267 (50)	0.270 (38)	0.261 (12)
C-1562T	CC	0.794 (81)	0.839 (157)	0.851 (120)	0.804 (37)
	CT	0.177 (18)	0.150 (28)	0.142 (20)	0.174 (8)
	TT	0.029 (3)	0.011 (2)	0.007 (1)	0.022 (1)
R(279)Q	RR	0.480 (49)	0.476 (89)	0.490 (69)	0.435 (20)
	RQ	0.402 (41)	0.406 (76)	0.411 (58)	0.391 (18)
	QQ	0.118 (12)	0.118 (22)	0.099 (14)	0.174 (8)
	Alleles				
−90(CA)$_n$	L	0.451 (92)	0.489 (183)	0.489 (138)	0.489 (45)
	H	0.549 (112)	0.511 (191)	0.511 (144)	0.511 (47)
C-1562T	C	0.882 (180)	0.914 (342)	0.922 (260)	0.891 (82)
	T	0.118 (24)	0.086 (32)	0.078 (22)	0.109 (10)
R(279)Q	R	0.681 (139)	0.679 (254)	0.695 (196)	0.630 (58)
	Q	0.319 (65)	0.321 (120)	0.305 (86)	0.370 (34)

MWA, migraine without aura; MA, migraine with aura.

4. Discussion

While most previous studies have focused on the possible predictive value of circulating MMP-9 levels in migraine attacks, this is the first study to investigate the possible association of functional MMP-9 polymorphisms with migraine. Moreover, no previous studies have examined how combinations of genetic markers (haplotypes) in the MMP-9 gene affect the circulating MMP-9 levels, or another relevant index of net MMP-9 activity (MMP-9/TIMP-1 ratio). The main finding of the present study is that a specific MMP-9 haplotype "C L Q" is associated with high MMP-9 concentrations in patients with migraine, although we found no significant associations between MMP-9 genetic polymorphisms and migraine. This finding may have relevant pharmacogenetic implications including the identification of a particular group of migraine patients that may benefit from the use of MMPs inhibitors.

Although we have not investigated the molecular mechanisms explaining how migraine patients carrying the "C L Q" haplotype have higher MMP-9 levels, we could speculate that enhanced MMP-9 levels associated with this haplotype may predispose these patients to increased vascular BBB permeability, thus promoting the development of an inflammatory environment in their central nervous systems, which contributes to migraine attacks (Gursoy-Ozdemir et al., 2004). Interestingly, we found that MMP-9/TIMP-1 tended ($p<0.10$) to be higher in subjects with migraine carrying the "C L Q" haplotype, thus suggesting that this haplotype associated with increased MMP-9 levels is possibly also associated with increased net MMP-9 activity (Belo et al., 2009; Fontana et al., 2011). It is possible that the increased MMP-9 levels found in these subjects may result in impaired BBB integrity because clinical (Castellanos et al., 2003; Rosell et al., 2006) and experimental (Sumii and Lo, 2002) studies suggest that MMP-9 is critically implicated in this alteration.

Previous studies reported enhanced plasma MMP-9 levels in migraine patients, either between or during migraine attacks (Leira et al., 2007; Imamura et al., 2008). Our findings reported here show increased MMP-9 levels in patients with MWA compared to patients with MA, and these findings are very similar to those previously shown in a smaller study (Martins-Oliveira et al., 2009). Interestingly, we found that MA patients, but not MWA patients, had lower MMP-9 and higher TIMP-1 concentrations than healthy controls, thus suggesting that higher TIMP-1 levels could be associated with MMP-9 plasma reduction in MA patients, although we have not determined the mechanisms possibly involved in these differences. Another study showed that MMP-9 levels were not correlated with the frequency or duration of

Table 3
Effects of genotype on plasma MMP-9 concentrations in controls and in migraine patients.

Polymorphism	Genotype	Controls (n = 102)	Migraine			P
			All (n = 187)	MWA (n = 141)	MA (n = 46)	
$-90(CA)_n$	LL	150.9 (100.2–404.4)	179.4 (126.2–275.6)	181.3 (133.6–274.6)	164.2 (73.8–434.6)	ns
	HL	162.1 (115.7–229.0)	155.3 (95.1–256.5)	164.7 (99.9–265.5)	105.6 (68.8–172.1)	ns
	HH	176.3 (101.9–332.9)	156.6 (100.2–267.1)	180.8 (101.7–320.0)	120.5 (85.7–162.4)	ns
C-1562T	CC	153.8 (105.1–244.7)	163.4 (100.1–258.0)	166.6 (107.8–266.2)	146.6 (73.2–212.5)	ns
	CT + TT	183.2 (130.0–348.5)	151.2 (103.1–292.3)	240.6 (121.5–382.8)	103.3* (86.0–140.8)	0.016
R(279)Q	RR	170.6 (113.5–336.8)	171.8 (102.7–250.1)	177.6 (116.2–250.1)	124.4 (61.2–261.7)	ns
	RQ	144.8 (91.2–191.2)	152.2 (95.6–260.2)	162.4 (97.7–282.8)	113.8 (71.8–169.4)	ns
	QQ	182.9 (124.5–408.8)	161.5 (114.7–368.8)	302.7 (137.4–467.3)	140.8 (94.0–162.4)	ns

MWA, migraine without aura; MA, migraine with aura. Values are expressed as median (interquartile range).
* $p<0.05$ vs. control group and MWA (Kruskall–Wallis test).

Table 4
Estimated MMP-9 haplotype frequencies in the control group and in migraine patients.

	Haplotypes	Controls	Migraine	MWA	MA
H1	CLR	0.429 (87)	0.453 (170)	0.442 (125)	0.467 (43)
H2	CHR	0.249 (51)	0.226 (84)	0.253 (71)	0.163 (15)
H3	CHQ	0.186 (38)	0.203 (76)	0.184 (52)	0.240 (22)
H4	THQ	0.112 (23)	0.082 (31)	0.073 (21)	0.108 (10)
H5	TLR	–	–	–	–
H6	CLQ	0.021 (4)	0.033 (12)	0.043 (12)	0.021 (2)
H7	THR	0.003 (1)	–	–	–
H8	TLQ	–	0.003 (1)	0.005 (1)	–

MWA, migraine without aura; MA, migraine with aura.

migraine attacks (Ashina et al., 2010). It is highly probable that some differences between studies may explain differences in MMP-9 levels. For example, we included only women in our present study, and this is not the case in many other studies which included both men and women. Moreover, it is possible that many environmental factors may also affect MMP-9 levels (Jacob-Ferreira et al., 2009, 2010a).

Another potential implication of the present findings is that migraine patients carrying the "C L Q" haplotype may be exposed at increased cardiovascular risk. Indeed, it is widely acknowledged that migraine affects the risk of cardiovascular events (Kurth et al., 2006; Bigal et al., 2009). Whether patients with migraine carrying the "C L Q" haplotype are exposed to increased cardiovascular risk associated with this specific haplotype is unknown. However, this suggestion is supported by growing evidence indicating that increased circulating MMP-9 levels are proportionally associated with increased cardiovascular risk (Garvin et al., 2008). Indeed, a complex long term study would be required to test this hypothesis. However, if this hypothesis is proven true, migraine patients with the "C L Q" haplotype would clearly benefit from the use of MMPs inhibitors.

Some limitations of the present study should be considered. Firstly, there were differences in the number of subjects in each group, and this may have decreased the power to detect significant differences

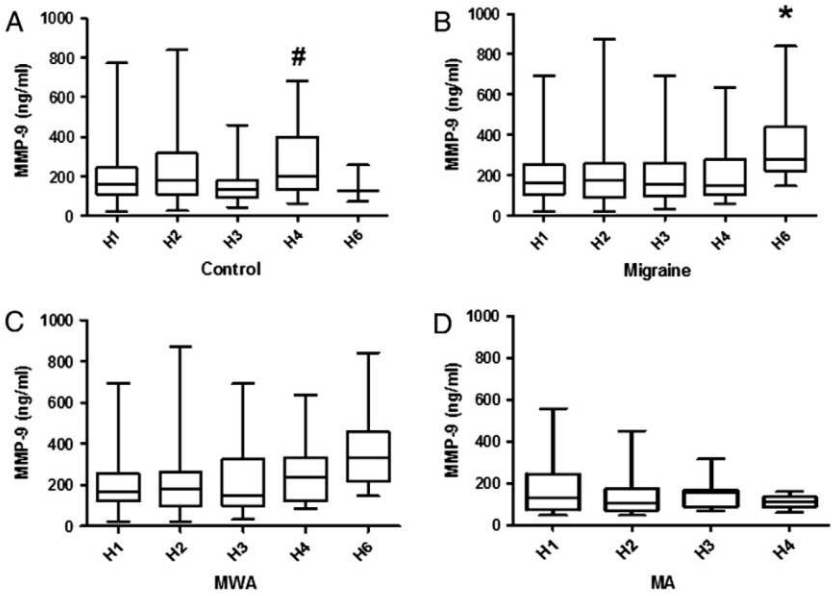

Fig. 1. Effects of MMP-9 haplotypes on plasma MMP-9 concentrations in the control (Panel A), migraine (Panel B), migraine without aura (MWA; Panel C) and migraine with aura (MA; Panel D) groups. The box and whisker plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values. #$p<0.05$ for H4 vs. H3 (panel A). *$p<0.05$ for H6 vs. H1, H2, H3 (panel B).

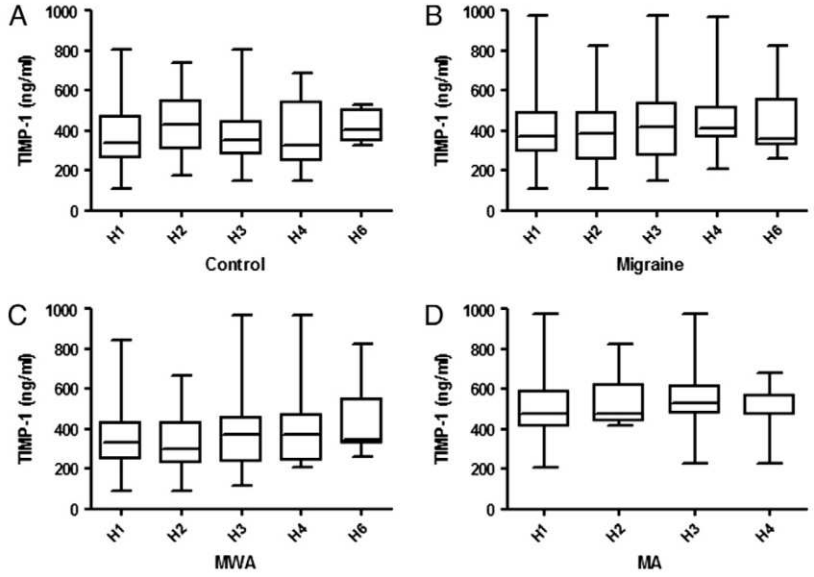

Fig. 2. Effects of MMP-9 haplotypes on plasma TIMP-1 CONCENTRATIONS in the control (Panel A), migraine (Panel B), migraine without aura (MWA; Panel C) and Migraine with aura (MA; Panel D) groups. The box and whisker plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values.

between groups; secondly, we studied only women. We decided to include only women in our study because the prevalence of migraine is higher in women than in men (Jensen and Stovner, 2008). The male:female ratio for migraine among adults varies from 1:2 to 1:3, thus facilitating the recruitment of patients for the study. Because we included only women in the present study, we can not necessarily extrapolate our findings to men, especially because there are many differences between man and women, particularly with respect to hormonal issues; thirdly, we have not evaluated clinical cardiovascular events in the present study or other parameters of the insulin and lipid metabolism that may affect MMPs in migraine patients (Bernecker et al., 2011). However, these limitations require additional studies.

In conclusion, our findings suggest that the "C L Q" haplotype is associated with highest MMP-9 levels in migraine patients, possibly

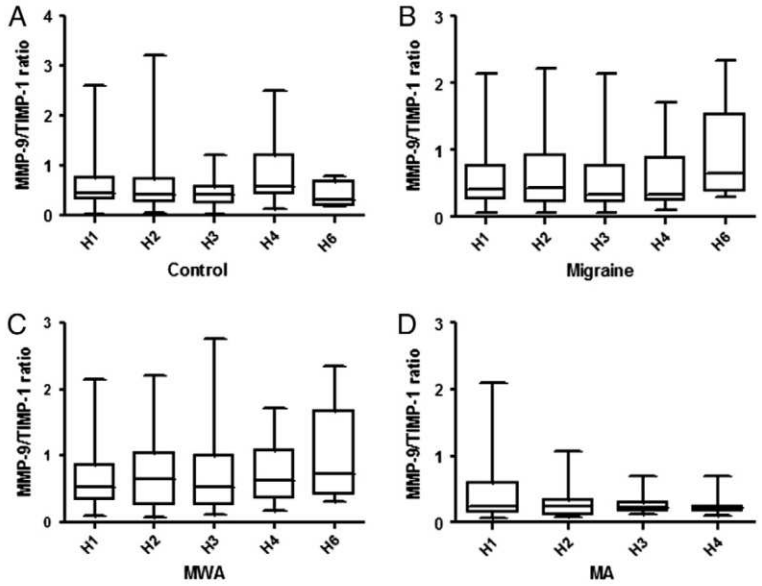

Fig. 3. Effects of MMP-9 haplotypes on MMP-9/TIMP-1 ratio in the control (Panel A), migraine (Panel B), migraine without aura (MWA; Panel C) and migraine with aura (MA; Panel D) groups. The box and whisker plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values.

contributing to proteolytic breakdown of the BBB. Patients with this specific haplotype may benefit from the use of MMPs inhibitors.

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3.2 - CAPÍTULO 2

MATRIX METALLOPROTEINASE (MMP)-2 GENE POLYMORPHISMS AFFECT CIRCULATING MMP-2 LEVELS IN PATIENTS WITH MIGRAINE WITH AURA

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Matrix metalloproteinase (MMP)-2 gene polymorphisms affect circulating MMP-2 levels in patients with migraine with aura

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ABSTRACT

Matrix metalloproteinases (MMP) are involved in the disruption of blood–brain barrier (BBB) during migraine attacks. In the present study, we hypothesized that two functional polymorphisms ($C^{-1306}T$ and $C^{-735}T$) in *MMP-2* gene and *MMP-2* haplotypes are associated with migraine and modify MMP-2 and tissue inhibitor of MMP (TIMP)-2 levels in migraine. Genotypes for *MMP-2* polymorphisms were determined by real time-PCR using Taqman allele discrimination assays. Haplotypes were inferred using the PHASE program. Plasma MMP-2 and TIMP-2 concentrations were measured by gelatin zymography and ELISA, respectively, in 148 healthy women without history of migraine and in 204 women with migraine (153 without aura; MWA, and 51 with aura; MA). Patients with MA had higher plasma MMP-2 concentrations and MMP-2/TIMP-2 ratios than patients with MWA and controls ($P<0.05$). While *MMP-2* genotype and haplotype distributions for the polymorphisms were similar among the groups ($P>0.05$), we found that the CC genotype for $C^{-735}T$ polymorphism and the CC haplotype were associated with higher plasma MMP-2 concentrations in MA group ($P<0.05$). Our findings may help to understand the role of MMP-2 and its genetic variants in the pathophysiology of migraine and to identify a particular group of migraine patients with increased MMP-2 levels that would benefit from the use of MMP inhibitors.

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1. Introduction

Migraine is a common, complex, disabling, neurovascular disorder affecting approximately 10% of the adult population worldwide, especially women (Jensen and Stovner, 2008). Special attention should be given to this clinical disorder because women with migraine, particularly those with aura, are exposed to an increased risk of cardiovascular diseases including ischemic stroke (Bigal et al., 2010; Rist et al., 2010). Although the underlying etiology of this condition remains unknown, cortical spreading depression (CSD), plasma protein extravasation, and neurogenic inflammation are mechanisms involved in the pathophysiology of migraine (Galletti et al., 2009; Pietrobon and Striessnig, 2003). In this respect, mounting evidence suggests that matrix metalloproteinases (MMP) may alter the vascular permeability of cerebral vessels and disrupt blood–brain barrier (BBB), thus leading to a migraine attacks (Ashina et al., 2010; Bernecker et al., 2011; Gursoy-Ozdemir et al., 2004; Martins-Oliveira et al., 2009). Importantly, increased circulating MMP-2 concentrations were shown in patients with migraine (Martins-Oliveira et al., 2009).

MMPs are a large family of zinc-dependent endopeptidases involved in the degradation of many components of the extracellular matrix (Sbardella et al., 2012). Accordingly, MMP activity is regulated at the levels of gene transcription, post-translational modifications, and by interactions with their endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs) (Fontana et al., 2012). Because an imbalance between MMP-2 and its endogenous inhibitor (TIMP-2) contributes to several pathologic conditions affecting both the cardiovascular and the central nervous systems (Ceron et al., 2012; Heo et al., 1999; Marson et al., 2012; Palei et al., 2012; Rosenberg, 2009), MMP inhibition has been suggested as an attractive therapeutic target in prevention of such diseases (Castro et al., 2011; Romi et al., 2012). Importantly, TIMP-2 is the most relevant MMP-2 inhibitor (Raffetto and Khalil, 2008) and therefore the study of circulating MMP-2/TIMP-2 ratio may be useful to assess reflect net MMP-2 activity in clinical studies, and therefore changes in TIMP-2 levels may increase MMP-2 activity favoring disease conditions.

Functional single nucleotide polymorphisms in the promoter region of the *MMP-2* gene (the $C^{-1306}T$/rs243865 and the $C^{-735}T$/

Abbreviations: MMPs, Matrix metalloproteinases; BBB, Blood–brain barrier; TIMPs, Tissue inhibitor of MMPs; CSD, Cortical spreading depression; MWA, Migraine without aura; MA, Migraine with aura; RT-PCR, Real time-polymerase chain reaction; ELISA, Enzyme-linked immunosorbent assay.

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rs2285053) affect MMP-2 expression or activity (Price et al., 2001; Yu et al., 2004) and may predispose to disease conditions (Lacchini et al., 2012; Saracini et al., 2012; Zhou et al., 2005), especially in those subjects carrying *MMP-2* variants associated with increased MMP-2 concentrations (Jacob-Ferreira et al., 2011; Marson et al., 2012). However, no previous study has examined how *MMP-2* gene polymorphisms affect MMP-2 concentrations in patients with migraine.

This study aimed at determining whether functional *MMP-2* polymorphisms are associated with migraine, and whether *MMP-2* polymorphisms affect MMP-2 concentrations in patients. Furthermore, because the analysis of combinations of genetic markers in a region of interest (haplotypes) may provide an improved genetic information (Crawford and Nickerson, 2005), the possibility that *MMP-2* haplotypes affect the circulating MMP-2 and TIMP-2 levels, and MMP-2/TIMP-2 ratio (a better index of net MMP-2 activity) in migraine patients was also examined.

2. Materials and methods

2.1. Study population

This study was approved by the Ethics Committee at Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil, and each subject provided written informed consent.

This study included 204 women with migraine enrolled at the Headache Clinic of the Division of Neurology, University Hospital of the Faculty of Medicine of Ribeirao Preto, and 148 healthy women without history of migraine. Among patients, 153 women were diagnosed with migraine without aura (MWA) and 51 with migraine with aura (MA). All study subjects underwent a complete medical history and physical examination. Diagnosis of migraine was made according to the International Classification of Headache Disorders criteria (2004). The control group included healthy women without headache randomly selected from the local population, unrelated to the patients. Furthermore, women with other diseases including inflammatory diseases, pregnant, or with other kinds of headache were excluded from the study.

After written, informed consent was obtained, venous blood samples were collected into vacutainer plastic tubes containing sodium/potassium EDTA. Blood samples were centrifuged at 1000 ×*g* for 10 min. Plasma samples were separated and immediately stored at −70 °C until used to measure plasma MMP-2 and TIMP-2 concentrations. In addition, aliquots of whole blood were separated and stored at −20 °C for genomic DNA extraction.

2.2. DNA isolation and genotype determination

Genomic DNA was extracted from the cellular component of 1 mL of whole blood through salting-out method and stored at −20 °C until analyzed.

Genotypes for the $C^{-1306}T$ (rs243865) and the $C^{-735}T$ (rs2285053) polymorphisms in the 5'-flanking region of *MMP-2* gene were determined by real time-polymerase chain reaction (RT-PCR), using Taqman Allele Discrimination assays (Applied Biosystems, Carlsbad, CA, USA). Probes and primers used for the $C^{-1306}T$ genotyping assay were customized as follows: forward 5′-GCCATTGTCAATGTTCCCTAAAACA-3′, reverse 5′-TGACTTCTGAGCTGAGACCTGAA-3′ and probes 5′-CAGC ACTC[T/C]ACCTCT-3′. TaqMan PCR was performed in a total volume of 12 μl (3 ng of DNA, 1× TaqMan master mix, 1× assay mix) placed in 96-well PCR plates. Fluorescence from PCR amplification was detected using Chromo 4 Detector (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with manufacturer's software. Probes and primers used in *MMP-2* $C^{-735}T$ assay were designed by Applied Biosystems (ID: C_26734093-20). TaqMan PCR and fluorescence reading were performed as described above for the $C^{-1306}T$ polymorphism (Lacchini et al., 2012).

2.3. Determination of plasma MMP-2 by SDS-PAGE gelatin zymography

To investigate the effects of *MMP-2* polymorphisms or haplotypes on the circulating levels of MMP-2, gelatin zymography of MMP-2 from plasma samples was performed as previously reported (Gerlach et al., 2007). Briefly, plasma samples were diluted in sample buffer (2% SDS, 125 mM Tris–HCl; pH 6.8, 10% glycerol and 0.001% bromophenol blue) and subjected to electrophoresis on 7% SDS-PAGE co-polymerized with gelatin (1%) as the substrate. After electrophoresis the gel was incubated for 1 h at room temperature in a 2% Triton X-100 solution, and incubated at 37 °C for 16 h in Tris–HCl buffer, pH 7.4, containing 10 mM $CaCl_2$. Gels were stained with 0.05% Coomassie brilliant blue G-250, and then unstained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin, by densitometry using ImageJ Program (National Institutes of Health, USA). MMP-2 was identified as a band at 72 kDa by direct comparison with relative mobility of Sigma SDS-PAGE LMW marker proteins. The intensity value for the MMP-2 band was calculated as relative activity according to the intensity of related MMP-2 standard (Gerlach et al., 2007).

2.4. Enzyme immunoassay of TIMP-2

Plasma TIMP-2 concentrations were measured in EDTA-plasma, using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. The reaction was evaluated using a μQuant™ microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Moreover, we calculated the MMP-2/TIMP-2 ratio because this ratio may be a better index of net MMP-2 activity (Martins-Oliveira et al., 2009).

2.5. Haplotype inference

Haplotypes were inferred using the Bayesian statistical based program PHASE version 2.1 (Stephens et al., 2001) (http://www.stat.washington.edu/stephens/software.html) to estimate the haplotype frequencies in the population and the most likely pairs of haplotypes for each individual. The possible haplotypes including genetic variants of two polymorphisms in the *MMP-2* gene studied, $C^{-1306}T$ and $C^{-735}T$ were: H1 (CC); H2 (CT); H3 (TC); H4 (TT). However, due to low frequency of the H4 haplotype, we excluded it from the analysis.

To assess differences in haplotype frequency distributions was used χ^2 test, and to compare haplotype frequencies in controls and migraine a value of $P<0.0125$ (0.05/4 − number of possible haplotypes) was considered significant to correct for the number of comparisons made.

2.6. Statistical analyses

The clinical data of study participants were compared by the Kruskall–Wallis test followed by the Dunn's multiple comparison test. The results were expressed as mean ± standard deviation. The categorical variables were compared by Fisher's exact test or χ^2 test and expressed as frequencies and percentages (StatView, Cary, NC, USA). A value of $P<0.05$ was considered to be statistically significant.

The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium, and differences in the genotypic and alleles frequencies of each polymorphism between the groups were also assessed using χ^2 tests. Due to the relatively low frequency of the TT genotype for both MMP-2 polymorphisms, the CT and TT genotypes (CT + TT group) were combined. To compare the effect of *MMP-2* genotypes on circulating levels of MMP-2, TIMP-2, and MMP-2/TIMP-2 ratios, the unpaired

Student's test (normally distributed variables) or Mann–Whitney test (not normally distributed variables) were used. A value of $P<0.05$ was considered the minimum level of statistical significance.

In addition, to examine whether *MMP-2* haplotypes affect the circulating levels of MMP-2, TIMP-2, and MMP-2/TIMP-2 ratios, the Kruskall–Wallis test followed by Dunn's Multiple Comparison test (non-parametric data) or ANOVA followed by Tukey's test (parametric data) were used.

3. Results

The data regarding clinical characteristics of subjects included in the present study are summarized in Table 1. There were no significant differences in the clinical parameters among groups ($P>0.05$; Table 1). Higher MMP-2 levels were found in patients in the MA group compared with the MWA group ($P<0.05$; Table 1). Lower TIMP-2 levels were found in both MA and MWA groups compared with controls ($P<0.05$; Table 1). These differences resulted in higher MMP-2/TIMP-2 ratios in MA and MWA groups compared with controls, and higher MMP-2/TIMP-2 ratios in the MA group compared with the MWA group (both $P<0.05$; Table 1).

The results showing *MMP-2* single-locus analysis and haplotypes distributions in the groups are shown in Supplementary Table 1 and Supplementary Table 2, respectively. The distribution of genotypes for the two *MMP-2* polymorphisms showed no deviation from Hardy–Weinberg equilibrium (all $P>0.05$). No significant differences in the genotype and allelic distribution for the two *MMP-2* polymorphisms were found when patients and controls were compared (all $P>0.05$; Supplementary Table 1). In line with these results, no significant differences in the distribution of *MMP-2* haplotypes frequencies among groups were found (all $P>0.05$; Supplementary Table 2).

The influence of the $C^{-1306}T$ and the $C^{-735}T$ *MMP-2* polymorphisms on plasma MMP-2, TIMP-2, and MMP-2/TIMP-2 ratios was determined in the three study groups (Figs. 1 and 2). While no major effects of genotypes for the $C^{-1306}T$ polymorphism on plasma MMP-2 levels in migraine patients were found ($P>0.05$; Fig. 1A), lower MMP-2 levels were found in controls with the CT or TT genotypes compared with controls with the CC genotype ($P<0.05$; Fig. 1A). Moreover, higher MMP-2 concentrations were found in MA patients with the CC genotype than in MWA patients with the same genotype ($P<0.05$; Fig. 1A). Lower TIMP-2 levels were found in the MWA and MA groups, independently of their genotypes for the $C^{-1306}T$ polymorphism (both $P<0.05$; Fig. 1B). While higher MMP-2/TIMP-2 ratio were found in MA patients with the CC genotype compared with MWA or controls with the same genotype (both $P<0.05$; Fig. 1C), higher MMP-2/TIMP-2 ratios were found in MWA and MA with CT and TT genotypes than in controls with the same genotypes ($P<0.05$; Fig. 1C).

Table 1
Clinical characteristics of study participants.

Parameters	Control ($N=148$)	MWA ($N=153$)	MA ($N=51$)
Age (years)	36.9 ± 11.7	38.2 ± 11.5	39.5 ± 10.6
BMI (kg/m^2)	25.7 ± 5.3	26.6 ± 5.8	25.9 ± 5.8
Ethnicity (%white)	70	62	58
Familial history (%)	–	68	80
Frequency (%)			
1–3/months	–	11	14
3–5/months	–	17	8
5–10/months	–	12	26
10–15/months	–	25	18
>15/months	–	35	34
Intensity (%)			
Mild	–	0	4
Moderate	–	33	26
Severe	–	67	70
Duration (%)			
<12 hours	–	15	14
12–24 hours	–	19	14
>24 hours	–	66	72
Headache free (%)	–	53	56
MMP-2 (A.U.)	0.99 ± 0.39	0.96 ± 0.38	1.16 ± 0.39*,#
TIMP-2 (ng/ml)	115.0 ± 28.3	95.4 ± 18.1*	92.5 ± 19.0*
MMP-2/TIMP-2	0.0089 ± 0.004	0.0102 ± 0.004*	0.0131 ± 0.005*,#

MWA, migraine without aura; MA, migraine with aura; BMI, body mass index. A.U., arbitrary units; Values are the mean ± S.D. or % number of subjects.
* $P<0.05$ *vs.* Control group.
\# $P<0.05$ *vs.* MWA group.

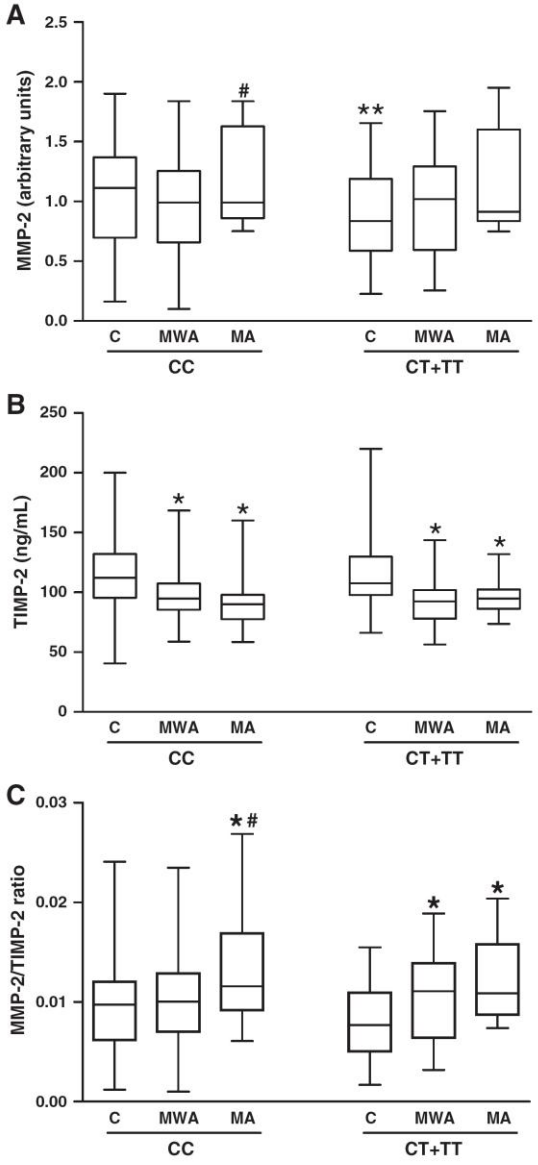

Fig. 1. Plasma MMP-2 (Panel A) and TIMP-2 (Panel B) concentrations, and MMP-2/TIMP-2 ratios (Panel C) in controls (C), in women with migraine without aura (MWA), and in women with migraine with aura (MA) with different MMP-2 genotypes for the $C^{-1306}T$ polymorphism. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values. *$P<0.05$ *vs.* the respective C group. #$P<0.05$ *vs.* the respective MWA group. **$P<0.05$ *vs.* C group with CC genotype.

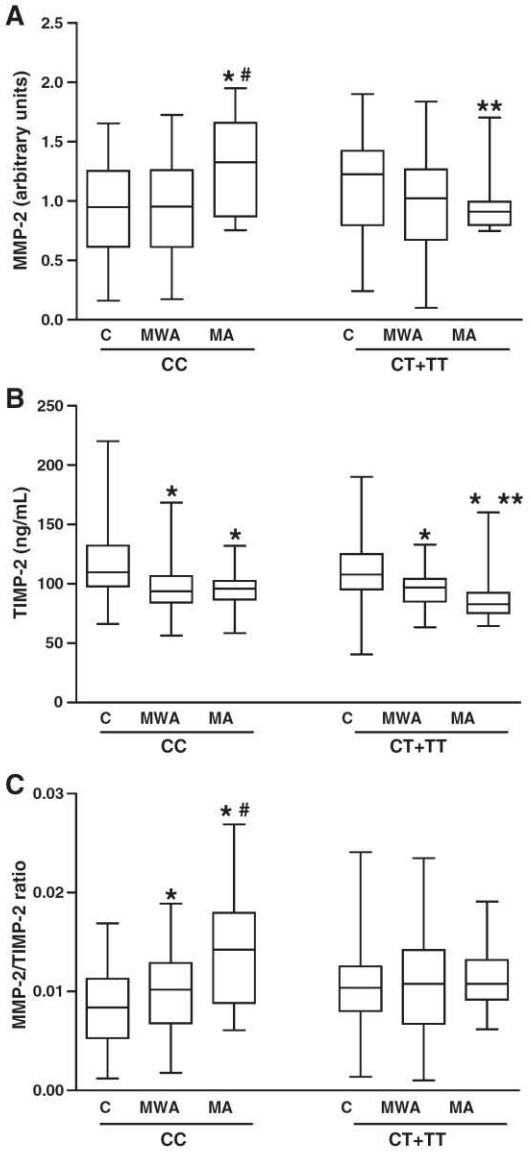

Fig. 2. Plasma MMP-2 (Panel A) and TIMP-2 (Panel B) concentrations, and MMP-2/TIMP-2 ratios (Panel C) in controls (C), in women with migraine without aura (MWA), and in women with migraine with aura (MA) with different MMP-2 genotypes for the $C^{-735}T$ polymorphism. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values. *$P<0.05$ *vs.* the respective C group. #$P<0.05$ *vs.* the respective MWA group. **$P<0.05$ *vs.* MA group with CC genotype.

With respect to the $C^{-735}T$ polymorphism, higher MMP-2 concentrations were found in MA patients with the CC genotype when compared with MWA patients and controls with the same genotype ($P<0.05$; Fig. 2A). Interestingly, higher MMP-2 levels were found in MA patients with the CC genotype than in MA patients with the CT or TT genotypes ($P<0.05$; Fig. 2A). Lower TIMP-2 levels were found in MWA or in MA patients than in controls, independently of genotypes ($P<0.05$; Fig. 2B), and MA patients with the CT or TT genotypes had lower TIMP-2 levels than MA patients with the CC genotype ($P<0.05$; Fig. 2B). Higher MMP-2/TIMP-2 ratios were found in MA or in MWA patients with the CC genotype ($P<0.05$), but not with the CT and TT genotypes (Fig. 2C).

The analysis of MMP-2 haplotypes showed higher plasma MMP-2 levels in MA patients with the CC (H1) haplotype compared with MWA and controls with the same haplotype ($P<0.05$; Fig. 3A). Patients with MA and the CT (H2) haplotype had lower MMP-2 levels

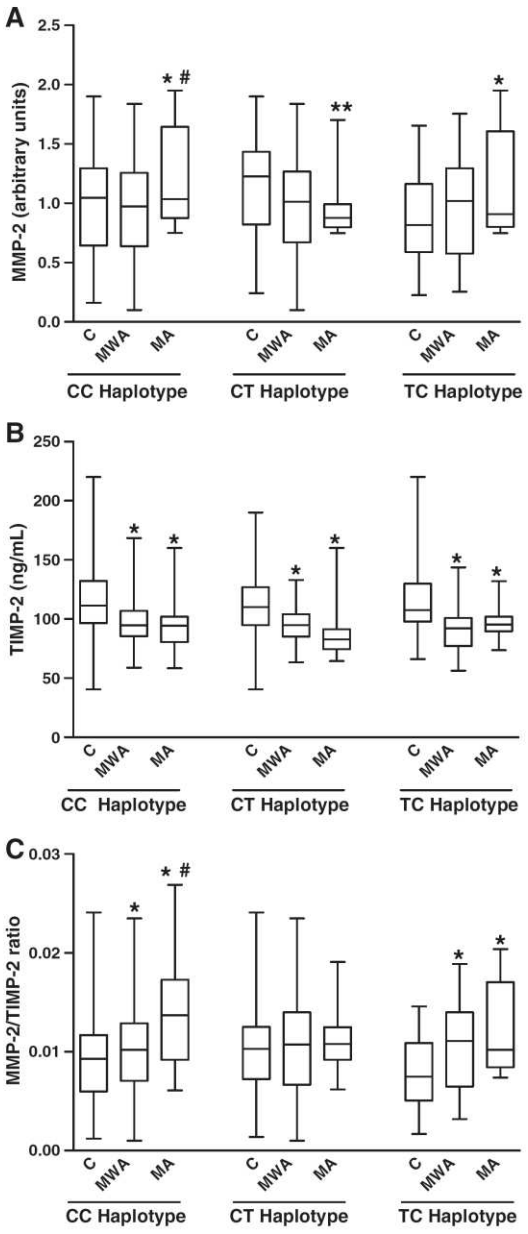

Fig. 3. Plasma MMP-2 (Panel A) and TIMP-2 (Panel B) concentrations, and MMP-2/TIMP-2 ratios (Panel C) in controls (C), in women with migraine without aura (MWA), and in women with migraine with aura (MA) with different MMP-2 haplotypes. The box and whiskers plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values. *$P<0.05$ *vs.* the respective C group. #$P<0.05$ *vs.* the respective MWA group. **$P<0.05$ *vs.* MA group with CC (H1) haplotype.

when compared with MA patients and the CC (H1) haplotype ($P<0.05$; Fig. 3A). Higher MMP-2 levels were found in MA patients with the TC (H3) haplotype compared with controls with the same haplotype ($P<0.05$; Fig. 3A). Interestingly, lower TIMP-2 levels were found in MWA and in MA groups compared with controls independently of their haplotypes (all $P<0.05$; Fig. 3B). Higher MMP-2/TIMP-2 ratios were found in MWA and in MA groups compared with controls carrying either the CC (H1) or the TC (H3) haplotype (both $P<0.05$; Fig. 3C), but not in subjects carrying the CT (H2) haplotype (Fig. 3C).

A power calculation analyses was performed using the Power for Genetic Association Analyses (PGA) software (http://dceg.cancer.gov/bb/tools/pga/). Given the sample size of this study, we obtained a statistical power of 80% with an alpha of 0.05 to detect a relative risk of 1.7 and 2.2 for the migraine total group (MWA and MA groups) and for the MA group, respectively.

4. Discussion

To our knowledge, this is the first study to show that functional *MMP-2* polymorphisms and haplotypes affect the circulating MMP-2 levels or MMP-2/TIMP-2 ratios in patients with migraine. While no significant associations between *MMP-2* genetic polymorphisms or haplotypes and migraine were found, the variations in MMP-2 and MMP-2/TIMP-2 ratios associated with different *MMP-2* genotypes and haplotypes show that these *MMP-2* polymorphisms affect relevant features of the disease. Given the relevance of MMP-2 levels in plasma, patients with higher MMP-2 levels associated with particular genotypes or haplotypes may be exposed at increased risk of cardiovascular complications.

Despite the fact that MMP-2 has been associated with changes in the BBB integrity in several pathologic conditions affecting the central nervous system (Feng et al., 2011; Liu et al., 2012; Yang and Rosenberg, 2011; Yang et al., 2007), MMP-dependent mechanisms involved in the disruption of BBB during migraine attacks have not been fully elucidated (Edvinsson and Tfelt-Hansen, 2008). However, previous studies suggest that CSD-related MMP activation may enhance cerebrovascular permeability and alter the BBB composition and function, thus generating a neuroinflammatory microenvironment that results in migraine symptoms (Gursoy-Ozdemir et al., 2004). Giving support to this suggestion, increased MMP-2 levels and MMP-2/TIMP-2 ratios (which indicate increased net MMP-2 activity) were found in women with migraine with aura, thus providing evidence for the increased net MMP-2 activity in those patients. Since abnormal MMP-2 activity clearly contributes to vascular dysfunction and may increase the incidence of cardiovascular events (Ceron et al., 2012; Dhillon et al., 2010; Ehrlich et al., 2011) we could speculate that this particular group of migraine patients may be exposed at higher risk of developing cardiovascular disease, especially those with genotypes or haplotypes associated with increased MMP-2 levels. In fact, recent epidemiological studies reported an association of migraine with aura not only with coronary events but also with ischemic stroke (Bigal et al., 2010; Kurth et al., 2012; Rist et al., 2010), and growing evidence implicates MMP-2 in neuronal injury resulting in BBB disruption, cerebral edema, and hemorrhage during the early phase of ischemic stroke (Rosenberg et al., 1998; Yang and Rosenberg, 2011). Moreover, MMP activity inhibition is effective in preserving BBB function and attenuates brain damage in the animal stroke models (Rosenberg et al., 1992; Wang et al., 2012; Yang et al., 2007). Therefore, our findings may have clinically relevant implications.

Because MMP-2 plays a central role in the degradation of extracellular matrix components, both in physiological and in pathophysiological conditions (Castro et al., 2011; Sbardella et al., 2012), there is growing interest in examining whether polymorphisms in the *MMP-2* gene are associated with differences in MMP-2 levels (Jacob-Ferreira et al., 2011; Lacchini et al., 2012; Marson et al., 2012; Palei et al., 2012). Interestingly, the allele −1306T or −735T disrupts a Sp-1 binding site within the promoter region of *MMP-2* gene, thus leading in lower MMP-2 promoter activity (Price et al., 2001; Yu et al., 2004). These observations support our findings showing that women with migraine with aura carrying the CC genotype for $C^{-735}T$ polymorphism have increased MMP-2 levels compared with those carrying the T allele.

A previous study showed that the *MMP-2* haplotype combining the C alleles for both polymorphisms is associated with increased MMP-2 promoter activity and mRNA expression (Yu et al., 2004). In line with this previous report, higher MMP-2 concentrations were found in women with migraine with aura carrying the CC haplotype. Although the molecular mechanisms explaining how patients with aura carrying the CC haplotype have higher MMP-2 levels are not clarified by our findings, we could speculate that increased MMP-2 levels may predispose to migraine attacks resulting of impaired BBB integrity. Furthermore, our findings may be clinically relevant because they may help to identify of a particular group of patients with migraine that would benefit from the use of MMP inhibitors. However, this issue would require a clinical trial and it would be interesting to replicate the present findings in different populations. It is also possible that other drugs used in the therapy of other disease conditions (Ceron et al., 2010; Sousa-Santos et al., 2012) may downregulate MMP-2 levels and therefore exert beneficial effects in migraine patients.

This study has some limitations. Migraine is more frequently found in women than in men (Jensen and Stovner, 2008). One limitation of the present study is that we have studied only women, and therefore our findings may not be extrapolated to men. In addition, differences between populations (Lacchini et al., 2010) may also affect the contribution of MMPs polymorphisms to migraine. Further studies should address those issues. Although this study has statistically significant findings, it would be interesting to carry out studies with bigger sample size. Finally, only two polymorphisms in the promoter region of *MMP-2* gene were included in the present study. Although these polymorphisms are functional, other polymorphisms should be considered in future studies.

In conclusion, despite the lack of significant association between genotypes or haplotypes for the *MMP-2* polymorphisms and migraine susceptibility, we found that functional *MMP-2* polymorphisms and haplotypes affect the circulating MMP-2 levels or MMP-2/TIMP-2 ratios in patients with migraine. Our findings offer biochemical evidence for a possible influence of *MMP-2* polymorphisms in the pathogenesis of migraine, particularly with aura. These findings may have relevant implications in clinical practice with respect to the possible use of MMP inhibitors when they become available for clinical use.

Conflict of interest statement

The authors declare no conflict of interest and have received no payment in preparation of this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2012.09.109.

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Supplementary data - Matrix metalloproteinase (MMP)-2 gene polymorphisms affect circulating MMP-2 levels in patients with migraine with aura

Supplementary Table 1 – MMP-2 genotypes and alleles frequencies distributions in the control and migraine women.

Genotype	***Control (N=148)***	***MWA (N=153)***	***P[a]***	***MA (N=51)***	***P[b]***	***P[c]***
($C^{-1306}T$)						
CC	98 (66)	112 (73)	0.187	35 (69)	0.752	0.528
CT+TT	50 (34)	41 (27)		16 (31)		
C *allele*	240 (81)	260 (85)	0.204	84 (82)	0.776	0.529
T *allele*	56 (19)	46 (15)		18 (18)		
($C^{-735}T$)						
CC	107 (72)	112 (73)	0.860	32 (63)	0.199	0.156
CT+TT	41 (28)	41 (27)		19 (37)		
C *allele*	248 (84)	264 (86)	0.392	80 (78)	0.221	0.059
T *allele*	48 (16)	42 (14)		22 (22)		

MWA. migraine without aura; MA. migraine with aura.

P[a] – P value for Control *versus* MWA. **P[b]** – P value for Control *versus* MA. **P[c]** – P value for MWA versus MA.

Chi-square tests were used to compare groups.

Supplementary Table 2 – MMP-2 haplotypes frequencies distributions in the control and migraine women.

	Haplotype	***Control (N=296)***	***MWA (N=306)***	***P[a]***	***MA (N=102)***	***P[b]***	***P[c]***
H1	CC	195 (66)	220 (72)	0.111	65 (64)	0.694	0.119
H2	CT	45 (15)	40 (13)	0.453	19 (18)	0.417	0.167
H3	TC	53 (18)	44 (14)	0.239	15 (15)	0.459	0.935
H4	TT	3 (1)	2 (1)	0.627	3 (3)	0.168	0.069

MWA. migraine without aura; MA. migraine with aura.

P[a] – P value for Control *versus* MWA. **P[b]** – P value for Control *versus* MA. **P[c]** – P value for MWA versus MA

Chi-square tests were used to compare groups.

4 - DISCUSSÃO

Apesar de alguns estudos avaliarem o envolvimento das MMPs, especialmente das gelatinases MMP-2 e MMP-9, na fisiopatologia da migrânea [30-33, 53, 54, 64, 65], o nosso estudo é o primeiro que investiga a possível associação entre polimorfismos funcionais nos genes da MMP-2 e da MMP-9 com a migrânea. Além disso, nenhum estudo prévio avaliou se esses polimorfismos genéticos, isoladamente ou combinados em haplótipos, poderiam afetar as concentrações circulantes das MMPs, bem como a razão MMPs/TIMPs (que é considerado um melhor índice para avaliar a atividade das MMPs) em pacientes com migrânea.

Em conjunto, os principais achados dos estudos 1 e 2 do presente trabalho foram:

1. As concentrações plasmáticas de MMP-9 estão diminuídas nas mulheres com MA, quando comparadas àquelas com MSA;
2. Os níveis plasmáticos de TIMP-1 estão significativamente aumentados, enquanto a razão MMP-9/TIMP-1 está diminuída nas mulheres com MA em relação às mulheres com MSA e às controles;
3. As maiores concentrações plasmáticas de MMP-2, assim como os maiores valores para a razão MMP-2/TIMP-2 foram encontrados nas mulheres com MA, quando comparadas àquelas com MSA e controles;

4. As concentrações plasmáticas de TIMP-2 estão significativamente diminuídas nas mulheres com migrânea, tanto MSA quanto MA, em relação às mulheres do grupo controle;

5. Não encontramos nenhuma associação significativa entre os polimorfismos estudados para a MMP-2 e para a MMP-9, isoladamente ou combinados na forma de haplótipos, com a migrânea;

6. Genótipos e haplótipos da MMP-2 e da MMP-9 são capazes de modular as concentrações plasmáticas das MMPs, -2 e -9, respectivamente, nas pacientes com migrânea;

7. Os maiores níveis plasmáticos de MMP-9 foram encontrados nas mulheres com migrânea com o haplótipo "C L Q" para a MMP-9;

8. O genótipo CC para o polimorfismo $C^{-735}T$ e o haplótipo "CC" foram associados às maiores concentrações plasmáticas de MMP-2 e também aos maiores valores para a razão MMP-2/TIMP-2 nas mulheres com MA.

Inicialmente, um importante achado deste trabalho é que as mulheres com migrânea possuem um desequilíbrio entres as concentrações plasmáticas de MMPs e TIMPs. Enquanto alguns estudos prévios reportaram um aumento dos níveis plasmáticos de MMP-9 em pacientes com migrânea, quando comparadas aos controles, tanto durante quanto entre o período de crises [54, 64, 65], nossos

resultados mostram uma diminuição dos níveis plasmáticos de MMP-9 nas pacientes com MA em relação às com MSA, mas nenhuma diferença em relação ao grupo controle. Esses resultados estão em contraste com os achados de *Gursoy-Ozdemir et al.* mostrando que em modelo animal de migrânea ocorre ativação e aumento significativo da expressão da MMP-9 na região cortical ipsilateral ao fenômeno da DCA [32]. Nesse sentido, um aumento acentuado dos níveis plasmáticos de MMP-9 seria esperado na migrânea, especialmente na presença de aura, já que a aura visual tem sido sugerida como uma manifestação clínica da DCA, dada à correlação temporal entre esses dois fenômenos [18].

É bem provável que esses resultados conflitantes possam ser parcialmente devido a diferenças entre as populações estudadas, o tamanho amostral de cada estudo, o tipo de amostra e as metodologias utilizadas para a quantificação das MMPs. No presente trabalho, incluímos apenas mulheres, enquanto que os demais estudos consideram, para as análises, pacientes de ambos os sexos. Além disso, alguns trabalhos utilizam amostras de soro para a determinação bioquímica das MMPs e esse tipo de amostra sabidamente não é o mais recomendado, uma vez que no soro, durante o processamento das amostras, ocorre a liberação de MMPs por plaquetas e/ou leucócitos, resultando em níveis de MMPs artificialmente aumentados em relação às concentrações plasmáticas reais [66, 67]. Finalmente, sabe-se que fatores ambientes podem alterar os níveis de MMP-9 e contribuir para essas diferenças [68, 69].

Além disso, outro resultado interessante do presente trabalho é que pacientes com MA tem um aumento significativo dos níveis plasmáticos de TIMP-1 e, consequentemente, uma diminuição significativa da razão MMP-9/TIMP-1. Atualmente, acredita-se que a razão entre MMPs e TIMPs seja um melhor indicador para a atividade das MMPs, em comparação aos valores de MMPs e TIMPs isoladamente. Isso porque é o equilíbrio crítico entre esses dois parâmetros quem determina o grau de degradação da matriz extracelular e das alterações estruturais e funcionais relacionadas a doenças cardiovasculares [41]. Como a atividade das MMPs é regulada, entre outros mecanismos, pela interação dos seus inibidores endógenos teciduais [34], podemos sugerir que os altos níveis plasmáticos de TIMP-1 encontrados nas mulheres com aura possam ser os responsáveis pela inibição significativa da MMP-9 e, consequentemente, pela redução acentuada de seus níveis circulantes nessas pacientes. É importante ainda, salientar que as ações dos TIMPs não se limitam apenas a inibir a ação proteolítica das MMPs, sendo que eles podem participar da regulação da proliferação, migração e apoptose celular [40, 70].

De forma interessante, todos os polimorfismos selecionados para o gene da MMP-9 nesse estudo de associação são funcionais, ou seja, possuem evidências *in vitro* de seus efeitos sobre a expressão e/ou a atividade da MMP-9. O primeiro polimorfismo, o SNP $C^{-1562}T$ do promotor da MMP-9, destrói um sítio de anelamento do tipo AP-1, que age reprimindo o gene. Dessa forma, a presença do alelo polimórfico T é responsável por uma maior expressão da MMP-9 [55]. O

microssatélite CA na posição -90 tem alelos que se distribuem mais frequentemente ao redor de 14 repetições (tratadas como *"L"*, *low*) e ao redor de 21-24 repetições (tratadas como *"H"*, *high*). Estudos *in vitro* mostraram que o aumento do número de repetições facilita a transcrição gênica, sendo que a maior expressão da MMP-9 é encontrada na presença de 21 repetições [56]. Por fim, o SNP no exon 6 da MMP-9, leva à substituição do aminoácido glutamina para arginina dentro de um domínio tipo fibronectina, responsável pelo reconhecimento ao colágeno tipo IV e ligação no sítio catalítico da enzima. Como esse domínio é altamente conservado, espera-se uma baixa afinidade da enzima pelo colágeno tipo IV quando ele é removido. Assim sendo, podemos imaginar que o alelo que gera a enzima com glutamina na posição 279 a faz ter alta afinidade pelo colágeno, resultando em sua atividade plena, enquanto que na presença do alelo que gera a enzima com arginina tem como consequência uma afinidade reduzida pelo colágeno [57].

Apesar de não encontrarmos associação entre os polimorfismos estudados no gene da MMP-9 e a migrânea, os mesmos de forma isolada ou em combinação dentro de um haplótipo, são capazes de modular as concentrações plasmáticas de MMP-9 nas pacientes com migrânea. Os maiores níveis plasmáticos de MMP-9 foram encontrados nas mulheres com migrânea portadoras do haplótipo "C L Q", respectivamente para os polimorfismos $C^{-1562}T$, $-90(CA)_n$ e R(279)Q no gene da MMP-9. Com base apenas em informações funcionais desses polimorfismos [55-57], não conseguimos explicar porque as mulheres com migrânea portadoras

deste haplótipo específico possuem os maiores níveis circulantes de MMP-9. Nesse caso, vale salientar que a migrânea é considerada uma doença complexa e, como tal, sua fisiopatologia não envolve apenas a participação das MMPs, cujo papel também não pode ser unicamente explicado pelo efeito da combinação de apenas três polimorfismos em um único gene. Além disso, pouco se sabe a respeito da interação entre efeitos genéticos, e entre genes e ambiente na migrânea.

No presente estudo, os mecanismos moleculares que levam às mulheres com migrânea portadoras do haplótipo "C L Q" a apresentarem as maiores concentrações plasmáticas de MMP-9 não foram investigados. Apesar disso, podemos sugerir que o aumento das concentrações plasmáticas da MMP-9 associadas a esse haplótipo específico poderia predispor essas pacientes a um aumento importante na permeabilidade vascular da BHE, que poderia promover no sistema nervoso central um ambiente inflamatório favorável ao agravamento de uma crise migranosa [32]. Nesse sentido, nós também observamos uma tendência ($P<0,10$) a encontrar os maiores valores para a razão MMP-9/TIMP-1 nas pacientes portadores deste mesmo haplótipo, sugerindo que o haplótipo "C L Q" associado aos maiores níveis plasmáticos de MMP-9, é também possivelmente relacionado à maior atividade da MMP-9. Ainda é possível sugerir que o aumento das concentrações plasmáticas da MMP-9 encontrado nessas mulheres possa resultar em comprometimento da integridade da BHE, já que evidências clínicas e experimentais têm mostrado o envolvimento da MMP-9 nessas alterações [71-73].

Assim como a MMP-9, a MMP-2 tem sido frequentemente associada com alterações na integridade da BHE em várias condições patológicas envolvendo o sistema nervoso central [50, 74-76]. A degradação de importantes constituintes da BHE pelas MMPs fornece um ambiente favorável para a infiltração de células inflamatórias no sistema nervoso central e o consequente estabelecimento de um processo neuroinflamatório local, além de poder também contribuir para processos envolvendo desmielinização e neurotoxicidade [77]. Ainda que os mecanismos dependentes de MMP-2 envolvidos no rompimento da BHE durante uma crise de migrânea não estejam bem estabelecidos [78] quanto àqueles envolvendo a ativação da MMP-9 [32], os resultados do nosso segundo estudo mostram um aumento significativo não só nos níveis circulantes de MMP-2, como também na razão MMP-2/TIMP-2, nas mulheres com MA quando comparadas àquelas com MSA e às controles, sugerindo haver nesse grupo de pacientes um aumento da atividade da MMP-2.

Além disso, neste trabalho observamos níveis plasmáticos diminuídos para o TIMP-2, tanto nas mulheres com MA quanto nas mulheres com MSA, em relação ao grupo controle. Este é um resultado interessante na medida em que o TIMP-2, quando em altas concentrações, inibe a MMP-2, mas em baixas concentrações pode participar da ativação da pró-MMP-2 juntamente com algumas MMPs do tipo membrana (MT-MMP): o TIMP-2, por meio de seu domínio inibitório N-terminal, liga-se à MT1-MMP (MMP-14) presente na superfície celular formando um complexo que age como um “receptor” para a pró-MMP-2 se ligar

posteriormente; a pró-MMP-2, dessa maneira, liga-se por meio de seu domínio hemopexina ao domínio C-terminal do TIMP-2 ligado à MT1-MMP, formando um complexo; posteriormente, uma segunda MT1-MMP ativa e livre de TIMP-2 é necessária e realiza a clivagem da região que está protegendo o sítio catalítico da MMP-2, tornando-a ativa [37, 79]. Portanto, as baixas concentrações plasmáticas de TIMP-2 encontradas nas mulheres com migrânea é um achado relevante, pois além de favorecer o desequilíbrio entre a razão MMP-2/TIMP-2, induzindo ao aumento da degradação de componentes da matriz extracelular e da lâmina basal da BHE, pode também favorecer a ativação da MMP-2.

Como a MMP-2 desempenha um importante papel na degradação de componentes da matriz extracelular em processos fisiológicos e patológicos [35, 80], há um crescente interesse em avaliar se polimorfismos funcionais no gene da MMP-2 estão associados a diferentes níveis circulantes dessa enzima. No presente estudo, avaliamos dois SNPs funcionais na região promotora do gene da MMP-2, o polimorfismo $C^{-1306}T$ e o polimorfismo $C^{-735}T$, ambos representados pela troca de uma citocina por uma timina. A substituição dessas bases nucleotídicas é capaz de abolir independentemente dois sítios de ligação do tipo SP-1, que são considerados como *enhancers* de expressão da MMP-2. Sendo assim, a presença do alelo C para esses polimorfismos aumentam a expressão da MMP-2, e o haplótipo unindo esses dois alelos parece ter efeito sinérgico sobre a expressão e atividade do gene [59, 60]. Essas evidências funcionais suportam nossos achados mostrando que mulheres com MA portadoras do genótipo CC para o polimorfismo

$C^{-735}T$ e/ou do haplótipo “CC” têm um aumento significativo na concentração circulante de MMP-2. Este aumento das concentrações plasmáticas da MMP-2 na migrânea com aura pode, em partes, predispor essas pacientes a crises de migrânea em função do comprometimento da integridade da BHE.

Os resultados dos nossos estudos relacionados às alterações das concentrações plasmáticas das MMPs e dos seus inibidores nas mulheres com migrânea devem ser interpretadas com prudência, pois esses dois marcadores podem ser liberados na corrente sanguínea oriundos de diversas fontes teciduais e nossos achados não nos permite apontar a principal fonte desses marcadores circulantes. A MMP-2 é constitutivamente expressa em vários tipos celulares, incluindo fibroblastos, células endoteliais, epiteliais e musculares lisas, astrócitos, fluidos cerebrospinal, neutrófilos, entre outras, enquanto que a MMP-9 é altamente induzida e principalmente encontrada em monócitos e macrófagos [35, 77]. Além disso, os níveis plasmáticos, tanto das MMPs quanto dos TIMPs, não necessariamente refletem a expressão e atividade tecidual das MMPs, pois além dessas enzimas serem produzidas por outras fontes, sua liberação do interstício para o sangue pode não ser necessariamente proporcional às concentrações internas ao tecido [81].

Como já dito anteriormente, há evidências consistentes de que níveis circulantes aumentados das gelatinases, a MMP-2 e a MMP-9, podem contribuir para disfunção endotelial e aumentar consideravelmente a incidência de eventos

cardiovasculares [44, 45, 82, 83], além de estarem associadas com importantes danos neuronais durante AVC isquêmico, resultantes do comprometimento da integridade da BHE e da formação de edemas cerebrais e hemorragias [76, 84], que são atenuados pela inibição da atividade dessas MMPs [50, 85, 86]. Somado a esses fatos, estudos epidemiológicos recentes têm mostrado uma associação positiva entre a migrânea, particularmente o subtipo com aura, com a ocorrência de eventos isquêmicos e coronarianos [9, 10, 87]. Os mecanismos pelos quais a migrânea com aura pode aumentar o risco de eventos isquêmicos são complexos e, apesar de especulativos, algumas hipóteses são bem plausíveis, como o fato de a DCA, que é o substrato para a aura migranosa, poder levar a uma diminuição tão intensa no fluxo sanguíneo cerebral, capaz de atingir um nível isquêmico e diretamente predispor o indivíduo às lesões cerebrais observadas durante um AVC isquêmico [9, 88, 89]. Já relação direta entre migrânea e a incidência de doenças cardiovasculares é mais difícil de ser explicada, mas alguns trabalhos vêm justificando essa associação através da presença de algumas comorbidades, incluindo obesidade, dislipidemia e síndrome metabólica, que estão frequentemente presentes na migrânea e que sabidamente são fatores de risco para os eventos cardiovasculares relatados nessas pacientes [88]. Essas comorbidades, se presentes na pacientes com migrânea, poderiam ter impactos também sobre as concentrações circulantes das MMPs [90, 91].

No entanto, o nosso estudo não nos permite inferir se as pacientes com migrânea portadoras de genótipos e haplótipos específicos para a MMP-2

(haplótipo “CC”) e/ou MMP-9 (haplótipo “C L Q”), associados os maiores níveis plasmáticos dessas enzimas, serão também expostas a maiores riscos de eventos cardiovasculares e isquêmicos futuros. Sendo assim, estudos prospectivos e mais complexos seriam interessantes de serem conduzidos nessa população e necessários para esclarecer essa hipótese.

Além disso, a replicações dos nossos achados em outras populações e ensaios clínicos futuros também são necessários para saber se o nosso estudo pode auxiliar na identificação de um grupo particular de pacientes com migrânea, que sejam refratárias a tratamentos convencionais, e que possivelmente tenham melhores chances de sucesso com o uso de inibidores para as MMPs. Na prática clínica, os inibidores das MMPs têm sido propostos e utilizados em algumas condições patológicas que envolvam processos inflamatórios agudos ou crônicos, como doenças periodontais, artrite reumatoide e câncer [92].

Algumas limitações do presente estudo devem ser consideradas. Entre elas, podemos citar as diferenças em relação ao número de indivíduos em cada grupo e o reduzido tamanho amostral, especialmente no grupo de mulheres com MA, que podem contribuir para a redução do poder em se detectar diferenças significativas entre os grupos, apesar da seleção e do pareamento criteriosos dos grupos. Em partes esse reduzido tamanho amostral pode ser justificado pela dificuldade no recrutamento de pacientes com MA, já que a aura está presente em apenas 20-30% das pacientes com migrânea [12, 14].

O cálculo do poder estatístico do nosso estudo foi realizado utilizando o programa PGA - *Power for Genetic Association Analyses* [93], disponível no site http://dceg.cancer.gov/bb/tools/pga. O cálculo foi feito para os diferentes grupos de migrânea, o total e o com aura (MA). Dessa forma, de posse da tabela com os dados brutos das genotipagens, fizemos o cálculo do grau efetivo de liberdade. A frequência da doença utilizada para o cálculo foi de 15% e 4%, respectivamente para os grupos de migrânea total e com aura [4, 12, 14]. Para o estudo da MMP-9 em migrânea, o grau efetivo de liberdade calculado foi de 2,57 e de 2,34 para os grupos de migrânea total e com aura, respectivamente; e a razão entre controles e casos incluídos no estudo foi de 0,545 e de 2,217, para os grupos de migrânea total e com aura, respectivamente. Em relação ao estudo da MMP-2, a frequência utilizada para o alelo mais raro foi de 15% e 14%, respectivamente para os polimorfismos $C^{-1306}T$ e $C^{-735}T$; o grau efetivo de liberdade calculado foi de 1,99 e de 2,00, respectivamente, para os grupos de migrânea total e com aura; e a razão entre controles e casos incluídos no estudo foi de 0,725 e de 2,902, respectivamente, para os grupos de migrânea total e com aura. Com isso, dado nosso tamanho amostral, para o estudo da MMP-2 é possível obter um poder estatístico de 80%, com alfa de 0,05 para detectar um risco relativo de 1,7 e de 2,2 para os grupos de migrânea total e de migrânea com aura, respectivamente. Em relação ao estudo da MMP-9, a baixa frequência encontrada no nosso grupo para o alelo mais raro do polimorfismo $C^{-1562}T$ (7,8%) nos possibilita obter um poder estatístico de 80%, com alfa de 0,05 para detectar um risco relativo de 2,2 e de 3,0

para os grupos de migrânea total e de migrânea com aura, respectivamente. Portanto, a falta de associação entre os polimorfismos da MMP-9 e da MMP-2 com migrânea deve ser vista com cautela. É possível que haja associações gerando riscos relativos abaixo dos valores encontrados, ocasionado a possibilidade de um erro tipo II por baixo tamanho amostral. Com isso, estudos considerando um número maior de indivíduos no grupo de migrânea com aura são necessários para validar nossos achados.

Outra limitação importante que deve ser considerada é o fato de não termos realizado o controle genômico para as amostras envolvidas no presente estudo. Esse é um procedimento muito interessante que deve ser feito em estudos de associação para garantir que as associações, quando encontradas, não são associações espúrias que ocorrem em função de estratificação das amostras, principalmente em populações tão heterogêneas como a população brasileira.

No nosso estudo incluimos apenas mulheres e desse modo, não sabermos se os nossos achados também se aplicam aos homens. Vale também ressaltar que apesar de selecionarmos apenas polimorfismos funcionais e clinicamente relevantes, tanto para o gene da MMP-2 quanto para o gene da MMP-9, outros polimorfismos devem ser considerados em estudos futuros, já que os polimorfismos aqui selecionados não tem o poder de avaliar todo o gene. Além disso, apesar de orientarmos as pacientes quanto aos medicamentos utilizados durante as crises de migrânea, não podemos afastar outra limitação do nosso

estudo que seria a influência de alguns desses medicamentos sob as concentrações plasmáticas das MMPs. Deve ser considerado também o fato de que neste estudo não avaliamos os eventos cardiovasculares e outros parâmetros relacionados ao metabolismo de insulina e lipídeos, que podem contribuir para alterações nos níveis circulantes das MMPs [31]. Finalmente, devemos também considerar uma limitação inerente a este tipo de estudo, onde a simples associação de um efeito com um genótipo ou haplótipo pode não atribui uma relação causal direta. Portanto, a validação das conclusões de nossos estudos só será possível após a replicação dos nossos achados em outras populações.

As principais conclusões do nosso estudo são que: 1) em comparação com o grupo controle, as mulheres com migrânea apresentam um desequilíbrio entre as concentrações plasmáticas de MMPs (MMP-2 e MMP-9) e TIMPs (TIMP-1 e TIMP-2); 2) não encontramos associação entre os polimorfismos nos genes das MMPs -2 e -9, estudados no presente trabalho, com a migrânea; 3) No entanto, esses polimorfismos genéticos, de forma isolada ou combinados dentro de haplótipos, são capazes de alterar as concentrações plasmáticas das MMPs.

5 - CONCLUSÕES

Em conclusão nosso trabalho sugere que apesar da falta de associação entre os polimorfismos genéticos da MMP-9 e da MMP-2, estudados no presente trabalho, com a migrânea, podemos concluir que diferentes polimorfismos nesses gene, de forma isolada ou em combinação dentro de um haplótipo, podem modular as concentrações plasmáticas de MMP-9 e de MMP-2 em mulheres com migrânea. O haplótipo “C L Q” foi associado aos maiores níveis plasmáticos de MMP-9 em pacientes com migrânea, enquanto o genótipo CC para ao polimorfismo $C^{-735}T$ da MMP-2 e o haplótipo “CC” foram associados às maiores concentrações plasmáticas de MMP-2 e aos maiores valores para a razão MMP-2/TIMP-2 em mulheres com migrânea com aura. Podemos sugerir que o aumento das MMPs poderiam expor essas pacientes a uma maior chance de degradação de componentes da BHE, contribuindo, desse modo, para o seu rompimento proteolítico. Esses achados oferecem evidências bioquímicas para uma possível influência de polimorfismos genéticos nos genes da MMP-2 e da MMP-9 na patogênese da migrânea.

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7 - ANEXOS

Critérios Diagnóstico para a Migrânea (Sociedade Internacional de Cefaleia).

1. Critério de Diagnóstico para Migrânea sem Aura:

A. Pelo menos cinco crises preenchendo os critérios de B a D
B. Cefaleia com duração de 4 a 72 horas (se não tratada ou com tratamento ineficaz)
C. A cefaleia apresentando pelo menos duas das seguintes características:
    1. Localização unilateral;
    2. Qualidade pulsátil;
    3. Intensidade moderada a grave;
    4. Agravada por atividade física rotineira (por exemplo: caminhar ou subir escadas).
D. Durante a cefaleia, pelo menos um dos sintomas seguintes:
    1. Náusea e /ou vômito;
    2. Fotofobia e fonofobia.
E. Não atribuída a outros transtornos.

2. Critério de Diagnóstico para Migrânea com Aura:

A. Pelo menos duas crises preenchendo os critérios de B a D
B. Aura consistindo em pelo menos uma das seguintes características, mas sem paresia:
    1. Sintomas visuais completamente reversíveis, incluindo características positivas (pontos brilhantes ou luminosos, manchas) e/ou negativas (perda de visão);
    2. Sintomas sensoriais completamente reversíveis, incluindo características positivas (formigamento) e/ou negativas (dormência);
    3. Distúrbios na fala completamente reversíveis.

C. Pelo menos duas das seguintes características:
    1. Sintomas visuais homônimos e/ou sintomas sensoriais unilaterais;
    2. Pelo menos um sintoma de aura desenvolve-se gradualmente em $\geq$ 5 minutos e/ou diferentes sintomas de aura que ocorrem em sucessão em ≥ 5 minutos;
    3. Cada sintoma dura ≥ 5 minutos e ≤ 60 minutos.
D. Cefaleia preenchendo os critérios de B a D para Migrânea sem Aura começa durante a aura ou a sucede com intervalo de até 60 minutos.
E. Não atribuída a outros transtornos.

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Prezado Professor,

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Lembramos que devem ser apresentados a este CEP, o Relatório Parcial e o Relatório Final da pesquisa.

Atenciosamente.

PROF. DR. SÉRGIO PEREIRA DA CUNHA
Coordenador do Comitê de Ética em Pesquisa do HCRP e da FMRP-USP

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