ANA CAROLINA TAVEIROS PALEI

"ASSOCIAÇÃO DE POLIMORFISMOS DA METALOPROTEINASE-9 DE MATRIZ EXTRACELULAR COM A SUSCEPTIBILIDADE E RESPOSTA FARMACOLÓGICA DE PACIENTES COM PRÉ-ECLÂMPSIA/HIPERTENSÃO ARTERIAL GESTACIONAL"

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

ORIENTADOR: PROF. DR. JOSÉ EDUARDO TANUS DOS SANTOS

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minhas avós Maria e Linda,
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"A ciência humana de maneira nenhuma nega a existência de Deus.
Quando considero quantas e
quão maravilhosas coisas o homem compreende,
pesquisa e consegue realizar,
então reconheço claramente que
o espírito humano é obra de Deus, e
a mais notável."

(Galileu Galilei)

"Tenho a impressão de ter sido uma criança brincando à beira-mar,
divertindo-me em descobrir uma pedrinha mais lisa ou
uma concha mais bonita que as outras,
enquanto o imenso oceano da verdade
continua misterioso diante de meus olhos."

(Isaac Newton)

"Algo só é impossível até que alguém duvide e
acabe provando o contrário."

(Albert Einstein)

RESUMO

A pré-eclâmpsia é uma síndrome caracterizada por hipertensão associada à proteinúria. As metaloproteinases de matriz extracelular (MMPs) são enzimas zinco-dependentes que degradam vários componentes da matriz extracelular, cuja atividade é modulada pelos inibidores teciduais de metaloproteinases (TIMPs). Uma vez que as MMP-2 e MMP-9 são fundamentais para os processos de formação e remodelamento dos tecidos feto-placentários e participam da regulação do tônus vascular, os níveis dessas enzimas podem estar alterados em desordens hipertensivas da gestação. É possível ainda que polimorfismos localizados no gene da MMP-9 possam influenciar na susceptibilidade a essas doenças. Logo, os objetivos desse trabalho foram: **1)** comparar as concentrações plasmáticas de MMP-2, MMP-9, TIMP-1 e TIMP-2 entre grávidas saudáveis (GS), grávidas com hipertensão arterial gestacional (HAG) e com pré-eclâmpsia (PE); **2)** comparar as frequências genotípicas e haplotípicas dos polimorfismos $C^{-1562}T$ e (CA)n da MMP-9 entre GS, HAG e PE. E também correlacionar as concentrações de MMP-9 aos genótipos e haplótipos da MMP-9; **3)** comparar as frequências genotípicas e haplotípicas desses polimorfismos entre HAG ou PE que respondem ou não à farmacoterapia com anti-hipertensivos. Inicialmente, determinaram-se os níveis de pro-MMP-9 e pro-MMP-2 no plasma, por zimografia, e as concentrações plasmáticas de TIMP-1 e TIMP-2 nos grupos GS, HAG e PE, por ELISA. Nossos resultados revelaram um aumento da atividade líquida de MMP-9 (relação pro-MMP-9/TIMP-1) em HAG, mas em PE, comparados com GS. Em seguida, extraiu-se o DNA das voluntárias GS, HAG e PE, e determinaram-se as frequências genotípicas dos polimorfismos $C^{-1562}T$ e (CA)n, por PCR seguida de eletroforese, e as frequências haplotípicas, pelo programa PHASE. Observou-se que o genótipo CT para o polimorfismo $C^{-1562}T$ e o haplótipo H4 (T H) estão mais frequentes em HAG, mas não em PE, comparados com GS. Quando avaliamos as concentrações plasmáticas de MMP-9 segundo as distribuições genotípicas e haplotípicas, não se observam diferenças estatisticamente significantes nos grupos GS e PE, apesar de que o genótipo LH do polimorfismo (CA)n foi associado positivamente com a concentração de MMP-9 em HAG. Por último, determinaram-se as frequências genotípicas e haplotípicas nas pacientes HAG e

PE classificadas conforme a responsividade à metildopa ou à terapia anti-hipertensiva total. Verificamos que os genótipos CT+TT estão mais frequentes nas pacientes HAG que não respondem aos anti-hipertensivos, comparadas com as HAG responsivas, em ambas as abordagens. O haplótipo H2 (C H) está mais frequente nas HAG responsivas à terapia total, enquanto o haplótipo H4 nas HAG não-responsivas à terapia total. Além disso, verificou-se que haplótipo H2 está mais frequente nas pacientes PE que não respondem aos anti-hipertensivos em ambas as abordagens, comparadas com as PE responsivas. Portanto, nossos resultados sugerem que a MMP-9 apresenta um papel relevante na fisiopatologia da HAG e que o polimorfismo $C^{-1562}T$ e o haplótipo H4 estão associados com a susceptibilidade e com a não-responsividade à terapia anti-hipertensiva dessa doença. E ainda que o haplótipo H2 está associado com a não-responsividade aos anti-hipertensivos em PE.

Palavras-chave: Metaloproteinases da matriz (MMPs), pré-eclâmpsia, hipertensão na gravidez, polimorfismo, farmacogenética.

ABSTRACT

Preeclampsia is a syndrome characterized by hypertension plus proteinuria. Matrix metalloproteinases (MMPs) are zinc-dependent enzymes that break down several extracellular matrix components, whose activity is modulated mainly by tissue inhibitors of metalloproteinases (TIMPs). Since MMP-2 and MMP-9 are essential for the processes of placental and uterine artery remodeling, and they can participate in vascular tone control, levels of these enzymes may be altered in hypertensive disorders of pregnancy. It is also possible that polymorphisms in the MMP-9 gene may influence susceptibility to these diseases. Thus, the objectives of this study were: **1)** to compare plasma MMP-2, MMP-9, TIMP-1 and TIMP-2 concentrations among healthy pregnant (HP), pregnant women with gestational hypertension (GH) and preeclampsia (PE); **2)** to compare the genotype and haplotype frequencies of MMP-9 polymorphisms ($C^{-1562}T$ and (CA)n) among HP, GH and PE. And to correlate the MMP-9 concentrations with MMP-9 genotypes and haplotypes too; **3)** to compare genotype and haplotype frequencies of these polymorphisms between GH or PE who respond or non-respond to pharmacotherapy with antihypertensives. To achieve our first goal, we determined the plasma pro-MMP-9 and pro-MMP-2 levels by zymography, and plasma TIMP-1 and TIMP-2 concentrations by ELISA in GS, HAG and PE. Our results showed a net increase in the MMP-9 activity (ratio pro-MMP-9/TIMP-1) in GH, but not in PE, compared with HP. Moreover, to achieve our second goal, we firstly extracted DNA from HP, GH and PE volunteers, and then we determined the genotype frequencies of $C^{-1562}T$ and (CA)n polymorphisms by PCR followed by electrophoresis, and the haplotype frequencies by the program PHASE. It was observed that CT genotype for the $C^{-1562}T$ polymorphism and H4 haplotype (T H) are more frequent in GH, but not in PE, compared with the HP. When we evaluated plasma MMP-9 concentrations according to genotype and haplotype distributions, no statistically significant differences were observed in HP and GH groups; although the LH genotype for (CA)n polymorphism was associated significantly and positively with GH. To approach our third goal, we determined the genotype and haplotype frequencies in GH and PE patients classified as responsiveness to methyldopa or to global antihypertensive treatment. We found

that CT+TT genotypes are more frequent in GH patients who do not respond to antihypertensives in both approaches, compared with GH who respond. We also found that H2 haplotype (C H) is more common in GH women who respond to global therapy, and that H4 haplotype is more common in GH women who do not respond to global therapy. In addition, it was found he haplotype H2 are more frequent in PE patients who do not respond to antihypertensives in both approaches, compared with PE who respond. Therefore, our results suggest that MMP-9 has a role in the pathophysiology of GH and the $C^{-1562}T$ polymorphism and H4 haplotype are associated with susceptibility and non-responsiveness to antihypertensive treatment of this disease. While the H2 haplotype is associated with non-responsiveness to antihypertensives in PE.

Key-words: Matrix metalloproteinases (MMPs), preeclampsia, hypertension in pregnancy, polymorphism, pharmacogenetic.

%- porcentagem

AT- angiotensina

CGRP- peptídeo relacionado ao gene da calcitonina

DC- débito cardíaco

DCV- doenças cardiovasculares

DNA- ácido desoxirribonucléico

ELISA- *enzyme linked immune sorbent assay*

FeBraSGO- Federação Brasileira das Sociedades de Ginecologia e Obstetrícia

GS- gestantes saudáveis

HA- hipertensão arterial

HAG- hipertensão arterial gestacional

HAP- haplótipo

HELLP- *hemolysis, elevated liver enzymes, low platelets*

iECA- inibidor de enzima conversora de angiotensina

IMC- índice de massa corpórea

MEC- matriz extracelular

mmHg- milímetros de mercúrio

MMP- metaloproteinase de matriz extracelular

MT - MMP- metaloproteinase de matriz extracelular do tipo membrana

PA- pressão arterial

PAD- pressão sanguínea diastólica

PAS- pressão sanguínea sistólica

PCR- reação em cadeia da polimerase

PE- pré-eclâmpsia

RCIU- restrição de crescimento intra-uterino

RPM- ruptura prematura pré-termo de membrana

RVP- resistência vascular periférica

SDS- dodecil sulfato de sódio

SNP- *single nucleotide polymorphism* -polimorfismo de base única

VS- volume sanguíneo

TIMP- inibidor tecidual de metaloproteinase

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1- INTRODUÇÃO GERAL

1.1- Adaptações cardiovasculares na gestação

Grandes adaptações cardiovasculares ocorrem na gravidez para garantir o suprimento de nutrientes e a eliminação de metabólitos do feto. Observa-se redução da resistência vascular periférica (RVP), redução da pressão arterial sistêmica (PA), aumento do débito cardíaco (DC) e expansão do volume sanguíneo (VS). Essas alterações hemodinâmicas iniciam-se logo no 1º trimestre de gestação e atingem um pico no 2º trimestre, mantendo-se constante até o parto [1, 2]. Elas são provocadas por mudanças em mecanismos humorais e neurais, e devido à interação dos tecidos maternos com substâncias vasoativas placentárias, que causam em conjunto vasodilatação e resposta diminuída à vasoconstritores [3, 4].

No entanto, a gravidez de algumas mulheres não é acompanhada pelas alterações que permitem a redução da PA. Observa-se aumento da RVP, diminuição do DC e a expansão do VS é impedida, conduzindo a quadros de hipertensão arterial [5, 6]. Em decorrência do perfil hemodinâmico peculiar, os critérios de diagnóstico de hipertensão arterial na gravidez são específicos e caracterizam-se por valores de pressão arterial sistólica (PAS) ≥140mmHg e pressão arterial diastólica (PAD) ≥90mmHg em duas aferições, com intervalo mínimo de 4h em repouso [7, 8]. A Federação Brasileira das Sociedades de Ginecologia e Obstetrícia (FeBraSGO) [8] classifica a hipertensão na gravidez em:

- **Pré-eclâmpsia (PE):** hipertensão (PA ≥140/90mmHg) acompanhada de proteinúria (excreção de proteína >0,3g em urina de 24h) iniciadas após a 20ª semana gestacional em mulher previamente normotensa;
- **Sindrome HELLP:** (*Hemolysis, Elevated Liver enzymes, Low Platelets*)**:** aparecimento de hemólise, elevação das enzimas hepáticas (aspartato aminotransferase e desidrogenase láctica) e plaquetopenia em paciente PE;
- **Eclâmpsia:** aparecimento de convulsões em paciente PE;
- **Hipertensão crônica:** hipertensão presente antes da gravidez ou diagnosticada pela primeira vez antes da 20ª semana gestacional;

- **Superimposição de pré-eclâmpsia em hipertensão crônica:** aumento agudo na pressão arterial e proteinúria significativa, iniciados após a 20ª semana gestacional em mulher com hipertensão crônica;

- **Hipertensão arterial gestacional (HAG):** hipertensão transitória iniciada após a 20ª semana gestacional, sem proteinúria, e normalizada após o parto.

Dados recentes do Ministério da Saúde apontam as desordens hipertensivas da gestação (PE, eclâmpsia e HAG) como a maior causa de morte materna e perinatal no Brasil, sendo responsável por cerca de 20% das 1.590 mortes maternas que ocorreram no ano de 2007 [9]. Estima-se que a PE afete 3-5% das gestações no mundo, refletindo em aproximadamente 10% da mortalidade e morbidade matero-fetais tanto em países pobres quanto ricos [10]. Existem alguns fatores de risco que aumentam a probabilidade de uma gestante desenvolver PE, como primigravidez, gravidez múltipla, história familiar de PE, hipertensão e diabetes pré-existentes, obesidade, etnia, entre outros [11].

1.2- Fisiopatologia da pré-eclâmpsia

Os trofoblastos são as células precursoras da placenta humana, cujas funções (implantação embrionária, produção de hormônios gestacionais, proteção imunológica fetal, aumento do fluxo sanguíneo vascular materno na placenta, parto, etc.) são críticas para o sucesso da gravidez. Durante o 1º trimestre de uma gravidez normal, os citotrofoblastos invadem o tecido uterino (da decídua até a terça parte do miométrio) e movem-se contra a corrente sanguínea em direção às artérias espiraladas maternas, onde sofrem diferenciação em células com fenótipo endotelial [12, 13]. A migração e a diferenciação dos citotrofoblastos devem-se às alterações nos perfis de expressão de moléculas de superfície celular (citocinas, moléculas de adesão, constituintes da matriz extracelular, integrinas, antígenos de histocompatibilidade) [14].

Nesse processo ocorre remodelando gradual da camada endotelial e destruição do tecido elástico-muscular das artérias e arteríolas, o que confere aos vasos uteroplacentais elevada distensibilidade para acomodar o aumento fluxo sanguíneo durante a gestação [15-17].

Na pré-eclâmpsia, entretanto, parece ocorrer uma diminuição da invasão trofoblástica, levando à modificação incompleta das artérias espiraladas maternas e, consequentemente, à redução da perfusão sanguínea placentária [18]. Inicialmente, a placenta compensa esse quadro isquêmico com hiperplasia dos trofoblastos na região de trocas, mas depois ela sofre hipoperfusão e começa a liberar vasopressores na circulação materna que modificam a função endotelial [19], por alterar o balanço entre substâncias vasodilatadoras (óxido nítrico, prostaciclina) e vasoconstrictoras (endotelina, resposta aumentada à angiotensina II) [15, 16]. A demanda placentária para corrigir o suprimento sanguíneo inadequado ao feto está em desacordo com o fluxo sanguíneo materno necessário para irrigar seus órgãos [20]. A síndrome torna-se sistêmica, então, afetando múltiplos órgãos maternos, e a perda do controle do tônus vascular conduz à hipertensão e aumenta a permeabilidade vascular glomerular, provocando a proteinúria [21, 22].

Portanto, embora os sintomas maternos apareçam tardiamente, a origem da pré-eclâmpsia inicia-se precocemente, a partir de distúrbios na placenta. Contudo, ainda desconhecem-se os motivos que comprometem a invasão trofoblástica, a qual causa hipóxia placentária e posterior disfunção endotelial materna generalizada.

1.3- Função das MMPs na gestação

Um pré-requisito para o sucesso da invasão trofoblástica é a degradação e o remodelamento da matriz extracelular (MEC) uterina. As metaloproteinases da matriz extracelular (MMPs) são uma família de enzimas estruturalmente relacionadas, zinco e cálcio-dependentes, que apresentam

funções essenciais na formação e reestruturação dos tecidos, por degradarem vários componentes da MEC, incluindo o conteúdo da membrana basal, o colágeno intersticial, a fibronectina e proteoglicanas [23, 24]. A degradação da MEC acontece tanto em processos fisiológicos (desenvolvimento embrionário, morfogênese, reprodução, reabsorção tecidual) quanto em processos patológicos (destruição de cartilagem em atrite, ruptura de placa aterosclerótica, reestenose miocárdica, aneurismas, metástase tumoral, entre outros) [25].

As MMPs são secretadas na forma de precursores inativos (zimogênios), cuja latência é mantida através da interação entre o resíduo de cisteína presente no domínio pró-peptídico com o zinco presente no domínio catalítico, bloqueando o acesso do sítio ativo pelo substrato. Posteriormente, elas são ativadas no tecido por clivagem do domínio pró-peptídico, deixando o sítio catalítico livre para interação com o substrato [26, 27]. A remoção do pró-peptídeo pode ocorrer através de degradação por proteases, como MMPs tipo membrana (MT-MMPs) e furina, ou através de ação não proteolítica, como estresse oxidativo e detergentes [27] - este é o fundamento para detecção de atividade enzimática tanto da forma ativa quanto inativa das MMPs na zimografia, que é uma técnica bioquímica eletroforética que usa o dodecil sulfato de sódio (SDS) no gel.

Especificamente, as MMP-2 e MMP-9 (ou gelatinases A e B, respectivamente) degradam principalmente colágenos tipo IV e V e gelatina [23], estando envolvidas na migração e proliferação de células musculares lisas vasculares, pois permitem que essas células rompam a barreira de tecido conjuntivo ao redor [28, 29]. A propriedade das gelatinases em degradar a membrana basal é importante para permitir a invasão trofoblástica que ocorre na gestação. De fato, expressão e atividade de MMP-2 e MMP-9 têm sido mostradas em citotrofoblastos de tecido placentário humano [30, 31]. Além disso, as MMPs podem regular o tônus vascular. A MMP-2, por exemplo, tem a capacidade de clivar a big-endotelina-1, com produção subsequente de endotelina-1 (potente peptídeo vasoconstritor) [32], ou a adrenomedulina (peptídeo

vasodilador), criando substâncias com ações vasodilatadoras e outras com ações vasoconstritoras [33], ou ainda o peptídeo relacionado ao gene da calcitonina (CGRP), abolindo sua ação vasodilatadora [34]. Já a MMP-9 tem a capacidade de aumentar a reatividade miogênica em pequenas artérias renais, após a administração do hormônio ovariano relaxina [35]. A MMP-9 pode ainda clivar receptores relacionados à vasodilatação, como os beta-2 adrenérgicos [36], e até receptores relacionados à angiogênese, como o VEGFR-2 [37].

A regulação da atividade proteolítica dessas enzimas pode ocorrer em vários níveis: **1)** transcrição; **2)** tradução; **3)** secreção dos zimogênios; **4)** ativação dos zimogênios nos tecidos; **5)** interação das MMPs com inibidores teciduais de metaloproteinases (TIMPs) [38, 39]. A atividade simultânea de MMPs e TIMPs determina a dinâmica da degradação da MEC, sendo fundamental haver um equilíbrio entre MMPs e TIMPs para a manutenção da homeostase dos tecidos [38, 40]. As anormalidades fisiopatológicas na MEC que ocorrem durante a hipertensão refletem em concentrações circulantes de MMPs e TIMPs alteradas, como demonstrado por trabalhos que observaram níveis de MMP-9 aumentados [41-45], MMP-2 aumentados [41, 45]/inalterado [42] e TIMP-1 aumentado [41, 44]/ inalterado [42]/diminuído [43] em pacientes com hipertensão. Tais alterações nos níveis de MMPs e TIMPs foram revertidas com o uso de anti-hipertensivos [42-44].

Ademais, o gene que codifica a MMP-9 apresenta polimorfismos genéticos que podem afetar a expressão gênica e atividade dessa enzima [46]. Dois deles, presentes na região promotora, parecem ser funcionalmente importantes: $C^{-1562}T$ e (CA)n. Em relação ao SNP $C^{-1562}T$, estudos *in vitro* mostraram que a substituição de uma citosina por uma timina na posição -1562 do gene resulta em perda da ligação de uma proteína nuclear repressora de transcrição a essa região, induzindo a transcrição gênica e, consequentemente, aumentando a expressão gênica de MMP-9 [47]. Já o microssatélite (CA)n em torno da posição -90 de gene apresenta uma distribuição bi-modal de frequências alélicas, com o primeiro pico no alelo $(CA)_{14}$ e o segundo pico nos alelos $(CA)_{21}$ e

$(CA)_{22}$ [48]. Sugere-se que esse polimorfismo facilite a transição da estrutura do DNA de B para Z, auxiliando a abertura da dupla-fita e a transcrição gênica de MMP-9. Estudos *in vitro* mostraram que 14 repetições CA conduzem à redução da expressão em cerca de 50%, comparado com 21 repetições CA [49, 50]. Ambos os polimorfismos têm sido associado com aumento de susceptibilidade às doenças cardiovasculares [47-49, 51].

Há evidências de que a concentração de MMP-9 esteja diminuída em extrato de placenta de gestantes PE, comparado com extrato de gestantes normotensas [52]. Na literatura, verificam-se alguns estudos que avaliaram os níveis plasmáticos de MMP-2 e MMP-9 em gestantes com doenças hipertensivas gestacionais [52-56], porém não é possível concluir se esses níveis estão inalterados ou aumentados. Verificam-se também dois trabalhos contraditórios que avaliaram o polimorfismo $C^{-1562}T$ da MMP-9 na PE [57, 58]. Portanto, novos estudos são necessários para avaliar se essas gelatinases realmente apresentam efeitos sobre o remodelamento útero-placentário e a disfunção endotelial que caracterizam a pré-eclâmpsia.

1.4- Tratamento anti-hipertensivo da pré-eclâmpsia

Todas as drogas anti-hipertensivas atravessam a placenta e atingem a circulação fetal. Nenhuma das drogas rotineiras mostrou-se teratogênica, apesar de que os inibidores da enzima conversora de angiotensina (iECA) e os bloqueadores do receptor AT1 da angiotensina II terem efeito fetotóxico. O objetivo do tratamento da hipertensão arterial na gravidez é proteger a mulher dos perigos provocados por aumentos na PA, bem como permitir a continuação da gravidez, o crescimento e a maturação fetal [59, 60]. Nesse sentido, as diretrizes do tratamento da hipertensão na gestação incluem recomendações terapêuticas baseadas no diagnóstico específico e no nível de pressão sanguínea alvo. A FeBrasGO [8] considera a metildopa como droga de primeira escolha, devido à ampla experiência e ausência de efeitos fetais. Caso o uso de metildopa não seja

bem tolerado, bloqueadores de canais de cálcio e β-bloqueadores constituem boas opções alternativas ou aditivas.

Acredita-se que o mecanismo de ação pelo qual a metildopa exerce seu efeito anti-hipertensivo seja através da retroalimentação auto-inibitória dos receptores α2-adrenérgicos pré-sinápticos centrais. Essa droga é primeiramente captada pelos neurônios noradrenérgicos e, então, convertida no falso neurotransmissor metilnoradrenalina. A enzima monoaminooxidase não é capaz de desaminar e, consequentemente, degradar esse falso neurotransmissor, de modo que ele se acumula na fenda sináptica, deslocando a noradrenalina dos receptores. A metilnoradrenalina age preferencialmente estimulando os receptores α2-adrenérgicos pré-sinápticos, tendo pouca ação sobre os receptores β1-adrenérgicos. Assim, a metildopa diminui o tônus simpático dos vasos e a resistência vascular periférica, com redução mínima do débito cardíaco [59]. O efeito da metildopa sobre as MMPs e os TIMPs ainda é desconhecido.

2- HIPÓTESE

O propósito do nosso trabalho foi testar a seguinte hipótese: que polimorfismos ($C^{-1562}T$ e (CA)n) no gene da metaloproteinase-9 de matriz extracelular (MMP-9) estão associados com pré-eclâmpsia (PE) e/ou hipertensão arterial gestacional (HAG), e ainda que esses polimorfismos estão associados com a responsividade ao tratamento anti-hipertensivo dessas desordens hipertensivas da gestação.

3- OBJETIVOS

Para testar a hipóteses do trabalho, nós propusemos os seguintes objetivos:

1- Comparar as concentrações plasmáticas de metaloproteinase-2 de matriz extracelular (MMP-2), MMP-9, inibidor tecidual-1 de metaloproteinase (TIMP-1) e TIMP-2 entre grávidas saudáveis (GS), grávidas com hipertensão arterial gestacional (HAG) e com pré-eclâmpsia (PE);

2- Comparar as frequências genotípicas e haplotípicas dos polimorfismos ($C^{-1562}T$ e (CA)n) da MMP-9 entre GS, HAG e PE. E também correlacionar as concentrações de MMP-9 aos genótipos e haplótipos da MMP-9;

3- Comparar as frequências genotípicas e haplotípicas desses polimorfismos entre HAG ou PE que respondem ou não à farmacoterapia com anti-hipertensivos.

4- CAPÍTULOS

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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

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Abstract

Objectives: To compare the circulating levels of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitors of metalloproteinase (TIMP)-1, TIMP-2, and the MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios in preeclampsia and gestational hypertension with those found in normotensive pregnancies.

Design and methods: We studied 83 pregnant women (30 healthy pregnant women with uncomplicated pregnancies, 26 with gestational hypertension, and 27 with preeclampsia) and 30 healthy nonpregnant women in a cross-sectional study. MMP and TIMP concentrations were measured in plasma samples by gelatin zymography and ELISA, respectively.

Results: We found higher plasma pro-MMP-9 levels, and higher pro-MMP-9/TIMP-1 ratios in women with gestational hypertension (95%-CI: 1.031 to 2.357, and 0.012 to 0.031, respectively), but not with preeclampsia, compared with those found in normotensive pregnant women (95%-CI: 0.810 to 1.350, and 0.006 to 0.013, respectively; both $P<0.05$). We found no significant differences in pro-MMP-2 levels ($P>0.05$).

Conclusions: The higher net MMP-9 (but not MMP-2) activity in gestational hypertension compared with normotensive pregnancy suggests that MMP-9 plays a role in the pathophysiology of gestational hypertension. Conversely, the lack of such alterations in preeclampsia is consistent with the notion that different pathophysiological mechanisms are involved in these hypertensive disorders.

Keywords: Gestational hypertension; Metalloproteinases; Preeclampsia; TIMPs

Introduction

Hypertensive disorders are common complications affecting 5% to 10% of pregnancies [1] and a major cause of preterm delivery [2]. While various hypotheses have been explored to explain pregnancy-induced hypertension (PIH; which includes preeclampsia and gestational hypertension) and chronic hypertension, the pathophysiology of these conditions remains to be determined [3]. In this regard, there is growing evidence suggesting that decreased activity of matrix metalloproteinases (MMPs) could result in poor trophoblast invasion of maternal spiral arteries, thus leading to poor fetoplacental unit perfusion and release of placental factors that affect the vascular tone and remodeling [4]. In addition, it has recently been suggested that MMPs (especially MMP-2) play a greater role in mediating vasodilation in preeclamptic pregnancies compared with normotensive pregnancies [5]. Together, these findings are consistent with the notion that MMPs play significant roles in both acute and chronic regulation of the cardiovascular system [6].

Giving support to these previous reports, a few clinical studies described changes in the circulating levels of MMPs (especially MMP-2 and MMP-9) and their endogenous inhibitors (tissue inhibitors of metalloproteinases; TIMPs) in women with PIH [7–10]. This is important because measuring the plasma levels

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of MMPs and TIMPs may help to elucidate important mechanisms possibly involved in the pathogenesis of PIH. Since TIMP-1 and TIMP-2 are major inhibitors of MMP-9 and MMP-2, respectively [11], the assessment of MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios may lead to improved information regarding the net activity of these two MMPs. In this regard, there is only one study showing lower MMP-9/TIMP-1 ratios in the plasma from women with gestational hypertension compared with those found in the plasma of normotensive pregnant women [7]. Unfortunately, MMP-2/TIMP-2 ratios were not examined in this study [7]. Therefore, additional information is necessary to evaluate the possible contribution of MMP-2 and MMP-9 to gestational hypertension. With respect to preeclampsia, only two studies showed similar circulating MMP-9 and TIMP-1 concentrations in preeclamptic and in normotensive pregnant women at 37–38 week gestation, although MMP-9/TIMP-1 ratios were not examined in these studies [8,10]. In addition, although two studies by the same group [8,9] showed increased MMP-2 levels in the plasma from preeclamptic women, no previous study has compared the circulating levels of TIMP-2 levels or MMP-2/TIMP-2 ratios in preeclamptic women with those found in normotensive pregnancies.

In the present study, we hypothesized that altered MMP-2/TIMP-2 and possibly MMP-9/TIMP-1 ratios would be found in PIH (both gestational hypertension and preeclampsia) compared with normotensive pregnancy. Therefore, the aim of our study was to compare the circulating levels of MMP-2, MMP-9, TIMP-1, TIMP-2, and the MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios in preeclampsia and gestational hypertension with those found in normotensive pregnancies. We have also measured these levels in a group of healthy nonpregnant women.

Methods

Subjects

Approval for use of human subjects was obtained from the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil. This is a cross-sectional study where there was one sampling around 32 weeks at the exception of non pregnant normotensive women. All patients were enrolled in the Department of Obstetrics and Gynecology, University Hospital of the Faculty of Medicine of Ribeirao Preto from October/2006 to February/2007. We studied 83 pregnant women (30 healthy pregnant women with uncomplicated pregnancies, 26 with gestational hypertension, and 27 with preeclampsia) and 30 healthy non pregnant women randomly selected from the local population and unrelated to the patients. Hypertensive disorders were defined in accordance with the guidelines of the NHBPEP (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy) [12]. Gestational hypertension was defined as pregnancy-induced hypertension ($\geq$140 mmHg systolic or $\geq$90 mmHg diastolic on 2 or more measurements at least 6 h apart) without significant proteinuria in a woman after 20 weeks of gestation, and returning to normal by 12 weeks post-partum. Preeclampsia was defined as increased blood pressure with significant proteinuria ($\geq$0.3 g/24 h) in a woman after 20 weeks of gestation. No women with pre-existing hypertension, with or without superimposed preeclampsia, were included in the present study. Exclusion criteria included twin or multiple pregnancies or any evidence of previous medical illness.

While the patients were followed as outpatients (at about 32 weeks of gestational age) maternal venous blood samples were collected into standard Vacutainer tubes (Becton-Dickinson, Brazil) containing sodium/potassium EDTA, and antihypertensive treatment with methydopa was begun whenever indicated. The main reason for sampling pregnant women at about 32 weeks was that hypertensive (both preeclamptic and gestational hypertensive) women are at high risk of preterm delivery, and we wanted to examine MMPs/TIMPs in plasma samples from women sampled at comparable gestational ages. The tubes were centrifuged immediately at room temperature and plasma samples were stored for about 3–4 months at −70 °C until used to measure plasma MMP-2, MMP-9, TIMP-1, and TIMP-2 concentrations.

SDS-polyacrilamide gel electrophoresis (PAGE) gelatin zymography of MMP-9 and MMP-2

Gelatin zymography of MMP-9 and MMP-2 from plasma was performed as previously described[13–16]. Briefly, plasma samples were subjected to electrophoresis on 7% SDS-PAGE co-polymerized with gelatin (1%) as the substrate. After electrophoresis was complete, the gel was incubated for 1 h at room temperature in a 2% Triton X-100 solution, and incubated at 37 °C for 16 h in Tris–HCl buffer, pH 7.4, containing 10 mmol/L $CaCl_2$. The gels were stained with 0.05% Coomassie Brilliant Blue G-250, 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. Enzyme activity was assayed by densitometry using a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Kodak, Rochester, NY) [17]. The pro form of MMP-2 and MMP-9 were identified as bands at 72 and 92 kDa, respectively, by the relation of log Mr to the relative mobility of Sigma SDS-PAGE LMW marker proteins. A representative zymogram of plasma samples is shown in Fig. 1.

Enzyme immunoassays of TIMP-1 and TIMP-2

The plasma concentrations of TIMP-1 and TIMP-2 were measured with commercially available enzyme-linked immunosorbent assay kits [18] (Amersham Biosciences UK Limited, UK) according to the manufacturer's instructions.

Statistical analysis

With basis on previous studies [7], we calculated sample size taking into consideration that differences in MMP-9 corresponding to 60% of S.D. would be meaningful. Therefore, for $\alpha<0.05$ and $\beta>0.20$, 20 or more subjects would be required.

Data were reported as the mean±S.D. or range and quartiles. The between group comparisons were assessed by Kruskall–

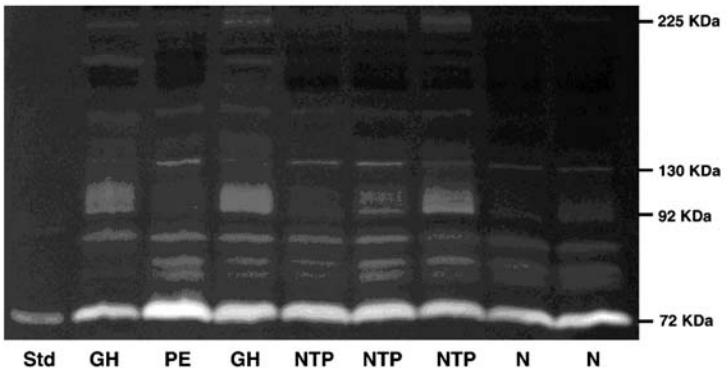

Fig. 1. Representative zymogram of plasma samples. The Marker lane shows the bands corresponding to gelatinases (225 kDa, 130 kDa, 92 kDa, and 72 kDa) from whole blood. Std shows the 72 kDa band (pro-MMP-2) from fetal bovine serum, which was used and a standard to normalize the data from all the gels, thus allowing between gel comparisons. N, NTP, GH, and PE correspond to plasma samples from nonpregnant, normotensive pregnant, gestational hypertensive, and preeclamptic women, respectively.

Wallis test, followed by Dunn's selected pair comparisons (StatView for Windows, Cary, NC, USA). A probability value <0.05 was considered the minimum level of statistical significance.

Results

Table 1 summarizes the clinical and laboratorial characteristics of the 113 subjects enrolled in the present study. There were no statistically significant differences in age, gestational age, heart rate, hemogolobin concentration, hematocrit, creatinine, and %nulliparous between hypertensive groups and the control group (all $P>0.05$). However, women with gestational hypertension had higher BMI than the other groups ($P<0.05$; Table 1). Higher systolic and diastolic blood pressure were found in women with gestational hypertension or with preeclampsia compared with the other groups (both $P<0.05$; Table 1). Lower birth weights and gestational ages at delivery were found in the group of preeclamptic women compared with the other groups ($P<0.05$; Table 1).

Gelatin zymography of plasma samples showed all forms of MMPs usually found in human plasma, including the homodimer of the pro-MMP-9 form (225 kDa), the pro-MMP-9 complexed with neutrophil gelatinase-associated lipocalin (NGAL) form (130 kDa), the pro-MMP-9 form (92 kDa) and the pro-MMP-2 (72 kDa) form (Fig. 1). Gelatinolytic activity was completely inhibited by 5 mM EDTA or 1 mM 1, 10-phenantroline (data not shown), thus confirming that these bands correspond to MMP activity. In addition, we have also found some bands between 92 and 72 kDa which were not inhibited by phenantroline and correspond to non-MMP gelatinases present in human plasma [19].

Interestingly, we found higher plasma levels of pro-MMP-9 in the group of women with gestational hypertension compared with those found in normotensive pregnant women ($P<0.05$; Fig. 2). In addition, women with gestational hypertension had higher pro-MMP-9/TIMP-1 ratios compared with those found in the normotensive pregnant controls ($P<0.05$; Fig. 2). No significant differences were found in pro-MMP-9 levels or in pro-MMP-9/TIMP-1 ratio when preeclamptic women were compared with normotensive pregnant women, although higher TIMP-1 levels were found in preeclamptic compared with normotensive pregnant women ($P<0.05$; Fig. 2).

We found no significant differences in pro-MMP-2 levels in the present study ($P>0.05$; Fig. 3). However, nonpregnant women had lower TIMP-2 levels and higher pro-MMP-2/TIMP-2 ratios than those found in normotensive pregnant controls (both $P<0.05$; Fig. 3).

Discussion

The main findings reported here are that women with gestational hypertension have higher plasma levels of pro-MMP-9

Table 1
Demographic characteristics of study participants

	Nonpregnant	Normotensive pregnant	Gestational hypertension	Preeclampsia
N	30	30	26	27
Age (years)	27.8±3.5	25.7±3.8	28.1±4.0	27.9±4.4
BMI (kg/m^2)	23.1±1.2	23.6±3.6	32.7±4.9*	24.9±4.8
GA (weeks)	–	32.9±3.5	32.2±4.9	32.4±3.6
SBP (mmHg)	113.9±5.7	107.3±6.8	132.6±10.3*	143.9±10.6*
DBP (mmHg)	77.1±4.8	70.0±4.1	82.9±8.4*	91.8±9.5*
HR (beats/min)	78.8±4.7	84.6±5.6	82.8±5.5	79.2±5.7
Hb (g/dL)	13.2±1.5	12.2±1.3	11.7±1.3	12.8±1.2
Hct (%)	38.6±3.1	34.7±3.8	35.0±3.7	37.7±3.2
24-h-Pr (mg/24 h)	ND	ND	127.6±67.9	1063.0±849.5
Creatinine (μmol/L)	61.2±19.5	56.7±15.9	54.1±11.5	57.6±13.3
Nulliparous (%)	35	40	31	41
Birth weight (g)	–	3223±540	3161±450	2200±462*
GAD (weeks)	–	39.3±0.9	38.7±0.7	34.2±1.8*

BMI, body mass index; GA, gestational age; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; Hb, hemoglobin concentration; Hct, hematocrit; GAD, gestational age at delivery; 24-h-Pr, 24-h proteinuria; ND: not determined (however, with negative dipstick test).
Values are the mean±S.D.
* $P<0.05$ *vs.* normotensive pregnant group.

and higher pro-MMP-9/TIMP-1 ratios compared with those found in the normotensive pregnant controls. In addition, although no significant differences were found in pro-MMP-2 levels, we found that normotensive pregnancy is associated with higher circulating TIMP-2 levels compared with those found in non pregnant women.

In the present study, we used gelatin zymography to examine whether PIH affects the circulating levels of MMP-2 and MMP-9 because altered expression or activity of these enzymes has been

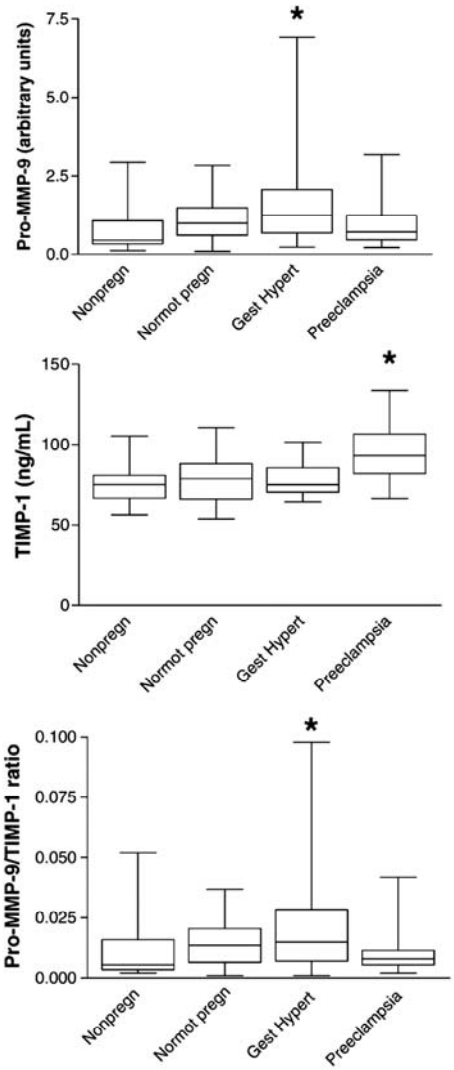

Fig. 2. Plasma Pro-MMP-9 and TIMP-1 concentrations, and Pro-MMP-9/TIMP-1 ratio in nonpregnant ($N=30$), normotensive pregnant ($N=30$), gestational hypertensive ($N=26$), and preeclamptic women ($N=27$). The box and whisker plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values. *$P<0.05$ *vs.* normotensive pregnant, by Kruskall–Wallis test, followed by Dunn's selected pair comparisons.

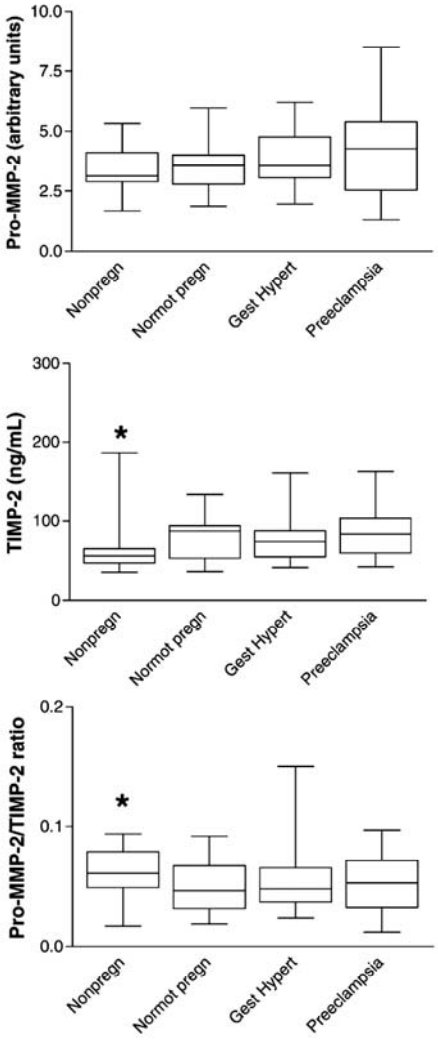

Fig. 3. Plasma Pro-MMP-2 and TIMP-2 concentrations, and Pro-MMP-2/TIMP-2 ratio in nonpregnant ($N=30$), normotensive pregnant ($N=30$), gestational hypertensive ($N=26$), and preeclamptic women ($N=27$). The box and whisker plots show range and quartiles. The boxes extend from the 25th percentile to the 75th percentile, with a line at the median. The whiskers show the highest and the lowest values. *$P<0.05$ *vs.* normotensive pregnant, by Kruskall–Wallis test, followed by Dunn's selected pairs comparisons.

reported to play a role in a variety of pathological conditions including gestational[7–10,20] and cardiovascular diseases including hypertension [17,21,22]. Indeed, the circulating level of MMP-9 has been suggested to be a clinically relevant blood-borne biochemical marker of diagnostic and prognostic value in cardiovascular diseases [21,22]. We have also measured the concentrations of TIMP-1 and TIMP-2 in order to evaluate the MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios, which may lead to improved information regarding the net MMP activities. Curiously, the increased pro-MMP-9 levels and pro-MMP-9/TIMP-1

ratios that we found in gestational hypertension are in contrast with previously reported results of a similar cross-sectional study [7]. Although we have no precise explanation for these conflicting results, differences in the methods (zymography *vs.* ELISA) used to measure the circulating MMP-9 levels may be involved. In addition, the circulating levels of MMP-9 correlated positively with gestational age [10], and it is possible that differences in gestational ages between studies may have affected the results. To our knowledge, there is no other study examining circulating MMPs in gestational hypertension. Importantly, the increased pro-MMP-9 levels and pro-MMP-9/TIMP-1 ratios in gestational hypertension reported here are consistent with previous studies showing higher MMP-9 levels in hypertensive patients compared with normotensive controls [17,23,24]. Further studies on gestational hypertension are needed to confirm these findings.

Consistent with two previous studies [8,10], we found no significant differences in circulating pro-MMP-9 levels when preeclamptic and normotensive pregnant women were compared. Our findings of similar MMP-9/TIMP-1 ratios in these two experimental groups give further support to the suggestion that net MMP-9 activity in plasma of preeclamptic women is similar to that found in normotensive pregnancies [8,10]. Taken together, these findings suggest that MMP-9 is not involved in the pathogenesis of preeclampsia.

The lack of significant differences in pro-MMP-2 levels between normotensive and hypertensive pregnancies (both gestational hypertension and preeclampsia) reported here are in contrast with two small previous studies by the same group [8,9]. Interestingly, these authors showed higher MMP-2 levels in preeclamptic women at 22 and at 36 week gestation, but not at 26 weeks [8], thus suggesting that gestational age has a major effect on MMP-2 levels. We have no obvious explanation for the differences between our results and those previously reported [8,9], it is possible that differences in gestational ages affect the results reported in these studies. However, in addition to the similar pro-MMP-2 concentrations, the comparable TIMP-2 levels and pro-MMP-2/TIMP-2 ratios in normotensive and hypertensive pregnancies reported here suggest that there are no differences in net MMP-2 activity among these groups.

It is well known that essential and chronic hypertensive women may become normotensive in pregnancy up to 32 weeks. However, although this is a cross-sectional study, the pregnant women enrolled in the present study were followed in our outpatient clinic from the beginning of their pregnancies. Therefore, it is not possible that other concurrent hypertensive conditions such as chronic hypertension may have affected the results reported here.

In conclusion, we found evidence indicating higher net MMP-9 (but not MMP-2) activity in gestational hypertension compared with that found in normotensive pregnancy. We also found lack of evidence for altered net MMP-9 or MMP-2 activities in preeclampsia. The lack of such alterations in preeclampsia is consistent with the notion that different pathophysiological mechanisms are involved in these hypertensive disorders. It is possible that higher net MMP-9 activities in gestational hypertensive pregnancies lead to accelerated cleaving of big endothelin-1 [25] to yield higher concentrations of medium endothelin-1, which is a more potent vasoconstrictor than endothelin-1 itself [6,26]. However, this hypothesis remains to be proved.

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Matrix metalloproteinase (MMP)-9 genotypes and haplotypes in preeclampsia and gestational hypertension

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ABSTRACT

Background: Abnormal production of matrix metalloproteinases (MMPs), especially MMP-9, may play a role in hypertensive disorders of pregnancy. These alterations may result from functional genetic polymorphisms in the promoter region of MMP-9 gene, which are known to change MMP-9 expression. We examined whether 2 MMP-9 polymorphisms ($C^{-1562}T$ and (CA)*n*) and haplotypes are associated with preeclampsia and/or gestational hypertension.

Methods: We studied 476 pregnant women: 176 healthy pregnant (HP), 146 pregnant with gestational hypertension (GH), and 154 pregnant with preeclampsia (PE). Genomic DNA was extracted from whole blood and genotypes for $C^{-1562}T$ and (CA)*n* polymorphisms were determined by PCR-RFLP. Haplotype frequencies were inferred using the PHASE ver. 2.1 program.

Results: For the g.−90(CA)13–25 polymorphism, no significant differences were found in genotype and allele distributions when PE or GH groups were compared with HP group. However, the CT genotype and T allele for g.−1562C>T polymorphism were more commonly found in GH subjects compared with the HP group (both $P<0.05$). Conversely, we found no differences in genotypes or allele distributions for the g.−1562C>T polymorphism when the PE and the HP groups were compared. No significant differences were found in overall distributions of haplotype frequencies when the GH or the PE group was compared with the HP group.

Conclusions: The $C^{-1562}T$ polymorphism in MMP-9 gene is associated with gestational hypertension, but not with preeclampsia. These findings may help to explain the higher plasma MMP-9 levels previously reported in GH compared with HP.

1. Introduction

Preeclampsia is an important hypertensive disorder of pregnancy and its pathophysiology remains unclear. Its origin probably lies in the placenta, since preeclampsia occurs only in the presence of placenta and delivery of the placenta remains the only definitive treatment [1]. Placentation is essential for a successful pregnancy [2]. Early in normal pregnancy, the cytotrophoblastic cells of the developing placenta invade the uterine tissue and disrupt the spiral arteries of the decidua and myometrium. However, the cytotrophoblastic invasion in preeclamptic women is impaired and the spiral arteries remain narrow [3]. This leads to hypoperfusion of the placenta and induces the release of vasopressors and other factors into the maternal circulation, thus involving multiple organs and becoming a systemic condition [4].

Matrix metalloproteinases (MMPs) are a family of structurally related, zinc-dependent enzymes that break down several extracellular matrix components [5]. Imbalanced MMP activity has been reported in clinical conditions affecting the cardiovascular system [6,7] including hypertensive disorders of pregnancy [8–11]. Indeed, altered MMP levels in hypertensive disorders of pregnancy may reflect abnormal invasive ability of trophoblastic cells [12,13], and upregulated MMPs may interact with increased oxidative stress and inflammatory mediators to produce endothelial dysfunction seen in preeclampsia [14].

Genetic polymorphisms in the MMP-9 gene affect MMP-9 transcription, and 2 of them are functional: the g.−1562C>T substitution (rs3918242) and the microsatellite g.−90(CA)13–25 (rs3222264) [15,16]. These functional MMP-9 polymorphisms have been associated with disease conditions, including cardiovascular diseases [15,17].

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However, there are 2 inconclusive studies examining whether the g.−1562C>T polymorphism is associated with hypertensive disorders of pregnancy. One of them shows that the T allele is less frequent in preeclampsia compared with healthy pregnancy [18], whereas another study suggests lack of association between this polymorphism and preeclampsia [19]. In the present study, we aimed at expanding these preliminary findings. We studied whether 2 functional polymorphisms (g.−90(CA)13–25 and g.−1562C>T) in the MMP-9 gene, either alone or combined within haplotypes, are associated with preeclampsia or with gestational hypertension.

2. Materials and methods

2.1. Subjects

Approval for use of human subjects was obtained from the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto. All volunteers were consecutively enrolled in the Department of Obstetrics and Gynecology, University Hospital of the Faculty of Medicine of Ribeirao Preto. We studied 476 pregnant women (176 healthy women with uncomplicated pregnancies, 146 women with gestational hypertension, and 154 women with preeclampsia). Hypertensive disorders were defined in accordance with the guidelines of the NHBPEP (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy) [20]. Gestational hypertension was defined as pregnancy-induced hypertension (≥140 mmHg systolic or ≥90 mmHg diastolic on ≥2 measurements at least 6 h apart) in a woman after 20 weeks of gestation, and returning to normal by 12 weeks post-partum. Preeclampsia was defined as increased blood pressure plus significant proteinuria (≥0.5 g/24 h) in a woman after 20 weeks of gestation. No women with pre-existing hypertension, with or without superimposed preeclampsia, were included in the present study.

At the time of clinic attendance, written informed consent was provided and maternal venous blood samples were collected. Genomic DNA was extracted from the cellular component of 1 ml of whole blood by a salting-out method and stored at −20 °C until analyzed.

2.2. Genotyping

Genotypes for the g.−1562C>T polymorphism (rs3918242) were determined by polymerase chain reaction (PCR) amplification using the primers 5′-GCC TGG CAC ATA GTA GGC CC-3′ (sense) and 5′-CTT CCT AGC CAG CCG GCA TC-3′ (antisense) and the conditions as previously described [21]. The amplified products were digested with Sph I restriction enzyme (New England Biolabs, Ipswich, MA) overnight at 37 °C, producing fragments of 247 bp and 188 bp in the case of a polymorphic variant (allele T), or an undigested 435 bp band in the case of a wild type allele (allele C). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized by silver staining.

To determine the genotypes for the g.−90(CA)13–25 polymorphism (rs3222264), a PCR was carried out using the primers 5′-GAC TTG GCA GTG GAG ACT GCG GGC A-3′ (sense) and 5′-GAC CCC ACC CCT CCT TGA CAG GCA A-3′ (antisense) and the conditions as previously described. The amplified products were separated in 7% polyacrylamide–8 M urea gel and visualized by silver staining. Differences in number of bases, from 144 bp (CA 13 repeats) to 168 bp (CA 25 repeats) were determined by comparison with migration of a 10 bp DNA ladder (Invitrogen, Carlsbad, CA) and with some samples from homozygotes that were sequenced. The alleles for the microsatellite g.−90(CA)13–25 polymophism were classified as "low" (L) count when the number of CA repeats was less than 21, and as "high" (H) when the number of CA repeats was ≥21 [22].

2.3. Statistical analysis

Statistical analysis was done using the SPSS 15.0 software (Chicago, IL). The clinical characteristics of women with gestational hypertension or preeclampsia were compared with those of healthy pregnant women by Mann–Whitney *U*-test, chi-square or Fisher exact, as appropriate. The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium, and differences in genotype and allele frequencies among groups were assessed using χ^2-tests or Fisher exact tests. A value of $P<0.05$ was considered statistically significant.

The Bayesian statistical based program PHASE ver. 2.1 was used to estimate the haplotypes frequencies in each group [23,24]. The possible haplotypes including genetic variants for 2 MMP-9 polymorphisms studied (C or T variants for the g.−1562C>T and H or L variants for g.−90(CA)13–25) were: H1 (CH), H2 (CL), H3 (TH) and H4 (TL). Differences in haplotype frequency were further tested using a contingency table. The minimum level of statistical significance was corrected for the number of comparisons made. Therefore, we considered significant a probability value of $P<0.05$/number of haplotypes ($P<0.05/4=0.0125$).

3. Results

Table 1 summarizes the characteristics of the 478 pregnant women enrolled in the present study. Healthy pregnant (HP), gestational hypertensive (GH) and preeclamptic (PE) women were matched by age, ethnicity, smoking, % primigravida, heart rate, fasting glucose, hemoglobin, and hematocrit (Table 1; all $P=$NS). As expected, PE and GH presented higher systolic and diastolic blood pressure compared with HP group (both $P=$NS). It should be noted, however, that most patients were receiving pharmacological therapy (methyldopa in most cases). Higher body mass index (BMI) was found in GH group compared with the other study groups ($P<0.05$). Lower gestational ages at delivery were found in GH and PE groups, and lower newborn weights were found only in PE compared with HP group (all $P<0.05$). Significant proteinuria was found in PE women.

Table 2 shows the results of the MMP-9 single-locus analysis. The frequencies of the MMP-9 genotypes in the control subjects were similar to those reported previously in healthy Brazilians [25]. The distribution of genotypes for the two polymorphisms studied here showed no deviation from Hardy–Weinberg equilibrium (all $P=$NS). For the g.−90(CA)13–25 polymorphism, no significant differences were found in genotype and allele distributions when PE or GH groups were compared with HP group (Table 2; all $P=$NS). However, the genotype and allele frequencies for the g.−1562C>T polymorphism were different in GH subjects as compared with HP subjects. The CT genotype and T allele were more commonly found in GH subjects compared with the HP group (Table 2; both $P<0.05$). Conversely, we found no differences in genotype or allele distributions for the g.−1562C>T polymorphism when the PE and the HP groups were compared (Table 2; both $P=$NS).

We estimated MMP-9 haplotype frequencies including the two polymorphisms for the three study groups (Table 3). No significant differences were found in overall distributions of haplotype frequencies when the GH or the PE group was compared with the HP group (Table 3; all $P=$NS).

4. Discussion

While maternal mortality has decreased around the world, the number of delivery hospitalizations with hypertensive disorders in pregnancy has increased [26]. Although preeclampsia is a transient condition, women who have had preeclampsia or gestational hypertension are at increased risk of hypertension, stroke, and coronary artery disease in their later lives [27,28]. In addition, recent studies have shown

Table 1
Demographic characteristics of study subjects.

Parameters	Healthy pregnant ($n=176$)	Gestational hypertension ($n=146$)	P	Preeclampsia ($n=154$)	P
Age (years)	25.8±5.6	27.1±6.6	NS	27.0±6.9	NS
Ethnicity (% White)	71.3	75.9	NS	68.8	NS
Current smoking (%)	13.2	11.0	NS	7.8	NS
Primigravida (%)	43.8	38.4	NS	42.5	NS
BMI (kg/m^2)	23.9±6.0	30.5±7.2*	0.00	26.5±6.1	NS
SBP (mmHg)	111.7±8.9	134.4±15.3*	0.00	143.8±17.1*	0.00
DPB (mmHg)	72.2±7.0	84.8±10.9*	0.00	89.6±11.1*	0.00
HR (beats/min)	82.1±6.0	81.9±7.4	NS	83.0±7.9	NS
Fasting glucose (mg/dl)	75.2±11.5	78.5±10.4	NS	76.4±12.5	NS
Hb (g/dl)	11.9±1.4	11.8±1.3	NS	12.0±1.6	NS
Hct (%)	35.8±5.0	35.5±3.6	NS	36.4±4.5	NS
Creatinine (μmol/l)	59.7±8.5	56.1±20.1	NS	60.6±15.1	NS
24-h-Pr (mg/24 h)	ND	141.7±79.6		1178.1±1412.5#	0.00
Newborn weight (g)	3342.2±458.4	3222.4±576.1	NS	2558.6±931.2*	0.00
GAD (weeks)	39.9±1.3	38.7±2.1*	0.00	36.0±4.3*	0.00

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; Hb, hemoglobin concentration; Hct, hematocrit; GAD, gestational age at delivery; 24-h-Pr, 24-h proteinuria; ND: not determined (however, with negative dipstick test).
Values are the mean ± S.D.
* $P<0.05$ *vs.* healthy pregnant group.
\# $P<0.05$ *vs.* gestational hypertension group.

Table 2
Genotype and allele frequencies for MMP-9 polymorphisms in healthy pregnant, gestational hypertension and preeclampsia.

Polymorphism	GENOTYPE OR ALLELE	Healthy pregnant ($n=176$)	Gestational hypertension ($n=146$)	OR (95% CI)	P	Preeclampsia ($n=154$)	OR (95% CI)	P
MMP-9	CC	143 (81%)	100 (68%)	1.0 (reference)		118 (77%)	1.0 (reference)	
$C^{-1562}T$	CT	31 (18%)	43 (29%)*	1.98 (1.17–3.36)	0.01	34 (22%)	1.33 (0.77–2.29)	NS
	TT	2 (1%)	3 (2%)	2.15 (0.35–13.08)	NS	2 (1%)	1.21 (0.17–8.74)	NS
				$\chi^2=7.02$	0.03		$\chi^2=1.07$	NS
	C	317 (90%)	243 (83%)			270 (88%)		
	T	35 (10%)	49 (17%)*	1.83 (1.15–2.91)	0.01	38 (12%)	1.28 (0.78–2.08)	NS
MMP-9	HH	67 (38%)	49 (34%)	1.0 (reference)		57 (37%)	1.0 (reference)	
(CA)*n*	LH	78 (44%)	71 (49%)	1.25 (0.76–2.03)	NS	65 (42%)	0.98 (0.60–1.59)	NS
	LL	31 (18%)	26 (18%)	1.15 (0.61–2.17)	NS	32 (21%)	1.21 (0.66–2.22)	NS
				$\chi^2=0.77$	NS		$\chi^2=0.54$	NS
	H	212 (60%)	169 (58%)			179 (58%)		
	L	140 (40%)	123 (42%)	1.10 (0.80–1.51)	NS	129 (42%)	1.09 (0.80–1.49)	NS

OR, odds ratio; CI, confidence interval.
* $P<0.05$ *vs.* healthy pregnant group.

increased incidence of cardiovascular events in the offspring born with maternal preeclampsia [29,30]. Together, these reports justify the search for genetic markers associated with hypertensive disorders of pregnancy, thus allowing early detection of increased susceptibility to disease conditions and increased morbidity and mortality [31–33].

In the present study, we analyzed the genotype and allele frequencies for two MMP-9 functional polymorphisms (g.−90(CA)13–25 and g.−1562C>T) in pregnant women with preeclampsia and, with gestational hypertension. The main finding reported here is that the T allele for g.−1562C>T polymorphism increases the susceptibility to gestational hypertension, but not to preeclampsia. Since this is the first study reporting on the possible association between the g.−1562C>T polymorphism and gestational hypertension, our positive finding requires further studies, especially in different populations. However, the association of the T allele with gestational hypertension is consistent with previous results showing increased MMP-9 levels in women with gestational hypertension [11]. This is because *in vitro* studies have associated the T allele with increased MMP-9 expression, thus possibly leading to increased circulating MMP-9 levels [15], although previous studies showed no effects in healthy subjects [21,25]. Therefore, it is possible that women with gestational hypertension have increased MMP-9 levels because the T allele of the g.−1562C>T polymorphism is more commonly found in women with this hypertensive disorder of pregnancy. However, the lack of significant association that we found between the g.−1562C>T polymorphism and preeclampsia, which confirms

Table 3
Estimated haplotype frequencies in healthy pregnant, gestational hypertension and preeclampsia.

	Haplotype $C^{-1562}T$	(CA)*n*	Healthy pregnant ($n=176$)	Gestational hypertension ($n=146$)	OR (95% CI)	P	Preeclampsia ($n=154$)	OR (95% CI)	P
H1	C	H	89 (50%)	61 (42%)	1.43 (0.92–2.22)	NS	71 (46%)	1.20 (0.78–1.85)	NS
H2	C	L	70 (40%)	61 (41%)	0.92 (0.59–1.44)	NS	64 (42%)	0.93 (0.60–1.44)	NS
H3	T	H	17 (10%)	23 (16%)	0.57 (0.29–1.12)	NS	19 (12%)	0.76 (0.38–1.52)	NS
H4	T	L	0	1 (1%)	0.28 (0.01–6.80)	NS	0 (0%)		
					$\chi^2=4.95$	NS		$\chi^2=0.94$	NS

OR, odds ratio; CI, confidence interval.
P was considered significant when <0.0125 (0.05/4 or 0.05/number of haplotypes).

previous findings reported by Fraser et al. [19], suggests that MMP-9 does not play a major role in preeclampsia. Interestingly, a previous study showed no significant differences in circulating MMP-9 concentrations in preeclamptic women compared with those found in healthy pregnant women [11], and gives further support to the lack of significant association between MMP-9 polymorphisms and preeclampsia [19].

The g.−90(CA)13–25 polymorphism has never been studied in hypertensive disorders of pregnancy before. The lack of significant association between the g.−90(CA)13–25 polymorphism and pre-eclampsia or gestational hypertension suggests that this MMP-9 polymorphism does not have any significant effect on the susceptibility to hypertensive disorders of pregnancy. This suggestion is in line with previous findings showing that this MMP-9 polymorphism does not modify the susceptibility to intrauterine growth retardation [34]. However, additional studies are necessary to confirm the lack of any effects of the g.−90(CA)13–25 polymorphism on the susceptibility to hypertensive disorders of pregnancy. Finally, since the placenta is mostly fetal tissue, it is possible that fetal genotypes affect the susceptibility to hypertensive disorders of pregnancy, and this issue should be addressed in further studies.

We found no significant associations between MMP-9 haplotypes and these conditions. Although haplotype analysis has been valued as a more powerful approach than the analysis of single polymorphisms, it may be less informative when a causal connection between genetic variations and a phenotype is truly driven by a single polymorphism [35]. This may well be the case of the present study. However, larger studies are required to confirm the haplotype findings reported here. While the present study had a limited power (40%) to detect differences between groups, the significant association between the g.−1562C>T polymorphism and gestational hypertension is relevant because it may help to explain the higher plasma MMP-9 levels previously reported in gestational hypertension compared with healthy pregnancies.

In conclusion, we found evidence indicating that g.−1562C>T polymorphism in MMP-9 gene is associated with gestational hypertension, but not with preeclampsia. While the lack of such association with preeclampsia is consistent with the notion that different pathophysiological mechanisms are involved in these hypertensive disorders, these findings require further confirmation.

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Association of Matrix metalloproteinase (MMP)-9 polymorphisms with plasma MMP-9 levels and with reponsiveness to anti-hypertensive therapy in preeclampsia and gestational hypertension

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ABSTRACT

Introduction: The irregular production of matrix metalloproteinases (MMPs), especially MMP-9, may be a manifestation of abnormal placentation and endothelial dysfunction such as in preeclampsia. We analyzed if two polymorphisms ($C^{-1562}T$ and (CA)n) in MMP-9 gene are associated with plasma MMP-9 levels and with responsiveness to antihypertensive therapy in preeclampsia and/or gestational hypertension.

Material and Methods: We studied 214 healthy pregnants (HP), 185 pregnants with gestational hypertension (GH) and 214 pregnants with preeclampsia (PE), who were stratified as responsive or nonresponsive to antihypertensive therapy, according to clinical and laboratorial parameters of therapeutic responsiveness. Genomic DNA was extracted from whole blood and genotyping for $C^{-1562}T$ and (CA)n polymorphisms were done by PCR without or with restriction, respectively. Haplotype frequencies were inferred using the program PHASE version 2.1. Plasma MMP-9 concentrations were measured by ELISA.

Results: Plasma MMP-9 concentrations were similar among genotypes for both polymorphisms and haplotypes in HP and PE groups. In GH patients, the HL genotype was significantly associated with plasma MMP-9 levels. For (CA)n polymorphism, the genotype and allele frequencies were not different between responsive and nonresponsive in GH and PE patients. However, significant differences were found for the $C^{-1562}T$ polymorphism in GH patients (CT+TT genotypes were more frequent in non-responsives than in responsives, $P<0.05$), but not in PE women. In addition, MMP-9 haplotype distributions were different in GH and PE groups (H4 and H2 haplotypes, respectively, were more frequent in non-responsives than in responsives, $P<0.05$).

Conclusion: Our results suggest that (CA)n polymorphism in MMP-9 is linked with significant plasma MMP-9 variations in gestational hypertension. Moreover, the $C^{-1562}T$ polymorphism and MMP-9 haplotypes may be associated with responsiveness to antihypertensive treatment in gestational hypertension and preeclampsia.

Keywords: polymorphism, MMP-9, antihypertensives, pharmacogenetics, preeclampsia, gestational hypertension.

INTRODUCTION

Preeclampsia (hypertension associated with proteinuria) is estimated to affect 3-5% of pregnancies, counting for approximately 10% of maternal-fetal mortality and morbidity worldwide [1]. This syndrome only develops during pregnancy and remits after delivery of the placenta, which suggests that its origin lies in the placenta [2]. The trigger in preeclampsia is postulated to involve reduced placental perfusion that leads to widespread maternal vascular endothelial dysfunction by mechanisms involving the placental release of vasopressors [3] and other factors related to oxidative stress and platelet activation [4] or to inflammation [5] into the maternal circulation.

Matrix metalloproteinases (MMPs) are a family of structurally related, calcium and zinc-dependent enzymes that break down several extracellular matrix components [6]. MMPs play significant roles in several physiological processes such as embryogenesis [7] and angiogenesis [8], but their imbalance expression and activity have been reported in many clinical conditions affecting the cardiovascular system including hypertensive disorders of pregnancy [9-13]. Specifically, MMP-2 and MMP-9 may be involved in placental and uterine artery remodeling [14, 15] and in vascular tone control [16, 17]. Moreover, evidences have also shown that MMPs participate in oxidative stress and inflammatory process, which can contribute for the endothelial dysfunction seen in preeclampsia [18].

MMP-9 activity is regulated at different levels including its transcription, translation, activation of latent forms of MMP, and inhibition by endogenous inhibitors such as TIMPs [19]. In addition, there are several polymorphisms in the MMP-9 gene that can thus affect MMP-9 transcription [20]. Two of them are known

to be functional: the SNP g.-1562C>T and the microsatellite g.-90(CA)13-25. *In vitro* studies showed that the “C” to “T” substitution at -1562 position results in an increase of MMP-9 expression [21]. However, the $(CA)_{14}$ allele in -90 position compared with the $(CA)_{21}$ allele causes approximately a 50% reduction in MMP-9 promoter activity [22, 23]. Both polymorphisms have been associated with preeclampsia and gestational hypertension [24, 25], thus suggesting that genetic variations may predispose to these hypertensive disorders of pregnancy.

Although antihypertensive drugs do not reverse the pathophysiological alterations of preeclampsia, they allow maintenance of pregnancy and increase the gestational age of delivery, thus decreasing adverse maternal and fetal outcomes [26]. In this regard, the guidelines for antihypertensive treatment of pregnant disorders include therapeutic recommendations based on specific diagnosis (mild-to-moderate hypertension, severe hypertension and preeclampsia) and on the targeted blood pressure level [27]. Typically, the therapy includes methyldopa and nifedipine. The ability of various antihypertensive drugs to modify MMP activity may serve as a potential pharmacologic mechanism for possible protective actions beyond arterial pressure lowering. Indeed, some calcium channel blockers can alter plasma MMP-9 levels in hypertensive patients [28-30]. No earlier study has examined whether MMP-9 genotypes or haplotypes could affect the antihypertensive effects of drugs used to treat hypertensive disorders of pregnancy.

Then, the main goals of this study were: **1)** to compare plasma MMP-9 concentrations in the genetic variants for g.-1562C>T and g.-90(CA)13-25 MMP-9 polymorphisms in gestational hypertension and preeclampsia, and **2)** to compare the distribution of the genetic variants for the same polymorphisms in gestational hypertensive and preeclamptic pregnant women who are responsive to antihypertensive therapy, with the distribution of these variants in corresponding pregnant women who are not responsive.

MATERIAL AND METHODS

Subjects

Approval for use of human subjects was obtained from the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto (FMRP). All volunteers were consecutively enrolled in the Department of Obstetrics and Gynecology, University Hospital of the FMRP. We studied 613 pregnants (214 healthy women with uncomplicated pregnancies, 185 women with gestational hypertension and 214 women with preeclampsia). Hypertensive disorders were defined in accordance with the NHBPEP (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy) [27]. Gestational hypertension (GH) was defined as pregnancy-induced hypertension (≥140mmHg systolic or ≥90mmHg diastolic on 2 or more measurements at least 6h apart) in a woman after 20 weeks of gestation, and returning to normal by 12 weeks post-partum. Preeclampsia (PE) was defined as gestational hypertension plus significant proteinuria (≥0.3g/24h). No women with pre-existing hypertension, with or without superimposed preeclampsia, were included in the present study.

At the time of clinic attendance, written informed consent was provided and maternal venous blood samples were collected. Genomic DNA was extracted from the cellular component of 1mL of whole blood by a salting-out method and stored at -20ºC until analyzed. Plasma were obtained from centrifugation of whole blood in EDTA at 2000g for 10min and stored at -70ºC until assayed.

Genotyping

Genotypes for the g.-1562C>T polymorphism (rs3918242) were determined by polymerase chain reaction (PCR) as previously described [21, 31], using the primers 5´-GCC TGG CAC ATA GTA GGC CC-3´ (sense) and 5´-CTT CCT AGC CAG CCG GCA TC-3´ (antisense). The amplified products were digested with *Sphl*restriction enzyme (New England Biolabs, Ipswich/MA/USA)

overnight at 37ºC, producing fragments of 247bp and 188bp in the case of a polymorphic variant (allele T), or an undigested 435bp band in the case of a wild type allele (allele C). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized by silver staining.

To determine the genotypes for the g.-90(CA)13-25 polymorphism (rs3222264), a PCR was carried out as previously described [31, 32], using the primers 5´-GAC TTG GCA GTG GAG ACT GCG GGC A-3´ (sense) e 5´-GAC CCC ACC CCT CCT TGA CAG GCA A-3´ (antisense). The amplified products were separated in 7% polyacrylamide-8M urea gel and visualized by silver staining. Differences in number of bases, from 144bp (CA 13 repeats) to 168bp (CA 25 repeats) were determined by comparison with migration of a 10bp DNA ladder (Invitrogen, Carlsbad/CA/USA) and with some samples from homozygotes that were sequenced. To make easier the interpretation of the bands in the gel, the alleles were classified in accordance with the biallelic distribution of this polymorphism [33]: “low” (L) when the number of CA repeats was less than 21, and “high” (H) when the number of CA repeats was 21 or more.

Antihypertensive treatment and drug response evaluation

The GH and PE patients in this study were monitored closely for signs and symptoms of preeclampsia, with careful fetal surveillance and laboratory tests at least twice weekly. Responsiveness to therapy was based on the evaluation of clinical and laboratory parameters (see below) in response to the administration of antihypertensive drugs. The initial antihypertensive drug of choice was methyldopa (1000-1500mg/day), followed by nifedipine (40-60mg/dia) and/or hydralazine (5-30mg/dia), which were added in case of lack of significant responses to methyldopa. The following clinical laboratory outcomes were considered as reflecting a lack of response to therapy [27]:

1- Clinical symptoms including blurred vision, persistent headache or scotomata, persistent right upper quadrant or epigastric pain;

2- Systolic blood pressure above 140mmHg and diastolic blood pressure above 90mmHg, as assessed by the blood pressure curve;

3- HELLP (hemolysis, elevated liver enzymes and a low platelet count) syndrome; proteinuria >2.0g/24h; creatinine >1.2mg/100mL or blood urea nitrogen >30mg/100mL; AST (aspartate aminotransferase) >40U/L and ALT (alanine aminotransferase) >60U/L;

4- Fetal hypoactivity or nonreactive fetus, as revealed by cardiotocography; intrauterine growth restriction, oligoamnio, abnormal biophysical profile score, doppler velocimetry abnormalities, as evaluated by ultrasound.

We excluded 4 GH and 1 PE patients from analysis because some laboratory tests were missing, making it impossible to classify them with certainty.

Enzyme immunoassays of plasma MMP-9 and TIMP-1

Plasma MMP-9 concentrations were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) methods with commercially available kit (R&D Systems, Minneapolis/MN/USA) according to manufacturer's instructions.

Statistical analysis

Statistical analysis was done using the Stat-View (SAS Institute, Cary/NC/USA). The clinical characteristics of women with GH or PE were compared with those of healthy pregnant women (HP) by Student unpaired *t*-test, Mann-Whitney U-test, or chi-square. The distribution of genotypes for each

polymorphism was assessed for deviation from the Hardy-Weinberg equilibrium, and differences in genotype and allele frequencies among groups were assessed using χ2-tests or Fisher exact tests. A value of $P<0.05$ was considered statistically significant.

The Bayesian statistical based program PHASE 2.1 [34, 35] was used to estimate the haplotypes frequencies in each group. The possible haplotypes including genetic variants for two MMP-9 polymorphisms studied (C or T variants for the g.-1562C>T and L or H variants for g.-90(CA)13-25) were: H1 (CL), H2 (CH), H3 (TL) and H4 (TH). Differences in haplotype frequency were further tested using contingency tables. The minimum level of statistical significance was corrected for the number of comparisons made. Therefore, we considered significant $P <0.05$/number of haplotypes ($P<0.05/3=0.0167$). We rejected the rare haplotype (H3 frequency <0.1%) in analysis.

Linear regression analysis and nonlinear fitting routines were performed using the software Jump 5.0.1a (SAS Institute, Cary/NC/USA) to assess univariate relations between variables. In addition, a bivariate analysis was also performed to assess the potential confounding influence of each covariate on the relation between MMP-9 genotypes/haplotypes and GH/PE. The variables of clinical importance were then included in the multiple linear regression models. Plasma MMP-9 concentrations/responsiveness to methyldopa/responsiveness to global treatment were considered as dependent variable. MMP-9 genotype/haplotypes, age, ethnicity, smoking, body mass index, primiparity, gestational age at sampling and pharmacologic responsiveness were considered as independent variables.

RESULTS

Table 1 summarizes the characteristics of all pregnants enrolled in the study. Healthy pregnant (HP), gestational hypertensive (GH) and preeclamptic (PE) women showed similar ethnicity (% white), % current smoking, heart rate, hemoglobin, hematocrit and creatinine (all $P>0.05$). As expected, PE and GH presented higher systolic (SBP) and diastolic blood pressure (DBP) compared with HP (both $P<0.05$). It should be noted, however, that most patients were receiving antihypertensive therapy. Higher age, body mass index (BMI) and fasting glucose was found in GH and PE patients compared with HP group ($P<0.05$). Lower gestational ages at delivery (GAD) in GH and PE, lower newborn weights in PE and lower % primiparity in GH were found (all $P<0.05$, compared with HP). Significant proteinuria was found in PE. We also found higher plasma MMP-9 concentrations in GH and PE patients compared with HP women ($P<0.05$), despites the difference in the gestation age at sampling (GAS) between PE and HP groups ($P<0.05$).

The results of the MMP-9 single-locus analysis are on table 2. The distribution of genotypes for the two polymorphisms studied showed no deviation from Hardy-Weinberg equilibrium (all $P>0.05$). For g.-90(CA)13-25 polymorphism, no significant differences were found in genotype and allele distributions when PE or GH groups were compared with HP group (all $P>0.05$). However, for g.-1562C>T polymorphism the genotype and allele frequencies in GH were different as compared with HP, being CT genotype and T allele more frequents in GH patients (both $P<0.05$). We did not find any difference in genotype or allele frequency for g.-1562C>T polymorphism between PE and HP groups ($P>0.05$).

The results of the MMP-9 haplotype analysis are on table 3. The distribution of haplotype frequencies in GH was different as compared with HP, being the H4 (TH) more frequent in GH women ($P<0.0167$). However, no significant differences were found in haplotype distribution between PE and HP groups ($P>0.0167$).

To determine the influence of MMP-9 genotypes on plasma MMP-9 levels, we performed a multiple linear regression analysis adjusting for age, ethnicity, current smoking, BMI (<25Kg/m^2), primiparity and GAS (table 4). No significant differences were found in plasma MMP-9 concentration among genotypes for both MMP-9 polymorphisms in HP and PE groups (table 4, all P>0.05). Curiously, the LH genotype for (CA)n polymorphism was significantly and positively associated with plasma MMP-9 levels in GH group (table 4, P<0.05).

To determine the influence of MMP-9 haplotypes on plasma MMP-9 levels, we performed another multiple linear regression analysis adjusting for the same factors cited above (table 5). We did not found significant differences in plasma MMP-9 concentration among haplotypes in HP, GH and PE patients (table 5, all P>0.05). Only GAS was significantly and negatively associated with plasma MMP-9 levels in HP group and ethnicity significantly and was positively associated with plasma MMP-9 levels in PE group (table 5, both P<0.05).

Table 6 summarizes the characteristics of the GH and PE second the reponsiveness to methyldopa. Responsive and non-responsive GH/PE showed similar age, ethnicity, current smoking, fasting glucose and primigravida (all P>0.05). Unsurprisingly, non-responsive GH and HAG presented higher SBP and DBP compared with responsive groups (both P<0.05). Lower BMI was found lower in non-responsive GH and PE compared with responsive patients (P<0.05). Lower GAD, lower newborn weight, and higher proteinuria were found in non-responsive PE women (all P<0.05, compared with responsives). We did not found differences in plasma MMP-9 concentrations when responsive and non-responsive GH or PE patients were compared (P>0.05), despites the difference in the GAS between responsive and non-responsive PE groups (P<0.05).

The Table 7 with the characteristics of the GH and PE according to total therapy reponsiveness shows basically the same pattern that Table 6. The changes are in lower newborn weight in non-response GH compared with responsive (P<0.05), and no significant difference in proteinuria when responsive and non-responsive PE women were compared (P>0.05).

Table 8 and table 9 show the results of the MMP-9 single-locus analysis second responsiveness to methyldopa and global antihypertensive therapy responsiveness, respectively, after adjusting for age, ethnicity, current smoking, BMI (<25 Kg/m^2) and primiparity in a multiple logistic regression. For g.-1562C>T polymorphism, the CT plus TT genotypes were more frequent in non-responsive GH than in responsive GH ($P<0.05$). No significant differences were found in genotype distribution when responsive and non-responsive PE were compared ($P>0.05$). For g.-90(CA)13-25 polymorphism, we did not find differences in genotype frequencies between responsive and non-responsive GH or PE groups ($P>0.05$). Intriguingly, for this polymorphism the genotype distribution in PE were borderline in both approaches, being HH genotype more frequent in non-responsives than in responsives ($P<0.05$).

Table 10 and 11 show the results of the MMP-9 haplotype analysis second responsiveness to methyldopa and global antihypertensive treatment, respectively, after adjusting for age, ethnicity, current smoking, BMI (<25Kg/m^2) and primiparity in a multiple logistic regression. The haplotype distributions in PE were different in both approaches, being H2 (C H) more frequent in non-responsives than in responsives to methyldopa (table 10, $P<0.05$) or to global anti-hypertensive treatment (table 11, $P<0.05$). The haplotype distribution in GH were different only when we considered the responsiveness to global antihypertensive treatment, being H2 (C H) more frequent in responsives than in non-responsives (table 10, $P<0.05$), and H3 (T H) more frequent in non-responsives than in responsives (table 10, $P<0.05$).

DISCUSSION

Despite its position as a leading cause of maternal-fetal mortality and morbidity, there is no effective drug treatment of preeclampsia, and current management have some limitations. Then, our report is relevant for understanding of preeclampsia pathophysiology and for investigation of genetic markers that

could early detect the susceptibility to hypertensive disorders of pregnancy, and predict who would respond or not respond the antihypertensive therapy.

With respect to plasma MMP-9 levels, the data obtained here is compatible with previously results, that showed higher MMP-9 in hypertensive disorders of pregnancy [10, 11]. Although in our earlier report we have only seen significant difference between GH and HP, now we also found higher plasma MMP-9 concentration in PE. However, the present study did not measure the tissue inhibitor of metalloproteinase-1 (TIMP-1, main endogenous inhibitor of MMP-9) to determine the net activity of MMP-9 in PE women [36]. A possible mechanism to contribution of MMP-9 for endothelial dysfunction is that endothelial and smooth muscle cells can released MMP-9 after stimulation by hypertension-related mechanical stress on the vascular wall. As a result, elastin is degraded more than collagen, which may increase fibrosis [37]. In light of these findings, the higher MMP-9 concentration in third trimester of pregnancy in GH and PE is understandable. These results are consistent with the notion that altered plasma MMP-9 levels may reflect a worse prognostic of disease, which could be based, at least in part, on a specific genetic background involving functional MMP-9 polymorphisms.

In regard to MMP-9 polymorphisms, the data related here confirm our prior study in a smaller population [25], i.e., the g.-1562C>T polymorphism is associated only with GH, and the g.-90(CA)13-25 polymorphism is not associated with both diseases. The T allele was more frequent in GH than in HP group, which means that T allele probably increases the susceptibility to gestational hypertension. As *in vitro* study that associated T allele with increased MMP-9 expression [21], the higher T allele frequency evidenced in GH is consistent with the higher circulating MMP-9 levels found in the same patients. Nevertheless, when we evaluate plasma MMP-9 concentration second genotype distributions, our results indicate significant effects only for g.-90(CA)13-25 polymorphism in GH group.

Concerning to MMP-9 haplotypes, our results in a larger population suggest that the H4 (combination of T allele from g.-1562C>T and H allele from g.-90(CA)13-25 polymorphism) increases the susceptibility to gestational hypertension. No significant difference was found in PE patients. When we evaluate plasma MMP-9 concentration second haplotype distribution, our data indicate no significant effects in GH, HP and PE groups. The predominant source of MMP-9 detected in the blood is unknown, but it could be from placental tissue [14, 15] or circulating neutrophils and monocytes as a consequence of a general inflammatory state in hypertensive disorders of pregnancy [18]. A way to examine whether the association between gestational hypertension and elevated plasma MMP-9 level might be causal was to evaluate the impact of functional variations in MMP-9 gene, but we did not observed differences in MMP-9 concentration among MMP-9 genotypes and haplotypes. Since the placenta is mostly fetal tissue, it is possible that fetal genome contributes for development of gestational hypertension, but this is still unclear [38].

In the present study, we investigated for the first time the effects of MMP-9 polymorphisms in responsiveness to methyldopa or to global antihypertensive therapy in hypertensive disorders of pregnancy. Our results suggest that the genotype distribution for g.-90(CA)13-15 polymorphism did not differ between responsive and non-responsive GH and PE groups in both approaches. However, for g.-1562C>T polymorphism, the presence of CT+TT genotypes probably decreases the susceptibility to methyldopa and to global therapy responses in gestational hypertension, but not in preeclampsia. Interestingly, the T allele that was associated with non-response to treatment in GH is the same allele that was associated with increased susceptibility to gestational hypertension. Although no earlier study has examined whether MMP-9 polymorphisms modify the effects of drugs used to treat chronic hypertension, Zhou et al. [39] showed that T allele increases the SBP, DBP and aortic pulse wave velocity.

In addition, we also firstly analyzed the effects of MMP-9 haplotypes in responsiveness to methyldopa or to global antihypertensive therapy. Our results suggest that H2 (combination of C allele from g.-1562C>T and H allele from g.-90(CA)13-25 polymorphism) decreases the susceptibility to methyldopa and to global therapy responses in preeclampsia. However, considering the global therapy responsiveness, the H2 was more frequent in responsive than in non-responsive GH, and the H3 (the same haplotype that increase the susceptibility to GH) decrease the susceptibility to respond. No significant difference was found in distribution of the MMP-9 haplotypes second responsiveness to methyldopa in GH patients.

While there is no evidence that methyldopa produces antihypertensive effects by mechanisms involving reduction of MMP-9 production, it has been shown that many calcium channel blockers can reduce plasma MMP-9 levels in chronic hypertension [28-30]. Therefore, it is possible that the drugs used to treat hypertensive disorders of pregnancy ameliorate clinical symptoms by decreasing circulating MMP-9, thus counteracting the impaired plasma MMP-9 concentration reported here. However, we did not observed significant differences in plasma MMP-9 levels between responsive and non-responsive, even in GH group, this may have been caused by the effect of antihypertensives (the vast majority of patients were under treatment).

It should be made clear that the criteria used in this study to evaluate the lack of response to antihypertensive therapy may have affected our conclusions. Although there is no clear definition about how to precisely assess the severity of these hypertensive disorders of pregnancy, it is possible that responsiveness to therapy is affected by disease severity. Further studies are needed to improve our understanding of these syndromes.

In conclusion, we found evidence indicating that gestational hypertension is associated with MMP-9 polymorphisms, isolated (g.-1562C>T) and combined in haplotypes (H2 and H4), which affects its responsiveness to antihypertensive therapy. The MMP-9 haplotype (H2) can also affect the

responsiveness to antihypertensives in preeclampsia. Moreover, our study shows no effects of MMP-9 polymorphisms or haplotypes on the plasma MMP-9 levels in preeclampsia, but g.-90(CA)13-25 polymorphism may alter MMP-9 concentration in gestational hypertension. Nevertheless, our findings require further confirmation.

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Table 1- Demographic characteristics of study subjects

Parameters	Healthy Pregnant	Gestational Hypertension	P	Preeclampsia	P
	(n = 214)	(n = 185)		(n = 214)	
Age (years)	24.5±0.4	27.0±0.5*	**0.000**	26.±0.5*	**0.003**
Ethnicity (% White)	71.5	73.3	0.704	69.9	0.718
Current Smoking (%)	12.6	11.2	0.689	8.7	0.205
BMI (Kg/m^2)	23.3±0.3	29.5±0.5*	**0.000**	27.2±0.4*	**0.000**
SBP (mmHg)	112.1±0.7	133.4±1.1*	**0.000**	142.3±1.1*	**0.000**
DPB (mmHg)	72.2±0.5	84.2±0.8*	**0.000**	88.9±0.7*	**0.000**
HR (beats/min)	82.3±0.6	82.0±0.5	0.749	82.7±0.5	0.680
Fasting Glucose (mg/dL)	75.1±1.0	79.2±1.1*	**0.005**	79.2±1.8*	**0.047**
Hb (g/dL)	11.9±0.1	11.9±0.1	0.885	12.0±0.1	0.718
Hct (%)	35.7±0.4	35.8±0.3	0.808	36.1±0.3	0.400
Creatinine (□mol/L)	58.9±2.6	55.1±0.8	0.190	62.6±1.4	0.746
24-h-Pr (mg/24h)	ND	134.5±9.3		1333.0±151.0#	**0.000**
Primiparity (%)	50.3	39.9*	**0.042**	44.5	0.243
GAD (weeks)	39.8±0.1	38.8±0.1*	**0.000**	36.0±0.3*	**0.000**
Newborn weight (g)	3316.0±34.6	3202.0±41.7	0.095	2546.0±64.7*	**0.000**
GAS (weeks)	36.8±0.2	36.0±0.4	0.330	34.2±0.4*	**0.000**
MMP-9 (ng/mL)	243.1±13.0	307.1±22.5*	**0.032**	295.6±17.5*	**0.031**

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; Hb, hemoglobin concentration; Hct, hematocrit; GAD, gestational age at delivery; 24-h-Pr, 24-h proteinuria; GAS, gestational age at sampling; MMP-9 (matrix metaloproteinase-9; ND: not determined (however, with negative dipstick test).

Values are the mean ± S.E.M.

* P<0.05 *vs.* healthy pregnant group.

# P<0.05 *vs.* gestational hypertension group.

Table 2- Genotype and allele frequencies for MMP-9 polymorphisms in HP, GH and PE

Poly morphism	Genotype or Allele	HP (n=214)	GH (n=185)	OR (95% CI)	P	PE (n=214)	OR (95% CI)	P
MMP-9	**CC**	176 (82%)	128 (69%)	1.00 (reference)		167 (78%)	1.00 (reference)	
$C^{-1562}T$	**CT**	34 (16%)	54 (29%)	2.18 (1.34-3.55)*	**0.002**	44 (21%)	1.36 (0.83-2.24)	0.259
	TT	4 (2%)	3 (2%)	0.97 (0.21-4.41)	0.970	3 (1%)	0.79 (0.17-3.59)	0.790
				χ^2 = 10.21	**0.006**		χ^2 = 1.66	0.436
	C	*386 (90%)*	*310 (84%)*	*1.00 (reference)*		*378 (88%)*	*1.00 (reference)*	
	T	*42 (10%)*	*60 (16%)*	*1.78 (1.17-2.71)**	***0.008***	*50 (12%)*	*1.22 (0.79-1.88)*	*0.440*
MMP-9	**LL**	39 (18%)	30 (16%)	1.00 (reference)		45 (21%)	1.00 (reference)	
(CA)n	**LH**	95 (45%)	97 (53%)	1.33 (0.76-2.31)	0.329	91 (43%)	0.83 (0.50-1.39)	0.513
	HH	80 (37%)	58 (31%)	0.94 (0.53-1.69)	0.882	78 (36%)	0.85 (0.50-1.44)	0.590
				χ^2 = 2.61	0.271		χ^2 = 0.54	0.763
	L	*162 (40%)*	*157 (42%)*	*1.00 (reference)*		*181 (42%)*	*1.00 (reference)*	
	H	*240 (60%)*	*213 (58%)*	*0.92 (0.69-1.22)*	*0.559*	*247 (58%)*	*0.92 (0.70-1.22)*	*0.573*

MMP-9, matrix metalloproteinase-9; HP, health pregnant; GH, gestational hypertension; PE, preeclampsia; OR, odds ratio; CI, confidence interval.

* P<0.05 *vs.* health pregnant group.

Table 3- Haplotype frequencies for MMP-9 polymorphisms in HP, GH and PE

Haplotype	HP (n=2x214)	GH (n=2x185)	OR (95% CI)	P	PE (n=2x214)	OR (95% CI)	P
H1 (C L)	173 (40%)	155 (42%)	1.06 (0.80-1.41)	0.718	179 (42%)	1.06 (0.81-1.39)	0.728
H2 (C H)	213 (50%)	155 (42%)	0.73 (0.55-0.96)	0.027	199 (47%)	0.88 (0.67-1.15)	0.374
H3 (T L)	0	2 (0%)	5.81 (0.28-121.60)	0.128	2 (0%)	5.02 (0.24-105.00)	0.157
H4 (T H)	42 (10%)	58 (16%)	1.71 (1.12-2.61)*	**0.014**	48 (11%)	1.16 (0.75-1.80)	0.578
			χ^2 = 10.53	**0.015**		χ^2 = 2.98	0.395

MMP-9, matrix metalloproteinase-9; HP, health pregnant; GH, gestational hypertension; PE, preeclampsia; OR, odds ratio; CI, confidence interval.

P were considered significant when < 0.0167 (0.05/3 or 0.05/number of haplotypes not rare).

*P<0.0167 *vs.* health pregnant group.

Table 4- Effects of MMP-9 genotypes on plasma MMP-9 level after adjusting for selected variables in HP, HG and PE patients

Groups	Health Pregnant		Gestational Hypetension		Preeclampsia	
	R^2	RMSE	R^2	RMSE	R^2	RMSE
Model	0.0291	0.3411	0.0916	0.3370	0.0287	0.3634
	B	P	B	P	B	P
Age (years)	0.2171	0.4751	-0.1324	0.6778	0.1066	0.7444
Ethnicity (% White)	0.0275	0.3679	-0.0039	0.9118	0.0437	0.1907
Current Smoking (%)	-0.0279	0.4445	0.0376	0.4021	0.0035	0.9391
BMI (< 25Kg/m^2)	0.0312	0.3002	-0.0270	0.4350	-0.0353	0.2458
Primiparity (%)	-0.0112	0.7165	0.0295	0.3995	0.0274	0.4440
GAS (weeks)	-0.0114	0.4400	-0.0032	0.6650	-0.0004	0.9529
$C^{-1562}T$ Polymorphism	P = 0.6847		P = 0.9249		P = 0.7495	
	B	P	B	P	B	P
CC	0.0148	0.6847	0.0032	0.9249	0.0123	0.7495
CT+TT	-0.0148	0.6847	-0.0032	0.9249	0.0123	0.7495
(CA)n Polymorphism	P=0.9026		**P=0.0096**		P=0.9003	
	B	P	B	P	B	P
LL	-0.0204	0.6712	-0.1012	0.1126	0.0189	0.7094
LH	0.0139	0.7119	0.1351*	**0.0025**	0.0008	0.9833
HH	0.0065	0.8729	-0.0339	0.5056	-0.0197	0.6576

MMP-9, matrix metalloproteinase-9; HP, heath pregnant; GH, gestational hypertension; PE, preeclampsia; BMI, body mass index; GAS, gestational age at sampling;

R^2, proportion of the variation in the response around the mean that can be attributed to terms in the model rather than to random error; RMSE, root mean square error; B, parameter estimates for each term;

* P<0.05 in gestational hypertension group.

Table 5- Effects of MMP-9 haplotypes on plasma MMP-9 level after adjusting for selected variables in HP, HG and PE patients

Groups	Health Pregnant		Gestational Hypertension		Preeclampsia	
Model	R^2	RMSE	R^2	RMSE	R^2	RMSE
	0.0431	0.3338	0.0140	0.3434	0.0308	0.3562
	B	P	B	P	B	P
Age (years)	0.2534	0.2363	-0.1650	0.4755	0.0795	0.7252
Ethnicity (% White)	0.0188	0.3852	0.0069	0.7848	0.0490#	**0.0365**
Current Smoking (%)	-0.0021	0.9404	0.0044	0.8932	-0.0049	0.8795
BMI (< 25Kg/m^2)	0.0340	0.1090	-0.0147	0.5564	-0.0385	0.0701
Primiparity (%)	-0.0129	0.5508	0.0256	0.3211	0.0211	0.3998
GAS (weeks)	-0.0276*	**0.0216**	0.0017	0.7622	-0.0006	0.9019
MMP-9 Haplotypes	P = 0.7798		P = 0.9695		P = 0.8426	
	B	P	B	P	B	P
H1 (C L)	-0.0196	0.4978	0.0066	0.8344	0.0101	0.7532
H2 (C H)	0.0055	0.8471	0.0029	0.9262	-0.0152	0.6245
H4 (T H)	0.0141	0.7292	-0.0095	0.8122	0.0052	0.9080

MMP-9, matrix metalloproteinase-9; HP, heath pregnant; GH, gestational hypertension; PE, preeclampsia; BMI, body mass index; GAS, gestational age at sampling.

R^2, proportion of the variation in the response around the mean that can be attributed to terms in the model rather than to random error; RMSE, root mean square error; B, parameter estimates for each term.

* P<0.05 in health pregnant group and # P<0.05 in preeclampsia group.

Table 6- Demographic characteristics according to methyldopa responsiveness in GH and PE patients

Parameters	GH - Methyldopa Responsiveness			PE - Methyldopa Responsiveness		
	Responsive (n = 125)	Nonresponsive (n = 56)	P[a]	Responsive (n = 61)	Nonresponsive (n = 152)	P[b]
Age (years)	26.8±0.6	27.6±1.0	0.439	27.3±0.8	26.4±0.6	0.387
Ethnicity (% White)	74.2	71.4	0.698	70.5	70.4	0.989
Current Smoking (%)	10.5	14.3	0.462	9.8	8.6	0.777
BMI (Kg/m^2)	30.5±0.7	27.4±0.8*	**0.007**	29.6±0.9	26.4±0.5$^{\#}$	**0.001**
SBP (mmHg)	129.6±1.1	142.8±2.4*	**0.000**	130.1±1.4	147.5±1.3$^{\#}$	**0.000**
DPB (mmHg)	81.5±0.8	90.3±1.6*	**0.000**	81.4±1.1	92.1±0.8$^{\#}$	**0.000**
Fasting Glucose (mg/dL)	79.7±1.2	76.6±2.4	0.207	79.7±1.9	79.1±2.5	0.280
24-h-Pr (mg/24h)	144.3±12.4	113.5±12.1	0.137	649.3±130.2	1564.0±192.0$^{\#}$	**0.004**
Primiparity (%)	37.6	46.4	0.263	36.1	47.7	0.123
GAD (weeks)	38.9±0.2	38.7±0.2	0.255	38.6±0.4	35.0±0.3$^{\#}$	**0.000**
Newborn weight (g)	3216.0±47.8	3175.0±84.6	0.653	3256.0±81.1	2245.0±74.3$^{\#}$	**0.000**
GAS (weeks)	35.4±0.5	37.1±0.4	0.060	35.3±0.7	33.8±0.4	**0.008**
MMP-9 (ng/mL)	323±28.4	277.7±37.0	0.405	328.9±34.8	286.9±20.5	0.287

GH, gestational hypertension; PE, preeclampsia; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; 24-h-Pr, 24-h proteinuria; GAD, gestational age at delivery; GAS, gestational age at sampling; MMP-9, matrix metalloproteinase-9.

Values are the mean ± S.E.M.

P[a] Nonresponsive gestation hypertension *vs.* Responsive gestational hypertension

P[b] Nonresponsive preeclampsia *vs.* Responsive preeclampsia

*P<0.05 *vs.* Responsive gestational hypertension and $^{\#}$P<0.05 *vs.* Responsive preeclampsia

Table 7- Demographic characteristics according to therapy responsiveness in GH and PE patients

Parameters	GH - Therapy Responsiveness			PE - Therapy Responsiveness		
	Responsive (n=159)	Nonresponsive (n=22)	P[a]	Responsive (n=114)	Nonresponsive (n=99)	P[b]
Age (years)	26.9±0.5	28.1±1.7	0.934	26.6±0.6	26.7±0.7	0.426
Ethnicity (% White)	74.7	63.6	0.272	71.1	69.7	0.829
Current Smoking (%)	10.8	18.2	0.310	12.3	5.1	0.068
BMI (Kg/m^2)	30.0±0.6	26.1±1.3*	**0.018**	28.7±0.6	25.8±0.5$^{\#}$	**0.001**
SBP (mmHg)	131.6±1.1	148.6±4.3*	**0.000**	135.1±1.8	151.0±1.6$^{\#}$	**0.000**
DPB (mmHg)	82.7±0.8	95.6±2.2*	**0.000**	84.7±0.9	93.9±1.0$^{\#}$	**0.000**
Fasting Glucose (mg/dL)	79.0±1.1	77.7±5.5	0.713	77.6±1.7	81.1±3.4	0.920
24-h-Pr (mg/24h)	138.3±10.7	114.4±17.0	0.383	1093.0±206.1	1585.0±218.7	0.092
Primiparity (%)	40.9	36.4	0.686	41.6	47.5	0.390
GAD (weeks)	38.9±0.2	38.5±0.3	0.235	38.0±0.3	33.8±0.5$^{\#}$	**0.000**
Newborn weight (g)	3244.0±44.4	2901.0±111.5*	**0.008**	2985.0±71.0	1998.0±90.5$^{\#}$	**0.000**
GAS (weeks)	35.8±0.4	36.8±0.7	0.477	35.3±0.4	32.7±0.5**#**	**0.000**
MMP-9 (ng/mL)	322.3±25.4	210.5±26.4	0.195	293.9±23.6	305.5±27.0	0.748

GH, gestational hypertension; PE, preeclampsia; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; 24-h-Pr, 24-h proteinuria; GAD, gestational age at delivery; GAS, gestational age at sampling; MMP-9, matrix metalloproteinase-9.

Values are the mean ± S.E.M.

P[a] Nonresponsive gestation hypertension *vs.* Responsive gestational hypertension.

P[b] Nonresponsive preeclampsia *vs.* Responsive preeclampsia.

*P<0.05 *vs.* Responsive gestational hypertension and $^{\#}$P<0.05 *vs.* Responsive preeclampsia.

Table 8- Genotype frequencies of MMP-9 polymorphisms according to responsiveness in GH and PE patients

Poly morphism	Genotype	GH - Methyldopa Responsiveness				PE - Methyldopa Responsiveness			
		R (n=125)	**NR** (n=56)	**OR (95% CI)**	**P**	**R** (n=61)	**NR** (n=152)	**OR (95% CI)**	**P**
MMP-9	**CC**	93 (74%)	33 (59%)	1.00 (reference)		46 (75%)	120 (79%)	1.00 (reference)	
$C^{-1562}T$	**CT+TT**	32 (26%)	23 (41%)	2.23 (1.06-4.73)*	**0.036**	15 (25%)	32 (21%)	0.62 (0.28-1.39)	0.240
		R (n=125)	**NR** (n=56)	**OR (95% CI)**	**P**	**R** (n=61)	**NR** (n=152)	**OR (95% CI)**	**P**
MMP-9	**LL**	21 (17%)	8 (14%)	1.00 (reference)		18 (29%)	26 (17%)	1.00 (reference)	
(CA)n	**LH**	65 (52%)	31 (56%)	1.27 (0.49-3.33)	0.629	26 (43%)	65 (43%)	1.23 (0.53-2.92)	0.637
	HH	39 (31%)	17 (30%)	0.79 (0.25-2.40)	0.677	17 (28%)	61 (40%)	2.61 (1.00-7.16)	0.054

MMP-9, matrix metalloproteinase-9; GH, gestational hypertension; PE, preeclampsia; R, responsive; NR, non-responsive; OR, odds ratio; CI, confidence interval

The genotype distribution was adjusted for age, ethnicity, current smoking, body mass index and primiparity

* P<0.05 *vs.* Responsive gestational hypertension

Table 9- Genotype frequencies of MMP-9 polymorphisms according to responsiveness in GH and PE patients

Poly morphism	Genotype	GH - Therapy Responsiveness				PE - Therapy Responsiveness			
		R (n=159)	**NR** (n=22)	**OR (95% CI)**	**P**	**R** (n=114)	**NR** (n=99)	**OR (95% CI)**	**P**
MMP-9	**CC**	114 (72%)	12 (55%)	1.00 (reference)		88 (77%)	78 (79%)	1.00 (reference)	
$C^{-1562}T$	**CT**	45 (28%)	10 (45%)	2.81 (1.00-8.10)*	**0.049**	26 (23%)	21 (21%)	0.66 (0.32-1.35)	0.259
		R (n=159)	**NR** (n=22)	**OR (95% CI)**	**P**	**R** (n=114)	**NR** (n=99)	**OR (95% CI)**	**P**
MMP-9	**LL**	25 (16%)	4 (18%)	1.00 (reference)		27 (24%)	17 (17%)	1.00 (reference)	
(CA)n	**LH**	85 (53%)	11 (50%)	0.76 (0.15-3.59)	0.817	50 (44%)	41 (42%)	1.02 (0.47-2.24)	0.963
	HH	49 (31%)	7 (32%)	0.85 (0.21-3.52)	0.725	37 (32%)	41 (41%)	2.31 (0.98-5.55)	0.058

MMP-9, matrix metalloproteinase-9; GH, gestational hypertension; R, responsive; NR, non-responsive; PE, preeclampsia; OR, odds ratio; CI, confidence interval.

The genotype distribution was adjusted for age, ethnicity, current smoking, body mass index and primiparity.

*P<0.05 *vs.* Responsive gestational hypertension.

Table 10- Haplotype frequencies of MMP-9 polymorphisms according to responsiveness in GH and PE patients

Haplotype	GH - Methyldopa Responsiveness				PE - Methyldopa Responsiveness			
	R (n=125x2)	**NR** (n=56x2)	**OR (95% CI)**	**P**	**R** (n=61x2)	**NR** (n=152x2)	**OR (95% CI)**	**P**
H1 (C L)	107 (43%)	46 (41%)	1.00 (reference)		62 (51%)	116 (38%)	1.00 (reference)	
H2 (C H)	110 (44%)	41 (37%)	0.57 (0.29-1.11)	0.099	44 (36%)	154 (51%)	2.04 (1.05-3.96)*	**0.034**
H4 (T H)	33 (13%)	24 (21%)	2.26 (0.97-5.18)	0.056	16 (13%)	33 (11%)	0.76 (0.32-1.92)	0.547

MMP-9, matrix metalloproteinase-9; GH, gestational hypertension; PE, preeclampsia; R, responsive; NR, non-responsive; OR, odds ratio; CI, confidence interval.

The haplotype distribution was adjusted for age, ethnicity, current smoking, body mass index and primiparity.

* P<0.05 *vs.* Responsive preeclampsia.

Table 11- Haplotype frequencies of MMP-9 polymorphisms according to responsiveness in GH and PE patients

Haplotype	GH - Therapy Responsiveness				PE - Therapy Responsiveness			
	R (n=159x2)	**NR** (n=22x2)	**OR (95% CI)**	**P**	**R** (n=114x2)	**NR** (n=99x2)	**OR (95% CI)**	**P**
H1 (C L)	134 (42%)	19 (43%)	1.00 (reference)		104 (46%)	74 (37%)	1.00 (reference)	
H2 (C H)	138 (43%)	13 (30%)	0.37 (0.13- 0.95)*	**0.043**	96 (42%)	102 (52%)	1.90 (1.06-3.45)#	**0.033**
H4 (T H)	45 (14%)	12 (27%)	3.45 (1.17- 9.58)*	**0.020**	28 (12%)	21 (11%)	0.73 (0.32-1.66)	0.454

MMP-9, matrix metalloproteinase-9; GH, gestational hypertension; PE, preeclampsia; R, responsive; NR, non-responsive; OR, odds ratio; CI, confidence interval.

The haplotype distribution was adjusted for age, ethnicity, current smoking, body mass index and primiparity.

* P<0.05 *vs.* Responsive gestational hypertension.

# P<0.05 *vs.* Responsive preeclampsia.

5- DISCUSSÃO GERAL

Apesar de a mortalidade materna ter diminuído no mundo, o número de internações hospitalares para o parto de gestantes com desordens hipertensivas tem aumentado [61]. Embora a pré-eclâmpsia (PE) seja uma condição transitória, as mulheres que desenvolvem essa síndrome apresentam risco aumentado de desenvolver hipertensão, acidente vascular cerebral e doença arterial coronariana no resto de suas vidas [62, 63]. Trabalhos recentes mostraram também um aumento da incidência de eventos cardiovasculares em filhos de mães que tiveram PE [64, 65]. Além disso, não há um tratamento medicamentoso efetivo para a pré-eclâmpsia e a terapia adotada atualmente possui algumas limitações. Assim, nosso estudo é relevante para a compreensão da fisiopatologia de desordens hipertensivas da gestação e para a investigação de marcadores genéticos que possam detectar precocemente a suscetibilidade a essas doenças e prever quem vai responder ou não responder a terapia anti-hipertensiva.

Os dados epidemiográficos de nossas voluntárias revelaram que a hipertensão na gestação é prevalente em mulheres mais velhas e obesas, ambos fatores de risco já reconhecidos para pré-eclâmpsia [11]. Entretanto, a etnicidade, o hábito de fumar e a primiparidade não diferiu entre as grávidas com HAG ou PE, comparadas com GS. Apesar dos valores de glicemia em HAG e PE serem estatisticamente diferentes dos valores observados em GS, nenhuma gestante diabética ou com outra comorbidade (nefropatias, coagulopatias, doenças imunológicas), foi incluída no estudo. Como consequência da hipertensão, o prognóstico materno-fetal piorou, sendo a semana gestacional do parto diminuída em HAG e PE e o peso do recém-nascido reduzido em PE, comparadas com GS.

Inicialmente, avaliou-se se as doenças hipertensivas da gestação afetam os níveis plasmáticos de MMP-2 e MMP-9, porque há relatos na literatura que a expressão e/ou a atividade dessas enzimas estão alteradas em hipertensão [41-45], inclusive em pré-eclâmpsia [53-56]. Ademais, tem-se sugerido que o nível circulante de MMP-9 é um marcador bioquímico clinicamente relevante para diagnóstico e prognóstico de doenças cardiovasculares [66, 67]. As concentrações de TIMP-1 e TIMP-2 também foram medidas no plasma, a fim de avaliar as

relações pro-MMP-9/TIMP-1 e pro-MMP-2/TIMP-2, as quais podem informar mais precisamente a atividade líquida dessas MMPs. Nossos principais achados foram que grávidas com HAG apresentam níveis plasmáticos de pro-MMP-9 e relação pro-MMP-9/TIMP-1 elevados, em comparação com GS. Além disso, embora não houve diferença estatística significante nos níveis plasmáticos de pro-MMP-2, demonstrou-se que a gravidez (normotensa ou hipertensa) está associada com níveis circulantes de TIMP-2 e razão pro-MMP-2/TIMP-2 maiores que mulheres não-grávidas.

Curiosamente, os níveis elevados de pro-MMP-9 que foram verificados em HAG estão em desacordo com um artigo publicado anteriormente [56]. Tayebjee et. al. [56] encontraram uma diminuição na relação MMP-9/TIMP-1 em doenças hipertensivas da gestação, não havendo diferença entre HAG e PE. Embora não se tenha uma explicação precisa, diferenças nos métodos empregados para determinar os níveis de MMP-9 (zimografia *versus* ELISA) podem ter ocasionado esses resultados conflitantes. Sabe-se que os níveis de MMP-9 estão correlacionados com a idade gestacional [52-54]; logo, diferenças na idade gestacional da coleta podem também ter afetado os resultados. O aumento da atividade líquida de MMP-9 em HAG é consistente com trabalhos que mostraram níveis elevados de MMP-9 em pacientes com hipertensão comparados com controles normotensos [41-45]. No entanto, outros estudos são necessários para confirmar nossos achados.

Corroborando com dois trabalhos anteriores [52, 54] e um artigo posterior ao nosso [53], não se verificou diferença nos níveis circulantes de pro-MMP-9 e na relação pro-MMP-9/TIMP-1 entre PE e GS. Esses achados sugerem que a atividade da MMP-9 no plasma de gestantes com PE é similar à atividade encontrada em GS e que, portanto, essa enzima não está envolvida na patogênese da fase clínica da PE (circulação hiperdinâmica e disfunção endotelial). Porém, ela pode ainda estar envolvida na fase inicial da doença, como demonstrado por Kolben et al. [52] que observaram concentrações de MMP-9 reduzidas em tecido placentário de pacientes com PE, comparado com tecido de GS.

A ausência de diferença estatisticamente significante nos níveis circulantes de pro-MMP-2 entre gestantes hipertensas (HAG e PE) e normotensas verificada aqui contraria dois trabalhos prévios [54, 55] e um posterior [53], os quais revelam níveis aumentados de MMP-2 em PE. Myers et al. [54] mostraram ainda que os níveis de MMP-2 estão elevados em PE na 22ª e 36ª semana gestacional, mas não na 26ª semana gestacional. Logo, embora não se tenha uma explicação precisa, é possível que diferenças na metodologia e na idade gestacional da coleta ocasionaram esses resultados divergentes. Contudo, como as concentrações de TIMP-2 e a relação pro-MMP-2/TIMP-2 foram similares em GS, HAG e PE, acredita-se que não existam diferenças na atividade líquida de MMP-2 entre esses grupos.

Com relação às concentrações plasmáticas de MMP-9 de todas as voluntárias grávidas estudadas, os resultados obtidos são compatíveis com os resultados anteriores. Embora em nossa análise prévia observamos diferença significativa apenas entre HAG e GS, agora nós também encontramos maiores níveis plasmáticos de MMP-9 em PE. No entanto, a concentração de TIMP-1 não foi avaliada no plasma dessas gestantes, a fim de se determinar a atividade líquida de MMP-9. Um mecanismo possível para a contribuição da MMP-9 na disfunção endotelial é que as células endoteliais e musculares lisas podem secretar MMP-9 após a estimulação pelo stress mecânico da hipertensão na parede vascular. Assim, a elastina é degradada mais que o colágeno, o que pode aumentar a fibrose [68]. Frente a esses achados, a maior concentração de MMP-9 reportada aqui no terceiro trimestre da gravidez em HAG e PE é compreensível. Isso é consistente com a noção de que alterações nos níveis plasmáticos de MMP-9 podem refletir em um pior prognóstico da doença, o que poderia basear-se, pelo menos em parte, em uma susceptibilidade genética específica envolvendo polimorfismos da MMP-9.

Analisaram-se, então, as frequências genotípicas e alélicas de dois polimorfismos funcionais no gene da MMP-9 ((CA)n e $C^{-1562}T$) em gestantes com HAG e PE, pois há relatos na literatura que evidenciaram a associação

desses polimorfismos com hipertensão [51], inclusive com pré-eclâmpsia [57]. Nossos principais achados foram que o genótipo CT e o alelo T do polimorfismo $C^{-1562}T$ estão mais frequentes em HAG, mas não em PE, comparadas com GS.

Visto que esse foi o primeiro estudo que avaliou a possível associação do polimorfismo $C^{-1562}T$ com HAG, nossos resultados requerem confirmação com trabalhos feitos em populações maiores e diferentes. Entretanto, a associação do alelo T com HAG é consistente com os níveis plasmáticos de MMP-9 elevados em HAG aqui demonstrados. Isso porque estudos *in vitro* têm associado o alelo T com expressão de MMP-9 elevada [47], levando provavelmente a um aumento dos níveis circulantes de MMP-9. Então, é possível que gestantes com HAG apresentem níveis elevados de MMP-9 porque o alelo T é mais frequentemente encontrado em grávidas com essa doença. A falta de associação significativa entre o polimorfismo $C^{-1562}T$ e PE, o que confirma os resultados reportados por Fraser *et. al.* [58], sugere que a MMP-9 não tenha um papel relevante na PE. Interessantemente, nós não encontramos diferenças nos níveis plasmáticos de MMP-9 em gestantes com PE, dando suporte para a falta de associação entre esse polimorfismo da MMP-9 e PE.

O polimorfismo (CA)n ainda não havia sido avaliado em gestantes com desordens hipertensivas da gestação. A falta de associação significativa entre o polimorfismo (CA)n e HAG ou PE sugere que esse polimorfismo da MMP-9 não tenha qualquer efeito sobre a susceptibilidade em desenvolver essas doenças. Trabalhos prévios investigaram a associação entre o polimorfismo (CA)n e ruptura prematura pré-termo de membrana (RPM) [69] ou restrição de crescimento intra-uterino (RCIU) [70]. Enquanto Ferrand et al. [69] observaram que o alelo com 14 repetições CA é mais frequente em recém-nascidos afro-americanos cujas mães desenvolveram RPM, comparados com aqueles bebês nascidos em termo, Gremlich et al. [70] não observaram diferenças nas frequencias alélicas entre recém-nascidos com RCIU e bebês compatíveis com a idade gestacional do nascimento. Embora seja incerto se o genoma fetal pode contribuir para o desenvolvimento de desordens hipertensivas da gestação [71], há um estudo

recente que demonstrou que a etnicidade pode influenciar na distribuição genotípica de polimorfismos da MMP-9 [72]. Assim, trabalhos adicionais são necessários para confirmar a falta de associação do polimorfismo (CA)n e HAG e PE.

Esse foi o primeiro estudo que avaliou a possível associação entre haplótipos da MMP-9 e desordens hipertensivas da gestação. Embora a associação entre os haplótipos da MMP-9 e HAG ou PE não tenha sido estatisticamente significativa em nossa primeira análise, quando se ampliou a população estudada, o haplótipo H4 (combinação dos alelos T e H), foi mais frequente em HAG, comparada com GS. Interessantemente, o alelo T do polimorfismo $C^{-1562}T$ e o alelo H do polimorfismo (CA)n são aqueles que os estudos *in vitro* têm associado com maiores níveis de expressão de MMP-9 [47, 50]. Mesmo que a análise haplotípica tenha sido considerada uma abordagem mais poderosa do que análise dos genótipos isoladamente [73], ela pode ser menos informativa quando uma associação casual entre variantes genéticas e um fenótipo é dirigida por um único polimorfismo [74]. O polimorfismo $C^{-1562}T$ parece ser mais relevante para o desenvolvimento de HAG, pois essa associação foi significativa mesmo quando se analisou uma população reduzida; entretanto, outros estudos são necessários para confirmar isso.

Em relação à associação dos polimorfismos $C^{-1562}T$ e (CA)n com as concentrações plasmáticas de MMP-9, não houve diferença estatisticamente significante entre os genótipos de ambos os polimorfismos nos grupos GS e PE. No entanto, quando avaliamos a concentração plasmática de MMP-9 segundo a distribuição genotípica para o polimorfismo (CA)n, nossos resultados indicaram uma inexplicável associação significativa e positiva apenas com o genótipo LH no grupo HAG. Embora alguns trabalhos tenham associado o alelo T com níveis plasmáticos aumentados de MMP-9 em hipertensão não tratada [51] e outras doenças cardiovasculares [66, 75], resultados negativos semelhantes ao nosso foram encontrados em uma população masculina saudável [76] e em síndrome coronariana aguda [77].

Quando avaliamos a concentração plasmática de MMP-9 conforme a distribuição haplotípica, não observamos efeitos significativos nos grupos GS, HAG e PE. A fonte predominante de MMP-9 no sangue é desconhecida, mas pode ser oriunda do tecido placentário [30, 31] ou dos neutrófilos e monócitos circulantes em decorrência do estado inflamatório generalizado que ocorre nas desordens hipertensivas da gestação [78]. Uma maneira de verificar se a associação entre hipertensão gestacional e níveis plasmáticos elevados de MMP-9 é causal foi avaliar o impacto de variações funcionais no gene MMP-9, mas nós não encontramos diferenças relevantes na concentração de MMP-9 entre os genótipos e haplótipos. Uma vez que a placenta é um tecido predominantemente fetal, é possível que o genoma do feto contribua para a produção anormal de MMP-9 em HAG e PE, mas isso requer confirmação.

No presente estudo, investigaram-se pela primeira vez os efeitos de polimorfismos da MMP-9 na resposta à metildopa ou à terapia anti-hipertensiva total em desordens hipertensivas da gestação. Nossos resultados sugerem que a distribuição genotípica para o polimorfismo (CA)n não diferiu entre pacientes responsivas e não responsivas nos grupos HAG e PE em ambas as abordagens. No entanto, para o polimorfismo $C^{-1562}T$, a presença dos genótipos CT+TT provavelmente reduz a suscetibilidade de resposta à metildopa e à terapia total em HAG, mas não em PE. Curiosamente, o alelo T, que foi associado com a não resposta ao tratamento em HAG, é o mesmo alelo que foi associado com o aumento da susceptibilidade em desenvolver HAG. Embora nenhum trabalho anterior tenha avaliado se polimorfismos da MMP-9 podem modificar os efeitos de drogas usadas para tratar a hipertensão arterial crônica, Zhou et al. [51] mostraram que o alelo T está associado com aumentos na PAS, PAD e velocidade da onda de pulso aórtico.

Além disso, analisaram-se também os efeitos de haplótipos da MMP-9 na resposta à metildopa ou à terapia anti-hipertensiva total. Nossos resultados sugerem que o haplótipo H2 (combinação dos alelos C e H) diminui a susceptibilidade de resposta à metildopa e à terapia total em PE. Entretanto,

em relação à resposta terapêutica total em HAG, o haplótipo H2 foi mais frequente nas pacientes responsivas do que nas não responsivas e o haplótipo H4 (o mesmo que aumenta a susceptibilidade em desenvolver HAG) diminui a susceptibilidade de resposta aos anti-hipertensivos. Nenhuma diferença significativa foi encontrada na distribuição haplotípica conforme a responsividade à metildopa em pacientes com HAG.

Embora não haja evidência de que a metildopa produza efeitos anti-hipertensivos por mecanismos que envolvam a redução da produção de MMP-9, foi demonstrado que os bloqueadores de canais de cálcio podem reduzir os níveis plasmáticos de MMP-9 em hipertensão arterial crônica [42, 79, 80]. Portanto, é possível que as drogas utilizadas para tratar desordens hipertensivas da gestação aliviam os sintomas clínicos via diminuição de MMP-9 circulante, anulando assim a concentração plasmática de MMP-9 aumentada aqui relatada. No entanto, não observamos diferenças significativas nos níveis de MMP-9 entre os grupos de responsivas e não-responsivas tanto em HAG quanto em PE; porém, isso já pode ter sido causado pelo efeito dos anti-hipertensivos que a grande maioria das pacientes estava tomando.

Deve ficar claro que os critérios empregados nesse estudo para avaliar a ausência de resposta à terapia anti-hipertensiva podem ter influenciado os nossos resultados. Embora não exista uma definição clara sobre como avaliar com precisão a gravidade das desordens hipertensivas da gestação, é possível que a resposta à terapia seja afetada pela gravidade da doença. Assim, mais estudos são necessários para melhorar a nossa compreensão dessas síndromes.

6- CONCLUSÃO GERAL

Em conclusão, nossas evidências sugerem que a atividade líquida de MMP-9, mas não de MMP-2, está aumentada em gestantes com HAG, mas não com PE. Nossos resultados indicam que o polimorfismo $C^{-1562}T$ e o haplótipo H4 (combinação dos alelos T e H) da MMP-9 estão associados com HAG, mas não com PE. Nós encontramos evidências de que a responsividade a anti-hipertensivos em HAG está associada com o polimorfismo $C^{-1562}T$ e os haplótipos H2 (combinação dos alelos C e H) e H4. O haplótipo H2 também pode afetar a capacidade de resposta aos anti-hipertensivos em PE. Apesar de o polimorfismo (CA)n poder alterar a concentração de MMP-9 em HAG, nosso estudo não mostrou efeitos relevantes de polimorfismos ou haplótipos da MMP-9 sobre as concentrações plasmáticas de MMP-9 em PE.

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8- ANEXOS

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14048-900 Ribeirão Preto SP

Ribeirão Preto, 20 de junho de 2006

Ofício nº 1759/2006
CEP/MGV

Prezado Professor:

O trabalho intitulado **"FARMACOGENÉTICA DO TRATAMENTO ANTI-HIPERTENSIVO DE PACIENTES COM PRÉ-ECLÂMPSIA: RELEVÂNCIA DO HAPLÓTIPO DA eNOS",** foi analisado pelo Comitê de Ética em Pesquisa, em sua 227ª Reunião Ordinária realizada em 12/06/2006, e enquadrado na categoria: **APROVADO, bem como o Termo de Consentimento Livre e Esclarecido,** de acordo com o Processo HCRP nº 4682/2006.

Aproveito a oportunidade para apresentar a Vossa Senhoria protestos de estima e consideração.

DRª. MARCIA GUIMARÃES VILLANOVA
Secretária do Comitê de Ética em Pesquisa do HCRP e da FMRP-USP

Ilustríssimo Senhor
PROF.DR. JOSE EDUARDO TANUS DOS SANTOS
VALÉRIA CRISTINA SANDRIM (Colaboradora)
Depto. de Farmacologia – FMRP-USP
Em mãos

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO -
ESTUDO

Projeto: "Farmacogenética do tratamento anti-hipertensivo de pacientes com pré-eclâmpsia: relevância do haplótipo da eNOS"

Responsáveis:

- Prof. Dr. José Eduardo Tanus dos Santos (FMRP-USP) - CREMESP 84.966
- Prof. Dr. Ricardo de Carvalho Cavalli - CREMESP 91.680
- Valéria Cristina Sandrim

Você está sendo convidada a participar de um estudo cujos detalhes são:

1) Este projeto pretende basicamente estudar se algumas variações comuns de alguns dos seus genes (que transmitem características hereditárias) podem contribuir no aumento da sua pressão sanguínea durante sua gravidez. Além disso, pretendemos estudar como estas variações podem modificar as quantidades de algumas substâncias produzidas pelo seu organismo e presentes no seu sangue. Isto poderá nos auxiliar no entendimento das alterações cardiovasculares que acontecem durante a gravidez e na obtenção de marcadores genéticos de susceptibilidade à pressão sanguínea elevada durante a gestação.

2) Sua participação neste estudo será:

Serão retirados, no máximo, 30 mL do seu sangue, coletados por punção venosa utilizando técnica adequada. Este volume de sangue é cerca de 15 (quinze) vezes menor do que o volume de sangue habitualmente doado

quando um indivíduo doa sangue para bancos de sangue. Este sangue será utilizado para realizar todos os estudos bioquímicos e genéticos mencionados acima. Uma segunda coleta (10 mL de sangue venoso) será realizada caso o seu médico prescreva para o tratamento da pressão alta o medicamento α-metildopa. Com isto, encerra-se a sua participação neste estudo.

3) Você terá direito a ressarcimento financeiro caso haja gastos gerados exclusivamente pela sua participação como voluntário desta pesquisa.

4) Caso haja dano comprovadamente decorrente da pesquisa você terá direito à indenização.

5) NÓS NÃO PODEMOS E NÃO GARANTIREMOS QUE VOCÊ RECEBERÁ QUALQUER BENEFÍCIO DIRETO DESTE ESTUDO.

6) Indiretamente, acreditamos que este estudo trará como benefício um entendimento da fisiopatologia da pré-eclâmpsia, bem como na obtenção de marcadores genéticos de susceptibilidade a pré-eclâmpsia e de resposta à α-metildopa.

7) Qualquer dado que possa ser publicado posteriormente em revistas científicas, não revelará a sua identidade. Entretanto, órgãos governamentais ligados à saúde podem solicitar informações a respeito da pesquisa e identidade dos voluntários nela envolvidos.

8) Você pode retirar o seu consentimento para participar deste estudo a qualquer momento, inclusive sem justificativas e sem qualquer prejuízo para você.

9) Você terá a garantia de receber a resposta a qualquer pergunta ou esclarecimento de qualquer dúvida a respeito dos procedimentos, riscos, benefícios e de outras situações relacionadas com a pesquisa e o tratamento a que será submetida. Qualquer questão a respeito do estudo ou de sua saúde deve ser dirigida ao Prof. Dr. José Eduardo Tanus dos Santos (telefone 0xx16 3602-3163), do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP), ao Dr. Ricardo de Carvalho Cavalli (telefone 0xx16 36022588), do Departamento de Ginecologia e Obstetrícia da Faculdade de Medicina de Ribeirão Preto (FMRP) ou à Doutoranda Valéria Cristina Sandrim (telefone 0xx16 36262851, 0xx16 36023329) do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP). O telefone do Comitê de Ética em Pesquisa da FMRP é 016-3602-2228.

Eu,_____, abaixo assinado, declaro que em ____/____/____ fui devidamente informada em detalhes pelo pesquisador responsável no que diz respeito ao objetivo da pesquisa, aos procedimentos que serei submetido, aos riscos e benefícios, à forma de ressarcimento no caso de eventuais despesas, bem como à indenização quanto por danos decorrentes da pesquisa. Declaro que tenho pleno conhecimento dos direitos e das condições que me foram asseguradas e acima relacionadas.

Declaro, ainda, que concordo inteiramente com as condições que me foram apresentadas e que, livremente, manifesto a minha vontade de participar do referido projeto.

Ribeirão Preto, _____ de _____ de _____.

_____ _____

Assinatura do voluntário Assinatura do investigador/testemunha

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO -

GRÁVIDAS CONTROLE

Projeto: "Farmacogenética do tratamento anti-hipertensivo de pacientes com pré-eclâmpsia: relevância do haplótipo da eNOS"

Responsáveis:

- Prof. Dr. José Eduardo Tanus dos Santos (FMRP-USP) - CREMESP 84.966
- Prof. Dr. Ricardo de Carvalho Cavalli - CREMESP 91.680
- Valéria Cristina Sandrim

Você está sendo convidada a participar deste estudo COMO UMA VOLUNTÁRIA APRESENTANDO GESTAÇÃO NORMAL. Sua participação visa oferecer dados CONTROLES.

Você está sendo convidada a participar deste estudo cujos detalhes são:

1) Este projeto pretende basicamente estudar se algumas variações comuns de alguns dos seus genes (que transmitem características hereditárias) podem contribuir em manter sua pressão sanguínea em níveis normais durante sua gravidez. Além disso, pretendemos estudar como estas variações podem modificar as quantidades de algumas substâncias produzidas pelo seu organismo e presentes no seu sangue. Isto poderá nos auxiliar no entendimento das alterações cardiovasculares que acontecem durante a gravidez e na obtenção de marcadores genéticos de susceptibilidade à pressão sanguínea elevada durante a gestação.

2) Sua participação neste estudo será:

Serão retirados, no máximo, 30 mL do seu sangue, coletados por punção venosa utilizando técnica adequada. Este volume de sangue é cerca de 15 (quinze) vezes menor do que o volume de sangue habitualmente doado quando um indivíduo doa sangue para bancos de sangue. Este sangue será utilizado para realizar todos os estudos bioquímicos e genéticos mencionados acima.

3) Você terá direito a ressarcimento financeiro caso haja gastos gerados exclusivamente pela sua participação como voluntário desta pesquisa.

4) Caso haja dano comprovadamente decorrente da pesquisa você terá direito à indenização.

5) NÓS NÃO PODEMOS E NÃO GARANTIREMOS QUE VOCÊ RECEBERÁ QUALQUER BENEFÍCIO DIRETO DESTE ESTUDO.

6) Indiretamente, acreditamos que este estudo trará como benefício um entendimento da fisiopatologia da pré-eclâmpsia, bem como na obtenção de marcadores genéticos de susceptibilidade a pré-eclâmpsia e de resposta à α-metildopa.

7) Qualquer dado que possa ser publicado posteriormente em revistas científicas, não revelará a sua identidade. Entretanto, órgãos governamentais ligados à saúde podem solicitar informações a respeito da pesquisa e identidade dos voluntários nela envolvidos.

8) Você pode retirar o seu consentimento para participar deste estudo a qualquer momento, inclusive sem justificativas e sem qualquer prejuízo para você.

9) Você terá a garantia de receber a resposta a qualquer pergunta ou esclarecimento de qualquer dúvida a respeito dos procedimentos, riscos, benefícios e de outras situações relacionadas com a pesquisa e o tratamento a que será submetida. Qualquer questão a respeito do estudo ou de sua saúde deve ser dirigida ao Prof. Dr. José Eduardo Tanus dos Santos (telefone 0xx16 3602-3163), do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP), ao Dr. Ricardo de Carvalho Cavalli (telefone 0xx16 3602-2588), do Departamento de Ginecologia e Obstetrícia da Faculdade de Medicina de Ribeirão Preto (FMRP) ou à Doutoranda Valéria Cristina Sandrim (telefone 0xx16 36262851, 0xx16 36023329) do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP). O telefone do Comitê de Ética em Pesquisa da FMRP é 016-3602-2228.

Eu, _____,
abaixo assinado, declaro que em ____/____/____ fui devidamente informada em detalhes pelo pesquisador responsável no que diz respeito ao objetivo da pesquisa, aos procedimentos que serei submetido, aos riscos e benefícios, à forma de ressarcimento no caso de eventuais despesas, bem como à indenização quanto por danos decorrentes da pesquisa. Declaro que tenho pleno conhecimento dos direitos e das condições que me foram asseguradas e acima relacionadas.

Declaro, ainda, que concordo inteiramente com as condições que me foram apresentadas e que, livremente, manifesto a minha vontade de participar do referido projeto.

Ribeirão Preto, _____ de _____ de _____.

_____ _____

Assinatura do voluntário Assinatura do investigador/testemunha

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO -
CONTROLE

Projeto: "Farmacogenética do tratamento anti-hipertensivo de pacientes com pré-eclâmpsia: relevância do haplótipo da eNOS"

Responsáveis:

- Prof. Dr. José Eduardo Tanus dos Santos (FMRP-USP) - CREMESP 84.966
- Prof. Dr. Ricardo de Carvalho Cavalli - CREMESP 91.680
- Valéria Cristina Sandrim

Você está sendo convidada a participar deste estudo COMO UMA VOLUNTÁRIA SADIA. Sua participação visa oferecer dados CONTROLES.

Você está sendo convidada a participar deste estudo cujos detalhes são:

1) Este projeto pretende basicamente estudar se algumas variações comuns de alguns dos seus genes (que transmitem características hereditárias) podem contribuir no aumento da pressão sanguínea durante a gravidez. Além disso, pretendemos estudar como estas variações podem modificar as quantidades de algumas substâncias produzidas pelo seu organismo e presentes no seu sangue. Isto poderá nos auxiliar no entendimento das alterações cardiovasculares que acontecem durante a gravidez e na obtenção de marcadores genéticos de susceptibilidade à pressão sanguínea elevada durante a gestação.

2) Sua participação neste estudo será:

Serão retirados, no máximo, 30 mL do seu sangue, coletados por punção venosa utilizando técnica adequada. Este volume de sangue é cerca de 15 (quinze) vezes menor do que o volume de sangue habitualmente doado quando um indivíduo doa sangue para bancos de sangue. Este sangue será utilizado para realizar todos os estudos bioquímicos e genéticos mencionados acima.

3) Você terá direito a ressarcimento financeiro caso haja gastos gerados exclusivamente pela sua participação como voluntário desta pesquisa.

4) Caso haja dano comprovadamente decorrente da pesquisa você terá direito à indenização.

5) NÓS NÃO PODEMOS E NÃO GARANTIREMOS QUE VOCÊ RECEBERÁ QUALQUER BENEFÍCIO DIRETO DESTE ESTUDO.

6) Indiretamente, acreditamos que este estudo trará como benefício um entendimento da fisiopatologia da pré-eclâmpsia, bem como na obtenção de marcadores genéticos de susceptibilidade a pré-eclâmpsia e de resposta à α-metildopa.

7) Qualquer dado que possa ser publicado posteriormente em revistas científicas, não revelará a sua identidade. Entretanto, órgãos governamentais ligados à saúde podem solicitar informações a respeito da pesquisa e identidade dos voluntários nela envolvidos.

8) Você pode retirar o seu consentimento para participar deste estudo a qualquer momento, inclusive sem justificativas e sem qualquer prejuízo para você.

9) Você terá a garantia de receber a resposta a qualquer pergunta ou esclarecimento de qualquer dúvida a respeito dos procedimentos, riscos, benefícios e de outras situações relacionadas com a pesquisa e o tratamento a que será submetida. Qualquer questão a respeito do estudo ou de sua saúde deve ser dirigida ao Prof. Dr. José Eduardo Tanus dos Santos (telefone 0xx16 3602-3163), do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP), ao Dr. Ricardo de Carvalho Cavalli (telefone 0xx16 3602-2588), do Departamento de Ginecologia e Obstetrícia da Faculdade de Medicina de Ribeirão Preto (FMRP) ou à Doutoranda Valéria Cristina Sandrim (telefone 0xx16 36262851, 0xx16 36023329) do Departamento de Farmacologia da Faculdade de Medicina de Ribeirão Preto (FMRP). O telefone do Comitê de Ética em Pesquisa da FMRP é 016-3602-2228.

Eu, _____,
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Declaro, ainda, que concordo inteiramente com as condições que me foram apresentadas e que, livremente, manifesto a minha vontade de participar do referido projeto.

Ribeirão Preto, _____ de _____ de _____.

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Assinatura do voluntário Assinatura do investigador/testemunha

OBSERVAÇÃO:

O PROJETO INTITULADO “Efeitos de haplótipos da metaloproteinase-9 da matriz extracelular sobre a susceptibilidade e resposta farmacológica de pacientes com pré-eclâmpsia/doença hipertensiva gestacional” É UM SUB-PROJETO DO PROJETO INTITULADO “Farmacogenética do tratamento anti-hipertensivo de pacientes com pré-eclâmpsia: relevância do haplótipo da eNOS”, O QUAL JÁ FOI APROVADO PELO Comitê de Ética em Pesquisa da Faculdade de Medicina de Ribeirão Preto-USP.

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