UNIVERSIDADE ESTADUAL DE CAMPINAS

Faculdade de Ciências Médicas

BERNARDO PAVINATO MARSON

INFLUÊNCIA DE POLIMORFISMOS GENÉTICOS SOBRE OS NÍVEIS CIRCULANTES DAS METALOPROTEINASES DE MATRIZ EXTRACELULAR 2 E 9 DURANTE HEMODIÁLISE

INFLUENCE OF GENETIC POLYMORPHISMS ON THE CIRCULATING LEVELS OF THE MATRIX METALLOPROTEINASES 2 AND 9 DURING HEMODIALYSIS

Campinas, 2012

UNIVERSIDADE ESTADUAL DE CAMPINAS

Faculdade de Ciências Médicas

INFLUÊNCIA DE POLIMORFISMOS GENÉTICOS SOBRE OS NÍVEIS CIRCULANTES DAS METALOPROTEINASES DE MATRIZ EXTRACELULAR 2 E 9 DURANTE HEMODIÁLISE

ORIENTAÇÃO: Prof. Dr. José Eduardo Tanus dos Santos

INFLUENCE OF GENETIC POLYMORPHISMS ON THE CIRCULATING LEVELS OF THE MATRIX METALLOPROTEINASES 2 AND 9 DURING HEMODIALYSIS

BERNARDO PAVINATO MARSON

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

Doctorade thesis presented to Post-Graduation Comission of Faculdade de Ciências Médicas da Universidade Estadual de Campinas - UNICAMP to attainment of the title of Doctor in Pharmacology.

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA POR BERNARDO PAVINATO MARSON, E ORIENTADA PELO PROF. DR. JOSÉ EDUARDO TANUS DOS SANTOS.

_____

José Eduardo Tanus dos Santos

Campinas, 2012

FICHA CATALOGRÁFICA ELABORADA POR
MARISTELLA SOARES DOS SANTOS – CRB8/8402
BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS
UNICAMP

M359i

Marson, Bernardo Pavinato, 1978-
Influência de polimorfismos genéticos sobre os níveis circulantes das metaloproteinases de matriz extracelular 2 e 9 durante hemodiálise / Bernardo Pavinato Marson. -- Campinas, SP : [s.n.], 2012.

Orientador : José Eduardo Tanus dos Santos.
Tese (Doutorado) - Universidade Estadual de Campinas, Faculdade de Ciências Médicas.

1. Diálise renal. 2. Metaloproteinases da matriz. 3. Polimorfismo genético. 4. Insuficiência renal crônica. 5. Nefrologia. I. Santos, José Eduardo Tanus dos. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

Informações para Biblioteca Digital

Título em inglês: Influence of genetic polymorphisms on the circulating levels of the matrix metalloproteinases 2 and 9 during hemodialysis.
Palavras-chave em inglês:
Renal dialysis
Matrix metalloproteinases
Polymorphism, genetic
Renal chronic failure
Nephrology
Área de concentração: Farmacologia
Titulação: Doutor em Farmacologia
Banca examinadora:
José Eduardo Tanus dos Santos [Orientador]
José Francisco Figueiredo
José Butori Lopes de Faria
Marcio Dantas
Joao Egídio Romão Junior
Data da defesa: 25-07-2012
Programa de Pós-Graduação: Farmacologia

Banca Examinadora de Tese de Doutorado

BERNARDO PAVINATO MARSON

Orientador: Prof. Dr. José Eduardo Tanus dos Santos

Membros:
Prof. Dr. José Eduardo Tanus dos Santos
Prof. Dr. José Francisco Figueiredo
Prof. Dr. Jose Butori Lopes de Faria
Prof. Dr. Marcio Dantas
Prof. Dr. João Egídio Romão Junior

Curso de pós-graduação em Farmacologia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 25/07/2012

Dedicatória

Dedico este trabalho aos pacientes em diálise, que diariamente batalham por suas vidas com dignidade e perseverança.

Agradeço ao meu paizão Ronaldo Antônio e a minha mãezinha Nívea Luiza. Tenho a sorte de ter pais que se amam de verdade, e que sempre lutaram com valentia as batalhas para que eu tivesse um futuro. Eles não só me deram a vida duas vezes, como realizaram um esforço de guerra em meio a intempéries econômicas abrindo mão de prazeres básicos para garantir que eu tenha uma vida ampla e tranquila.

Agradeço ao meu irmão Fernando, sempre comigo, e que com seu amor tanto me salvou. Eu certamente seria uma pessoa muito inferior sem a sua existência.

Agradeço ao Dr. José Eduardo Tanus dos Santos, o grande responsável pela minha formação como pesquisador. Exemplo de profissional de elevado caráter e sabedoria. Suas peculiares críticas e observações tornaram claro um caminho outrora nebuloso. A confiança a mim dispensada é motivo de orgulho.

Agradeço aos pacientes que aceitaram participar deste projeto. Estas são pessoas sofridas, suas lutas para estarem vivos no dia seguinte os tornam heróis. Suas trajetórias, seja com alegria ou com dor, me ensinaram muito sobre o sentido da vida.

Agradeço aos professores Carlos Eduardo Poli de Figueiredo e Bartira Pinheiro da Costa. Este projeto não teria existido sem suas preciosas colaborações, ensinando lições fundamentais e ajudando a me tornar um pesquisador. À Samantha e à Silvia, nossa equipe de coletas que realizou intenso trabalho em várias cidades do Rio Grande com desprendimento e alegria; e à Annerose Barros, à Manuela Boeira e ao Guilherme Menegon pelo árduo trabalho no computador. Tenho grande dívida com o Riccardo Lacchini e a Sandra, que me introduziram no ambiente múltiplo da bancada. O Riccardo mostrou ser um grande estatístico e ao mesmo tempo estar no front da biologia molecular. Às clínicas de hemodiálise e suas equipes de enfermagem, essenciais na logística do trabalho.

Agradeço ao serviço de nefrologia da PUCRS, onde conheci pessoas que me apresentaram esta especialidade maravilhosa e talharam a minha prática médica. Sem eles, não sei que tipo de médico eu seria.

Agradeço ao Marco Pacheco, por todo o amadurecimento e honestidade nesta longa caminhada. Difícil transmitir minha admiração em palavras.

Agradeço a Andréia Fochesatto, nela conheci uma alma verdadeira. Seu apoio e atitudes em momentos decisivos deste projeto servem de exemplo que não esquecerei.

Agradeço às agências de fomento que permitiram a execução destas tarefas. À Fundação de Amparo à Pesquisa do Estado de São Paulo, pelo apoio financeiro.

Agradeço aos membros da banca examinadora pelas sugestões e considerações.

E agradeço a vida numa acepção geral. Ela me permitiu ter uma família linda e numerosa, com tios, tias, primos, primas, sem os quais a vida teria menos sabor. Ela me permitiu ter amigos e amigas surpreendentes, pessoas que tornam meus dias mais leves e interessantes. Agradeço profundamente a todos eles.

“... and now, as you graduate to begin anew, I wish that for you.

Stay Hungry. Stay Foolish”.

Steve Jobs

A insuficiência renal crônica é uma complicação grave de diferentes doenças, como diabetes, hipertensão e glomerulopatias. Quando os rins entram em falência, é preciso substituir a função renal, terapia geralmente feita através de hemodiálise. A população de pacientes dependentes de hemodiálise, cujo número cresce de forma geométrica, está exposta a taxas extremamente elevadas de eventos cardiovasculares fatais e não fatais. Fatores de risco clássicos e específicos da uremia se somam conferindo alterações patológicas severas na parede dos vasos. A própria sessão de hemodiálise ativa a inflamação e induz aterogênese. A degradação da elastina e a apoptose das células musculares lisas evoluem para a mudança do fenótipo da camada média, que se expande reduzindo o lúmem em um processo de acentuada calcificação arterial. Alterações na atividade das metaloproteinases de matriz extracelular (MMPs) 2 e 9 e desequilíbrios com seus inibidores endógenos, os TIMPs, ajudam a compor este cenário ao estimular o remodelamento cardiovascular e reorganizar a matriz extracelular, permitindo a expansão tecidual e o depósito de cálcio. Os níveis circulantes de MMP-2 e -9 estão relacionados com maior severidade de doenças cardiovasculares na hemodiálise. Diversos polimorfismos genéticos foram associados com alterações na concentração e/ou atividade destas enzimas, e é possível que diferenças nas distribuições dos polimorfismos ajudem a discriminar indivíduos expostos a níveis plasmáticos mais elevados de MMP-2 e -9 tanto antes como após a sessão de hemodiálise. Os principais polimorfismos são: um polimorfismo de nucleotídeo único (SNP) ($C^{-1562}T$) e um microssatélite (-90 CA_{14-24}) na região promotora, e um SNP no exon 6 ($A^{855}T$, Q279R) da MMP-9, e dois SNPs ($C^{-1306}T$ e $C^{-735}T$) no promotor da MMP-2. Como estes polimorfismos também foram associados com diversas doenças, o propósito deste estudo foi avaliar se eles influem na concentração plasmática de MMP-2 e MMP-9 em pacientes submetidos à hemodiálise crônica, e se afetam o efeito que a sessão de hemodiálise tem sobre os seus níveis circulantes.

Para atingir nosso objetivo, estudamos 98 pacientes com idades entre 18 e 65 anos e submetidos à hemodiálise há mais de 3 meses. Amostras de sangue venoso foram coletadas em dois momentos, antes do início e após o término da sessão de hemodiálise. As concentrações de MMP-2 foram determinadas

por zimografia e as de MMP-9, TIMP-1 e TIMP-2 foram analisadas por ELISA. O DNA genômico foi extraído a partir do sangue total e amostras foram então genotipadas para os polimorfismos da MMP-2 e MMP-9. As frequências dos haplótipos da MMP-2 e -9 foram estimadas pelo programa PHASE. Nossos resultados mostraram que a sessão de hemodiálise reduz os níveis circulantes de MMP-2 e não altera o TIMP-2, ao passo que os níveis de MMP-9 e TIMP-1 se encontram aumentados ao final da sessão. Encontramos uma associação entre os genótipos que envolvem o alelo de análise T do polimorfismo $C^{-735}T$ e o haplótipo CT demonstrando níveis pré hemodiálise significativamente aumentados de MMP-2 (P= 0,0077 e P= 0,01, respectivamente), mas não de TIMP-2. Os genótipos da MMP-2 não alteram o efeito da sessão da hemodiálise, que reduziu a MMP-2 e o TIMP-2 independente de marcadores genéticos. Marcadores genéticos da MMP-9 mostraram estar associados a níveis maiores de MMP-9 após a hemodiálise: os genótipos CC e QQ (P= 0,0081 e P= 0,0415, respectivamente) e o haplótipo CLQ (P= 0,0012). As concentrações de TIMP-1 aumentaram significativamente após a hemodiálise nos genótipos HH e QR (P= 0,0375 e P= 0,0113, respectivamente) e no haplótipo CHR (P= 0,0008). Adicionalmente, marcadores genéticos da MMP-9 não alteraram os níveis basais de MMP-9 e TIMP-1. Estes achados sugerem que marcadores genéticos da MMP-2 e -9 interferem nos níveis circulantes destas proteases no contexto da hemodiálise.

Palavras-chave: Hemodiálise. Metaloproteinases de matriz extracelular. Polimorfismos genéticos. Doença renal em estádio terminal. Nefrologia.

ABSTRACT

Chronic renal disease is a serious complication which may occur in patients who suffer from a vast range of diseases, such as diabetes, hypertension and glomerulonephritis, among others. When the kidneys fail, it becomes necessary to substitute the renal function, which is usually made through hemodialysis. The population of patients that are dependent of hemodialysis are rapidly growing in number. These patients are exposed to extremely high rates of cardiovascular events. Both traditional and uremic specific factors account for severe pathologic alterations on the walls of the vessels. The session of hemodialysis itself stimulates inflammation and induces atherogenesis. The degradation of elastin and the apoptosis of smooth muscle cells eventually progresses to a change in the phenotype of the media layer, which expands, thus reducing the arterial lumen in a process of accelerated calcification. Alteration in the activity of the matrix metalloproteinases (MMPs) 2 and 9 and imbalancements with its endogenous inhibitors - the TIMPs - help to set the scenario for cardiovascular diseases by stimulating cardiovascular remodelling and reorganizing the extracellular matrix, allowing tissue expansion and calcium deposits. The circulating levels of MMP-2 and -9 are associated with greater severity of cardiovascular diseases on patients undergoing hemodialysis. Diverse genetic polymorphisms were associated with alterations on the concentration and with the activity of these enzymes, and it is possible that differences on the distribution of these polymorphisms may help to discriminate individuals exposed to increased plasmatic levels of MMP-2 and -9, both before and after hemodialysis. The main polymorphisms known are: one single nucleotide polymorphism (SNP) ($C^{-1562}T$) and one microsatellite (-90 CA_{14-24}) on the promoter region, and one SNP on exon 6 ($A^{855}G$, Q279R) of MMP-9, and two SNPs ($C^{-1306}T$ and $C^{-735}T$) on the promoter of MMP-2. These polymorphisms were also associated with a number of diseases. Our purpose was to study whether they influence the circulating levels of MMP-2 and -9 in patients undergoing hemodialysis, and whether they affect the levels of these proteases after hemodialysis.

In order to reach our aim, we have studied 98 patients whose ages ranged between 18 and 65 years of age and who were undergoing chronic hemodialysis for at least 3 months. Venous samples were collected in two

moments, before and after hemodialysis. The concentrations of MMP-2 were assayed with gelatin zymography, and MMP-9, TIMP-1, and TIMP-2 were analyzed with ELISA. Genomic DNA were extracted and samples were genotypied for MMP-2 and -9 polymorphisms. The haplotypic frequencies were analyzed by the PHASE software. Our results show that sessions of hemodialysis reduce the levels of MMP-2, however, it does not alter TIMP-2, while MMP-9 and TIMP-1 suffer an increase after the hemodialysis session. We found an association amidst the genotypes with the variant allele T on the SNP $C^{-735}T$ and on the haplotype CT showing elevated pre hemodialysis levels of MMP-2 (P= 0,0077 and P= 0,01, respectively), but not on TIMP-2. The MMP-2 genotypes do not modify the effect of a hemodialysis session. Genetic markers of the MMP-9 were associated with enhanced levels of MMP-9 after hemodialysis: the CC and QQ genotypes (P= 0,0081 and P= 0,0415, respectively) and the haplotype CLQ (P= 0,0012). The concentrations of TIMP-1 increased significantly after hemodialysis on the genotypes HH and QR (P= 0,0375 and P= 0,0113, respectively) and on the haplotype CHR (P= 0,0008). Furthermore, genetic markers of the MMP-9 have not altered the basal levels of MMP-9 and TIMP-1. These findings suggest that the genetic markers of MMP-2 and -9 interfere on circulating levels of these proteases on the hemodialysis setting.

Key-words: Hemodialysis. Matrix metalloproteinases. Polymorphisms. End stage kidney disease. Nephrology.

% - porcentagem

C - citosina

DCV – doença cardiovascular

H – conjunto de alelos do microssatélite – 90 CA_{14-24} da MMP-9 englobando todos os alelos acima de 21 repetições do dinucleotídeo

IRC – insuficiência renal crônica

L - conjunto de alelos do microssatélite – 90 CA_{14-24} da MMP-9 englobando todos os alelos abaixo de 21 repetições do dinucleotídeo

MEC – matriz extracelular

mmHg – milímetros de mercúrio

MMP – metaloproteinase de matriz extracelular

PA – pressão arterial

PCR – *Polimerase chain reaction* - reação em cadeia da polimerase

pmp – pacientes por milhão da população

T - timina

TIMP – *tissue inhibitor of metalloproteinases* – inibidores teciduais de metaloproteinase de matriz extracelular

SNP – *single nucleotide polymorphism* – polimorfismo de nucleotídeo único

SUMÁRIO

INTRODUÇÃO

Insuficiência renal crônica

O surgimento da lesão renal crônica tem causas bastante distintas, como a hiperglicemia, elevados níveis pressóricos, isquemia, obstrução do fluxo urinário ou injúria auto-imune [1]. Contudo, independentemente da origem, a medida que a insuficiência renal se estabelece surge um fenótipo comum manifestado por anemia, acidose, hipervolemia, hipertensão, hiperparatireoidismo, hipovitaminose D e osteíte fibrosa cística [1-3]. Além disso, alterações eletrolíticas frequentes como hiperpotassemia e hiperfosfatemia carregam alto potencial de letalidade [1]. À medida que o dano progride aparecem sinais e sintomas clínicos de falência terminal como pericardite, anorexia, vômitos, disfunção plaquetária e neuropatia urêmica, tornando necessária a substituição da função renal. Entre as opções de terapia substitutiva, existem a hemodiálise, a diálise peritoneal e o transplante renal [4, 5]. A hemodiálise é a mais disponível e desponta como a terapia de escolha na maioria dos casos [5]. De fato, mais de 90 % dos pacientes em diálise no Brasil estão em hemodiálise [6].

A prevalência da IRC é alta e tem aumentado nas últimas décadas [7]. Na mesma linha, a população dependente de hemodiálise vem apresentando crescimento importante nos últimos anos, representando um elevado custo econômico e social [8, 9]. De fato, o Censo Brasileiro de 2010 aponta que o número de pacientes em hemodiálise aumentou mais de 100%, com prevalência de 483 pacientes por milhão da população (pmp) [6]. Estes são pacientes expostos a taxas alarmantes de mortalidade, visto que um indivíduo de 25 anos em hemodiálise tem a mesma chance de morrer que um indivíduo de 85 anos com função renal normal [9]. Entre as variadas causas que ajudam

a montar este cenário, as principais responsáveis são as doenças cardiovasculares (DCV) [9-11]. Extensa literatura aponta que elevadas taxas de morte súbita, cardiomiopatia, insuficiência cardíaca, acidente vascular cerebral e infarto do miocárdio agregam acentuada morbimortalidade para os pacientes urêmicos [10-15].

A DCV que acompanha a insuficiência renal crônica (IRC) tem manifestações histológicas exuberantes [13, 15, 16]. Ainda nas fases iniciais da IRC, a vasculopatia urêmica se inicia com a reorganização da matriz extracelular (MEC) e a degradação da elastina, proteína fundamental para a elasticidade e complacência das grandes artérias de condução [17]. Os peptídeos derivados da elastina estimulam profundas mudanças no fenótipo da parede vascular, conduzindo à morte celular e espessamento médiointimal [17, 18]. Enquanto parte da população de células musculares lisas da camada média sofre apoptose; enquanto as sobreviventes, sob a influência de fatores osteogênicos, se hipertrofiam adotando características osteoblásticas [10, 16, 17]. Em estágios mais avançados ocorre o depósito inapropriado de minerais de cálcio e fósforo (calcificação vascular) em artérias, miocárdio e valvas cardíacas, preferencialmente nas áreas de maior elastinólise [16]. A calcificação arterial clinicamente detectável se correlaciona fortemente com DCV e é um fator prognóstico de mortalidade na hemodiálise [19]. Suas manifestações clínicas envolvem modificações funcionais como elevada pressão de pulso e velocidade de onda de pulso [15, 20]. Este enrijecimento arterial é a base de alterações comuns na IRC, como hipertrofia de ventrículo esquerdo, disfunção sistólica e placas ateroscleróticas em maior número e mais instáveis [21].

Fatores de risco tradicionais como hipertensão, idade, história familiar, tabagismo, diabetes e hiperlipidemia explicam apenas em parte as complexas alterações vistas na vasculopatia urêmica [11, 15]. Uma extensa lista de fatores de risco específicos da uremia contribuem para este cenário [10]. Entre elas, alterações do metabolismo mineral ósseo como a hiperfosfatemia tem papel preponderante [16]. A desnutrição, a anemia e a presença de toxinas urêmicas com proeminente inflamação e estresse oxidativo ajudam a compor um cenário de acentuado risco vascular [13]. Alterações em enzimas do metabolismo da MEC, como as metaloproteinases de matriz extracelular (MMPs) e as catepsinas, também tem relação com este quadro [18, 22].

Metaloproteinases de matriz extracelular

As MMPs são uma família de endopeptidases dependentes de zinco, secretadas como zimogênio e presentes no plasma na forma de homodímeros [23]. São capazes de degradar diversas proteínas, incluindo componentes da membrana basal e da matriz extracelular, como a fibronectina, a elastina, a gelatina (colágeno desnaturado), laminina, proteoglicanos e colágeno tipo IV [24]. Citocinas, fatores de crescimento, e moléculas de adesão celular também são substratos para a ação das MMPs [23, 25]. Sua regulação ocorre primariamente pela transcrição, sendo também relevantes na sua atividade as modificações pós translacionais e a interação com inibidores endógenos, os inibidores teciduais de metaloproteinases (TIMPs) [23, 26-28]. Entre as diferentes classes de MMPs, se destacam a MMP-2 (gelatinase A, 72 kDa) e a MMP-9 (gelatinase B, 92 kDa) [27, 29]. Elas desempenham papéis chave na patogênese da IRC [30, 31] e em diversas patologias cardiovasculares em

diferentes populações [26, 32-36]. Recentemente, foi demonstrado que a MMP-2 também é capaz de degradar alvos intracelulares [29].

Enquanto a MMP-2 é constitutivamente produzida e regulada por outras MMPs, a MMP-9 é indutível por células do sistema imune, especialmente os macrófagos [25, 37]. As MMPs são constituídas por um pró-domínio autoinibitório, sítio catalítico, domínio tipo fibronectina, região complexadora do zinco, região da dobradiça e domínio tipo hemopexina. A remoção proteolítica do resíduo de cisteína do pró-peptídeo expõe o zinco ativando a enzima para o substrato [23, 24, 29]. Alternativamente, elas também podem ser ativadas por alterações conformacionais no pró-domínio por subprodutos do estresse oxidativo, como o peroxinitrito, levando a exposição do sítio catalítico sem a clivagem do pró-peptídeo [29].

Em condições fisiológicas as MMPs são relevantes para o desenvolvimento normal do indivíduo, na cicatrização e na adaptação deste a mudanças ambientais [23]. Contudo, sob estímulo da inflamação e do estresse oxidativo, elas participam de alterações teciduais [24]. As ações das MMPs levam a degradação de pacotes de colágeno, estimulando a síntese de novas fitas de colágeno e promovendo a fibrose tecidual [32]. Além disso, a degradação de proteínas da MEC abre espaço para a migração das células musculares lisas e a penetração de macrófagos levando a mais inflamação local, agravando o quadro de fibrose [25]. O balanço entre a degradação e a síntese de elastina pode favorecer a hipertrofia celular e a calcificação arterial [38]. Juntas, as MMPs têm efeitos sinérgicos que parecem estimular o remodelamento cardiovascular [39].

Metaloproteinases de matriz extracelular na hemodiálise

Estudos recentes têm apontado relevantes alterações nas MMPs na vasculopatia urêmica. Diferentes modelos animais de IRC mostram que tanto a MMP-2 como a MMP-9 tem papel fundamental no início do processo arteriosclerótico (através da elastocalcinose) como em fases mais avançadas (participando ativamente do acentuado depósito de cálcio que ocorre em áreas de morte celular) [22, 40, 41]. Interessantemente, estes dados encontram correspondência em humanos. Vasos obtidos de um elegante modelo comparando receptores de transplante (casos) com doadores (controles) mostram que os depósitos de MMP-2 são intensos nos sítios de calcificação e estão associados a alterações funcionais como maior enrijecimento arterial e velocidade de onda de pulso [20, 38]. Quando comparado com pacientes sem histórico de hemodiálise prévia, aqueles com exposição dialítica crônica demonstraram significativos aumentos na ativação da MMP-2 e nas alterações fenotípicas da artéria [38].

Níveis circulantes aumentados de MMP-2 e -9 têm sido associados com marcadores de aterosclerose na IRC [18, 42, 43]. Contudo, trabalhos prévios que procuraram determinar se os níveis destas gelatinases estão alterados na hemodiálise trouxeram resultados contraditórios. Comparados com controles saudáveis, a MMP-2 está aumentada na maioria dos estudos [42, 44-48], ao passo que a MMP-9 tem resultados conflitantes [37, 42, 44-47, 49]. A análise das concentrações circulantes destas proteases ao final da sessão de hemodiálise também têm sido alvo de interesse. A MMP-2 parece reduzir após uma sessão regular de 4 horas [46, 47, 50]. Por outro lado, os níveis pós hemodiálise da MMP-9 não alteraram ou estavam reduzidos nestas séries [44,

46, 47, 49, 50]. As diferenças nos estudos podem se dever a fatores genéticos. De fato, um crescente corpo de evidências tem demonstrado que diferenças na distribuição de polimorfismos com papéis funcionais em doenças [51-53], mostrando que a magnitude da influência dos fatores genéticos pode ajudar a explicar variações nos níveis circulantes das MMPs.

Polimorfismos genéticos da MMP-2 e -9 envolvidos em alterações cardiovasculares

Polimorfismos genéticos são variações específicas na sequência de bases nitrogenadas do DNA, cuja prevalência na população seja igual ou superior a 1%. O tipo mais comum de variação genética é a substituição de apenas uma base nitrogenada por outra, chamado de polimorfismo de nucleotídeo único (SNP - *single nucleotide polymorphysms*). Tal modificação pode promover várias alterações como na sequência de aminoácidos que codifica a proteína ou modificações na taxa de transcrição gênica [54].

Dentre os polimorfismos identificados para o gene da MMP-9, que se localiza no cromossomo 20, na região 20q11.2-q13.1, três são amplamente estudados e apresentam grande relevância clínica: um SNP na região promotora na posição -1562, que pode conter uma citosina (alelo mais comum) ou uma timina (alelo mais raro); um microssatélite na posição -90 do promotor, constituindo-se de repetições do dinucleotídeo CA entre 14 e 24 vezes (sendo a distribuição bimodal em torno de 14 repetições e em torno de 21-24 repetições); por último, um SNP no exon 6 do gene, constituindo-se de uma troca de adenina para guanina na posição 855. Este último gera uma troca de

glutamina (aminoácido polar não carregado) para arginina (aminoácido polar positivamente carregado) na posição 279 da proteína.

O gene da MMP-2 está localizado no cromossomo 16, na região 16q13-q21. Os polimorfismos mais estudados são um SNP gerando a troca de uma base C para uma T na posição -1306 da região promotora da MMP-2, e um SNP também gerando a troca de C para T na posição -735 do promotor.

Todos estes polimorfismos são funcionais e alteram a atividade e a concentração das MMP-2 e -9 [28, 55-59]. Eles têm sido associados com maior risco a diversas doenças ou desfechos cardiovasculares bastante prevalentes na IRC, como falência cardíaca [60], aterosclerose [61], risco de eventos cardiovasculares futuros [62], recuperação pós infarto [63], ou outras doenças que envolvem remodelamento tecidual, como câncer [59, 64] e rejeição a transplantes [65].

Apesar de vários estudos mostrarem a relevância destes polimorfismos em diversos contextos de DCV, eles ainda não foram analisados em pacientes urêmicos. Como variações na sequência genética destas MMPs podem levar a diferenças nos seus níveis circulantes, é plausível esperar que eles influenciem o remodelamento tecidual durante aterogênese e o curso da progressão da lesão vascular na IRC.

OBJETIVOS

Os objetivos do nosso estudo foram:

2.1 - Avaliar o efeito dos polimorfismos da MMP-2 e MMP-9 nos seus níveis plasmáticos em pacientes renais crônicos submetidos a hemodiálise por fistula arteriovenosa.

2.2 - Avaliar as possíveis associações de marcadores genéticos no gene da MMP-2 e -9 com as oscilações induzidas pela hemodiálise nos níveis circulantes destas enzimas ao final da sessão.

CAPÍTULO 1

BCPT Basic & Clinical Pharmacology & Toxicology

Basic & Clinical Pharmacology & Toxicology, 2012, **110**, 409–415 Doi: 10.1111/j.1742-7843.2012.00863.x

MiniReview

Imbalanced Matrix Metalloproteinases in Cardiovascular Complications of End-Stage Kidney Disease: A Potential Pharmacological Target

Bernardo P. Marson[1], Carlos E. Poli de Figueiredo[2] and Jose E. Tanus-Santos[3]

[1]Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas, São Paulo, Brazil, [2]Faculty of Medicine/IPB/HSL, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil and [3]Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, Brazil

(Received 16 October 2011; Accepted 11 January 2012)

Abstract: End-stage kidney disease (ESKD) is a major health problem associated with very high morbidity and mortality secondary to cardiovascular complications, especially in ESKD patients on dialysis. Therefore, exploring key mechanisms underlying cardiovascular alterations associated with ESKD may offer reasonable pharmacological targets that may benefit these patients. Imbalanced matrix metalloproteinases (MMP) activities have been implicated in many cardiovascular diseases, and growing evidence now indicates that excessive MMP activities contribute to cardiovascular complications in ESKD patients. However, there is no study on the effects of MMP inhibitors (MMPIs) in such patients. MMPIs may prevent against the vascular and cardiac changes associated with ESKD. In this MiniReview, we aimed at reviewing current evidence supporting the idea that pharmacological inhibition of imbalanced MMP activities in ESKD may decrease the morbidity and mortality associated with cardiovascular complications in ESKD patients. However, MMPs have variable effects during different phases of kidney disease, and therefore optimal timing for MMP inhibition during a disease process may vary significantly and is largely undetermined. While current research shows that MMPs play a role in the pathogenesis of the cardiovascular alterations found in ESKD patients, clinical studies are required to validate the idea of using MMPIs in ESKD.

Chronic kidney disease (CKD) is a major health problem worldwide, and the increasing prevalence of diabetes and hypertension will further enhance the number of patients developing end-stage kidney disease (ESKD) [1]. Regardless of the initial insult, CKD comprises a complex pathophysiology with progressive interstitial fibrosis, glomerulosclerosis, renal and systemic vascular stiffening, and calcification [2]. Tubular epithelial cells become profibrotic, mesenchymal scarring cells, with fibroblast and cytokine activation leading to extracellular matrix (ECM) remodelling [2]. Importantly, a disequilibrium between increased synthesis of ECM components and decreased ECM degradation occurs as a result of imbalanced matrix metalloproteinases (MMP) activities [3], and this promotes vascular, glomerular and tubular alterations associated with CKD [4–7].

In this MiniReview, we aim at showing evidence that pharmacological inhibition of imbalanced MMPs in ESKD may decrease the morbidity and mortality associated with cardiovascular diseases, a major cause of death in ESKD patients [1].

Author for correspondence: Jose E. Tanus-Santos, Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirao Preto, SP, Brazil (fax +55 16 3602 0220, e-mails tanus@fmrp.usp.br; tanussantos@yahoo.com).

ESKD Patients Are at Increased Risk of Developing Cardiovascular Complications

Chronic kidney disease is an independent risk factor of cardiovascular diseases, and cardiovascular diseases promote CKD, thus resulting in a vicious cycle [8, 9]. As detailed in fig. 1, multiple risk factors interact, resulting in extremely high rates of cardiovascular events and mortality associated with a more aggressive natural history in ESKD patients [7, 10, 11]. Importantly, cardiovascular abnormalities increase gradually with progressive decreases in glomerular filtration rate [9, 10]. Risk factors typically related to mortality in general population, such as body mass index and cholesterol levels, are positively associated with improved survival in dialysis patients, highlighting particular mechanisms involved in the vascular disease in ESKD patients [10]. The main issue in these patients is not the number or volume of atherosclerotic plaques but its composition (reduced fibrous component and greater calcification), which makes them more unstable and prone to rupture [10–12].

Two different patterns of vascular injury co-exist in uraemic patients: arteriolosclerosis and atherosclerosis [9–11]. The former is a premature ageing that involves loss of elastic fibres, reduced cushioning function and increased stiffness with

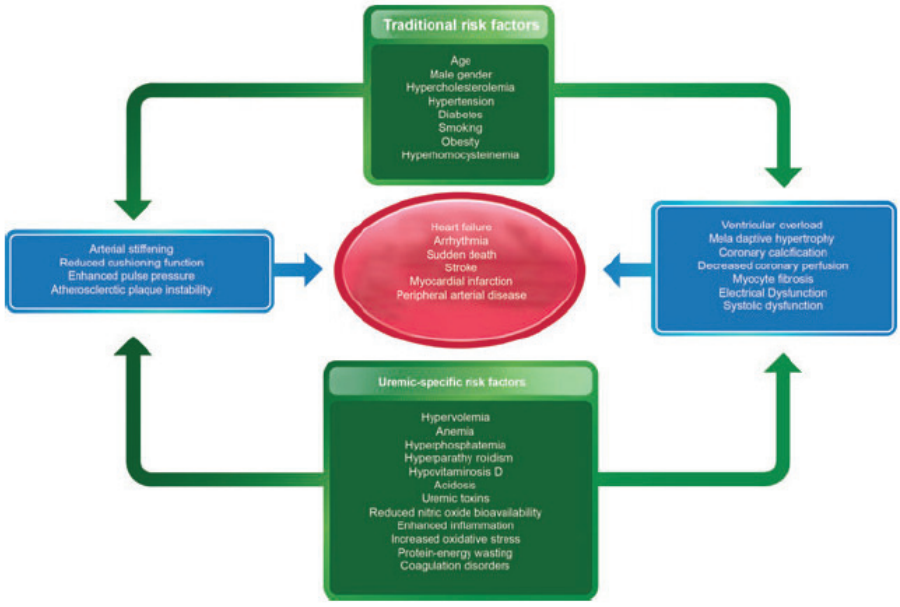

Fig. 1. Multiple risk factors, both traditional and uraemic-specific, interact to promote the vascular and cardiac alterations, which eventually cause clinical outcome in the centre of this figure.

arterial medial calcification and larger pulse pressure. The latter corresponds to intima-media thickening and altered conduit function because of obstructive plaques. Renal failure probably does not induce atherosclerosis, and rather aggravates pre-existent lesions [10], and maladaptive vascular remodelling is the final result of a complex conjunction of classic risk factors and specific ESKD features [13].

Uremic patients with alterations including increased volume overload, left ventricular hypertrophy and diastolic dysfunction [10] are more exposed to ischaemic damage resulting from reduced coronary reserve and ventricular dilation, which worsens microvascular oxygen diffusion [11, 14, 15].

MMPs and Cardiovascular Alterations in ESKD

Matrix metalloproteinases are enzymes involved in tissue remodelling and are usually secreted as pro-MMPs that are eventually activated [16]. They are regulated at multiple levels including gene transcription, interaction with their endogenous inhibitors [the tissue inhibitors of MMP (TIMPs)], and by other factors including oxidative stress [17].

The catalytic domain of MMPs has a zinc-binding site, and the prodomain contains a cysteine in coordination with the zinc in the catalytic domain, which keeps the enzyme in its inactive form. MMPs are activated by propeptide cleavage by proteases, or by detachment of the prodomain after the interaction between the prodomain and the catalytic site is disrupted, thus exposing the catalytic domain [18]. Many compounds may exert such effect, including reactive oxygen species, peroxynitrite, alkylating agents, heavy metals and disulphides [19].

Increased MMP activities enhance the degradation of ECM components, as well as the processing of non-matrix substrates including cytokines, cell adhesion molecules and growth factors [20, 21]. Although other MMPs may be relevant, this review is mainly focused on gelatinases (MMP-2, 72 kDa; MMP-9, 92 kDa) because these enzymes play important roles in the cardiovascular alterations associated with different cardiovascular diseases in different populations [22–27]. They cleave denatured collagen (gelatin), elastin and laminin [21, 24, 28], and abnormal MMP activity is a key feature in cardiovascular remodelling [26, 28, 29].

Atherosclerotic conditions such as metabolic syndrome, obesity and hypertension have been associated with increased MMP activities [30–32]. Both MMP-2 and MMP-9 were shown at increased levels in hypertension and may be involved in both vascular and cardiac remodelling of hypertension [33–35]. Moreover, imbalanced MMP activities may increase aortic stiffness, an independent marker of mortality in ESKD [36], and vascular MMP up-regulation is probably a result of activation of nuclear factor kappa-B pro-inflammatory pathways [21, 37]. Importantly, MMP-9 levels strongly correlated with carotid atherosclerosis burden independently of other factors in early, moderate and advanced CKD [38]. Similarly, the circulating levels of MMP-2 have been strongly linked to intima-media thickness in ESKD patients on haemodialysis [39]. These findings emphasize the role of imbalanced activities of pro-inflammatory proteases in the development of vascular alterations in CKD [21, 39]. In fact, inflammatory markers predict clinical events in ESKD patients and trigger sudden cardiac death by inducing plaque instability

or by directly affecting the myocardium electrical conduction system [14, 40].

The vascular alterations in ESKD include vascular thickening, smaller elastic fibres, calcification, vasomotor dysfunction and hypertrophy and apoptosis of smooth muscle cells, which may be related to MMP-2 activation because of mineral imbalance typically found in ESKD [7, 26, 41–43] (fig. 2). Supporting this idea, MMP-2 and MMP-9, two potent elastases, were up-regulated in arteries from diabetic ESKD patients, and these alterations correlated with vascular stiffness [25]. Interestingly, progressive CKD increased the circulating MMP-2 levels in association with increasing MMP-2, MMP-9 and TIMP-1 expression in the aorta [26]. Fragmented elastic lamellae may predispose to calcification, especially in the presence of uraemic factors such as hyperphosphataemia. Indeed, calcified deposits that develop in the media are associated with regions of elastin disorganization [25, 43]. In line with these results, uraemic rats showed increased blood pressure, arterial medial calcification, elastin degradation and increased vascular MMP-2 expression [42]. Moreover, MMP-2 over-expression and vascular smooth muscle cell phenotype change found in elastocalcinosis were shown as early events in CKD and preceded cell loss and arterial medial calcification [43].

MMP Levels in ESKD Patients and the Effect of Dialysis

A number of studies have been carried out to examine MMP/TIMP levels in ESKD patients on dialysis, and table 1 summarizes their findings. However, conflicting results have been reported, and MMP-9 levels were not different in three studies [39, 44, 45], higher in two studies [46, 47] and lower in one study [48], compared with healthy controls. These differences among studies may be explained by differences in ethnicity, age, type of dialysis membrane, causal diseases or other clinical conditions [49].

Matrix metalloproteinase-9 was positively correlated with severe hypertension in blacks, and significantly elevated when compared with healthy controls, although MMP-9 levels were similar to those found in hypertensive patients with normal renal function [47].

Most studies showed increased MMP-2 levels in ESKD compared with controls [39, 44, 46, 47] and maybe associated

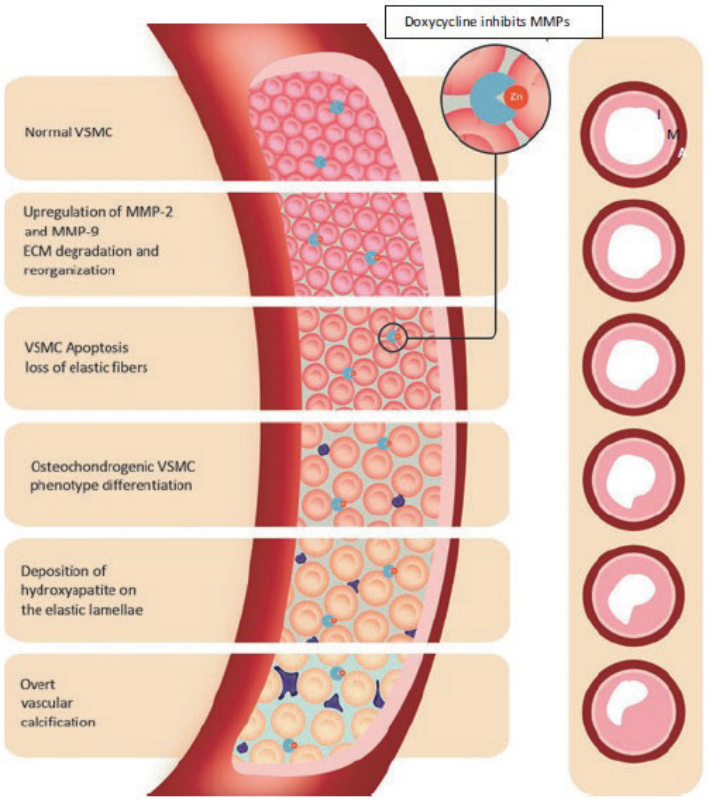

Fig. 2 Vascular alterations begin very early in chronic kidney disease (CKD) in a continuum in which MMPs are up-regulated, thus affecting the relation between vascular smooth muscle cells (VSMC) and the extracellular matrix (ECM). With time, arterial medial calcification becomes clinically detectable. Note the dark calcium deposits scattered in the later stages. Doxycycline may prevent these alterations and protect against the vascular structural modifications associated with CKD. Intima (I), media (M) and adventitia (A).

Table 1.

Studies to compare matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 levels in end-stage kidney disease (ESKD) patients on haemodialysis (HD) with those found in healthy controls (upper signals), or to compare the levels of these markers after HD with those before a HD session (lower signals).

References	Pat/Con	Post-HD measurements	MMP-2	MMP-9	TIMP-1	TIMP-2
[48]	18/15	Yes	=	↓	↓	↓
			↓↓	↔	↓↓	↔
[46]	19/30	Yes	↑	↑	↑	↑
			↓↓	↓↓	↓↓	↓↓
[44]	23/18	Yes	↑	=	NA	NA
			↔	↔		
[47]	30/18	No	↑	↑	NA	NA
[45]	21/20	Yes	NA	=	NA	NA
				↔		
[39]	40/20	No	↑	=	↑	↑
[50]	17/10	No	=	NA	NA	NA
[49]	40/–	Yes	NA	NA	NA	NA
			↓↓	↓↓	↔	↑↑

Upper signals: ↑, higher levels than healthy controls; =, similar levels compared with healthy controls; ↓, lower levels than healthy controls. Lower signals: ↑↑, haemodialysis increases the levels; ↔, haemodialysis has no effects on the levels; ↓↓, haemodialysis decreases the levels. Pat/Con, number of ESKD patients/number of healthy controls; HD, haemodialysis; NA, not available.

with organ damage but not with hypertension [47]. However, two studies showed similar MMP-2 levels [48, 50] in patients compared with healthy controls (table 1). Moreover, TIMP-1 and -2 comparisons between ESKD and controls have also shown conflicting results [46, 48]. As detailed in table 1, a single session of haemodialysis apparently does not affect or may decrease both MMP-2 and MMP-9 levels, as well as TIMP-1 and TIMP-2 levels [44–46, 48, 49]. Collectively, these studies suggest increased MMP levels in ESKD patients, and dialysis may decrease, particularly MMP-2 levels. The potential reason for this decrease is not known, because MMP-2 is a large molecule (>60–70 kDa for the different forms), and this feature should preclude its dialysability. Measuring systemic MMP levels may not reflect precisely MMP activities at tissue level. Furthermore, care must be taken to avoid artificial results caused by lack of pre-analytical care, because there are remarkable differences between serum and plasma samples [51, 52], and the use of appropriate samples is of major relevance [33].

It is possible that genetic factors involving MMP-2 and MMP-9 may interact with ESKD to modulate MMP levels. Indeed, it has been suggested that genetic factors may interact with environmental and disease factors and affect MMP levels in some groups of patients [23, 53–55]. Further research is required to understand how MMP polymorphisms may modify the effects of long-term dialysis on plasma MMPs. The study of genetic MMP polymorphisms may help to identify patients with worse prognosis and that could respond to pharmacological interventions [56], possibly including MMP inhibitors (MMPIs).

Uncertain Effects of MMP Inhibitors in Non-dialytic CKD

Matrix metalloproteinase inhibitors have been studied in diabetic and hypertensive nephropathy, retardation of CKD, transplantation and attenuation of cystic diseases [2, 4, 26, 57–62]. Doxycycline, a non-selective MMPI that is easily available, improved elastic fibre integrity and reduced arterial stiffness in Marfan syndrome [63]. While earlier clinical trials of MMPIs in chronic cardiovascular disorders failed to show clear effects [64], doxycycline reduced proteinuria both in short open trials with patients and in animal models of diabetic nephropathy [60–62]. In line with these findings, chronic administration of MMPIs delayed CKD progression in hypertensive and diabetic nephropathy [59]. However, these findings were not reproduced in other forms of kidney diseases [65]. Moreover, the test of candesartan in the adriamycin kidney injury model resulted in regression of established glomerulosclerosis associated with increased glomerular MMP-2 activity, and such effect was attenuated by pre-treatment with doxycycline or by targeted deletion of MMP-2 gene [65], thus suggesting a protective effect of MMP-2 activity. These findings suggest that MMPs may not be deleterious in all kidney diseases, although most of the studies suggest that this may be true.

A selective MMPI for gelatinases was tested in a kidney transplantation model of chronic allograft nephropathy [58]. Adding complexity to the understanding of how MMPs affect kidney diseases, early MMP inhibition resulted in significantly lower-grade chronic allograft nephropathy in that study, whereas late inhibition induced higher proteinuria and more severe chronic allograft nephropathy [58], thus suggesting a time-dependent effect for the MMPI. Another important factor that should be taken into consideration to explain discrepancies between studies is that differences in the MMPI dosage scheme or species may affect drug responses [22], and further studies are required to better define useful doses of MMPIs, especially in human beings.

Increased MMP-2 and diminished MMP-9 activities were shown in patients with different stages of CKD [66]. There is now evidence for imbalanced MMP/TIMP activity and increased deposition of ECM in CKD at intermediate stages of disease [2, 5]. However, MMPs may have different and maybe

apparently contrasting effects during different disease phases [2]. Indeed, MMPs may play dual roles in a number of primary nephropathies, with an acute, harmful effect contributing to damage in the early phases and a protective, compensatory effect against deleterious ECM deposition in later-phases [4, 58]. Therefore, optimal timing for MMPs inhibition during a disease process may vary significantly and is largely undetermined [4]. Moreover, MMP expression varies according to localization, and therefore MMPI may have different effects along the entire nephron [5, 58]. A major challenge for future therapeutic interventions using MMPIs will certainly be how to achieve therapeutic effects without causing any harm. More detailed studies on the involvement of MMPs and TIMPs in CKD will help to improve our understanding of how MMPIs may be helpful.

It should be noted that a number of drugs affecting the cardiovascular system may down-regulate MMP activities. These drugs include diuretics [31], calcium channel blockers [67–70], angiotensin converting enzyme inhibitors [71] and statins [72], among others. However, it remains to be determined how these drugs may affect MMP activities in ESKD.

MMP Inhibition May Protect ESKD Patients against Cardiovascular Complications

Typical features of ageing and cardiovascular diseases in the general population are utterly accelerated in ESKD patients, leading to far amplified occurrence of CV events in dialysis patients [8, 73]. Despite receiving the best available therapy, a considerable proportion of ESKD patients die from cardiovascular issues [10]. Therefore, exploring key mechanisms underlying specific renal atherosclerotic events may offer reasonable pharmacological targets that may benefit these patients [74]. While there is evidence that imbalanced MMP activities contribute to cardiovascular diseases in ESKD patients, there is no study on the effects of MMPIs in such patients.

Imbalanced MMP activities in CKD may promote cardiovascular disease, and therefore it is possible that MMPIs may exert beneficial effects by postponing cardiac remodelling and vascular events more clearly in patients than in individuals with normal renal function. In line with this suggestion, doxycycline attenuated aortic calcification in a CKD animal model [26] and prevented elastin degradation caused by early MMP-9 activation [75], thus suggesting that inhibiting MMPs is a potential therapeutic strategy to protect against the vascular alterations commonly found in patients with ESKD. However, the use of MMPIs in the non-dialytic CKD may be risky [4]. The risk of accelerated CKD progression after MMP inhibition in non-dialytic patients may preclude them from using MMPIs to improve cardiovascular health. This is because MMPs apparently preserve residual kidney function in such patients [65]. Moreover, patients with moderate CKD have less severe cardiovascular diseases, and the possible benefits of MMPIs would probably be less evident in these patients [10].

Hypervolaemia is an important issue in patients with anuria, and it is aggravated by the presence of arteriovenous fistula because the fistula increases cardiac output by approximately 20%. Increased MMP activities preceded left ventricular remodelling induced by experimental chronic volume overload [76], and MMP inhibition attenuated this effect and prevented left ventricular dilation and hypertrophy, thus preserving ventricular function [76]. Whether this protective effect associated with MMP inhibition is found in ESKD patients on haemodialysis is not known.

In conclusion, mounting evidence indicates that cardiovascular complications deserve pharmacological intervention in ESKD patients. Taking into consideration that abnormal cholesterol levels are not the major issue in the pathogenesis of cardiovascular diseases in these patients, the development of new tangible targets has been encouraged [74]. Current data suggest that excessive degradation of the ECM is a critical step in the pathogenesis of the vascular alterations found in ESKD patients, and imbalanced MMP activities contribute to these alterations. While there is now reasonable evidence supporting the use of MMPIs to prevent these alterations, clinical studies are required to validate this suggestion. Genetic research may add important evidence because genetic polymorphisms may help to identify patients with worse prognosis that may have better responses to MMPIs.

Acknowledgements

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

1 Coresh J, Selvin E, Stevens LA, Manzi J, Kusek JW, Eggers P *et al.* Prevalence of chronic kidney disease in the United States. JAMA 2007;**298**:2038–47.
2 Boor P, Sebekova K, Ostendorf T, Floege J. Treatment targets in renal fibrosis. Nephrol Dial Transplant 2007;**22**:3391–407.
3 Aresu L, Benali S, Garbisa S, Gallo E, Castagnaro M. Matrix metalloproteinases and their role in the renal epithelial mesenchymal transition. Histol Histopathol 2011;**26**:307–13.
4 Ronco P, Chatziantoniou C. Matrix metalloproteinases and matrix receptors in progression and reversal of kidney disease: therapeutic perspectives. Kidney Int 2008;**74**:873–8.
5 Catania JM, Chen G, Parrish AR. Role of matrix metalloproteinases in renal pathophysiologies. Am J Physiol Renal Physiol 2007;**292**:F905–11.
6 Cheng S, Lovett DH. Gelatinase A (MMP-2) is necessary and sufficient for renal tubular cell epithelial-mesenchymal transformation. Am J Pathol 2003;**162**:1937–49.
7 Chung AW, Yang HH, Kim JM, Sigrist MK, Chum E, Gourlay WA *et al.* Upregulation of matrix metalloproteinase-2 in the arterial vasculature contributes to stiffening and vasomotor dysfunction in patients with chronic kidney disease. Circulation 2009;**120**:792–801.
8 Weiner DE, Tabatabai S, Tighiouart H, Elsayed E, Bansal N, Griffith J *et al.* Cardiovascular outcomes and all-cause mortality: exploring the interaction between CKD and cardiovascular disease. Am J Kidney Dis 2006;**48**:392–401.
9 Briet M, Collin C, Karras A, Laurent S, Bozec E, Jacquot C *et al.* Arterial remodeling associates with CKD progression. J Am Soc Nephrol 2011;**22**:967–74.
10 Drueke TB, Massy ZA. Atherosclerosis in CKD: differences from the general population. Nat Rev Nephrol 2010;**6**:723–35.

11 Schwarz U, Buzello M, Ritz E, Stein G, Raabe G, Wiest G *et al.* Morphology of coronary atherosclerotic lesions in patients with end-stage renal failure. Nephrol Dial Transplant 2000;**15**:218–23.

12 Pelisek J, Assadian A, Sarkar O, Eckstein HH, Frank H. Carotid plaque composition in chronic kidney disease: a retrospective analysis of patients undergoing carotid endarterectomy. Eur J Vasc Endovasc Surg 2010;**39**:11–6.

13 Coll B, Rodriguez JA, Craver L, Orbe J, Martinez-Alonso M, Ortiz A *et al.* Serum levels of matrix metalloproteinase-10 are associated with the severity of atherosclerosis in patients with chronic kidney disease. Kidney Int 2010;**78**:1275–80.

14 Shamseddin MK, Parfrey PS. Sudden cardiac death in chronic kidney disease: epidemiology and prevention. Nat Rev Nephrol 2011;**7**:145–54.

15 Guerin AP, Pannier B, Marchais SJ, London GM. Cardiovascular disease in the dialysis population: prognostic significance of arterial disorders. Curr Opin Nephrol Hypertens 2006;**15**:105–10.

16 Das S, Mandal M, Chakraborti T, Mandal A, Chakraborti S. Structure and evolutionary aspects of matrix metalloproteinases: a brief overview. Mol Cell Biochem 2003;**253**:31–40.

17 Castro MM, Rizzi E, Rodrigues GJ, Ceron CS, Bendhack LM, Gerlach RF *et al.* Antioxidant treatment reduces matrix metalloproteinase-2-induced vascular changes in renovascular hypertension. Free Radic Biol Med 2009;**46**:1298–307.

18 Woessner JF Jr. Matrix metalloproteinase inhibition. From the Jurassic to the third millennium. Ann N Y Acad Sci 1999;**878**: 388–403.

19 Ra HJ, Parks WC. Control of matrix metalloproteinase catalytic activity. Matrix Biol 2007;**26**:587–96.

20 Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 2007;**8**:221–33.

21 Newby AC. Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability. Arterioscler Thromb Vasc Biol 2008;**28**:2108–14.

22 Guimaraes DA, Rizzi E, Ceron CS, Oliveira AM, Oliveira DM, Castro MM *et al.* Doxycycline dose-dependently inhibits MMP-2-mediated vascular changes in 2K1C hypertension. Basic Clin Pharmacol Toxicol 2011;**108**:318–25.

23 Lacchini R, Jacob-Ferreira AL, Luizon MR, Coeli FB, Izidoro-Toledo TC, Gasparini S *et al.* Matrix metalloproteinase 9 gene haplotypes affect left ventricular hypertrophy in hypertensive patients. Clin Chim Acta 2010;**411**:1940–4.

24 Fontana V, Silva PS, Belo VA, Antonio RC, Ceron CS, Biagi C *et al.* Consistent alterations of circulating matrix metalloproteinases levels in untreated hypertensives and in spontaneously hypertensive rats: a relevant pharmacological target. Basic Clin Pharmacol Toxicol 2011;**109**:130–7.

25 Chung AW, Yang HH, Sigrist MK, Brin G, Chum E, Gourlay WA *et al.* Matrix metalloproteinase-2 and -9 exacerbate arterial stiffening and angiogenesis in diabetes and chronic kidney disease. Cardiovasc Res 2009;**84**:494–504.

26 Chen NX, O'Neill KD, Chen X, Kiattisunthorn K, Gattone VH, Moe SM. Activation of Arterial Matrix Metalloproteinases Leads to Vascular Calcification in Chronic Kidney Disease. Am J Nephrol 2011;**34**:211–9.

27 Lacchini R, Metzger IF, Luizon M, Ishizawa M, Tanus-Santos JE. Interethnic differences in the distribution of matrix metalloproteinases genetic polymorphisms are consistent with interethnic differences in disease prevalence. DNA Cell Biol 2010;**29**:649–55.

28 Castro MM, Rizzi E, Figueiredo-Lopes L, Fernandes K, Bendhack LM, Pitol DL *et al.* Metalloproteinase inhibition ameliorates hypertension and prevents vascular dysfunction and remodeling in renovascular hypertensive rats. Atherosclerosis 2008;**198**:320–31.

29 Neto-Neves EM, Dias-Junior CA, Rizzi E, Castro MM, Sonego F, Gerlach RF *et al.* Metalloproteinase inhibition protects against cardiomyocyte injury during experimental acute pulmonary thromboembolism. Crit Care Med 2011;**39**:349–56.

30 Belo VA, Souza-Costa DC, Lana CM, Caputo FL, Marcaccini AM, Gerlach RF *et al.* Assessment of matrix metalloproteinase (MMP)-2, MMP-8, MMP-9, and their inhibitors, the tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in obese children and adolescents. Clin Biochem 2009;**42**:984–90.

31 Ceron CS, Castro MM, Rizzi E, Montenegro MF, Fontana V, Salgado MC *et al.* Spironolactone and hydrochlorothiazide exert antioxidant effects and reduce vascular matrix metalloproteinase-2 activity and expression in a model of renovascular hypertension. Br J Pharmacol 2010;**160**:77–87.

32 Goncalves FM, Jacob-Ferreira AL, Gomes VA, Casella-Filho A, Chagas AC, Marcaccini AM *et al.* Increased circulating levels of matrix metalloproteinase (MMP)-8, MMP-9, and pro-inflammatory markers in patients with metabolic syndrome. Clin Chim Acta 2009;**403**:173–7.

33 Gerlach RF, Meschiari CA, Marcaccini AM, Palei AC, Sandrim VC, Cavalli RC *et al.* Positive correlations between serum and plasma matrix metalloproteinase (MMP)-2 or MMP-9 levels in disease conditions. Clin Chem Lab Med 2009;**47**:888–91.

34 Castro MM, Rizzi E, Prado CM, Rossi MA, Tanus-Santos JE, Gerlach RF. Imbalance between matrix metalloproteinases and tissue inhibitor of metalloproteinases in hypertensive vascular remodeling. Matrix Biol 2010;**29**:194–201.

35 Rizzi E, Castro MM, Prado CM, Silva CA, Fazan R Jr, Rossi MA *et al.* Matrix metalloproteinase inhibition improves cardiac dysfunction and remodeling in 2-kidney, 1-clip hypertension. J Card Fail 2010;**16**:599–608.

36 Chue CD, Townend JN, Steeds RP, Ferro CJ. Republished paper: arterial stiffness in chronic kidney disease: causes and consequences. Postgrad Med J 2010;**86**:560–6.

37 Cau SB, Guimaraes DA, Rizzi E, Ceron CS, Souza LL, Tirapelli CR *et al.* Pyrrolidine dithiocarbamate down-regulates vascular matrix metalloproteinases and ameliorates vascular dysfunction and remodelling in renovascular hypertension. Br J Pharmacol 2011; **164**:372–81.

38 Addabbo F, Mallamaci F, Leonardis D, Tripepi R, Tripepi G, Goligorsky MS *et al.* Searching for biomarker patterns characterizing carotid atherosclerotic burden in patients with reduced renal function. Nephrol Dial Transplant 2007;**22**:3521–6.

39 Pawlak K, Pawlak D, Mysliwiec M. Serum matrix metalloproteinase-2 and increased oxidative stress are associated with carotid atherosclerosis in hemodialyzed patients. Atherosclerosis 2007; **190**:199–204.

40 Benedetto FA, Tripepi G, Mallamaci F, Zoccali C. Rate of atherosclerotic plaque formation predicts cardiovascular events in ESRD. J Am Soc Nephrol 2008;**19**:757–63.

41 Amann K, Wolf B, Nichols C, Tornig J, Schwarz U, Zeier M *et al.* Aortic changes in experimental renal failure: hyperplasia or hypertrophy of smooth muscle cells? Hypertension 1997;**29**: 770–5.

42 Kumata C, Mizobuchi M, Ogata H, Koiwa F, Kondo F, Kinugasa E *et al.* Involvement of matrix metalloproteinase-2 in the development of medial layer vascular calcification in uremic rats. Ther Apher Dial 2011;**15**(Suppl 1):18–22.

43 Pai A, Leaf EM, El-Abbadi M, Giachelli CM. Elastin degradation and vascular smooth muscle cell phenotype change precede cell loss and arterial medial calcification in a uremic mouse model of chronic kidney disease. Am J Pathol 2011;**178**:764–73.

44 Pawlak K, Mysliwiec M, Pawlak D. Peripheral blood level alterations of MMP-2 and MMP-9 in patients with chronic kidney disease on conservative treatment and on hemodialysis. Clin Biochem 2011;**44**:838–43.

45 Polañska B, Makulska I, Augustyniak D, Niemczuk M, Zwolińska D, Jankowski A. Serum levels of MMP-9 in children and young

adults with chronic kidney disease treated conservatively and undergoing hemodialysis. Central Eur J Immunol 2007;**32**:66–71.
46 Musial K, Zwolinska D. Matrix metalloproteinases and soluble Fas/FasL system as novel regulators of apoptosis in children and young adults on chronic dialysis. Apoptosis 2011;**16**:653–9.
47 Friese RS, Rao F, Khandrika S, Thomas B, Ziegler MG, Schmid-Schonbein GW *et al*. Matrix metalloproteinases: discrete elevations in essential hypertension and hypertensive end-stage renal disease. Clin Exp Hypertens 2009;**31**:521–33.
48 Rysz J, Banach M, Stolarek RA, Mikhailidis DP, Cialkowska-Rysz A, Pokoca L *et al*. Serum metalloproteinases MMP-2, MMP-9 and metalloproteinase tissue inhibitors TIMP-1 and TIMP-2 in patients on hemodialysis. Int Urol Nephrol 2011;**43**:491–8.
49 Chou FP, Chu SC, Cheng MC, Yang SF, Cheung WN, Chiou HL *et al*. Effect of hemodialysis on the plasma level of type IV collagenases and their inhibitors. Clin Biochem 2002;**35**:383–8.
50 Preston GA, Barrett CV, Alcorta DA, Hogan SL, Dinwiddie L, Jennette JC *et al*. Serum matrix metalloproteinases MMP-2 and MMP-3 levels in dialysis patients vary independently of CRP and IL-6 levels. Nephron 2002;**92**:817–23.
51 Gerlach RF, Demacq C, Jung K, Tanus-Santos JE. Rapid separation of serum does not avoid artificially higher matrix metalloproteinase (MMP)-9 levels in serum versus plasma. Clin Biochem 2007;**40**:119–23.
52 Gerlach RF, Uzuelli JA, Souza-Tarla CD, Tanus-Santos JE. Effect of anticoagulants on the determination of plasma matrix metalloproteinase (MMP)-2 and MMP-9 activities. Anal Biochem 2005; **344**:147–9.
53 Jacob-Ferreira AL, Lacchini R, Gerlach RF, Passos CJ, Barbosa F Jr, Tanus-Santos JE. A common matrix metalloproteinase (MMP)-2 polymorphism affects plasma MMP-2 levels in subjects environmentally exposed to mercury. Sci Total Environ 2011;**409**:4242–6.
54 -Jacob-Ferreira AL, Passos CJ, Gerlach RF, Barbosa F Jr, Tanus-Santos JE. A functional matrix metalloproteinase (MMP)-9 polymorphism modifies plasma MMP-9 levels in subjects environmentally exposed to mercury. Sci Total Environ 2010;**408**:4085–92.
55 Demacq C, Vasconcellos VB, Marcaccini AM, Gerlach RF, Silva WA, Tanus-Santos JE. Functional polymorphisms in the promoter of the matrix metalloproteinase-9 (MMP-9) gene are not linked with significant plasma MMP-9 variations in healthy subjects. Clin Chem Lab Med 2008;**46**:57–63.
56 Demacq C, Vasconcellos VB, Marcaccini AM, Gerlach RF, Machado AA, Tanus-Santos JE. A genetic polymorphism of matrix metalloproteinase 9 (MMP-9) affects the changes in circulating MMP-9 levels induced by highly active antiretroviral therapy in HIV patients. Pharmacogenomics J 2009;**9**:265–73.
57 Osten L, Kubitza M, Gallagher AR, Kastner J, Olbrich H, de Vries U *et al*. Doxycycline accelerates renal cyst growth and fibrosis in the pcy/pcy mouse model of type 3 nephronophthisis, a form of recessive polycystic kidney disease. Histochem Cell Biol 2009; **132**:199–210.
58 Lutz J, Yao Y, Song E, Antus B, Hamar P, Liu S *et al*. Inhibition of matrix metalloproteinases during chronic allograft nephropathy in rats. Transplantation 2005;**79**:655–61.
59 Williams JM, Zhang J, North P, Lacy S, Yakes M, Dahly-Vernon A *et al*. Evaluation of metalloprotease inhibitors on hypertension and diabetic nephropathy. Am J Physiol Renal Physiol 2011;**300**:F983–98.
60 Naini AE, Harandi AA, Moghtaderi J, Bastani B, Amiran A. Doxycycline: a pilot study to reduce diabetic proteinuria. Am J Nephrol 2007;**27**:269–73.
61 Aggarwal HK, Jain D, Talapatra P, Yadav RK, Gupta T, Kathuria KL. Evaluation of role of doxycycline (a matrix metalloproteinase inhibitor) on renal functions in patients of diabetic nephropathy. Ren Fail 2010;**32**:941–6.
62 Kumar Bhatt L, Addepalli V. Minocycline with aspirin: an approach to attenuate diabetic nephropathy in rats. Ren Fail 2011;**33**:72–8.
63 Chung AW, Yang HH, Radomski MW, van Breemen C. Long-term doxycycline is more effective than atenolol to prevent thoracic aortic aneurysm in marfan syndrome through the inhibition of matrix metalloproteinase-2 and -9. Circ Res 2008;**102**:e73–85.
64 Dorman G, Cseh S, Hajdu I, Barna L, Konya D, Kupai K *et al*. Matrix metalloproteinase inhibitors: a critical appraisal of design principles and proposed therapeutic utility. Drugs 2010;**70**: 949–64.
65 Hayashi K, Sasamura H, Ishiguro K, Sakamaki Y, Azegami T, Itoh H. Regression of glomerulosclerosis in response to transient treatment with angiotensin II blockers is attenuated by blockade of matrix metalloproteinase-2. Kidney Int 2010;**78**:69–78.
66 Chang HR, Yang SF, Li ML, Lin CC, Hsieh YS, Lian JD. Relationships between circulating matrix metalloproteinase-2 and -9 and renal function in patients with chronic kidney disease. Clin Chim Acta 2006;**366**:243–8.
67 Martinez ML, Castro MM, Rizzi E, Fernandes K, Demacq C, Bendhack LM *et al*. Lercanidipine reduces matrix metalloproteinase-2 activity and reverses vascular dysfunction in renovascular hypertensive rats. Eur J Pharmacol 2008;**591**:224–30.
68 Martinez ML, Lopes LF, Coelho EB, Nobre F, Rocha JB, Gerlach RF *et al*. Lercanidipine reduces matrix metalloproteinase-9 activity in patients with hypertension. J Cardiovasc Pharmacol 2006;**47**:117 –22.
69 Martinez ML, Rizzi E, Castro MM, Fernandes K, Bendhack LM, Gerlach RF *et al*. Lercanidipine decreases vascular matrix metalloproteinase-2 activity and protects against vascular dysfunction in diabetic rats. Eur J Pharmacol 2008;**599**:110–6.
70 Marcal DM, Rizzi E, Martins-Oliveira A, Ceron CS, Guimaraes DA, Gerlach RF *et al*. Comparative study on antioxidant effects and vascular matrix metalloproteinase-2 downregulation by dihydropyridines in renovascular hypertension. Naunyn Schmiedebergs Arch Pharmacol 2011;**383**:35–44.
71 Takai S, Yamamoto D, Jin D, Inagaki S, Yoshikawa K, Tanaka K *et al*. Inhibition of matrix metalloproteinase-9 activity by lisinopril after myocardial infarction in hamsters. Eur J Pharmacol 2007; **568**:231–3.
72 Izidoro-Toledo TC, Guimaraes DA, Belo VA, Gerlach RF, Tanus-Santos JE. Effects of statins on matrix metalloproteinases and their endogenous inhibitors in human endothelial cells. Naunyn Schmiedebergs Arch Pharmacol 2011;**383**:547–54.
73 Hage FG, Venkataraman R, Zoghbi GJ, Perry GJ, DeMattos AM, Iskandrian AE. The scope of coronary heart disease in patients with chronic kidney disease. J Am Coll Cardiol 2009; **53**:2129–40.
74 Zoccali C, Seck S. What makes plaques vulnerable in CKD?: a fresh look at metalloproteinases. Kidney Int 2010;**78**:1206–8.
75 Bouvet C, Moreau S, Blanchette J, de Blois D, Moreau P. Sequential activation of matrix metalloproteinase 9 and transforming growth factor beta in arterial elastocalcinosis. Arterioscler Thromb Vasc Biol 2008;**28**:856–62.
76 Chancey AL, Brower GL, Peterson JT, Janicki JS. Effects of matrix metalloproteinase inhibition on ventricular remodeling due to volume overload. Circulation 2002;**105**:1983–8.

CAPÍTULO 2

Original Report: Patient-Oriented, Translational Research

American Journal of Nephrology

Am J Nephrol 2012;35:209–215
DOI: 10.1159/000336108

Received: November 7, 2011
Accepted: November 22, 2011
Published online: February 1, 2012

Matrix Metalloproteinase (MMP)-2 Genetic Variants Modify the Circulating MMP-2 Levels in End-Stage Kidney Disease

Bernardo P. Marson[a] Riccardo Lacchini[b] Vanessa Belo[b] Samantha Dickel[c]
Bartira P. da Costa[c] Carlos E. Poli de Figueiredo[c] Jose E. Tanus-Santos[b]

[a]Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas, Campinas,
[b]Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, and
[c]Faculty of Medicine/IPB/HSL of Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil

Key Words
End-stage kidney disease · Haplotypes · Hemodialysis · Matrix metalloproteinase-2 · Polymorphisms · Tissue inhibitor of metalloproteinases-2

Abstract
Background: Matrix metalloproteinases (MMPs) play important roles in the pathophysiology of renal diseases, and imbalanced MMP-2 and its endogenous inhibitor (the tissue inhibitor of metalloproteinases-2; TIMP-2) are implicated in the vascular alterations of end-stage kidney disease (ESKD) patients. We have examined whether MMP-2 gene polymorphisms and haplotypes modify MMP-2 and TIMP-2 levels in ESKD patients as well as the effects of hemodialysis on the concentrations of these biomarkers. ***Methods:*** We determined MMP-2 and TIMP-2 plasma levels by gelatin zymography and ELISA, respectively, in 98 ESKD patients and in 38 healthy controls. Genotypes for two relevant MMP-2 polymorphisms ($C^{-1306}T$ and $C^{-735}T$ in the promoter region) were determined by TaqMan® allele discrimination assay and real-time polymerase chain reaction. The software program PHASE 2.1 was used to estimate the haplotype frequencies. ***Results:*** We found increased plasma MMP-2 and TIMP-2 levels in ESKD patients compared to controls ($p < 0.05$), and hemodialysis decreased MMP-2 (but not TIMP-2) levels ($p < 0.05$). The T allele for the $C^{-735}T$ polymorphism and the C-T haplotype were associated with higher MMP-2 (but not TIMP-2) levels ($p < 0.05$), whereas the $C^{-1306}T$ had no effects. Hemodialysis decreased MMP-2 (but not TIMP-2) levels independently of MMP-2 genotypes or haplotypes ($p < 0.05$). ***Conclusions:*** MMP-2 genotypes or haplotypes modify MMP-2 levels in ESKD patients, and may help to identify patients with increased MMP-2 activity in plasma. Hemodialysis reduces MMP-2 levels independently of MMP-2 genetic variants.

Introduction

Matrix metalloproteinases (MMPs) are a wide family of zinc-dependent proteases that regulate tissue remodeling, cell proliferation and angiogenesis by cleaving many components of the extracellular matrix [1]. Whereas their activities are balanced by their interactions with endogenous inhibitors (the tissue inhibitors of metalloproteinases; TIMPs), there is now clear evidence that they play important roles in the pathophysiology of renal diseases [2].

B.P. Marson and R. Lacchini contributed equally to this work.

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0250–8095/12/0353–0209$38.00/0

Accessible online at:
www.karger.com/ajn

Jose E. Tanus-Santos, MD, PhD
Department of Pharmacology
Faculty of Medicine of Ribeirão Preto, University of São Paulo
Av. Bandeirantes, 3900, Ribeirão Preto, SP 14049-900 (Brazil)
Tel. +55 16 3602 3163, E-Mail tanus@fmrp.usp.br

End-stage kidney disease (ESKD) patients have unstable atherosclerotic plaques that are prone to rupture [3]. This process includes enhanced arterial calcification and activation of fibroblasts and cytokines, eventually leading to vascular extracellular matrix remodeling [4, 5]. While growing evidence suggests that MMP abnormalities are involved in the vascular changes associated with kidney failure [6], a particular imbalance between MMP-2 and its endogenous inhibitor, TIMP-2, has been implicated in the vascular alterations of ESKD [7]. Previous studies showed altered MMP-2 and TIMP-2 levels in dialysis patients, thus suggesting a mechanism for cardiovascular disease (CVD) complications in these patients [8–12]. However, inconsistent results have been reported with respect to the effects of hemodialysis on the circulating MMP levels. Some studies suggested that plasma MMP-2 and TIMP-2 are unaltered or even reduced in uremic subjects compared to healthy controls [13, 14]. In addition, conflicting results have been reported with respect to the effects of a single hemodialysis session on MMP-2 and TIMP-2 levels [10, 11, 14, 15].

There is now evidence that a single nucleotide polymorphism (SNP) in the MMP-2 gene may affect MMP-2 expression or activity [16, 17]. Two SNPs in the promoter region of the MMP-2 gene apparently affect MMP-2 expression [18, 19] (the $C^{-1306}T$; rs 243865, and the $C^{-735}T$; rs 2285053) and have been associated with malignancies and CVD [20–22]. Nevertheless, no previous study has examined how these functional MMP-2 polymorphisms, or their combinations within haplotypes, affect MMP-2 levels in ESKD patients. Moreover, no previous study has examined how these MMP-2 polymorphisms may modify the effects of a hemodialysis session on MMP-2/TIMP-2 levels.

We compared MMP-2 and TIMP-2 plasma levels in healthy volunteers with those found in ESKD patients on hemodialysis, and we examined the effects of a single hemodialysis session on these biochemical markers. We hypothesized that MMP-2 alleles, genotypes, and haplotypes could alter the circulating levels of MMP-2 and TIMP-2 and modify the effects of a single hemodialysis session on these biochemical markers in ESKD patients.

Subjects and Methods

Patients and Healthy Controls

This cross-sectional, observational study was approved by the Research Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), and informed consent was obtained from each participant.

We studied 98 patients with end-stage renal disease on chronic hemodialysis followed in three dialysis clinics (ESKD group). We included patients aged between 18 and 65 years who were on regular treatment for at least 3 months and were clinically stable. Patients with previous CVD or using medications were excluded from our sample because we aimed at sampling representative patients on hemodialysis. The etiology for ESKD and concomitant CVD are described in supplementary table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000336108). The patients were routinely dialyzed three times a week for 4 h with a polysulfone hollow-fiber membrane, bicarbonate dialysate, and standard heparin anticoagulation. Reverse osmosis was used for water treatment and the dialysate was regularly checked for the presence of endotoxin. Dialysis adequacy was evaluated by measuring Kt/V. Blood pressure was measured using a calibrated sphygmomanometer with appropriated cuff size.

A group of 38 healthy volunteers with normal renal function was recruited among blood donors at the blood bank of the University Hospital (PUCRS) as a control group. These subjects were matched for age and gender with the patients in the ESKD group. All subjects provided a complete health history and underwent physical examination and laboratory analysis to exclude subjects with hypertension, diabetes mellitus, other concomitant CVDs, respiratory, hepatic, renal, or hematological dysfunction.

Venous blood samples from each subject were collected into EDTA Vacutainer tubes (Becton-Dickinson, São Paulo, Brazil) by venipuncture. Patients in the ESKD group were sampled immediately before and at the end of the hemodialysis session. The blood samples were centrifuged at 1,000 *g* for 10 min and plasma fractions were immediately stored at –70°C until used for biochemical measurements. Venous blood samples were also collected to extract genomic DNA.

Hematological and biochemical parameters were determined by routine techniques using an automated analyzer (Johnson Vitros Chemistry 5.1 SS). LDL cholesterol was calculated using Friedewald's formula.

Assessment of MMP-2 Levels in Plasma by SDS-Polyacrylamide Gel Electrophoresis Gelatin Zymography

Gelatin zymography of MMP-2 of plasma samples was performed as previously described [23]. Briefly, plasma samples were subjected to electrophoresis on 7% SDS-polyacrylamide gel electrophoresis (PAGE) co-polymerized with gelatin (1%) as the substrate. After electrophoresis was complete, the gels were 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 mmol/l $CaCl_2$. The gels were stained with 0.05% Coomassie Brilliant Blue G-250 for 3 h, and then destained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. The gels were scanned and the digital images were obtained from the scanner. The intensity of the band corresponding to MMP-2 (72 kDa) was analyzed with an image analysis software (Image J 1.43u). The intensity value for the MMP-2 band was calculated as relative activity according to the intensity of related MMP-2 standard [23].

Enzyme Immunoassay of TIMP-2

Plasma TIMP-2 concentrations were measured with commercially available ELISA assay kits (R&D Systems, Minneapolis, Minn., USA), according to the manufacturer's instructions.

Genotyping for MMP-2 Polymorphisms

Genomic DNA was extracted from the cellular component of 1 ml of whole blood and stored at –20°C until analyzed. Genotypes for the $C^{-1306}T$ (rs 243865) and the $C^{-735}T$ (rs 2285053) in the 5′-flanking region of the MMP-2 gene were determined by TaqMan® allele discrimination assay (Applied Biosystems, Carlsbad, Calif., 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′-CAGCACTC[T/C]ACCTCT-3′. TaqMan polymerase chain reaction (PCR) was performed in a total volume of 12 μl (3 ng of dried DNA, 1× TaqMan master mix, 900 nM of each primer, and 200 nM of each probe) placed in 96-well PCR plates. Fluorescence from PCR amplification was detected using Chromo 4 Detector (Bio-Rad Laboratories, Hercules, Calif., USA) and analyzed with the manufacturer's software. Probes and primers used in the 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 [24].

Statistical Analysis

Clinical features were compared between the groups using unpaired Student's t or Mann-Whitney's tests. The distribution of the genotypes for each polymorphism was assessed for deviation from the Hardy-Weinberg equilibrium using χ^2 tests. To examine the effects of the MMP-2 genotypes and haplotypes on circulating levels of MMP-2, TIMP-2, or MMP-2/TIMP-2 ratios, we used unpaired Student's t test and one-way ANOVA (followed by Tukey's posttest), respectively. Comparisons of MMP-2, TIMP-2, or MMP-2/TIMP-2 ratios in the ESKD group before and after hemodialysis were made with paired Student's t test.

Haplotype frequencies were estimated using PHASE software (http://depts.washington.edu/uwc4c/express-licenses/assets/phase/). Only haplotypes with frequencies higher than 5% were taken into consideration. The possible haplotypes including genetic variants of two polymorphisms in the MMP-2 gene studied ($C^{-1306}T$ and $C^{-735}T$) were: H1 (C,C); H2 (C,T); H3 (T,C), and H4 (T,T). Because the TT genotype for both polymorphisms was very rare, we grouped the CT and TT genotypes. A value of $p < 0.05$ was considered statistically significant.

Table 1. Demographic and clinical features of healthy controls and ESKD patients

Clinical features	Controls (n = 38)	ESKD patients (n = 98)	p
Age, years	50 ± 9	51 ± 11	NS
Race, white/non-white	30/8	81/17	NS
Male/female	19/19	55/43	NS
Current smokers	6	45	0.0011
SBP, mm Hg	120 ± 11	141 ± 30	<0.0001
DBP, mm Hg	79 ± 9	81 ± 14	NS
Diabetes mellitus	0	33	<0.0001
Hypertension	0	77	<0.0001
BMI	27.7 ± 5.1	25.4 ± 5.8	0.0077
Total cholesterol, mg/dl	198.5 ± 45.2	158.9 ± 55.9	0.0002
HDL cholesterol, mg/dl	50.6 ± 17.6	38.0 ± 12.8	<0.0001
LDL cholesterol, mg/dl	119.5 ± 42.6	86.3 ± 42.6	<0.0001
Triglycerides, mg/dl	115.1 ± 61.6	194.5 ± 151.2	0.0169
Hemoglobin, g/dl	13.4 ± 1.02	10.6 ± 2.5	<0.0001
Hematocrit, %	40.3 ± 3.4	31.7 ± 6.0	<0.0001
Leukocytes, $\times 10^3/\mu l$	6.0 ± 7.5	6.7 ± 2.1	NS
Creatinine, mg/dl	0.86 ± 0.29	9.03 ± 3.22	<0.0001
Calcium, mg/dl	9.20 ± 0.76	8.90 ± 1.10	NS
Phosphorus, mg/dl	3.18 ± 0.70	5.91 ± 1.60	<0.0001
Potassium, mg/dl	4.19 ± 0.33	5.32 ± 0.96	<0.0001
PTH, pg/ml	NA	530 ± 655	–
Albumin, mg/dl	NA	3.91 ± 0.34	–

Results

Clinical features of studied subjects are shown in table 1. Although the groups were matched by age, gender, and race distributions (all $p > 0.05$), significant differences were found in arterial blood pressure, BMI, lipid fractions, hemoglobin, hematocrit, creatinine, phosphorus, and potassium concentrations (all $p < 0.05$). Further details with respect to the etiology of ESKD etiology or previous CVD diagnoses are shown in online supplementary table S1.

The distributions of allele, genotype, and haplotype frequencies in ESKD patients are shown in online supplementary table S2. The distribution of genotypes for each polymorphism showed no deviation from Hardy-Weinberg equilibrium ($p > 0.05$).

The plasma concentrations of MMP-2 and TIMP-2 were evaluated both in controls and in ESKD patients (before and after hemodialysis). While we found higher MMP-2 and TIMP-2 levels in ESKD patients compared to healthy controls (fig. 1a, b, respectively; both $p < 0.0001$), no significant differences between the groups were found in MMP-2/TIMP-2 ratios ($p = 0.0575$; fig. 1c). Interestingly, hemodialysis decreased MMP-2 levels ($p < 0.0001$; fig. 1a) without changing TIMP-2 concentrations ($p = 0.8916$; fig. 1b), thus lowering MMP-2/TIMP-2 ratios ($p < 0.0001$; fig. 1c).

When ESKD patients were divided according to the genotypes for the $C^{-1306}T$ polymorphism, we found no differences in MMP-2 or TIMP-2 levels before hemodialysis (both $p > 0.05$; fig. 2a, b). Hemodialysis decreased MMP-2 (but not TIMP-2) levels independently of the genotypes for the $C^{-1306}T$ polymorphism ($p < 0.0001$; fig. 2a, b).

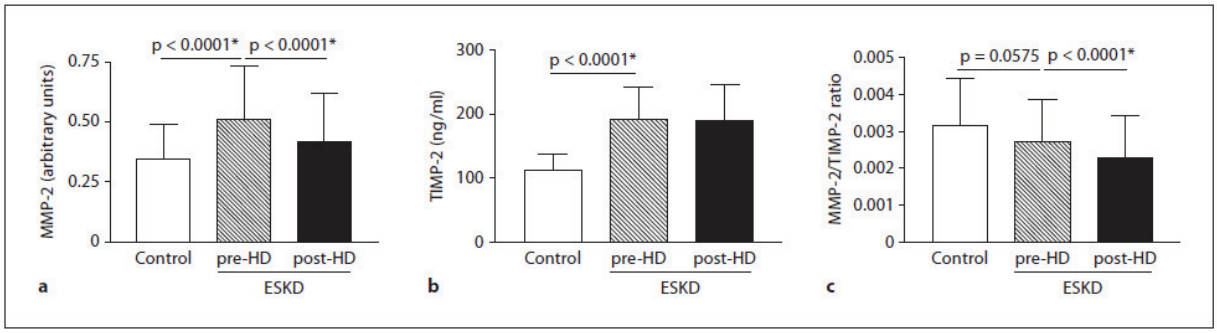

Fig. 1. Effects of hemodialysis on plasma MMP-2 and TIMP-2 levels, and on MMP-2/TIMP-2 ratios. Concentrations of MMP-2, TIMP-2, and MMP-2/TIMP-2 ratios in healthy controls and in ESKD patients before (pre) and after (post) hemodialysis (HD). * Statistically significant.

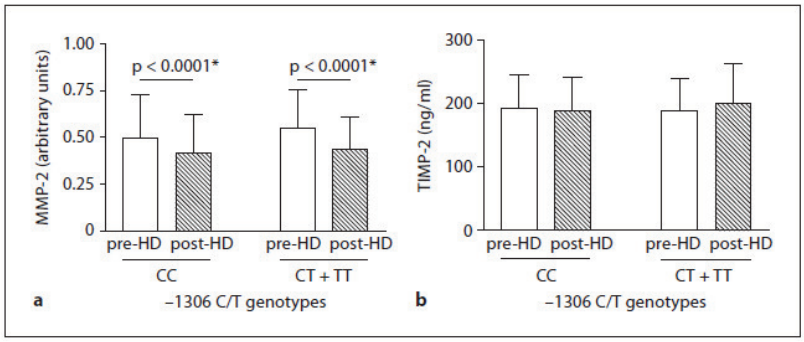

Fig. 2. Plasma concentrations of MMP-2 and TIMP-2 according to *MMP-2* $C^{-1306}T$ genotypes before (pre) and after (post) hemodialysis (HD). * Statistically significant.

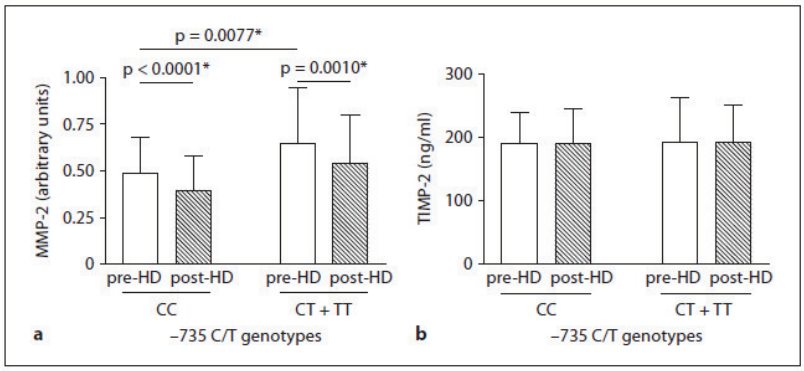

Fig. 3. Plasma concentrations of MMP-2 and TIMP-2 according to *MMP-2* $C^{-735}T$ genotypes before (pre) and after (post) hemodialysis (HD). * Statistically significant.

In contrast to the $C^{-1306}T$ polymorphism, when ESKD patients were divided according to the genotypes for the $C^{-735}T$ polymorphism, we found higher MMP-2 (but not TIMP-2) levels before hemodialysis in subjects with the CT/TT genotypes compared with those found in subjects with the CC genotype ($p = 0.0077$; fig. 3a, b). However, in parallel with the $C^{-1306}T$ polymorphism, hemodialysis decreased MMP-2 (but not TIMP-2) levels independently of the genotypes for the $C^{-735}T$ polymorphism ($p < 0.0010$; fig. 3a, b).

The analysis of haplotypes showed higher MMP-2 (but not TIMP-2) levels in ESKD patients with the C-T haplo-

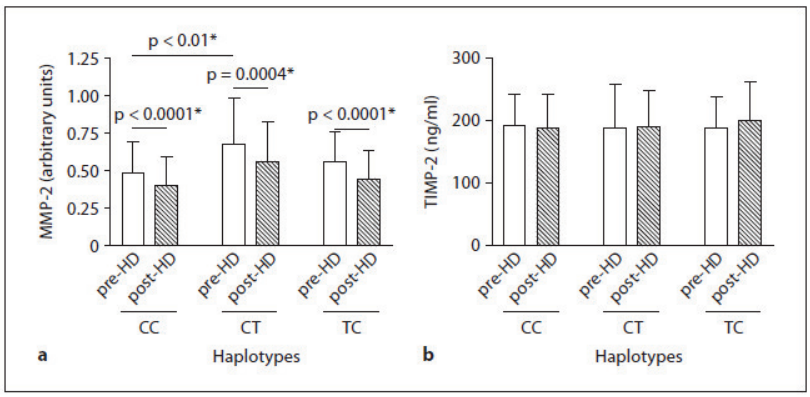

Fig. 4. Plasma concentrations of MMP-2 and TIMP-2 according to MMP-2 haplotypes before (pre) and after (post) hemodialysis (HD). * Statistically significant. The haplotype H4 (T,T) was not observed.

type compared with those with the C-C haplotype (the most common haplotype; $p < 0.01$; fig. 4a, b). Hemodialysis decreased MMP-2 (but not TIMP-2) levels in all MMP-2 haplotype groups ($p < 0.0004$; fig. 4a, b).

Discussion

The main findings of the present study were: (i) patients with ESKD have higher circulating MMP-2 and TIMP-2 levels than healthy controls; (ii) ESKD patients carrying the T allele for the $C^{-735}T$ polymorphism or the C-T haplotype have higher plasma MMP-2 levels than those without these genetic markers, and (iii) hemodialysis decreases plasma MMP-2 (but not TIMP-2) concentrations in ESKD patients. However, these effects are not modified by MMP-2 polymorphisms. This is the first study to examine how genetic MMP-2 variants may affect MMP-2/TIMP-2 levels in ESKD patients or modify the effects of hemodialysis.

Abnormal MMP-2 activity clearly contributes to the vasculopathy found in ESKD patients [7, 25]. Interestingly, experimental evidence showed an early upregulation of MMP-2 expression in areas of elastin degradation and smooth muscle cells phenotype change in chronic kidney disease course, which is associated with increased circulating MMP-2 levels [4–6]. These alterations clearly promote vascular medial layer calcification [6], and the increases in MMP-2 levels correlated positively with vascular stiffness and phosphate concentrations in chronic kidney disease patients [25]. In line with our results showing increased MMP-2 and TIMP-2 levels in ESKD patients, elevated circulating MMP-2 and TIMP-2 levels have been described as an indicator of CVD in dialysis patients [8, 9]. It is possible that TIMP-2 levels increase in order to protect against abnormal proteolytic activity in patients on dialysis, which could promote excessive extracellular matrix remodeling, as previously suggested [9, 15]. While most of the previous studies agree with our findings [8, 10–12], two studies detected no significant changes in these markers [13, 14], and one study showed lower TIMP-2 levels and augmented MMP-2/TIMP-2 ratios in ESKD [14]. Probably, the exclusion of CVD patients from that study may have affected the conclusions drawn by the authors [14]. The explanation for such discrepancies between studies may be explained by differences in the studied populations, ethnicity, age, sample size, and etiology for ESKD [15].

Several studies analyzed the effects of a hemodialysis session on the circulating levels of MMP-2. However, conflicting results have been reported. In line with our findings, most studies showed that hemodialysis reduced MMP-2 levels, with one exception [10, 11, 14, 15]. Variable results have also been reported for the effects of hemodialysis on TIMP-2 levels [10, 14, 15]. However, this study is the first to report significant reductions in MMP-2/TIMP-2 ratios after a dialysis session, whereas MMP-2/TIMP-2 ratios were not affected in another study [14]. It is not clear why TIMP-2 (a 21-kDa molecule) levels are not altered by hemodialysis, whereas the concentrations of MMP-2 (72 kDa) decrease. The larger size of MMP-2 should have precluded its filtration, thus suggesting that a mechanism other than ultrafiltration is certainly involved. Interestingly, although hemodialysis activates inflammatory responses, which promote MMP release [11], no study showed increased MMP-2 levels after a session.

Genetic markers may contribute to the variability in MMP-2 in ESKD patients. Given the importance of

MMP-2 to CVD [6, 26–28], MMP-2 polymorphisms could affect MMP-2 levels in uremic patients and therefore influence the prevalence of CVD in uremic patients. Whereas no previous study has examined this possibility in ESKD patients, we studied the effects of two functionally relevant MMP-2 SNPs in the promoter of MMP-2. The $C^{-735}T$ and $C^{-1306}T$ SNPs disrupt the Sp1 regulatory element in the promoter site (CCACC box), thus affecting MMP-2 expression in an allele-specific manner [18]. In line with the idea that the C allele for the $C^{-1306}T$ polymorphism increases MMP-2 activity, higher MMP-2 levels and MMP-2/TIMP-2 ratios were reported in subjects exposed to mercury and carrying this allele [16]. However, this polymorphism had no significant effects on MMP-2 levels in our uremic patients. This apparent discrepancy between studies is probably explained by differences between clinical conditions, with different factors, possibly with MMP-2 gene variants.

We found that the T allele for the $C^{-735}T$ polymorphism and the C-T haplotype were associated with increased MMP-2 (but not TIMP-2) levels in ESKD patients. While our results may not be consistent with previous molecular findings discussed above [18], little is known about the complex regulation of MMPs, especially MMP-2 in ESKD patients. In vivo clinical findings may differ significantly from in vitro molecular studies. In line with our findings, previous studies showed that the C-C haplotype was more commonly found in hypertensive patients with a lower left ventricular mass index [22], and this clinical finding also contrasts with molecular studies [18]. Although further studies are required to clarify the mechanisms underlying these clinical associations, it is interesting that we found no effects of MMP-2 polymorphisms on the changes in MMP-2 levels associated with a hemodialysis. These findings suggest that hemodialysis decreases MMP-2 levels independently of genetic factors.

The present study has some limitations. First, although this cross-sectional study allows the detection of associations, causality is not properly addressed. Second, we studied a relatively small number of patients, and this may have limited our chances to detect differences between the groups. Third, the patients included in the present study were under pharmacological treatment, and this may have altered MMP-2 and/or TIMP-2 levels [29, 30]. However, it is clearly unacceptable not to treat uremic patients.

In conclusion, we found that the MMP-2 genotypes or haplotypes modify MMP-2 levels in ESKD patients, and may help to identify patients with increased levels of MMP-2 while TIMP-2 remains unaltered. Our findings show that hemodialysis reduces MMP-2 levels independently of MMP-2 genetic variants.

Acknowledgements

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo and Conselho Nacional de Desenvolvimento Científico e Tecnológico (Brazil).

Disclosure Statement

The authors have no conflicts of interest to disclose.

References

1 Page-McCaw A, Ewald AJ, Werb Z: Matrix metalloproteinases and the regulation of tissue remodelling. Nat Rev Mol Cell Biol 2007; 8:221–233.
2 Catania JM, Chen G, Parrish AR: Role of matrix metalloproteinases in renal pathophysiologies. Am J Physiol Renal Physiol 2007; 292:F905–F911.
3 Hage FG, Venkataraman R, Zoghbi GJ, Perry GJ, DeMattos AM, Iskandrian AE: The scope of coronary heart disease in patients with chronic kidney disease. J Am Coll Cardiol 2009;53:2129–2140.
4 Kumata C, Mizobuchi M, Ogata H, Koiwa F, Kondo F, Kinugasa E, Akizawa T: Involvement of matrix metalloproteinase-2 in the development of medial layer vascular calcification in uremic rats. Ther Apher Dial 2011; 15(suppl 1):18–22.
5 Pai A, Leaf EM, El-Abbadi M, Giachelli CM: Elastin degradation and vascular smooth muscle cell phenotype change precede cell loss and arterial medial calcification in a uremic mouse model of chronic kidney disease. Am J Pathol 2011;178:764–773.
6 Chen NX, O'Neill KD, Chen X, Kiattisunthorn K, Gattone VH, Moe SM: Activation of arterial matrix metalloproteinases leads to vascular calcification in chronic kidney disease. Am J Nephrol 2011;34:211–219.
7 Chung AW, Yang HH, Kim JM, Sigrist MK, Chum E, Gourlay WA, Levin A: Upregulation of matrix metalloproteinase-2 in the arterial vasculature contributes to stiffening and vasomotor dysfunction in patients with chronic kidney disease. Circulation 2009; 120:792–801.
8 Pawlak K, Pawlak D, Mysliwiec M: Serum matrix metalloproteinase-2 and increased oxidative stress are associated with carotid atherosclerosis in hemodialyzed patients. Atherosclerosis 2007;190:199–204.
9 Pawlak K, Tankiewicz J, Mysliwiec M, Pawlak D: Systemic levels of MMP2/TIMP2 and cardiovascular risk in CAPD patients. Nephron Clin Pract 2010;115:C251–C258.
10 Musial K, Zwolinska D: Matrix metalloproteinases and soluble Fas/FasL system as novel regulators of apoptosis in children and young adults on chronic dialysis. Apoptosis 2011;16:653–659.

11 Pawlak K, Mysliwiec M, Pawlak D: Peripheral blood level alterations of MMP-2 and MMP-9 in patients with chronic kidney disease on conservative treatment and on hemodialysis. Clin Biochem 2011;44:838–843.
12 Friese RS, Rao F, Khandrika S, Thomas B, Ziegler MG, Schmid-Schonbein GW, O'Connor DT: Matrix metalloproteinases: discrete elevations in essential hypertension and hypertensive end-stage renal disease. Clin Exp Hypertens 2009;31:521–533.
13 Preston GA, Barrett CV, Alcorta DA, Hogan SL, Dinwiddie L, Jennette JC, Falk RJ: Serum matrix metalloproteinases MMP-2 and MMP-3 levels in dialysis patients vary independently of CRP and IL-6 levels. Nephron 2002;92:817–823.
14 Rysz J, Banach M, Stolarek RA, Mikhailidis DP, Cialkowska-Rysz A, Pokoca L, Piechota M, Baj Z: Serum metalloproteinases mmp-2, mmp-9 and metalloproteinase tissue inhibitors TIMP-1 and TIMP-2 in patients on hemodialysis. Int Urol Nephrol 2011;43:491–498.
15 Chou FP, Chu SC, Cheng MC, Yang SF, Cheung WN, Chiou HL, Hsieh YS: Effect of hemodialysis on the plasma level of type IV collagenases and their inhibitors. Clin Biochem 2002;35:383–388.
16 Jacob-Ferreira AL, Lacchini R, Gerlach RF, Passos CJ, Barbosa F Jr, Tanus-Santos JE: A common matrix metalloproteinase (MMP)-2 polymorphism affects plasma MMP-2 levels in subjects environmentally exposed to mercury. Sci Total Environ 2011;409:4242–4246.
17 Singh R, Srivastava P, Srivastava A, Mittal RD: Matrix metalloproteinase (MMP-9 and MMP-2) gene polymorphisms influence allograft survival in renal transplant recipients. Nephrol Dial Transplant 2010;25:3393–3401.
18 Yu C, Zhou Y, Miao X, Xiong P, Tan W, Lin D: Functional haplotypes in the promoter of matrix metalloproteinase-2 predict risk of the occurrence and metastasis of esophageal cancer. Cancer Res 2004;64:7622–7628.
19 Price SJ, Greaves DR, Watkins H: Identification of novel, functional genetic variants in the human matrix metalloproteinase-2 gene: role of Sp1 in allele-specific transcriptional regulation. J Biol Chem 2001;276:7549–7558.
20 Manso H, Krug T, Sobral J, Albergaria I, Gaspar G, Ferro JM, Oliveira SA, Vicente AM: Variants of the matrix metalloproteinase-2 but not the matrix metalloproteinase-9 genes significantly influence functional outcome after stroke. BMC Med Genet 2010;11:40.
21 Langers AM, Verspaget HW, Hommes DW, Sier CF: Single-nucleotide polymorphisms of matrix metalloproteinases and their inhibitors in gastrointestinal cancer. World J Gastrointest Oncol 2011;3:79–98.
22 Lacchini R, Jacob-Ferreira AL, Luizon MR, Gasparini S, Ferreira-Sae MC, Schreiber R, Nadruz W Jr, Tanus-Santos JE: Common matrix metalloproteinase 2 gene haplotypes may modulate left ventricular remodelling in hypertensive patients. J Hum Hypertens 2011 (E-pub ahead of print).
23 Gerlach RF, Demacq C, Jung K, Tanus-Santos JE: Rapid separation of serum does not avoid artificially higher matrix metalloproteinase (MMP)-9 levels in serum versus plasma. Clin Biochem 2007;40:119–123.
24 Lacchini R, Metzger IF, Luizon M, Ishizawa M, Tanus-Santos JE: Interethnic differences in the distribution of matrix metalloproteinases genetic polymorphisms are consistent with interethnic differences in disease prevalence. DNA Cell Biol 2010;29:649–655.
25 Chung AW, Yang HH, Sigrist MK, Brin G, Chum E, Gourlay WA, Levin A: Matrix metalloproteinase-2 and -9 exacerbate arterial stiffening and angiogenesis in diabetes and chronic kidney disease. Cardiovasc Res 2009;84:494–504.
26 Rizzi E, Castro MM, Prado CM, Silva CA, Fazan R Jr, Rossi MA, Tanus-Santos JE, Gerlach RF: Matrix metalloproteinase inhibition improves cardiac dysfunction and remodeling in 2-kidney, 1-clip hypertension. J Card Fail 2010;16:599–608.
27 Chancey AL, Brower GL, Peterson JT, Janicki JS: Effects of matrix metalloproteinase inhibition on ventricular remodeling due to volume overload. Circulation 2002;105:1983–1988.
28 Guimaraes DA, Rizzi E, Ceron CS, Oliveira AM, Oliveira DM, Castro MM, Tirapelli CR, Gerlach RF, Tanus-Santos JE: Doxycycline dose-dependently inhibits MMP-2-mediated vascular changes in 2K1C hypertension. Basic Clin Pharmacol Toxicol 2011;108:318–325.
29 Ceron C, Castro M, Rizzi E, Montenegro M, Fontana V, Salgado M, Gerlach R, Tanus-Santos J: Spironolactone and hydrochlorothiazide exert antioxidant effects and reduce vascular matrix metalloproteinase-2 activity and expression in a model of renovascular hypertension. Br J Pharmacol 2010;160:77–87.
30 Martinez ML, Rizzi E, Castro MM, Fernandes K, Bendhack LM, Gerlach RF, Tanus-Santos JE: Lercanidipine decreases vascular matrix metalloproteinase-2 activity and protects against vascular dysfunction in diabetic rats. Eur J Pharmacol 2008;599:110–116.

Erratum

In the above article by Marson et al. entitled 'Matrix Metalloproteinase (MMP)-2 Genetic Variants Modify the Circulating MMP-2 Levels in End-Stage Kidney Disease' [Am J Nephrol 2012;35:209–215], the following error occured on page 210 under Subjects and Methods, right column, third sentence: 'Patients with previous CVD or using medications were excluded.', it should read 'Patients with previous CVD or using medications were not excluded.'

SUPPLEMENTARY TABLES

Table S1

Etiology and previous cardiovascular (CVD) diagnosis of end stage kidney disease patients included in our study

Etiology	Number of patients (98)
Diabetes Mellitus (n)	28
Essential hypertension (n)	17
Glomerulonephritis (n)	15
Polycystic kidney disease (n)	14
Urologic causes (n)	5
Chronic interstitial nephritis (n)	4
Unknown (n)	15

Previous CVD diagnosis	Number of patients (38)
Ischemic heart disease (n)	24
Stroke (n)	14
Peripheral artery disease (n)	13

Other features	
Currently on anti-hypertensive treatment (n)	65
Mean time of chronic HD (months)	59 ± 62
Residual diuresis (n)	43

Table S2

Distribution of MMP-2 genotypes, alleles, and haplotypes frequencies in end stage kidney disease patients.

Genotypes		Alleles		Haplotypes	
$C^{-1306}T$	n (%)	$C^{-1306}T$	n (%)		n (%)
CC	73 (74.5)	C	170 (86.7)	H1 (C,C)	154 (78.6)
CT	24 (24.5)	T	26 (13.3)	H2 (C,T)	16 (8.2)
TT	1 (1)			H3 (T,C)	26 (13.2)
$C^{-735}T$		$C^{-735}T$		H4 (T,T)	0 (0)
CC	83 (84.7)	C	180 (91.8)		
CT	14 (14.3)	T	16 (8.2)		
TT	1 (1)				

CAPÍTULO 3

Clinica Chimica Acta 414 (2012) 46–51

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Functional matrix metalloproteinase (MMP)-9 genetic variants modify the effects of hemodialysis on circulating MMP-9 levels

Bernardo P. Marson [a,1], Riccardo Lacchini [a,1], Vanessa Belo [b], Silvia G. Mattos [c], Bartira P. da Costa [c], Carlos E. Poli-de-Figueiredo [c], Jose E. Tanus-Santos [a,*]

[a] Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas, Campinas, Brazil
[b] Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of São Paulo, Ribeirão Preto, Brazil
[c] Faculty of Medicine/IPB/HSL of Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil

ARTICLE INFO

Article history:
Received 29 April 2012
Received in revised form 9 August 2012
Accepted 14 August 2012
Available online 21 August 2012

Keywords:
End stage kidney disease
Haplotypes
Hemodialysis
Matrix metalloproteinase-9
Polymorphism

ABSTRACT

Background: Altered levels of matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), are involved in cardiovascular alterations associated with end stage kidney disease (ESKD). Genetic polymorphisms in *MMP-9* gene affect MMP-9 levels. We examined how *MMP-9* polymorphisms and haplotypes affect the changes in plasma MMP-9 and TIMP-1 levels found in patients with ESKD undergoing hemodialysis.

Methods: We studied 94 ESKD patients undergoing hemodialysis for at least 3 months. MMP-9 and TIMP-1 were measured by ELISA in plasma from blood samples collected before and after a session of hemodialysis. Genotypes for three MMP-9 polymorphisms ($C^{-1562}T$, rs3918242; −90 $(CA)_{14-24}$, rs2234681; and Q279R, rs17576) were determined by Taqman® Allele Discrimination Assay and real-time polymerase chain reaction. Haplotype frequencies were determined with the software program PHASE 2.1.

Results: Hemodialysis increased MMP-9 and TIMP-1 levels ($P<0.05$). Genotypes had no effects on baseline MMP-9 and TIMP-1 levels ($P>0.05$). Hemodialysis increased MMP-9 and TIMP-1 levels in subjects with the CC (but not CT or TT) genotype for the $C^{-1562}T$ polymorphism ($P<0.05$), and increased MMP-9 levels in subjects with the QQ (but not QR or RR) genotype for the Q279R polymorphism ($P<0.05$), whereas the $CA(n)_{14-24}$ polymorphism had no major effects. While MMP-9 haplotypes had no effects on baseline MMP-9 levels ($P>0.05$), hemodialysis increased MMP-9 levels and MMP-9/TIMP-1 ratios in subjects carrying the CLQ haplotype ($P=0.0012$ and $P=0.0045$, respectively).

Conclusion: ESKD patients with the QQ genotype for the Q279R polymorphism or with the CLQ haplotype are exposed to more severe increases in MMP-9 levels after hemodialysis. Such patients may benefit from the use of MMP inhibitors.

1. Introduction

Patients with end stage kidney disease (ESKD) bear a heavy burden of cardiovascular diseases (CVD) [1], and hemodialysis may trigger a maladaptive process leading to arterial medial calcification, stiffness, and loss of function mimicking the atherogenic effects of uremic factors and inflammation [1,2]. These vascular alterations found in ESKD are linked to extracellular matrix remodeling and elastocalcinosis [2,3] and involve imbalanced matrix metalloproteinase (MMP) activity [4].

MMPs are structurally related, zinc dependent, enzymes that degrade the extracellular matrix and other non-extracellular matrix-related substrates [5]. They are regulated at transcriptional and post-translational levels, and their activity is also dependent on endogenous inhibitors, the tissue inhibitors of MMPs (TIMPs) [6]. Mounting evidence indicates that imbalanced MMP activity contributes to diseased vessels in patients with renal failure [3,7–9], particularly MMP-9, which is associated with atherosclerosis in non-dialytic chronic kidney disease (CKD) [7,8]. Moreover, increased MMP-9 expression has been reported in monocytes from patients with ESKD [10], and recent studies have shown altered MMP-9 and TIMP-1 levels in patients on dialysis [11–15]. Nevertheless, inconsistent findings have been reported regarding the effects of hemodialysis on the circulating MMP levels. Some studies suggest that plasma MMP-9 remains unaltered [11,12,16] or decreases after a hemodialysis session [13,17].

MMP-9 activity is highly dependent on its expression levels [6,18], and functional genetic polymorphisms in the *MMP-9* gene may affect MMP-9 concentrations [19], possibly modifying the susceptibility to cardiovascular diseases [18,20,21]. Therefore, we hypothesized that

* Corresponding author at: Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Av. Bandeirantes, 3900 14049-900 Ribeirao Preto, SP, Brazil. Tel.: +55 16 3602 3163; fax: +55 16 3633 2301.
E-mail addresses: tanus@fmrp.usp.br, tanussantos@yahoo.com (J.E. Tanus-Santos).
[1] These authors contributed equally to this manuscript.

0009-8981/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2012.08.014

2 functional polymorphisms in the promoter region of *MMP-9* gene (the $C^{-1562}T$ — rs3918242, and the microsatellite $(CA)_{14-24}$ in −90 position — rs2234681), and one polymorphism in exon 6 (the Q279R; rs17576) could affect the changes in MMP-9 levels associated with hemodialysis. In support of this hypothesis, these polymorphisms have been associated with variable MMP-9 levels in other clinical conditions [22–24]. While previous works have studied MMP-2 [25] and MMP-9 [26] polymorphisms in dialysis, no previous study has examined how functional MMP-9 polymorphisms, or their combinations within haplotypes, affect the changes in MMP-9 and TIMP-1 levels during a hemodialysis session.

2. Materials and methods

2.1. Patients

The present study was carried out in accordance with the Helsinki Declaration ethical guidelines. Approval for use of human blood was obtained from the Research Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul, and informed consent was obtained from each participant. Ninety-four patients with ages from 18 to 65 y were studied in 3 hemodialysis units. ESKD was defined as glomerular filtration rate <15 ml/min associated with clinical signs of uremic syndrome requiring dialysis. The patients were on continuous therapy for at least 3 months and were stable (without clinical complications). Clinical data were based on medical history, physical examination, and routine analytical tests. While the patients included in the present study had different causes for their clinical condition, diabetes mellitus, hypertension, glomerulonephritis, and polycystic kidney disease were the main causal diseases. The dialysis schedule included three 4-h dialysis sessions per week with a polysulfone hollow-fiber membrane, bicarbonate dialysate, and regular heparin anticoagulation. Reverse osmosis was used for water treatment and the dialysate was regularly checked for the presence of endotoxin. Dialysis adequacy was evaluated by measuring Kt/V. Blood pressure was measured using a calibrated sphygmomanometer with appropriated cuff size.

Blood samples were collected into EDTA vaccutainer tubes (Becton-Dickinson, São Paulo, Brazil) by venipuncture of the arteriovenous fistula before and after a hemodialysis session. The blood samples were centrifuged at 1000×*g* for 10 min and plasma fractions were immediately stored at −70 °C until used for biochemical measurements. Blood samples were also collected to extract genomic DNA. The biochemical and hematological parameters were determined by routine techniques using an automated analyzer (Johnson Vitros Chemistry 5.1 SS). LDL-cholesterol was calculated using the Friedewald's formula.

2.2. Genotyping

Genotypes for the $C^{-1562}T$ (rs3918242) polymorphism in the 5′-flanking region of MMP-9 were determined by polymerase chain reaction (PCR) amplification using the primers: 5′-GCCTGGCAC ATAGTAGGCCC-3′ (sense) and 5-CTTCCTAGCCAGCCGGCATC-3 (antisense), and PCR conditions as described elsewhere [27]. The amplified products were digested with SphI (New England Biolabs, Ipswich, MA) overnight at 37 °C, producing fragments of 247 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). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized after silver staining.

The −90 $(CA)_{14-24}$ (rs3222264) microsatellite was detected by polymerase chain reaction as described previously [19], using the primers 5′-GACTTGGCAGT GGAGACTGCGGGCA-3′ (sense) and 5′-GAC CCCACCCCTCCTTGACAGGCAA-3′ (antisense). Amplified products were separated in a 7% polyacrylamide–urea gel and visualized after silver staining. Differences in molecular weight (or number of bases), from 144 bp to 168 bp, were determined by a comparison with migration of a 10-bp DNA ladder (Invitrogen, Carlsbad, CA) and to homozygous samples previously sequenced. In order to make easier the analysis of the bands in the gel, the alleles were classified in accordance with the biallelic distribution of this polymorphism: alleles were grouped as "low" (L) when the number of CA repeats was less than 21, and as "high" (H) when the number of CA repeats was ≥21 [28].

Genotypes for the MMP-9 Q279R (rs17576) polymorphism were determined by Taqman® Allele Discrimination assay. Probes and primers used in the MMP-9 assay were designed by Applied Biosystems (ID: C_11655953_10). TaqMan polymerase chain reaction was performed in a total volume of 12 μl (3 ng of DNA, 1× TaqMan master mix, 900 nmol/l of each primer and 200 nmol/l of each probe) placed in 96-well PCR plates. Fluorescence from polymerase chain reaction amplification was detected using Chromo 4 Detector (Bio-Rad Laboratories, Hercules, CA) and analyzed with the manufacturer's software [23].

2.3. Measurement of plasma MMP-9 and TIMP-1 concentrations

The plasma MMP-9 and TIMP-1 concentrations were measured with commercially available enzyme-linked immunosorbent assay kits (DY911 and DY970, respectively; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.4. Statistical analysis

Statistical analysis was carried out using the Stat-View (SAS Institute, Cary, NC). The clinical characteristics, plasma MMP-9 and TIMP-1 concentrations, and MMP-9/TIMP-1 ratios were compared with Student's paired *t*-test (parametric data), Mann–Whitney *U*-test (non-parametric data) or chi-square (categorical data). The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium by using chi-squared tests. Difference in the alleles, genotypes, and haplotype frequencies was assessed with chi-squared tests. Because the TT genotype for the $C^{-1562}T$ polymorphism was very rare, we combined this genotype with the heterozygous CT genotype. A $P<0.05$ was considered statistically significant.

The Bayesian statistical-based program Phase 2.1 was used to estimate the haplotype frequencies in each group (http://depts.washington.edu/uwc4c/express-licenses/assets/phase/). The possible haplotypes including genetic variants of three MMP-9 polymorphisms studied ($C^{-1562}T$, $90(CA)_{14-24}$, and Q279R) were H1 (C, L, Q); H2 (C, L, R); H3 (C, H, Q); H4 (C, H, R); H5 (T, L, Q); H6 (T, L, R); H7 (T, H, Q); and H8 (T, H, R). Differences in haplotype frequency distributions were further tested using contingency tables, and to compare specific haplotype frequencies, a value of $P<0.01$ (0.05/number of observed haplotypes) was considered significant to correct for multiple comparisons. We excluded the rare haplotypes H5, H6 and H7 from the analysis.

3. Results

Clinical characteristics of patients are shown in Table 1. Detailed information regarding the etiology of renal failure and coexistent cardiovascular diseases and medications is provided in Supplementary Table S1. The distribution of allele, genotype, and haplotype frequencies in hemodialysis subjects are shown in Supplementary Table S2. The distribution of genotypes for each polymorphism showed no deviation from Hardy–Weinberg equilibrium ($P>0.05$).

The plasma MMP-9 and TIMP-1 concentrations were evaluated in ESKD patients, both before and after hemodialysis. While the session of hemodialysis increased MMP-9 and TIMP-1 plasma concentrations (Fig. 1; $P=0.0087$ and $P=0.0148$, respectively), the MMP-9/TIMP-1

Table 1
Clinical and demographic characteristics of the patients.

Clinical characteristics	Patients, N = 94
Age, y	51 ± 10.8
Race (white/non-white)	79/15
Sex (masculine/feminine)	52/42
Smoking	44.39
SAP, mm Hg	141.4 ± 40.3
DAP, mm Hg	78.8 ± 12.5
Diabetes mellitus	34.6
Hypertension	79.6
BMI, kg/m^2	25.29 ± 5.18
Total cholesterol, mg/dl	157.8 ± 54.6
HDL cholesterol, mg/dl	38.4 ± 11.9
LDL cholesterol, mg/dl	86.6 ± 40.9
Triglycerides, mg/dl	196.1 ± 150.6
Hemoglobin, g/dl	10.4 ± 2.8
Hematocrit, %	31.9 ± 6.1
Leukocytes, × 10^3/μl	6518 ± 2137
Creatinine, mg/dl	9.6 ± 3.2
Calcium, mg/dl	8.8 ± 1
Phosphorus, (mg/dl)	6.6 ± 4.2
Potassium, (mg/dl)	5.2 ± 1.2
PTH (pg/ml)	519.2 ± 452
Albumin (mg/dl)	3.82 ± 0.32

ratios tended to increase after hemodialysis (Fig. 1; P = 0.0561). We found no significant differences when patients with primary kidney diseases and secondary kidney diseases were compared (data not shown).

When ESKD patients were divided according to genotypes, we found no significant effects of genotypes on MMP-9 and TIMP-1 levels measured before hemodialysis (Figs. 2, 3, and 4; all P > 0.05). However, hemodialysis increased MMP-9 levels in some genotype groups. Specifically, we found significant increases in both MMP-9 and TIMP-1 levels in subjects with the CC genotype for the C^{-1562}T polymorphism (Fig. 2; both P < 0.05), but not in subjects with CT or TT genotypes for this polymorphism. When the patients were divided according the genotypes for the CA(n)$_{14–24}$ polymorphism, we found that TIMP-1 levels increased in subjects with the HH genotype (Fig. 3; P = 0.0375). However, this increase in TIMP-1 levels was not associated with significant changes in MMP-9/TIMP-1 ratios (Fig. 3; P > 0.05). Interestingly, we found a trend for increased MMP-9 after hemodialysis in subjects with the LL or the HL genotypes for this polymorphism (Fig. 3; P = 0.0668 and P = 0.0730, respectively). When the patients were divided according to genotypes for the Q279R polymorphism, we found significant increases in MMP-9 levels and in MMP-9/TIMP-1 ratios in subjects with the QQ genotype for this polymorphism (Fig. 4; both P < 0.05). While we found significant increases in TIMP-1 levels in subjects with the QR genotype, this change was not associated with significant changes in MMP-9/TIMP-1 ratios (Fig. 4; P > 0.05).

The analysis of haplotypes showed no effects of MMP-9 haplotypes on both MMP-9 and TIMP-1 levels measured before hemodialysis (Fig. 5; P > 0.05). However, we found significant increases in MMP-9 (but not TIMP-1) levels in subjects carrying the CLQ haplotype (Fig. 5; P = 0.0012), thus resulting in increased MMP-9/TIMP-1 ratio in this particular haplotype group (Fig. 5; P = 0.0045). While hemodialysis was associated with increased TIMP-1 levels in subjects with the CHR haplotype, no significant changes were found in MMP-9/TIMP-1 ratio (Fig. 5; P > 0.05).

4. Discussion

This study examined how MMP-9 genetic polymorphisms affect the changes in MMP-9 and TIMP-1 levels associated with a hemodialysis session in ESKD patients. While we found that MMP-9 polymorphisms do not significantly affect baseline MMP-9 or TIMP-1 levels in ESKD patients, we found a significant genetic contribution of MMP-9 polymorphisms and haplotypes to hemodialysis-induced changes in MMP-9 levels and MMP-9/TIMP-1 ratios. Our findings may help to understand the relevance of MMP-9 genetic polymorphisms to the pathophysiology of cardiovascular complications associated with ESKD and hemodialysis.

MMPs (particularly MMP-9) are important for extracellular matrix remodeling and play key roles in cardiovascular diseases [5,15,20,29]. MMP-9 activation promotes TGF-B signaling and a sequence of events

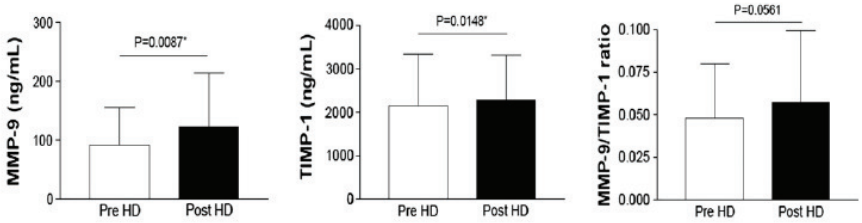

Fig. 1. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios in end stage kidney disease patients before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

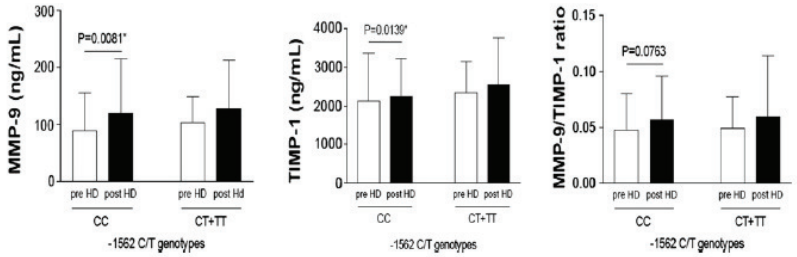

Fig. 2. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios in end stage kidney disease patients according to their genotypes for the *MMP-9* C^{-1562}T polymorphism. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios are shown before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

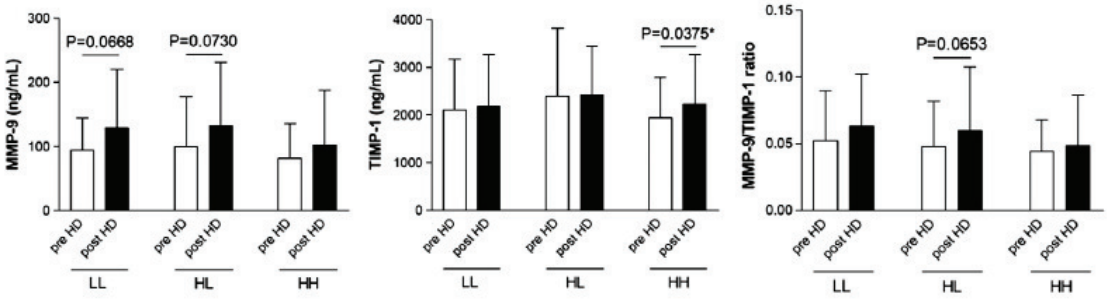

Fig. 3. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios in end stage kidney disease patients according to their genotypes for the *MMP-9* 90(CA)$_{14-24}$ polymorphism. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios are shown before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

that leads to elastocalcinosis and vascular stiffness [29]. Indeed, recent studies showed that upregulated MMP-9, without proper balance of TIMP-1, disrupts elastin and affects normal vascular smooth muscle cells allowing the extracellular matrix to expand and calcify, eventually leading to overt hydroxyapatite deposition and vascular dysfunction [3,7,9].

Our results showing that hemodialysis elevated the circulating levels of MMP-9 and TIMP-1 differ from previous reports, which described reduced or unaltered MMP-9 and TIMP-1 levels after hemodialysis [11,13,16,17]. We found minor increases in MMP-9/TIMP-1 ratios, and this finding agrees with previous results [12]. It is likely that inconsistencies in reported plasma MMP levels reflect the complex regulation of inflammation and MMPs, as well as differences in sample size, populations, anticoagulation regimen, vascular access, type of membrane and causal diseases for ESKD. In addition, pre-analytical issues may also explain dissimilarities, since modifications in processing the samples may alter MMP-9 levels [30,31]. MMP-9 is expressed constitutively at very low levels in bone marrow-derived cells, however it is highly inducible under oxidative and inflammatory conditions [6,10]. Since hemodialysis activates inflammatory and coagulation pathways, it is plausible to find increased MMP-9 levels after hemodialysis.

MMP-9 gene polymorphisms may modify MMP-9 concentrations and affect the development of cardiovascular complications in ESKD, as previously suggested [1]. Indeed, the C^{-1562}T polymorphism results in loss of a nuclear repressor protein binding site and enhanced MMP-9 mRNA and protein levels when the T allele is present [18,21]. The microsatellite 90(CA)14–24 in the promoter region of the MMP-9 gene was shown to reduce MMP-9 promoter activity when the (CA) 14 allele is present as compared with the (CA)21 allele [32]. The Q279R polymorphism causes an aminoacid residue substitution, thus affecting MMP-9 activity [22,33]. Our results showing similar baseline MMP-9 and TIMP-1 levels suggest that these polymorphisms do not have major effects in ESKD patients, at least in terms of circulating MMP-9 levels.

We found that hemodialysis increased MMP-9 and TIMP-1 levels in subjects with the CC (but not CT or TT) genotypes for the C^{-1562}T polymorphism, and increased MMP-9 (but not TIMP-1) levels in subjects with the QQ genotype for the Q279R polymorphism, thus increasing MMP-9/TIMP-1 ratios. However, the haplotypic analysis may be much more effective and informative than the single locus analysis [20]. While baseline MMP-9 and TIMP-1 levels were not affected by MMP-9 haplotypes, subjects with the CLQ haplotype showed increased MMP-9 (but not TIMP-1) levels after hemodialysis. Interestingly, this particular haplotype combines the C allele for C^{-1562}T polymorphism, and L allele for the 90(CA)$_{14-24}$ polymorphisms, which have been associated with lower MMP-9 upregulation [18,21,32]. While we have not examined the molecular mechanisms possibly explaining these findings, our results suggest that the CLQ haplotype combines a group of MMP-9 genetic markers that may lead to the highest rates of cardiovascular complications associated with abnormal MMP-9 activity in ESKD patients undergoing hemodialysis. It remains to be determined whether MMP-9 inhibition is a suitable pharmacologic approach to prevent or postpone cardiovascular in this highly susceptible population. In fact, pharmacological blockade of MMPs has been suggested in hemodialysis patients [4] and experimental evidence suggests that non-specific MMP inhibition may prevent cardiovascular alterations through complex mechanisms [34,35].

This study has some limitations that should be taken into consideration. The findings reported here may be quantitatively small because the differences that we found were not of major magnitude and some of our findings may not resist correction for multiple comparisons.

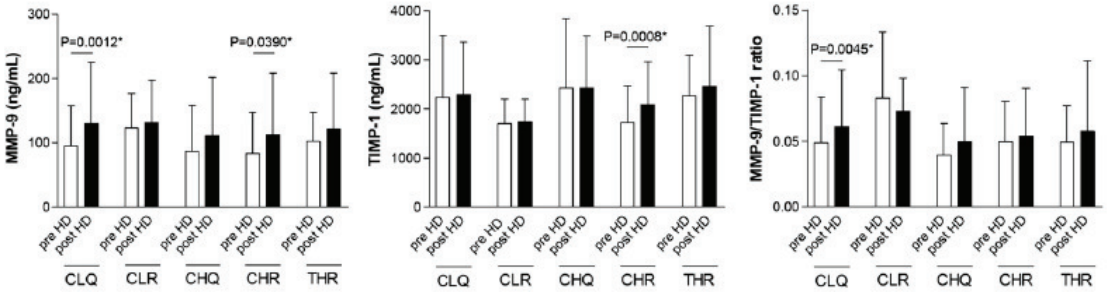

Fig. 4. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios in end stage kidney disease patients according to their genotypes for the *MMP-9* Q^{-279}R polymorphism. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios are shown before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

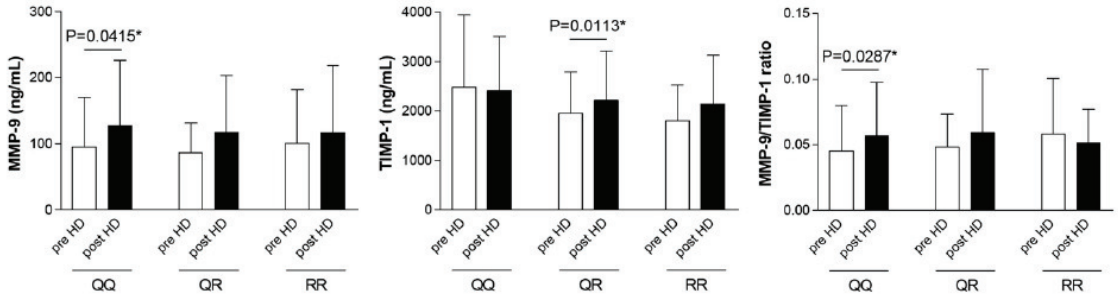

Fig. 5. Effects of hemodialysis (HD) on the circulating MMP-9 and TIMP-1 concentrations and on MMP-9/TIMP-1 ratios in end stage kidney disease patients according to their *MMP-9* haplotypes. Concentrations of MMP-9, TIMP-1 and MMP-9/TIMP-1 ratios are shown before (Pre HD) and after (Post HD) a session of HD. Data are shown as mean ± SD. *Statistically significant.

However, it is common sense that the contribution of a single polymorphism to any clinical condition is usually of minor magnitude, and our findings suggest that MMP-9 polymorphisms may have some minor effects on MMP-9 changes induced by dialysis. Moreover, the assays used in the present study are based on antigen detection and may not reflect activity, particularly where a genotypic difference may affect activity.

In conclusion, we found evidence supporting the idea that MMP genetic polymorphisms affect MMP alterations in ESKD patients [25] undergoing hemodialysis. ESKD patients with the QQ genotype for the Q279R polymorphism or with the CLQ haplotype are exposed to more severe increases in MMP-9 levels after hemodialysis. These findings may indicate a group of patients that will have worse cardiovascular prognosis when undergoing hemodialysis. Whether such patients would benefit from the use of MMP inhibitors remains to be elucidated.

Acknowledgments

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo and Conselho Nacional de Desenvolvimento Científico e Tecnológico.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2012.08.014.

References

[1] Stenvinkel P, Pecoits-Filho R, Lindholm B. Coronary artery disease in end-stage renal disease: no longer a simple plumbing problem. J Am Soc Nephrol 2003;14:1927-39.
[2] Mizobuchi M, Towler D, Slatopolsky E. Vascular calcification: the killer of patients with chronic kidney disease. J Am Soc Nephrol 2009;20:1453-64.
[3] Pai A, Leaf EM, El-Abbadi M, Giachelli CM. Elastin degradation and vascular smooth muscle cell phenotype change precede cell loss and arterial medial calcification in a uremic mouse model of chronic kidney disease. Am J Pathol 2011;178:764-73.
[4] Marson BP, de Figueiredo CE, Tanus-Santos JE. Imbalanced matrix metalloproteinases in cardiovascular complications of end-stage kidney disease: a potential pharmacological target. Basic Clin Pharmacol Toxicol 2012;110:409-15.
[5] Fontana V, Silva PS, Gerlach RF, Tanus-Santos JE. Circulating matrix metalloproteinases and their inhibitors in hypertension. Clin Chim Acta 2012;413:656-62.
[6] Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). Crit Rev Biochem Mol Biol 2002;37:375-536.
[7] Chung AW, Yang HH, Sigrist MK, et al. Matrix metalloproteinase-2 and -9 exacerbate arterial stiffening and angiogenesis in diabetes and chronic kidney disease. Cardiovasc Res 2009;84:494-504.
[8] Addabbo F, Mallamaci F, Leonardis D, et al. Searching for biomarker patterns characterizing carotid atherosclerotic burden in patients with reduced renal function. Nephrol Dial Transplant 2007;22:3521-6.
[9] Chen NX, O'Neill KD, Chen X, Kiattisunthorn K, Gattone VH, Moe SM. Activation of arterial matrix metalloproteinases leads to vascular calcification in chronic kidney disease. Am J Nephrol 2011;34:211-9.
[10] Ebihara I, Nakamura T, Tomino Y, Shimada N, Koide H. Metalloproteinase-9 mRNA expression in monocytes from patients with chronic renal failure. Am J Nephrol 1998;18:305-10.
[11] Pawlak K, Mysliwiec M, Pawlak D. Peripheral blood level alterations of MMP-2 and MMP-9 in patients with chronic kidney disease on conservative treatment and on hemodialysis. Clin Biochem 2011;44:838-43.
[12] Rysz J, Banach M, Stolarek RA, et al. Serum metalloproteinases MMP-2, MMP-9 and metalloproteinase tissue inhibitors TIMP-1 and TIMP-2 in patients on hemodialysis. Int Urol Nephrol 2011;43:491-8.
[13] Chou FP, Chu SC, Cheng MC, et al. Effect of hemodialysis on the plasma level of type IV collagenases and their inhibitors. Clin Biochem 2002;35:383-8.
[14] Pawlak K, Pawlak D, Mysliwiec M. Circulating beta-chemokines and matrix metalloproteinase-9/tissue inhibitor of metalloproteinase-1 system in hemodialyzed patients—role of oxidative stress. Cytokine 2005;31:18-24.
[15] Friese RS, Rao F, Khandrika S, et al. Matrix metalloproteinases: discrete elevations in essential hypertension and hypertensive end-stage renal disease. Clin Exp Hypertens 2009;31:521-33.
[16] Polañska B, Makulska I, Augustyniak D, Niemczuk M, Zwolińska D, Jankowski A. Serum levels of MMP-9 in children and young adults with chronic kidney disease treated conservatively and undergoing hemodialysis. Cent Eur J Immunol 2007;32:66-71.
[17] Musial K, Zwolinska D. Matrix metalloproteinases and soluble Fas/FasL system as novel regulators of apoptosis in children and young adults on chronic dialysis. Apoptosis 2011;16:653-9.
[18] Zhang B, Ye S, Herrmann SM, et al. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. Circulation 1999;99:1788-94.
[19] Metzger IF, Luizon MR, Lacchini R, Tanus-Santos JE. Genetic variants in matrix metalloproteinase-9 gene modify metalloproteinase-9 levels in black subjects. DNA Cell Biol 2012;31:504-10.
[20] Lacchini R, Jacob-Ferreira AL, Luizon MR, et al. Matrix metalloproteinase 9 gene haplotypes affect left ventricular hypertrophy in hypertensive patients. Clin Chim Acta 2010;411:1940-4.
[21] Medley TL, Cole TJ, Dart AM, Gatzka CD, Kingwell BA. Matrix metalloproteinase-9 genotype influences large artery stiffness through effects on aortic gene and protein expression. Arterioscler Thromb Vasc Biol 2004;24:1479-84.
[22] Blankenberg S, Rupprecht HJ, Poirier O, et al. Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease. Circulation 2003;107:1579-85.
[23] Palei AC, Sandrim VC, Amaral LM, et al. Matrix metalloproteinase-9 polymorphisms affect plasma MMP-9 levels and antihypertensive therapy responsiveness in hypertensive disorders of pregnancy. Pharmacogenomics J in press, http://dx.doi.org/10.1038/tpj.2011.31.
[24] Belo VA, Souza-Costa DC, Luizon MR, et al. Matrix metalloproteinase-9 genetic variations affect MMP-9 levels in obese children. Int J Obes (Lond) 2012;36:69-75.
[25] Marson BP, Lacchini R, Belo V, et al. Matrix metalloproteinase (MMP)-2 genetic variants modify the circulating MMP-2 levels in end-stage kidney disease. Am J Nephrol 2012;35:209-15.
[26] Hirakawa S, Lange EM, Colicigno CJ, Freedman BI, Rich SS, Bowden DW. Evaluation of genetic variation and association in the matrix metalloproteinase 9 (MMP9) gene in ESRD patients. Am J Kidney Dis 2003;42:133-42.
[27] Demacq C, de Souza AP, Machado AA, Gerlach RF, Tanus-Santos JE. Genetic polymorphism of matrix metalloproteinase (MMP)-9 does not affect plasma MMP-9 activity in healthy subjects. Clin Chim Acta 2006;365:183-7.
[28] Demacq C, Vasconcellos VB, Marcaccini AM, Gerlach RF, Silva Jr WA, Tanus-Santos JE. Functional polymorphisms in the promoter of the matrix metalloproteinase-9 (MMP-9) gene are not linked with significant plasma MMP-9 variations in healthy subjects. Clin Chem Lab Med 2008;46:57-63.
[29] Bouvet C, Moreau S, Blanchette J, de Blois D, Moreau P. Sequential activation of matrix metalloproteinase 9 and transforming growth factor beta in arterial elastocalcinosis. Arterioscler Thromb Vasc Biol 2008;28:856-62.

[30] Souza-Tarla CD, Uzuelli JA, Machado AA, Gerlach RF, Tanus-Santos JE. Methodological issues affecting the determination of plasma matrix metalloproteinase (MMP)-2 and MMP-9 activities. Clin Biochem 2005;38:410-4.
[31] Gerlach RF, Uzuelli JA, Souza-Tarla CD, Tanus-Santos JE. Effect of anticoagulants on the determination of plasma matrix metalloproteinase (MMP)-2 and MMP-9 activities. Anal Biochem 2005;344:147-9.
[32] Shimajiri S, Arima N, Tanimoto A, et al. Shortened microsatellite d(CA)21 sequence down-regulates promoter activity of matrix metalloproteinase 9 gene. FEBS Lett 1999;455:70-4.
[33] Zhang B, Henney A, Eriksson P, Hamsten A, Watkins H, Ye S. Genetic variation at the matrix metalloproteinase-9 locus on chromosome 20q12.2–13.1. Hum Genet 1999;105:418-23.
[34] Castro MM, Rizzi E, Ceron CS, et al. Doxycycline ameliorates 2K-1C hypertension-induced vascular dysfunction in rats by attenuating oxidative stress and improving nitric oxide bioavailability. Nitric Oxide 2012;26:162-8.
[35] Castro MM, Tanus-Santos JE, Gerlach RF. Matrix metalloproteinases: targets for doxycycline to prevent the vascular alterations of hypertension. Pharmacol Res 2011;64:567-72.

SUPPLEMENTARY TABLES

Table S1

Etiology and previous cardiovascular (CVD) diagnosis of end stage kidney disease patients included in our study

Etiology	Number of patients (94)
Diabetes Mellitus (n)	28
Essential hypertension (n)	15
Glomerulonephritis (n)	15
Polycystic kidney disease (n)	14
Urologic causes (n)	5
Chronic interstitial nephritis (n)	4
Unknown (n)	13
Previous CVD diagnosis	**Number of patients (38)**
Ischemic heart disease (n)	24
Stroke (n)	13
Peripheral artery disease (n)	13
Other features	
Currently on anti-hypertensive treatment (n)	65
Mean time of chronic HD (months)	58 ± 9
Residual diuresis (n)	42

Table S2

Distribution of MMP-9 genotypes, alleles, and haplotypes frequencies in end stage kidney disease patients.

Genotypes		Alleles		Haplotypes	
$C^{-1562}T$	n (%)	**$C^{-1562}T$**	n (%)		n (%)
CC	76 (80.9)	**C**	169 (89.9)	**H1 (C,H,Q)**	39 (20.7)
CT	17 (18.1)	**T**	19 (10.1)	**H2 (C,H,R)**	40 (21.3)
TT	1 (1)			**H3 (C,L,Q)**	86 (45.7)
CA_{14-24}		**CA_{14-24}**		**H4 (C,L,R)**	4 (2.1)
HH	30 (32)	**H**	98 (52.1)	**H5 (T,H,Q)**	0 (0)
HL	38 (40.4)	**L**	90 (47.9)	**H6 (T,H,R)**	19 (10.1)
LL	26 (27.6)			**H7 (T,L,Q)**	0 (0)
Q279R				**H8 (T,L,R)**	0 (0)
QQ	42 (44.7)	**Q**	125 (66,5)		
QR	41 (43.6)	**R**	63 (33.5)		
RR	11 (11.7)				

DISCUSSÃO

Os artigos apresentados nos Capítulos 2 e 3 foram os primeiros estudos a investigar a importância dos polimorfismos genéticos das MMPs no contexto da hemodiálise. No caso da MMP-2, os marcadores genéticos que envolvem o alelo de análise T do polimorfismo da posição -735 estão associados com maiores níveis basais de MMP-2, porém sem alterar os níveis pós hemodiálise. Por outro lado, polimorfismos e haplótipos da MMP-9, enquanto não modificam os níveis pré hemodiálise, contribuem para as alterações nas concentrações de MMP-9 e TIMP-1 notadas após a sessão.

Todos estes polimorfismos que estudamos têm reconhecida funcionalidade por evidências *in vitro* de efeitos sobre a expressão e/ou atividade da MMP-2 ou da MMP-9. O SNP $C^{-1562}T$ do promotor da MMP-9 resulta na perda de um sítio repressor de anelamento do tipo AP-1, o qual age reduzindo a expressão do gene. Desta forma, o alelo de análise T gera maiores níveis de MMP-9 [28, 66]. O microssatélite -90 $CA_{(14-24)}$ da região promotora tem alelos que se distribuem mais frequentemente ao redor de 14 repetições (tratadas nesta tese como “L”, *low*) e ao redor de 21-24 repetições (tratadas nesta tese como “H”, *high*). Os alelos com maiores repetições (H) tem maior atividade do promotor, provavelmente por afetar o distanciamento entre sítios de ligação de fatores de transcrição [56]. O polimorfismo Q279R ($A^{855}G$) do exon 6 da MMP-9 causa uma substituição do aminoácido Glutamina para Arginina dentro de um domínio tipo fibronectina, assim afetando a atividade da MMP-9 [55]. Este domínio é altamente conservado entre as espécies e é responsável pelo reconhecimento ao colágeno e sua ligação ao sítio catalítico da enzima. Alterações na sua estrutura leva a uma enzima com baixa afinidade pelo colágeno [57]. Desta forma, podemos imaginar que o alelo que gera a

enzima com Glutamina na posição 279 a faz ter alta afinidade pelo colágeno, resultando em atividade plena da enzima associada a maiores níveis circulantes de MMP-9 com potencial implicação na DCV [62]. Dois polimorfismos funcionais na região promotora da MMP-2, $C^{-735}T$ e o $C^{-1306}T$, são associados a alterações funcionais da MMP-2 por abolirem independentemente dois sítios regulatórios tipo SP-1, na região promotora, que agem como *enhancers* de expressão da MMP-2 [58]. Interessantemente, o haplótipo unindo os dois alelos C destes SNPs parece ter efeito sinérgico sobre a expressão do gene [59].

Uma doença crônica requer uma complexa teia de mecanismos para surgir e se estabelecer. Condições facilmente mensuráveis e modificáveis (glicose, pressão arterial, colesterol) se juntam a influências comportamentais (tabagismo, etilismo, sedentarismo) e ambientais (exposição a agentes nocivos) na armação do quadro patológico. Contudo, ainda assim existe um grau elevado de suscetibilidade individual que não pode ser predito pelos fatores acima. Até hoje, este componente é genericamente referido como história familiar. O avanço no entendimento de como pequenas variações genéticas colaboram para o processo de doença vem aperfeiçoando este conceito [55]. Desta forma, polimorfismos genéticos influenciando a expressão/atividade das MMPs podem contribuir para o risco genético global que ajuda a determinar o fenótipo da doença. Apesar de os mecanismos moleculares exatos serem incertos, é provável que o efeito destes polimorfismos causando propensão para eventos CV ocorra de várias formas. Por exemplo, é plausível que uma maior expressão de MMP-9 associada ao alelo -1562T amplie a migração das células musculares lisas na formação das

placas ateroscleróticas. Estes indivíduos também podem estar predispostos a uma maior instabilidade da placa aterosclerótica devido a degradação da matriz e terem uma maior predisposição para o desenvolvimento de complicações clínicas como resultado da reorganização fibrótica em trombos murais.

Nós mostramos que variantes genéticas das MMPs modificam o potencial proteolítico do plasma ao alterar as MMPs sem um aumento proporcional de seus inibidores. Pacientes em hemodiálise portadores do polimorfismo QQ tem um desbalanceamento entre a MMP-9 com seu inibidor TIMP-1 após a sessão, achado condizente com a idéia que este genótipo está relacionado com maiores níveis de MMP-9. No entanto, alguns resultados foram inesperados. Enquanto o alelo -735T da MMP-2 aumentou os níveis pré hemodiálise de MMP-2 e também da relação MMP-2/TIMP-2, estudos moleculares mostram que este alelo está relacionado a menores taxas de transcrição. O haplótipo CLQ, que também está relacionado com MMP-9 e MMP-9/TIMP-1 aumentadas após a hemodiálise, combina dois alelos cujas evidências moleculares apontam para menores níveis de MMP-9 (o alelo -1562C e o alelo L do microssatélite $CA_{(n)}$). Esta aparente discrepância entre os estudos é provavelmente explicada por diferenças entre condições clínicas e fatores envolvidos, pois achados *in vivo* podem se distinguir significativamente de achados *in vitro*. Por exemplo, o alelo -1306C, relacionado a maiores níveis de MMP-2, está associado com menores massas do ventrículo esquerdo em pacientes hipertensos [67], refletindo a complexa e ainda pouco conhecida regulação das MMPs na IRC. Independentemente destas aparentes inconsistências, estes resultados sugerem que marcadores genéticos podem levar a taxas maiores de complicações cardiovasculares associadas a

atividades aumentadas das MMP-2 e -9 em pacientes em hemodiálise crônica. Cabe ressaltar que, embora não tenhamos encontrado efeito em alguns dos SNPs estudados, ainda assim é possível que eles tenham ação levando a maior formação tecidual de MMP, não sendo refletido através da avaliação dos níveis circulantes, conforme discussão abaixo.

A hemodiálise, apesar de atenuar algumas das anormalidades relacionadas a uremia, introduz outras perturbações no plasma. E qualquer distúrbio na homeostase celular vai levar a mudanças na expressão gênica em uma tentativa de se adaptar frente a novas demandas. O paciente urêmico convive com o *milieu* invariavelmente inflamado, que piora durante a sessão e causa uma série de mudanças moleculares [68]. Genes inflamatórios, como os das MMPs, são particularmente ativados [13]. Isto torna mais perceptível o efeito que os SNPs são capazes de exercer na produção de determinada enzima. Fatores genéticos são presumivelmente mais importantes para a MMP-9 do que para a MMP-2, ao menos nos níveis circulantes após uma sessão de hemodiálise. Como o gene da MMP-9 é altamente controlado por fatores de transcrição relacionados à inflamação como o NF-κβ [27], é plausível que sua ativação ocorra mais do que a MMP-2 durante a sessão.

Nossos resultados mostram uma redução na MMP-2 e na relação MMP-2/TIMP-2 circulante após a hemodiálise, enquanto o TIMP-2 não sofre alteração. Por outro lado, a MMP-9 e o TIMP-1 aumentam após a hemodiálise na mesma população. Diversos estudos clínicos anteriores que avaliaram os níveis circulantes das gelatinases na doença renal terminal apresentam resultados conflitantes, conforme descrito na Tabela 1 do Capítulo 1. Enquanto para a MMP-2 a maioria das séries é concordante com nosso achado, existem

maiores discordâncias quanto a MMP-9. A inconsistência de tais achados pode se dever a diversos fatores de confusão, como diferenças nos cuidados pré-analíticos durante o processo de coleta [69-71]. Por exemplo, a utilização de amostras de soro ao invés de plasma causa a liberação artificial de MMP-9 leucocitária levando a alterações nos valores [71, 72]. Na mesma linha, os esquemas de anticoagulação utilizados na sessão de hemodiálise não foram uniformes, o que também pode interferir com os resultados obtidos [71, 73]. Adicionalmente, não é possível realizar a sessão sem a "heparinização a pleno", o que incute um inevitável viés de aferição, ao menos na coleta pós hemodiálise. O tipo de membrana empregada também pode alterar as MMPs pós hemodiálise [50]. Uma ampla gama de fármacos cardiovasculares utilizadas por nossos pacientes modificam os níveis circulantes das proteinases, o que reduz a homogeneidade entre os estudos e pode induzir a comparações equivocadas [74-77]. Naturalmente, não é factível realizar estudos na IRC sem a presença de uma extensa lista de medicações. Diferenças na composição étnica da população estudada [78] e no tamanho amostral também podem também desempenhar um papel relevante nas diferenças observadas. Enquanto nós procuramos atingir uma população próxima a 100 pacientes, o tamanho amostral foi menor em todas as demais séries. Os antecedentes das populações estudadas também podem justificar dissimilaridades, visto que as doenças de base podem ter diferenças na concentração/expressão das MMPs [20, 45, 50]. Nós procuramos representar a distribuição habitual de doenças causais que compõe as clínicas de diálise. Assim, diabetes e hipertensão constituem o grupo majoritário em nossa amostra. Na maioria dos estudos anteriores foram estudadas coortes com

predomínio de glomerulopatias, rins policísticos e nefrites intersticiais. Além disso, a ausência de pacientes com histórico de DCV em algumas séries impede uma comparação mais adequada [46, 47].

A determinação dos níveis circulantes das MMPs pode ser realizada tanto com ELISA como por zimografia de gelatina [27, 79]. Ambas técnicas podem indicar a concentração das MMPs com alta confiabilidade e tem boa correlação, como observado em nosso laboratório. Optamos por realizar a zimografia no caso da MMP-2, um ensaio de atividade que mostra mais que o ELISA, tentando observar se ao final da sessão haveria o surgimento de outras bandas associadas a MMP-2, o que acabou não ocorrendo.

Um ponto decisivo para a nossa idéia de pesquisa é a relevância dos níveis circulantes destas proteases para o contexto da vasculopatia urêmica. A concentração plasmática de MMPs não necessariamente refletem a expressão e atividade das MMPs teciduais e podem não ser relacionados diretamente à degradação da MEC e ao enrijecimento arterial [20]. Não está claro quais células são os maiores contribuidores para as MMPs circulantes [32]. Enquanto células imunológicas são as principais fontes para a MMP-9 [27], existem muitas outras fontes possíveis para as demais MMPs, como observado na Figura 1. Além disto, a liberação de MMPs do interstício para o sangue não necessariamente é proporcional às concentrações internas do tecido.

Um crescente número de estudos tem demonstrado um desbalanceamento na atividade das MMPs tanto em modelos experimentais de vasculopatia urêmica como em humanos [22, 38, 40, 41], e isto tem estimulado a investigação das MMPs como potenciais biomarcadores de severidade da

doença. É possível que a concentração circulante destas enzimas possam ser associadas a complicações cardiovasculares e com prognóstico, sendo assim de utilidade clínica. Por exemplo, níveis elevados de MMP-9 circulantes foram relacionados com severidade de aterosclerose na IRC [43]. Na mesma linha, a MMP-2 plasmática foi relacionada com prevalência de DCV e aterosclerose carotídea na hemodiálise [42] e com maior velocidade de onda de pulso e enrijecimento aórtico na IRC pré-dialítica [18]. Assim, sugerimos que estas MMPs sejam avaliadas como possíveis desfechos substitutos (*surrogate endpoints*) para a DCV na hemodiálise. Não sabemos neste momento se modificações nos níveis circulantes das MMPs através de terapia medicamentosa refletiria em mudanças num desfecho clinicamente significativo.

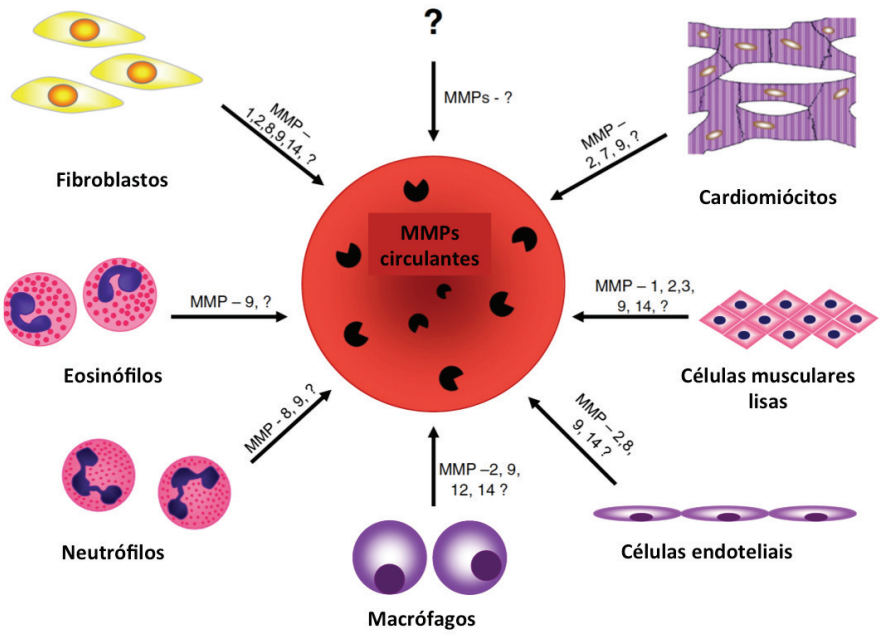

Figura 1. Potenciais fontes circulantes das MMPs encontradas no plasma.

Adaptado de Fontana et al., Clin Chim Acta, 2012.

As alterações do vaso que acometem os pacientes renais crônicos provavelmente são uma reação inicialmente adaptativa frente ao acúmulo de substâncias urêmicas, à hiperfosfatemia e à injúria hipertensiva imposta pela congestão hídrica. Mesmo sob este ponto de vista, a tentativa de reparação se torna um mecanismo mal adaptado que paradoxalmente contribui para o crescimento da disfunção cardíaca e vascular, impondo a busca de inibidores farmacológicos na tentativa de evitar ou postergar a progressão da DCV. De fato, a inibição das MMPs pode ser um horizonte terapêutico e tem sido alvo de intensa pesquisa, como mostrado no Capítulo 1. Nossa pesquisa pode ajudar a identificar subgrupos genéticos de pacientes expostos a maiores níveis de MMPs e com maior potencial benefício do emprego de inibição farmacológica na prevenção de eventos cardiovasculares.

Tomados juntos, esses resultados indicam que polimorfismo $C^{-735}T$ da MMP-2 junto com o polimorfismo Q279R e o haplótipo CLQ da MMP-9 modulam o equilíbrio entre as MMP-2 e -9 e seus inibidores na hemodiálise. Estas variações tem a perspectiva de se tornarem biomarcadores genéticos de severidade da vasculopatia urêmica.

CONCLUSÃO

CONCLUSÃO

Em conclusão, nós identificamos marcadores genéticos que parecem afetar moderadamente, porém de forma significativa, a concentração plasmática das MMPs no contexto da hemodiálise. Nossos achados indicam que a sessão de hemodiálise reduz os níveis circulantes de MMP-2 e a relação MMP-2/TIMP-2 enquanto aumenta os níveis de MMP-9 e TIMP-1. Nossas evidências sugerem que pacientes portadores do alelo T para o polimorfismo $C^{-735}T$ ou o haplótipo C-T tem maiores níveis pré hemodiálise de MMP-2 e da relação MMP-2/TIMP-2. Na mesma linha, pacientes que carregam o polimorfismo QQ do SNP Q279R ou o haplótipo CLQ apresentam maiores aumentos da MMP-9 e da relação MMP-9/TIMP-1 ao final da sessão. Nossos achados indicam um grupo de pacientes em hemodiálise que podem ter pior prognóstico cardiovascular. Enquanto intervenções farmacológicas na via MMPs/TIMPs podem eventualmente beneficiar estes pacientes, estudos clínicos e experimentais são necessários para validar esta sugestão.

REFERÊNCIAS BIBLIOGRAFICAS

REFERÊNCIAS BIBLIOGRAFICAS

1. Moranne, O., et al., *Timing of onset of CKD-related metabolic complications.* Journal of the American Society of Nephrology : JASN, 2009. **20**(1): p. 164-71.
2. Uhlig, K., et al., *KDOQI US commentary on the 2009 KDIGO Clinical Practice Guideline for the Diagnosis, Evaluation, and Treatment of CKD-Mineral and Bone Disorder (CKD-MBD).* American journal of kidney diseases : the official journal of the National Kidney Foundation, 2010. **55**(5): p. 773-99.
3. *KDOQI Clinical Practice Guideline and Clinical Practice Recommendations for anemia in chronic kidney disease: 2007 update of hemoglobin target.* American journal of kidney diseases : the official journal of the National Kidney Foundation, 2007. **50**(3): p. 471-530.
4. Abecassis, M., et al., *Kidney transplantation as primary therapy for end-stage renal disease: a National Kidney Foundation/Kidney Disease Outcomes Quality Initiative (NKF/KDOQITM) conference.* Clinical journal of the American Society of Nephrology : CJASN, 2008. **3**(2): p. 471-80.
5. Gomez, C.G., et al., *Validity of a standard information protocol provided to end-stage renal disease patients and its effect on treatment selection.* Peritoneal dialysis international : journal of the International Society for Peritoneal Dialysis, 1999. **19**(5): p. 471-7.
6. Sesso, R.C., et al., *2010 report of the Brazilian dialysis census.* Jornal brasileiro de nefrologia : órgao oficial da Sociedade Brasileira de Nefrologia, 2011. **33**(4): p. 442-7.
7. Coresh, J., et al., *Prevalence of chronic kidney disease in the United States.* JAMA, 2007. **298**(17): p. 2038-47.
8. Abra, G. and M. Kurella Tamura, *Timing of initiation of dialysis: time for a new direction?* Current opinion in nephrology and hypertension, 2012. **21**(3): p. 329-33.
9. *US Renal Data System: USRDS 2011 Annual Data Report; Atlas of End-Stage Renal Disease in the United States, Bethesda, National Institutes of Health; 2011. Available:* http://www.usrds.org/adr.htm*. Acessado em junho 2012.*
10. Drueke, T.B. and Z.A. Massy, *Atherosclerosis in CKD: differences from the general population.* Nat Rev Nephrol, 2010. **6**(12): p. 723-35.
11. Hage, F.G., et al., *The scope of coronary heart disease in patients with chronic kidney disease.* J Am Coll Cardiol, 2009. **53**(23): p. 2129-40.
12. Shamseddin, M.K. and P.S. Parfrey, *Sudden cardiac death in chronic kidney disease: epidemiology and prevention.* Nat Rev Nephrol, 2011. **7**(3): p. 145-54.
13. Stenvinkel, P., R. Pecoits-Filho, and B. Lindholm, *Coronary artery disease in end-stage renal disease: no longer a simple plumbing problem.* Journal of the American Society of Nephrology : JASN, 2003. **14**(7): p. 1927-39.
14. Pelisek, J., et al., *Carotid plaque composition in chronic kidney disease: a retrospective analysis of patients undergoing carotid endarterectomy.* Eur J Vasc Endovasc Surg, 2010. **39**(1): p. 11-6.
15. Chue, C.D., et al., *Republished paper: Arterial stiffness in chronic kidney disease: causes and consequences.* Postgrad Med J, 2010. **86**(1019): p. 560-6.
16. Shroff, R.C., et al., *Dialysis accelerates medial vascular calcification in part by triggering smooth muscle cell apoptosis.* Circulation, 2008. **118**(17): p. 1748-57.
17. Pai, A.S. and C.M. Giachelli, *Matrix remodeling in vascular calcification associated with chronic kidney disease.* Journal of the American Society of Nephrology : JASN, 2010. **21**(10): p. 1637-40.
18. Smith, E.R., et al., *Elastin degradation is associated with progressive aortic stiffening and all-cause mortality in predialysis chronic kidney disease.* Hypertension, 2012. **59**(5): p. 973-8.

19. Mizobuchi, M., D. Towler, and E. Slatopolsky, *Vascular calcification: the killer of patients with chronic kidney disease.* Journal of the American Society of Nephrology : JASN, 2009. **20**(7): p. 1453-64.
20. Chung, A.W., et al., *Matrix metalloproteinase-2 and -9 exacerbate arterial stiffening and angiogenesis in diabetes and chronic kidney disease.* Cardiovascular research, 2009. **84**(3): p. 494-504.
21. Nakano, T., et al., *Association of kidney function with coronary atherosclerosis and calcification in autopsy samples from Japanese elders: the Hisayama study.* American journal of kidney diseases : the official journal of the National Kidney Foundation, 2010. **55**(1): p. 21-30.
22. Pai, A., et al., *Elastin degradation and vascular smooth muscle cell phenotype change precede cell loss and arterial medial calcification in a uremic mouse model of chronic kidney disease.* The American journal of pathology, 2011. **178**(2): p. 764-73.
23. Page-McCaw, A., A.J. Ewald, and Z. Werb, *Matrix metalloproteinases and the regulation of tissue remodelling.* Nat Rev Mol Cell Biol, 2007. **8**(3): p. 221-33.
24. Murphy, G. and H. Nagase, *Progress in matrix metalloproteinase research.* Molecular aspects of medicine, 2008. **29**(5): p. 290-308.
25. Newby, A.C., *Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability.* Arterioscler Thromb Vasc Biol, 2008. **28**(12): p. 2108-14.
26. Galis, Z.S. and J.J. Khatri, *Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly.* Circulation research, 2002. **90**(3): p. 251-62.
27. Van den Steen, P.E., et al., *Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9).* Crit Rev Biochem Mol Biol, 2002. **37**(6): p. 375-536.
28. Zhang, B., et al., *Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis.* Circulation, 1999. **99**(14): p. 1788-94.
29. Schulz, R., *Intracellular targets of matrix metalloproteinase-2 in cardiac disease: rationale and therapeutic approaches.* Annual review of pharmacology and toxicology, 2007. **47**: p. 211-42.
30. Catania, J.M., G. Chen, and A.R. Parrish, *Role of matrix metalloproteinases in renal pathophysiologies.* Am J Physiol Renal Physiol, 2007. **292**(3): p. F905-11.
31. Aresu, L., et al., *Matrix metalloproteinases and their role in the renal epithelial mesenchymal transition.* Histol Histopathol, 2011. **26**(3): p. 307-13.
32. Fontana, V., et al., *Circulating matrix metalloproteinases and their inhibitors in hypertension.* Clinica chimica acta; international journal of clinical chemistry, 2012.
33. Palei, A.C., et al., *Comparative assessment of matrix metalloproteinase (MMP)-2 and MMP-9, and their inhibitors, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in preeclampsia and gestational hypertension.* Clinical biochemistry, 2008. **41**(10-11): p. 875-80.
34. Ceron, C.S., et al., *Time course involvement of matrix metalloproteinases in the vascular alterations of renovascular hypertension.* Matrix biology : journal of the International Society for Matrix Biology, 2012.
35. Neto-Neves, E.M., et al., *Metalloproteinase inhibition protects against cardiomyocyte injury during experimental acute pulmonary thromboembolism.* Critical care medicine, 2011. **39**(2): p. 349-56.
36. Belo, V.A., et al., *Assessment of matrix metalloproteinase (MMP)-2, MMP-8, MMP-9, and their inhibitors, the tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 in obese children and adolescents.* Clinical biochemistry, 2009. **42**(10-11): p. 984-90.
37. Ebihara, I., et al., *Metalloproteinase-9 mRNA expression in monocytes from patients with chronic renal failure.* American journal of nephrology, 1998. **18**(4): p. 305-10.

38. Chung, A.W., et al., *Upregulation of matrix metalloproteinase-2 in the arterial vasculature contributes to stiffening and vasomotor dysfunction in patients with chronic kidney disease.* Circulation, 2009. **120**(9): p. 792-801.
39. Castro, M.M., J.E. Tanus-Santos, and R.F. Gerlach, *Matrix metalloproteinases: targets for doxycycline to prevent the vascular alterations of hypertension.* Pharmacological research : the official journal of the Italian Pharmacological Society, 2011. **64**(6): p. 567-72.
40. Kumata, C., et al., *Involvement of matrix metalloproteinase-2 in the development of medial layer vascular calcification in uremic rats.* Therapeutic apheresis and dialysis : official peer-reviewed journal of the International Society for Apheresis, the Japanese Society for Apheresis, the Japanese Society for Dialysis Therapy, 2011. **15 Suppl 1**: p. 18-22.
41. Chen, N.X., et al., *Activation of Arterial Matrix Metalloproteinases Leads to Vascular Calcification in Chronic Kidney Disease.* American journal of nephrology, 2011. **34**(3): p. 211-219.
42. Pawlak, K., D. Pawlak, and M. Mysliwiec, *Serum matrix metalloproteinase-2 and increased oxidative stress are associated with carotid atherosclerosis in hemodialyzed patients.* Atherosclerosis, 2007. **190**(1): p. 199-204.
43. Addabbo, F., et al., *Searching for biomarker patterns characterizing carotid atherosclerotic burden in patients with reduced renal function.* Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 2007. **22**(12): p. 3521-6.
44. Pawlak, K., M. Mysliwiec, and D. Pawlak, *Peripheral blood level alterations of MMP-2 and MMP-9 in patients with chronic kidney disease on conservative treatment and on hemodialysis.* Clinical biochemistry, 2011. **44**(10-11): p. 838-43.
45. Friese, R.S., et al., *Matrix metalloproteinases: discrete elevations in essential hypertension and hypertensive end-stage renal disease.* Clinical and experimental hypertension, 2009. **31**(7): p. 521-33.
46. Musial, K. and D. Zwolinska, *Matrix metalloproteinases and soluble Fas/FasL system as novel regulators of apoptosis in children and young adults on chronic dialysis.* Apoptosis : an international journal on programmed cell death, 2011. **16**(7): p. 653-9.
47. Rysz, J., et al., *Serum metalloproteinases MMP-2, MMP-9 and metalloproteinase tissue inhibitors TIMP-1 and TIMP-2 in patients on hemodialysis.* International urology and nephrology, 2011. **43**(2): p. 491-8.
48. Preston, G.A., et al., *Serum matrix metalloproteinases MMP-2 and MMP-3 levels in dialysis patients vary independently of CRP and IL-6 levels.* Nephron, 2002. **92**(4): p. 817-23.
49. Polañska, B., et al., *Serum levels of MMP-9 in children and young adults with chronic kidney disease treated conservatively and undergoing hemodialysis.* Central European Journal of Immunology, 2007. **32**(2): p. 66-71.
50. Chou, F.P., et al., *Effect of hemodialysis on the plasma level of type IV collagenases and their inhibitors.* Clinical biochemistry, 2002. **35**(5): p. 383-8.
51. Sandrim, V.C., et al., *Vascular endothelial growth factor genotypes and haplotypes are associated with pre-eclampsia but not with gestational hypertension.* Molecular human reproduction, 2009. **15**(2): p. 115-20.
52. Rezende, V.B., et al., *Haplotypes of vitamin D receptor modulate the circulating levels of lead in exposed subjects.* Archives of toxicology, 2008. **82**(1): p. 29-36.
53. Palei, A.C., et al., *Association between matrix metalloproteinase (MMP)-2 polymorphisms and MMP-2 levels in hypertensive disorders of pregnancy.* Experimental and molecular pathology, 2012. **92**(2): p. 217-221.

54. Silva, P.S., et al., *Pharmacogenetic implications of the eNOS polymorphisms for cardiovascular action drugs.* Arquivos brasileiros de cardiologia, 2011. **96**(2): p. e27-34.
55. Zhang, B., et al., *Genetic variation at the matrix metalloproteinase-9 locus on chromosome 20q12.2-13.1.* Hum Genet, 1999. **105**(5): p. 418-23.
56. Shimajiri, S., et al., *Shortened microsatellite d(CA)21 sequence down-regulates promoter activity of matrix metalloproteinase 9 gene.* FEBS Lett, 1999. **455**(1-2): p. 70-4.
57. Allan, J.A., et al., *Binding of gelatinases A and B to type-I collagen and other matrix components.* Biochem J, 1995. **309 (Pt 1)**: p. 299-306.
58. Price, S.J., D.R. Greaves, and H. Watkins, *Identification of novel, functional genetic variants in the human matrix metalloproteinase-2 gene: role of Sp1 in allele-specific transcriptional regulation.* The Journal of biological chemistry, 2001. **276**(10): p. 7549-58.
59. Yu, C., et al., *Functional haplotypes in the promoter of matrix metalloproteinase-2 predict risk of the occurrence and metastasis of esophageal cancer.* Cancer research, 2004. **64**(20): p. 7622-8.
60. Mizon-Gerard, F., et al., *Prognostic impact of matrix metalloproteinase gene polymorphisms in patients with heart failure according to the aetiology of left ventricular systolic dysfunction.* Eur Heart J, 2004. **25**(8): p. 688-93.
61. Fiotti, N., et al., *MMP-9 microsatellite polymorphism and susceptibility to carotid arteries atherosclerosis.* Arterioscler Thromb Vasc Biol, 2006. **26**(6): p. 1330-6.
62. Blankenberg, S., et al., *Plasma concentrations and genetic variation of matrix metalloproteinase 9 and prognosis of patients with cardiovascular disease.* Circulation, 2003. **107**(12): p. 1579-85.
63. Manso, H., et al., *Variants of the Matrix Metalloproteinase-2 but not the Matrix Metalloproteinase-9 genes significantly influence functional outcome after stroke.* BMC Med Genet, 2010. **11**: p. 40.
64. Rollin, J., et al., *Influence of MMP-2 and MMP-9 promoter polymorphisms on gene expression and clinical outcome of non-small cell lung cancer.* Lung Cancer, 2007. **56**(2): p. 273-80.
65. Singh, R., et al., *Matrix metalloproteinase (MMP-9 and MMP-2) gene polymorphisms influence allograft survival in renal transplant recipients.* Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 2010. **25**(10): p. 3393-401.
66. Medley, T.L., et al., *Matrix metalloproteinase-9 genotype influences large artery stiffness through effects on aortic gene and protein expression.* Arteriosclerosis, thrombosis, and vascular biology, 2004. **24**(8): p. 1479-84.
67. Lacchini, R., et al., *Common matrix metalloproteinase 2 gene haplotypes may modulate left ventricular remodelling in hypertensive patients.* Journal of human hypertension, 2011.
68. Carrero, J.J. and P. Stenvinkel, *Persistent inflammation as a catalyst for other risk factors in chronic kidney disease: a hypothesis proposal.* Clinical journal of the American Society of Nephrology : CJASN, 2009. **4 Suppl 1**: p. S49-55.
69. Souza-Tarla, C.D., et al., *Methodological issues affecting the determination of plasma matrix metalloproteinase (MMP)-2 and MMP-9 activities.* Clin Biochem, 2005. **38**(5): p. 410-4.
70. Jung, K., R.F. Gerlach, and J.E. Tanus-Santos, *Preanalytical pitfalls of blood sampling to measure true circulating matrix metalloproteinase 9 and tissue inhibitors of matrix metalloproteinases.* Clinica chimica acta; international journal of clinical chemistry, 2006. **373**(1-2): p. 180-1; author reply 182.

71. Gerlach, R.F., et al., *Rapid separation of serum does not avoid artificially higher matrix metalloproteinase (MMP)-9 levels in serum versus plasma.* Clinical biochemistry, 2007. **40**(1-2): p. 119-23.
72. Mannello, F., et al., *Silicate increases the release of MMP-9 forms in peripheral blood: why gelatin zymography differs significantly in citrate plasma and serum obtained with or without clot activators.* Clinical chemistry, 2007. **53**(11): p. 1981-2.
73. Rababah, M., et al., *Anticoagulants affect matrix metalloproteinase 9 levels in blood samples of stroke patients and healthy controls.* Clinical biochemistry, 2012. **45**(6): p. 483-9.
74. Izidoro-Toledo, T.C., et al., *Effects of statins on matrix metalloproteinases and their endogenous inhibitors in human endothelial cells.* Naunyn-Schmiedeberg's archives of pharmacology, 2011. **383**(6): p. 547-54.
75. Marcal, D.M., et al., *Comparative study on antioxidant effects and vascular matrix metalloproteinase-2 downregulation by dihydropyridines in renovascular hypertension.* Naunyn-Schmiedeberg's archives of pharmacology, 2011. **383**(1): p. 35-44.
76. Ceron, C.S., et al., *Spironolactone and hydrochlorothiazide exert antioxidant effects and reduce vascular matrix metalloproteinase-2 activity and expression in a model of renovascular hypertension.* British journal of pharmacology, 2010. **160**(1): p. 77-87.
77. Yiqin, Y., et al., *Aspirin inhibits MMP-2 and MMP-9 expression and activity through PPARalpha/gamma and TIMP-1-mediated mechanisms in cultured mouse celiac macrophages.* Inflammation, 2009. **32**(4): p. 233-41.
78. Lacchini, R., et al., *Interethnic differences in the distribution of matrix metalloproteinases genetic polymorphisms are consistent with interethnic differences in disease prevalence.* DNA and cell biology, 2010. **29**(11): p. 649-55.
79. Kleiner, D.E. and W.G. Stetler-Stevenson, *Quantitative zymography: detection of picogram quantities of gelatinases.* Analytical biochemistry, 1994. **218**(2): p. 325-9.

ANEXOS

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

Ofício 0799/07-CEP

Porto Alegre, 18 de julho de 2007.

Senhor(a) Pesquisador(a):

O Comitê de Ética em Pesquisa da PUCRS apreciou e aprovou seu protocolo de pesquisa registro CEP 07/03787, intitulado: "**Avaliação de marcadores genéticos e bioquímicos de suscetibilidade a insuficiência renal crônica em pacientes em terapia de substituição renal".**

Sua investigação está autorizada a partir da presente data.

Relatórios parciais e final da pesquisa devem ser encaminhados a este CEP.

Atenciosamente,

Prof Dr José Roberto Goldim
COORDENADOR DO CEP-PUCRS

Ilmo(a) Sr(a)
Dr(a) Carlos Eduardo Poli de Figueiredo
N/Universidade

PUCRS
Campus Central
Av. Ipiranga, 6690 - 3ºandar - CEP: 90610-000
Sala 314 - Fone Fax: (51) 3320-3345
E-mail: cep@pucrs.br
www.pucrs.br/prppg/cep

Observação: O projeto “Influência de polimorfismos genéticos sobre os níveis circulantes das metaloproteinases de matriz extracelular 2 e 9 durante hemodiálise” é um desdobramento do projeto “Avaliação de marcadores genéticos e bioquímicos de suscetibilidade a insuficiência renal crônica em pacientes em terapia de substituição renal” realizado em colaboração com o professor Carlos Eduardo Poli de Figueiredo do Serviço de Nefrologia do Hospital São Lucas da PUCRS.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

LINHA DE PESQUISA EM NEFROLOGIA:

Avaliação de Marcadores Genéticos e Bioquímicos de Suscetibilidade a

Insuficiência Renal Crônica em Pacientes em Terapia de Substituição Renal

Pesquisadores Responsáveis: Carlos Eduardo Poli de Figueiredo, Bartira Ercila Pinheiro da Costa, Bernardo Pavinato Marson, José Eduardo Tanus dos Santos.

Entrevistador da Equipe de Pesquisa**:**

_____

Nome do (a) paciente:

_____

SOBRE A PESQUISA: A presente pesquisa avalia aspectos da insuficiência renal crônica, como a pressão alta, na busca do aumento do conhecimento, alívio do sofrimento e melhora da saúde dos pacientes.

Nos estudos serão avaliados diversos aspectos que podem influenciar na doença, tais como: marcadores presentes no sangue; função dos vasos sangüíneos; função das células; função de órgãos, como os rins; e fatores genéticos que possam estar relacionados à suscetibilidade a essa doença.

A idéia é estudar fatores que possam ser importantes para a ocorrência da doença renal crônica. Estes testes poderão ajudar a diagnosticar as pessoas em risco ou com esta condição, ou eventualmente auxiliar na formulação de novos tratamentos.

O QUE SERÁ FEITO: Você será convidado(a) para uma entrevista com um dos membros da equipe de pesquisa. O pesquisador lhe dirá de que se trata a linha de pesquisa e o estudo que está sendo oferecido. Então será perguntado se deseja participar da pesquisa.

Caso concorde, após assinar este Termo de Consentimento Livre e Esclarecido, serão perguntados dados de sua história médica, coletado um volume de sangue venoso antes da sessão de hemodiálise ou da troca da bolsa de CAPD, além das coletas dos exames de rotina. Se você for indivíduo do grupo controle a coleta de sangue será através de punção de veia periférica. Em alguns estudos, serão avaliados a presença de marcadores genéticos. Os genes a serem estudados são extraídos do sangue, tentando identificar especificamente os possíveis causadores desta doença. Estas avaliações não interferirão nas suas avaliações e cuidados rotineiros.

O material biológico da pesquisa será coletado e congelado até a análise pelos colaboradores do Laboratório de Nefrologia da PUCRS. Os resultados serão publicados em revistas de circulação no meio médico e em congressos.

Para que os estudos possam ser realizados, é necessário que você faça a opção autorizando ou não a coleta dos diferentes materiais ou realização dos exames:
Sangue: _____AUTORIZO (Favor escrever SIM ou NÃO).
Análise genética: _____AUTORIZO (Favor escrever SIM ou NÃO).

*OBS.: Nem todos os testes acima serão necessariamente realizados.

CONFIDENCIALIDADE: Os registros serão mantidos em segredo.

MATERIAL EM ESTUDO E ARMAZENAMENTO: O material poderá ser utilizado apenas para esta pesquisa, ou também ser armazenado para emprego em futuros estudos. É necessário que você faça a opção autorizando ou não o armazenamento para emprego futuro: _____ AUTORIZO (Favor escrever SIM ou NÃO).

Se houver possibilidade de fazermos novas análises com o material coletado, será novamente solicitada a aprovação das Comissões de Ética em Pesquisa para realizar a avaliação adicional. Os estudos são desenvolvidos de forma anônima. Os resultados da pesquisa estarão disponíveis a você em qualquer momento por qualquer motivo. Questionamos se você gostaria de ser comunicado(a) sobre o resultado do estudo. É necessário que você faça a opção escrevendo SIM ou NÃO: _____ QUERO SABER DO RESULTADO DA PESQUISA.

RISCOS E BENEFÍCIOS: Os riscos ou desconfortos dessa pesquisa são considerados mínimos. Este estudo não lhe trará nenhum tipo de discriminação individual ou coletiva. A presente pesquisa se propõe a colaborar com o conhecimento sobre a insuficiência renal crônica e suas doenças relacionadas e a pressão arterial, não trazendo benefícios diretos para os(as) pacientes participantes.

LIBERDADE: A sua participação na pesquisa é totalmente voluntária e você pode desistir a qualquer momento, sem prejuízo do tratamento e sem a necessidade de explicar o motivo.

Eu, _____fui informado(a) pelo(a) _____ dos objetivos e justificativas dessa pesquisa de forma bem clara e detalhada. Recebi informações sobre cada passo que estarei envolvido(a). Todas as minhas dúvidas foram respondidas com clareza, e sei que poderei solicitar novos esclarecimentos a qualquer momento. Estou ciente que as informações por mim fornecidas serão mantidas em segredo e usadas somente conforme opção acima. Fui informado(a) que se existirem danos a minha saúde, causados diretamente pela pesquisa, terei direito a tratamento médico e indenização, conforme estabelece a lei. Também sei que não terei nenhum custo que seja relacionado à pesquisa.

Caso tiver novas perguntas sobre este trabalho, posso chamar os pesquisadores pelos seguintes telefones (051) 33367700 ou 33203000 - Ramais 3174 ou 2344, para qualquer dúvida como participante deste estudo.

Esta pesquisa tem aprovação do Comitê de Ética em Pesquisa da PUCRS. Sob as condições acima mencionadas, concordo em participar do presente estudo. Declaro que recebi cópia do presente Termo de Consentimento Livre e Esclarecido, aprovando-o e assinando-o após lê-lo com todo o cuidado possível.

Porto Alegre, ____ de _____ de _____.

_____	_____
Paciente ou Responsável	**Investigador**
CI	**CI/CRM**

***EQUIPE PARTICIPANTE: Carlos Eduardo Poli de Figueiredo, Bartira Ercila Pinheiro da Costa, Bernardo Pavinato Marson, José Eduardo Tanus dos Santos, Samantha Dickel, Silvia Mattos.**

Title: Imbalanced Matrix Metalloproteinases in Cardiovascular Complications of End-Stage Kidney Disease: A Potential Pharmacological Target

Author: Bernardo P. Marson,Carlos E. Poli de Figueiredo,Jose E. Tanus-Santos

Publication: Basic & Clinical Pharmacology & Toxicology

Publisher: John Wiley and Sons

Date: Mar 2, 2012

Order Completed

Thank you very much for your order.

This is a License Agreement between Bernardo Marson ("You") and John Wiley and Sons ("John Wiley and Sons"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

Get the printable license.

License Number	2992130071800
License date	Sep 18, 2012
Licensed content publisher	John Wiley and Sons
Licensed content publication	Basic & Clinical Pharmacology & Toxicology
Licensed content title	Imbalanced Matrix Metalloproteinases in Cardiovascular Complications of End-Stage Kidney Disease: A Potential Pharmacological Target
Licensed content author	Bernardo P. Marson,Carlos E. Poli de Figueiredo,Jose E. Tanus-Santos
Licensed content date	Mar 2, 2012
Start page	409
End page	415
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Order reference number	
Total	0.00 USD

Order Completed

Thank you very much for your order.

This is a License Agreement between Bernardo Marson ("You") and Elsevier ("Elsevier"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

Get the printable license.

License Number	2992110933510
License date	Sep 18, 2012
Licensed content publisher	Elsevier
Licensed content publication	Clinica Chimica Acta
Licensed content title	Functional matrix metalloproteinase (MMP)-9 genetic variants modify the effects of hemodialysis on circulating MMP-9 levels
Licensed content author	Bernardo P. Marson,Riccardo Lacchini,Vanessa Belo,Silvia G. Mattos,Bartira P. da Costa,Carlos E. Poli-de-Figueiredo,Jose E. Tanus-Santos
Licensed content date	24 December 2012
Licensed content volume number	414
Number of pages	6
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	Influência de polimorfismos genéticos sobre os níveis circulantes das metaloproteinases de matriz extracelular 2 e 9 durante hemodiálise
Expected completion date	Oct 2012
Estimated size (number of pages)	75
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD / 0.0 GBP
Total	0.00 USD

Assunto:	AW: Permission for my thesis
De:	Rights and Permissions (permission@karger.ch)
Para:	bernardomarson@yahoo.com.br;
Data:	Terça-feira, 18 de Setembro de 2012 9:35

Dear Mr Pavinto Marson,

thank you for your request below.

As to it, permission is granted herewith to reuse your article

Marson, B.P. et al: Am J Nephrol 2012;35:209-215

in your PhD thesis in print as well as to be published and stored on the University's Thesis website and on the University's Library (Universidade Estadual de Campinas – UNICAMP, Campinas, SP, BRAZIL), provided that full credit is given to the original source and that S. Karger AG, Basel is mentioned.

Please note that any further use, edition, translation or distribution either in print or electronically requires written permission again, as this permission is valid for the above mentioned purpose only.

Thank you for your cooperation and understanding.

I hope to have been of service to you.

With my very best wishes to Brazil,

Tatjana

Tatjana Sepin

Rights and Permissions
t +41 61 306 12 88

permission@karger.ch

S. Karger AG, Medical and Scientific Publishers, Allschwilerstrasse 10, 4009 Basel, Switzerland

t +41 61 306 12 88, f +41 61 306 12 34, www.karger.com