RICCARDO LACCHINI

"INFLUÊNCIA DE FATORES GENÉTICOS RELACIONADOS ÀS METALOPROTEINASES DA MATRIZ EXTRACELULAR SOBRE A SUSCEPTIBILIDADE À HIPERTROFIA CARDÍACA EM HIPERTENSOS"

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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 Doutorado em Farmacologia

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"We're living in a constant fight / Too much pressure everywhere and all around
Don't let it pull you down / Hold on tight / Cling to your dreams
Don't you depend on someone else/ Think for yourself and win the race

The chance you got comes never twice / Do your best, (and) do it right
Time will come but don't you hide / You are on your way

You're led by God but you're not a pawn / Should live your life, don't care 'bout
any scorn / Just let them fool around / No need to cry / Don't give up
Try to be as smart as you can be / I know you own so much ability

The chance you got comes never twice / Do your best, (and) do it right
Time will come but don't you hide / You are on your way"

"The Chance" – HELLOWEEN

Resumo

A hipertensão é uma doença muito comum, e está associada à alta morbidade e mortalidade cardiovascular. Em relação às doenças associadas à hipertensão, se observam diferenças étnicas, sendo que negros são expostos a maior risco cardiovascular que os brancos. A manutenção de níveis pressóricos elevados leva a alterações teciduais em vasos e no coração em um processo chamado remodelamento cardiovascular. No coração este processo leva à hipertrofia cardíaca. Esta condição causa progressiva perda na eficiência de contração do coração, e em casos avançados pode levar à morte dos pacientes. O remodelamento cardíaco envolve a participação de metaloproteinases de matriz extracelular (MMPs), principalmente a MMP-2 e MMP-9. Diversos polimorfismos genéticos foram associados com alterações na expressão e/ou atividade destas enzimas, e é possível que diferenças nas distribuições dos polimorfismos ajudem a explicar as diferenças étnicas quanto à morbidade e mortalidade cardiovascular. Os principais polimorfismos da MMP-9 e MMP-2 são: um polimorfismo de base única (SNP) ($C^{-1562}T$) e um microssatélite (-90 $CA_{(14-24)}$) no promotor, e um SNP no exon 6 ($A^{855}G$ – Q279R) da MMP-9, e dois SNPs ($C^{-1306}T$ e $C^{-735}T$) no promotor da MMP-2. Como estes polimorfismos também foram associados com diversas doenças, o propósito deste estudo foi avaliar se polimorfismos da MMP-9 e MMP-2 estão associados à hipertensão, a alterações ecocardiográficas em hipertensos e se diferem entre populações de brancos e de negros.

Em nosso estudo avaliamos 173 hipertensos submetidos a eco cardiografia e 137 indivíduos normotensos, pareados para idade gênero e raça. Avaliamos também 140 indivíduos brancos e 177 indivíduos negros recrutados aleatoriamente. Nossos resultados mostraram uma associação do alelo H e do genótipo HH do microssatélite -90 $CA_{(14-24)}$ com hipertensão (P= 0,0058 e P=0,0085, respectivamente), e os portadores destes alelos apresentaram maior risco a esta doença, quando comparados com o alelo L (*odds ratio,* ou razão de chances (OR) =1,581) e genótipo LL (OR=2,32). Haplótipos da MMP-9 foram capazes de exercer efeitos protetores (H3, P=0,049) e deletérios (H7; P=0,0015) sobre o diâmetro diastólico final e protetores (H3, P=0,0367) e deletérios (H7; P=0,0057) sobre o

índice de massa do ventrículo esquerdo. Não encontramos associações dos polimorfismos da MMP-2 com hipertensão, porém os haplótipos H1 e H3 mostraram efeitos protetores sobre diâmetro diastólico final e índice de massa do ventrículo esquerdo (H1; P=0,0290 e P=0,0318, respectivamente) e deletérios sobre e índice de massa do ventrículo esquerdo (H3; P=0,0187). Nós encontramos diferenças grandes nas distribuições dos polimorfismos estudados entre brancos e negros. O alelo H e genótipo HH do microssatélite -90 $CA_{(14-24)}$ é mais frequente em negros (P<0.05), o que poderia explicar em parte a maior incidência de hipertensão nestes indivíduos. Além disto, diversos haplótipos da MMP-9 e genótipos da MMP-2, cujos efeitos foram demonstrados sobre a hipertrofia cardíaca (incluindo os haplótipos H3 e H7 da MMP-9 e genótipo CC do $C^{-1306}T$ da MMP-2) tiveram frequências significativamente diferentes (P<0.05) entre brancos e negros, o que também ajuda a explicar diferenças étnicas observadas quanto à morbidade e mortalidade cardiovascular entre estes dois grupos.

Palavras-chave: Metaloproteinase 9 da matriz. Metaloproteinase 2 da matriz. Polimorfismo (Genética). Hipertrofia. Hipertensão arterial

Abstract

Hypertension is a very common disease and is associated with high cardiovascular morbidity and mortality. There are inter-ethnic differences regarding the risk to hypertension-related diseases, as black subjects are at higher cardiovascular risk than whites. The maintenance of high blood pressure levels trigger a process called cardiovascular remodeling, which causes several histological and functional changes in arteries and in myocardium. The myocardium remodeling usually causes cardiac hypertrophy. This condition leads to progressive loss in efficiency of heart pumping, and in advanced stages, it may result in death. Cardiac remodeling involves matrix metalloproteinases (MMPs) actions, and MMP-2 and MMP-9 are the most important. Several genetic polymorphisms were associated with differences in enzyme expression or activity and it is possible that differences in distribution of these polymorphisms may help to explain the observed inter-ethnic differences in cardiovascular risk observed between blacks and whites. The main polymorphisms of MMP-9 and MMP-2 are: a single nucleotide polymorphism (SNP) ($C^{-1562}T$) and a microsatellite (-90 CA $_{(14-24)}$) in promoter and a SNP in exon 6 ($A^{855}G$ – Q279R) on MMP-9 gene, and two SNPs ($C^{-1306}T$ e $C^{-735}T$) at MMP-2 promoter. As these polymorphisms were already associated with several diseases, the purpose of this study was to evaluate whether polymorphisms of MMP-9 and MMP-2 are associated with hypertension, with echocardiographic alterations and if they are different between blacks and whites.

This study included 173 hypertensive patients which were submitted to echocardiography and 137 age, race and gender matched healthy volunteers. Besides that, we also included 140 white subjects and 177 black subjects for the ethnic study. Our results shown an association of the H allele and HH genotype of -90 CA $_{(14-24)}$ microsatellite with hypertension (P= 0.0058 and P=0.0085, respectively),, and that carriers of these allele (odds ratio (OR) =1.581) and genotype (OR=2,32) may be at higher risk to hypertension. MMP-9 haplotypes exerted protective (H3; P=0.0490) or detrimental (H7; P=0.0015) effects on end-diastolic diameter and protective (H3; P=0.0367) or detrimental (H7; P=0.0057) effects on left ventricular mass index (LVMI). Although we didn't observe an

association of MMP-2 polymorphisms with hypertension, we have found that H1 and H3 may have protective effects on end-diastolic diameter and LVMI (H1; P=0.0290 and P=0.0318, respectively) and detrimental effects on LVMI (H3; P=0.0187). We have found that the studied polymorphisms differed significantly in their distributions between white and black groups. The H allele and HH genotype of the -90 $CA_{(14-24)}$ was more common in blacks($P<0.05$) which could help to explain why this group have higher risk to hypertension. Besides that, several MMP-9 haplotypes and MMP-2 genotypes differed significantly ($P<0.05$) in their frequencies between blacks and whites, including some that shown effects on heart hypertrophy (H3 and H7 of MMP-9 and CC genotype of $C^{-1306}T$ of MMP-2). This may also help to explain ethnic differences in cardiovascular morbidity and mortality between these groups.

Key-words: Metalloproteinase 9. Metalloproteinase 2. Polymorphisms. Hypertrophy. Hypertension.

% - porcentagem

PA - pressão arterial

mmHg - milímetros de mercúrio

MMPs - metaloproteinases de matriz extracelular

TIMPs - *tissue inhibitors of metalloproteinases* – inibidores teciduais de metaloproteinases

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

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

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

IMVE - índice de massa do ventrículo esquerdo

OR – *odds ratio* - razão de chances

SUMÁRIO

INTRODUÇÃO

Introdução

A hipertensão é uma condição clínica muito comum, que afeta aproximadamente 30% dos brasileiros(1). Ela é caracterizada pela manutenção de níveis de pressão arterial (PA) elevados de maneira permanente, mesmo em repouso, acima de 140 mmHg (pressão arterial sistólica) e/ou acima de 90 mmHg (pressão arterial diastólica). A manutenção destes níveis pressóricos elevados leva a uma série de processos associados à morbidade e mortalidade cardiovascular (1-3).

As estatísticas das doenças relacionadas à hipertensão são alarmantes: em 2001, cerca de 7,6 milhões de mortes em todo o mundo foram atribuídas à elevação da PA (1, 3). No Brasil somente em 2007 ocorreram mais de 300 mil óbitos por doenças do aparelho cardiovascular (4). Doenças relacionadas à hipertensão também geram altos custos para o sistema de saúde. De fato, no ano inteiro de 2007 foram contabilizadas mais de um milhão de internações no sistema único de saúde brasileiro(1). Em um único mês em 2009 foram aproximadamente 92 mil internações, gerando um custo de mais de 165 milhões de reais para o governo (5).

1- Patogênese do ventrículo esquerdo

O aumento das tensões e forças físicas às quais a parede arterial é exposta durante a hipertensão leva a um processo de hipertrofia da camada da íntima média destes vasos de condutância (6). Isto permite sua adaptação inicial ao maior stress mecânico, porém reduz sua complacência às ondas de pulso. Desta forma, as ondas de pulso são transmitidas de forma mais rápida pela árvore arterial, o que acaba sendo prejudicial por dois mecanismos: aumento do pico de

pressão nas artérias de resistência (podendo levar a dano em órgãos alvo como rins, fígado, encéfalo, entre outros), e reflexão precoce das ondas de pulso, que ao voltarem pela parede arterial encontram o coração durante o processo de sístole (levando a uma sobrecarga extra no ventrículo esquerdo) (6).

As lesões em órgãos alvo são responsáveis por grande parte da morbidade e mortalidade cardiovascular. A disfunção endotelial induzida pela hipertensão crônica tem papel fundamental na aterosclerose; os picos de pressão podem levar a rupturas em arteríolas ou capilares nos rins, prejudicando sua função. Rupturas de arteríolas no encéfalo podem levar a acidentes vasculares hemorrágicos. No miocárdio, as lesões induzidas pela hipertensão (7) se manifestam de forma diferente. O aumento da pressão arterial leva a um aumento no trabalho cardíaco para bombear o sangue e manter a perfusão dos tecidos nas mesmas condições de uma situação normal. O ventrículo esquerdo exerce uma força maior para vencer a pressão da aorta proximal, e assim abrir a válvula da aorta ejetando o sangue. O aumento do trabalho cardíaco leva a um maior consumo de oxigênio, e é um dos fatores determinantes para o processo de hipertrofia cardíaca. (8). O consumo de oxigênio, na cadeia respiratória das células, leva a uma produção, normalmente baixa, de moléculas com elétrons não pareados, chamadas espécies reativas de oxigênio, dentre elas, o ânion superóxido. Essas moléculas são radicais livres, com altíssima reatividade com todos os componentes celulares (tanto proteínas, membranas, DNA, e inclusive outros radicais livres), e são capazes de desencadear processos pró-apoptóticos, dentre outros. Além do dano direto causado pelo ânion superóxido, este é capaz de sequestrar um componente crucial para manutenção da circulação coronariana, o óxido nítrico. Essa molécula

deixa de ser disponível, prejudicando a oxigenação do tecido e aumentando a produção de radicais livres pelo desacoplamento de enzimas da cadeia respiratória. Além disso, o óxido nítrico capturado é transformado em peroxinitrito, por sua vez também tóxico e capaz de causar danos em proteínas e ativar as metaloproteinases de matriz extracelular independentemente da clivagem do seu pró-peptídeo (9). De fato, este parece ser um mecanismo importante para o desencadeamento do remodelamento vascular e cardíaco, e a redução do estado pró-oxidante do tecido pela ação de antioxidantes parecem reverter os efeitos do remodelamento cardiovascular (10-13).

A hipertrofia ocorre inicialmente de forma concêntrica, com o espessamento do septo e parede posterior do coração em detrimento da luz do ventrículo. Neste estágio, o coração mantém indicadores de função cardíaca, como a fração de ejeção e a capacidade de encurtamento das fibras inalteradas, de tal forma que este processo já foi denominado como “adaptativo”. A manutenção de níveis pressóricos elevados neste estágio pode levar à evolução para a hipertrofia excêntrica. Neste processo, ocorre um estiramento das paredes posterior e septo, de tal forma que a espessura dessas paredes é reduzida sensivelmente, e a capacidade contrátil das mesmas diminui. Este estiramento das paredes é acompanhado com um aumento do volume interno do ventrículo. Em conjunto, estes processos levam a uma redução da capacidade contrátil do ventrículo esquerdo, e uma redução na fração de ejeção do coração, culminando em uma queda na eficiência de bombeamento do coração (8, 14). A hipertrofia excêntrica em estado avançado pode exigir que o paciente passe por um transplante

cardíaco, ou o mesmo pode ir a óbito. Na figura 1 está ilustrado o processo de hipertrofia concêntrica e excêntrica

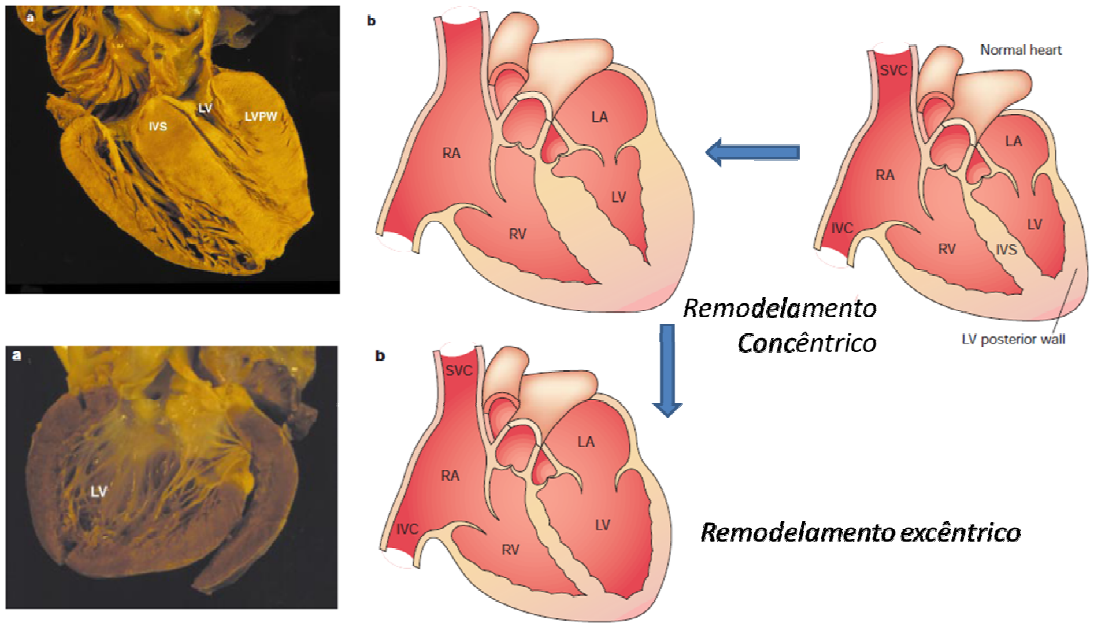

Figura 1. Processo de hipertrofia cardíaca. Adaptado de Towbin, et al., Nature 2002

No ponto de vista histológico, a hipertrofia cardíaca é caracterizada por notável aumento na deposição de colágeno, e remodelamento tecidual, que podem levar a uma desorganização deste tecido (6, 15). Uma vez que este depende de sua estrutura altamente especializada para desempenhar suas funções com eficiência, fica claro que o processo de hipertrofia cardíaca prejudica a função cardíaca em longo prazo, apesar de o processo inicial parecer adaptativo.

2- Efeito da etnicidade sobre o risco a eventos cardiovasculares

Estudos têm mostrado diferenças étnicas importantes em relação à hipertensão. A incidência de hipertensão é duas vezes maior em indivíduos de cor

não branca do que indivíduos da cor branca (1). Estudos brasileiros mostraram que a incidência de hipertensão é maior em mulheres negras quando comparadas com mulheres brancas (1, 16).

Além da incidência de hipertensão, diferenças étnicas foram demonstradas em relação a mortes por doença arterial coronariana, onde negros tiveram maiores índices de óbitos que brancos (17). A taxa anual de primeiro infarto também parece ser maior em negros, o que parece também ser afetado independentemente por gênero e idade (17). Além disto, foi mostrado que hipertensão maligna e complicações renais são mais frequentes em negros que em brancos (18). Por fim, sabe-se que negros tem um perfil de resposta farmacológica ao tratamento anti-hipertensivo diferente dos brancos. Negros respondem menos à monoterapia com betabloqueadores, inibidores da enzima conversora de angiotensina e bloqueadores de receptor de angiotensina II quando comparados com brancos. Por outro lado, negros respondem melhor a diuréticos e bloqueadores de canais de cálcio, e à terapia não farmacológica (1, 2). Além disto, as menores taxas de controle da pressão arterial com terapia anti-hipertensiva ocorrem em mulheres negras, enquanto que as maiores taxas de controle da pressão arterial são atingidas por indivíduos masculinos, não negros (19).

As diferenças étnicas podem ocorrer por diferenças em fatores socioeconômicos, ambientais ou genéticos. Em relação aos fatores genéticos, um crescente corpo de evidências tem demostrado diferenças étnicas na distribuição de polimorfismos com papéis funcionais em doenças (20-23), o que mostra que a magnitude da influência dos fatores genéticos pode ser diferente entre os grupos étnicos.

3- Metaloproteinases de matriz extracelular

As metaloproteinases de matriz extracelular (MMPs) são uma família de proteases dependentes de zinco, capazes de degradar diversas proteínas, incluindo componentes da membrana basal e matriz extracelular. Entre as MMPs, se destacam a MMP-2 e a MMP-9 (gelatinase A e gelatinase B, respectivamente), as quais desempenham papeis chave na patogênese de diversas doenças cardiovasculares (8).

As MMPs 2 e 9 são capazes de degradar diversos componentes da matriz extracelular, como laminina, elastina, colágeno tipo IV e fibronectina. Recentemente foi demonstrado que a MMP-2 também é capaz de degradar alvos intracelulares, como cadeia leve de miosina, o que pode ter impacto direto na contratilidade cardíaca (9, 24).

Diversas células do sistema imune secretam MMPs-9, especialmente os macrófagos. Além disso, sabe-se que o tecido vascular e cardíaco por si só são capazes de produzir MMP-2. As MMPs são secretadas na forma de precursores inativos, cujo sítio catalítico é coberto por um pró-peptídeo que impede a interação do substrato com o sítio catalítico (25). As enzimas podem ser ativadas por clivagem proteolítica do pró-peptídeo (26, 27), ou pela reação de um resíduo cisteína do pró-peptídeo com subprodutos do stress oxidativo, o que expõe o sítio catalítico sem clivagem do pró-peptídeo (9). Outros processos importantes são a exocitose de estoques intracelulares, ligação a inibidores (TIMPs) e expressão gênica.

Já foi demonstrado tanto em modelos animais (15, 28, 29) como em humanos (30, 31) que o processo de hipertrofia cardíaca envolve aumentos

transientes ou permanentes nos níveis e atividade das MMPs, e que a inibição inespecífica das MMPs é capaz de reverter os processos de remodelamento vascular (13) e cardíaco (11).

A ação das MMPs leva a degradação de pacotes de colágeno, o que pode estimular a síntese de novas fitas de colágeno e promover a fibrose tecidual. Além disso, a degradação de proteínas de matriz extracelular abre espaço para a penetração de macrófagos levando à inflamação local, o que pode agravar o quadro de fibrose. O balanço entre a degradação, e síntese de colágeno pode favorecer a hiperplasia e hipertrofia celular. Juntos, os efeitos das MMPs parecem estimular as alterações presentes no remodelamento cardiovascular (8).

4- Polimorfismos da MMP-9 e MMP-2

O gene da MMP-9 encontra-se no cromossomo 20, na região 20q11.2-q13.1. Diversos polimorfismos neste gene têm sido estudados, sendo os principais: um polimorfismo de base única (*single nucleotide polymorphism* – SNP) constituindo-se de uma troca de citosina para timina na posição -1562 do promotor do gene; um microssatélite na posição -90 do promotor, constituindo-se de repetições do dinucleotídeo CA entre 14 e 24 vezes (sendo a distribuição bimodal em torno de 14 repetições e em torno de 21-24 repetições (32)); por último, um SNP no exon 6 do gene, constituindo-se de uma troca de adenina para guanina na posição 855. Este último gera uma troca de Glutamina para Arginina na posição 279 da proteína.

O gene da MMP-2 está localizado no cromossomo 16, na região 16q13-q21. Os polimorfismos mais estudados deste gene são: um SNP gerando a troca

de citosina para timina na posição -1306 do promotor da MMP-2; um SNP gerando a troca de citosina para guanina na posição -735 do promotor da MMP-2.

Todos estes polimorfismos são funcionais (33-37), e já foram associados com maior risco a diversas doenças ou desfechos cardiovasculares associados à hipertensão, como falência cardíaca (38), aterosclerose (32), risco de eventos cardiovasculares futuros (31), recuperação pós infarto (39) ou outras doenças que envolvem remodelamento tecidual, como câncer (40) e rejeição a transplantes (41).

Devido ao papel intrínseco das MMPs em doenças que envolvem remodelamento tecidual, podemos imaginar que polimorfismos que afetem a função das MMPs possam ter algum efeito sobre a hipertensão e sobre o remodelamento cardíaco.

OBJETIVOS

Os objetivos do presente estudo foram:

1. Avaliar a associação de genótipos e haplótipos de polimorfismos funcionais da MMP-9 e da MMP-2 com hipertensão

2. Avaliar os efeitos de genótipos e haplótipos da MMP-9 e da MMP-2 sobre parâmetros indicativos de hipertrofia cardíaca

3. Avaliar diferenças étnicas na distribuição de genótipos e haplótipos que possam ajudar a explicar diferenças interetnicas observadas no risco cardiovascular.

CAPÍTULOS

Estrutura da tese.

Esta tese foi desenvolvida de 2009 a 2011, e resultou em três artigos, publicados em revistas internacionais. A ordem da apresentação dos artigos não respeita a ordem cronológica das publicações. O primeiro e segundo artigos avaliam a mesma casuística, com uma pequena redução no número de indivíduos no segundo, por esgotamento de amostras de DNA de pacientes não mais acompanhados pelo ambulatório do Professor Doutor Wilson Nadruz Jr. Os critérios de inclusão destes pacientes foram: pressão arterial sistólica acima de 140 mmHg, pressão arterial diastólica acima de 90mmHg, ou uso de anti-hipertensivos. Os critérios de exclusão destes pacientes foram: Idade abaixo dos 18 anos, evidência de valvulopatias cardíacas moderadas ou severas, cardiomiopatia hipertrófica, infarto do miocárdio prévio, doenças neoplásicas, e suspeita de hipertensão secundária.

O terceiro estudo foi feito avaliando duas populações brasileiras isoladas geograficamente: uma população de indivíduos com auto declaração como brancos, coletada na região de Ribeirão Preto (SP), e uma população de indivíduos com auto declaração como negros, coletada na região de Salvador (BA).

O primeiro e segundo artigos avaliam efeitos de polimorfismos dos genes da MMP-9 e MMP-2, respectivamente, sobre parâmetros de eco cardiografia em hipertensos. Estes dois estudos foram inicialmente desenvolvidos como um único manuscrito que foi submetido para a revista “Heart” (fator de impacto em 2011: 5.385). Durante o período de revisão por pares, finalizamos os experimentos e as análises do terceiro artigo (avaliando o efeito da etnicidade sobre a distribuição dos polimorfismos dos genes da MMP-9 e da MMP-2), submetido e aceito pela revista “DNA and Cell Biology”. Em seguida recebemos uma resposta negativa da revista Heart, e decidimos submeter os dados da MMP-9 para a revista “Clinica Chimica Acta”, e aprofundar o estudo da MMP-2 (até então somente o polimorfismo -1306 CT tinha sido estudado). Após alguns meses recebemos uma resposta positiva da revista “Clinica Chimica Acta”, e por fim submetemos os resultados completos da MMP-2 para a revista “Journal of Human Hypertension”.

CAPÍTULOS

A ordem de apresentação dos artigos nos capítulos a seguir segue a ordem cronológica em que os trabalhos foram concebidos e experimentos iniciados.

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Matrix metalloproteinase 9 gene haplotypes affect left ventricular hypertrophy in hypertensive patients

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ABSTRACT

Background: Matrix metalloproteinases (MMPs) are involved in cardiac remodeling and are encoded by genes showing genetic polymorphisms that have functional implications. We examined whether MMP-9 genetic polymorphisms are associated with hypertension and with left ventricular (LV) remodeling in hypertensive patients.
Methods: We studied 173 hypertensive patients and 137 age, race and gender matched healthy controls. Heart echocardiography was performed in all patients and the following MMP-9 genetic polymorphisms were analyzed: $C^{-1562}T$ (rs3918242), −90 $(CA)_{14-24}$ (rs2234681) and Q279R (rs17576). Haplo.stats analysis was used to assess whether MMP-9 haplotypes are associated with hypertension. Linear regression analysis was performed to assess whether MMP-9 haplotypes affect LV mass index (LVMI) and other echocardiography parameters.
Results: MMP-9 −90 $(CA)_{14-24}$ "HH" genotype (H allele defined by number of CA repeats ≥21) was associated with hypertension (P = 0.0085; OR = 2.321, 95% confidence interval = 1.250 to 4.309). While one MMP-9 haplotype ("C, H, Q") protects against LVMI and end-diastolic diameter increases due to remodeling (P = 0.0490 and P = 0.0367), another MMP-9 haplotype apparently has detrimental effects over both parameters in hypertensive patients ("T, H, Q", P = 0.0015 and P = 0.0057, respectively).
Conclusion: Genetic polymorphisms in MMP-9 gene may modify the susceptibility of hypertensive patients to LV remodeling. Further studies are necessary to examine whether these polymorphisms affect clinical events in hypertensive patients.

1. Introduction

Hypertension is a major health problem usually leading to cardiovascular complications. Chronic pressure overload results in left ventricle hypertrophy (LVH), which is a maladaptive process associated with myocardial apoptosis, fibrosis and loss of function [1]. LVH may progress to left ventricle dilatation and cardiac failure, a well established risk factor for cardiovascular mortality [2]. Matrix metalloproteinases (MMP) are structurally related, zinc dependent, enzymes that degrade several components of extracellular matrix [3]. Among several other MMPs, MMP-2 and MMP-9 are involved in cardiac remodeling [4] and may be related to progression of cardiac hypertrophy [5]. Indeed, increased levels of MMPs were shown in patients with heart failure without corresponding increases in their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) [6]. In addition, while high circulating MMP-9 levels predict worse outcome and increased mortality in patients with heart failure [7,8], experimental evidence suggests that non specific inhibition of MMPs prevents hypertensive cardiovascular remodeling [9,10].

MMP activities are regulated at different levels including transcription, activation of latent forms of MMPs, and inhibition by TIMPs [5,11]. Their effects may be modified by genetic polymorphisms that affect MMP expression or activity in several pathological situations [12,13], although no differences in MMP activity were shown in healthy subjects [14]. While several polymorphisms in MMP-9 genes have been described, there is limited information about their functionality. In the present report, we studied functional polymorphisms in MMP-9 gene: the $C^{-1562}T$ SNP (rs3918242) and the microsatellite $(CA)_{14-24}$ in −90 position (rs2234681), both in the promoter and the SNP $A^{855}G$ (Q279R, rs17576) in exon 6. These

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Table 1
Clinical features of healthy controls and hypertensive subjects.

Clinical features	Healthy controls	Hypertensive	P
N	137	173	–
Gender (male/female)	65/72	69/104	NS
Race (white/black)	96/41	129/44	NS
Age (years)	55.9 ± 12.46	54.5 ± 10.7	NS
BMI (kg/m^2)	27.5 ± 7.2	31.0 ± 5.9	<0.0001[a]
SBP (mm Hg)	123 ± 15	152 ± 26	<0.0001[a]
DBP (mm Hg)	81 ± 11	89 ± 15	<0.0001[a]
Total cholesterol (mg/dl)	183 ± 33	189 ± 44	NS
HDL cholesterol (mg/dl)	43 ± 9	51 ± 16	<0.0001[a]
LDL cholesterol (mg/dl)	94 ± 27	108 ± 35	0.0001[a]
Triglycerides (mg/dl)	159 ± 67	157 ± 110	NS
Glycemia (mg/dl)	105 ± 22	114 ± 50	0.0507
LVMI ($g/m^{2.7}$)	–	75 ± 24	–
Septal thickness (mm)	–	11 ± 1.7	–
Posterior wall thickness (mm)	–	11 ± 1.7	–
Relative wall thickness (mm)	–	0.43 ± 0.08	–
End-diastolic diameter (mm)	–	51 ± 6	–
Hypertensive treatment			
Diuretics (%)	–	83.2	–
ACEi or ARB (%)	–	80.3	–
β-blockers (%)	–	53.2	–
Ca^{2+} channel blockers (%)	–	57.2	–

Data are expressed as means ± SD or number of subjects. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ACEi, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers.
Student's *t* test or χ^2 test were used to compare groups.
[a] Statistically significant.

Table 3
MMP-9 haplotypes in healthy control and hypertensive groups.

MMP-9 haplotypes	Hap-score	p	Control (RF)	Hypertensive (RF)	OR	95% confidence interval
H 1 C, L, Q	−1.8914	0.0586	0.4530	0.3787	1.0000	(Reference)
H 2 C, L, R	−2.1541	0.0312	0.0737	0.0252	0.4924	0.2049 to 1.1836
H 3 C, H, Q	1.3903	NS	0.2358	0.2802	1.3766	0.9300 to 2.0377
H 4 C, H, R	1.4865	NS	0.1317	0.1886	1.6523	1.0297 to 2.6513
H 5 T, L, Q	NA	NA	NA	0.0118	2.8462	0.5144 to 15.7494
H 6 T, L, R	NA	NA	0.0062	0.0033	2.8462	0.5144 to 15.7494
H 7 T, H, Q	NA	NA	0.0047	0.0038	2.8462	0.5144 to 15.7494
H 8 T, H, R	0.6260	NS	0.0950	0.1083	1.3490	0.7547 to 2.4111

RF: relative frequencies; OR: odds ratio; NA: not available.
Global analysis parameters: global-stat = 11.072, df = 5, p-val = 0.04998.
A value of P<0.00625 (0.05/number of haplotypes) was considered significant to correct for the number of comparisons made in haplotype analysis (Bonferroni's correction).

diameter and ejection fraction (P<0.05, Table 4). BMI was also significantly and positively associated with all parameters (P<0.05, Table 4), but relative wall thickness and ejection fraction. Septal and posterior wall thickness, LV end-diastolic diameter and LVMI were significantly lower in women (P<0.05; Table 4), while relative wall thickness and ejection fraction were significantly higher in this group (P<0.05, Table 4). After adjustment for the selected variables, we found that both LVMI and LV end-diastolic diameter were significantly influenced by MMP-9 haplotypes (P<0.05, Table 4). The analysis of haplotypes showed that the rare H7 haplotype was associated with increases in LVMI and LV end-diastolic diameter (P = 0.0015 and P = 0.0057, respectively, Table 4), whereas the H3 haplotype was associated with reduced LVMI and LV end-diastolic diameter (P = 0.0490 and P = 0.0367, respectively, Table 4). No other echocardiography parameters were affected by the studied polymorphisms.

4. Discussion

MMP activities are of major importance for collagen turnover and heart remodeling [4]. Given the particular relevance of MMP-9 in these processes, functional genetic polymorphisms of these genes may predispose to LVH in hypertensive patients. In fact, the functionality of the polymorphisms studied here has been shown in previous studies. The $C^{-1562}T$ MMP-9 polymorphism results in the loss of a nuclear repressor protein binding site, which decreases MMP-9 expression when the T allele is present, thus increasing the enzyme expression 1.5 fold compared to the C allele [15]. The microsatellite −90 $(CA)_{14-24}$ in the promoter of the MMP-9 gene causes a 50% reduction in promoter activity when the $(CA)_{14}$ allele is present, as compared to the $(CA)_{21}$ allele [16]. Finally, the Q279R polymorphism

Table 2
MMP-9 genotypes and allele relative frequencies in healthy control and hypertensive groups.

		Healthy control n (%)	Hypertensive n (%)	P	Odds ratio	95% confidence interval
Genotypes						
$C^{-1562}T$	C, C	110 (0.80)	129 (0.75)	–	1.000	(Reference)
	C, T	25 (0.18)	44 (0.25)	NS	1.501	0.8633 to 2.609
	T, T	2 (0.02)	0 (0.00)	NS	0.171	0.0081 to 3.595
$(CA)_{14-24}$	L, L	42 (0.31)	34 (0.20)	–	1.000	(Reference)
	H, L	62 (0.45)	77 (0.44)	NS	1.534	0.8741 to 2.693
	H, H	33 (0.24)	62 (0.36)	0.0085[a]	2.321	1.250 to 4.309
Q279R	Q, Q	69 (0.50)	82 (0.47)	–	1.000	(Reference)
	Q, R	52 (0.38)	72 (0.42)	NS	1.165	0.7214 to 1.882
	R, R	16 (0.12)	19 (0.11)	NS	0.999	0.4776 to 2.091
Alleles						
$C^{-1562}T$	C	245 (0.89)	302 (0.87)	–	1.000	(Reference)
	T	29 (0.11)	44 (0.13)	NS	1.231	0.7478 to 2.026
$(CA)_{14-24}$	L	146 (0.53)	145 (0.42)	–	1.000	(Reference)
	H	128 (0.47)	201 (0.58)	0.0058[a]	1.581	1.149 to 2.176
Q279R	Q	190 (0.69)	236 (0.68)	–	1.000	(Reference)
	R	84 (0.31)	110 (0.32)	NS	1.054	0.7486 to 1.485

Data are expressed as number of subjects (and relative frequencies).
χ^2 tests were used to compare alleles and genotype distributions.
[a] Statistically significant.

Table 4
Effects of MMP-9 haplotypes on echocardiography parameters after adjusting for selected variables in hypertensive patients.

Source	Septal (mm)		Thickness		Posterior wall thickness (mm)		LVMI (g/m^{2,7})		Relative thickness		Wall end-diastolic diameter		Ejection fraction	
	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE
Model	0.1606	1.5059	0.1789	1.5299	0.2294	21.3495	0.0976	0.0760	0.3038	5.3893	0.0819	0.1106		
	B	P	B	P	B	P	B	P	B	P	B	P		
Female (vs male)	−0.3643	<0.0001[a]	−0.3816	<0.0001[a]	−2.8774	0.0214[a]	0.0088	0.0466[a]	−2.8910	<0.0001[a]	0.0263	<0.0001[a]		
Age (y)	−0.0016	NS	0.0014	NS	0.0969	NS	0.0003	NS	−0.0358	NS	0.0003	NS		
BMI (kg/m^2)	0.0804	<0.0001[a]	0.0904	<0.0001[a]	1.5119	<0.0001[a]	0.0012	NS	0.2704	<0.0001[a]	−0.0006	NS		
ACEi or ARB (y)	0.0495	NS	0.0986	NS	−0.0635	NS	0.0030	NS	0.0417	NS	−0.0108	NS		
Diuretics (y)	0.0322	NS	−0.0115	NS	3.6342	0.0305[a]	−0.0170	0.0045[a]	1.8274	<0.0001[a]	0.0101	NS		
β-blockers (y)	0.0092	NS	−0.0048	NS	−1.9357	NS	0.0021	NS	−0.4050	NS	−0.0018	NS		
CCB (y)	0.0764	NS	0.1146	NS	1.1671	NS	0.0054	NS	−0.1953	NS	0.0003	NS		
MAP (mm Hg)	0.0175	0.0007[a]	0.0166	0.0015[a]	0.1832	0.0118[a]	0.0007	0.0045[a]	0.0087	NS	−0.0001	NS		
MMP-9 haplotypes														
	P=0.8644		P=0.7623		P=0.0159[a]		P=0.6715		P=0.0149[a]		P=0.5295			
	B	P	B	P	B	P	B	P	B	P	B	P		
H1 C, L, Q	−0.0346	NS	−0.1052	NS	−4.6681	NS	0.0046	NS	−0.8937	NS	−0.0312	NS		
H2 C, L, R	0.7976	NS	0.9678	NS	0.7867	NS	0.0498	NS	−1.6546	NS	−0.0009	NS		
H3 C, H, Q	−0.0437	NS	−0.1258	NS	−9.2683	0.0490[a]	0.0160	NS	−2.5064	0.0367[a]	0.0008	NS		
H4 C, H, R	0.1146	NS	0.0726	NS	−1.4736	NS	0.0034	NS	−0.1679	NS	−0.0288	NS		
H5 T, L, Q	−0.8905	NS	−0.7681	NS	−10.6012	NS	−0.0305	NS	0.4062	NS	0.0932	NS		
H6 T, L, R	−0.5944	NS	−0.4761	NS	−4.6167	NS	−0.0136	NS	−1.2926	NS	−0.0108	NS		
H7 T, H, Q	0.6889	NS	0.5664	NS	37.2929	0.0015[a]	−0.0407	NS	8.2044	0.0057[a]	−0.0086	NS		
H8 T, H, R	−0.0378	NS	−0.1318	NS	−7.4518	NS	0.0110	NS	−2.0954	NS	−0.0139	NS		

LVMI, left ventricular mass index; BMI, body mass index; MAP, mean arterial pressure; ACEi, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers, CCB, Ca^{2+} channel blockers; y: yes, using that class of drugs; H1 to H8, different MMP-9 haplotypes.
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.
The final regression model was adjusted for gender, body mass index, age, mean arterial pressure and anti-hypertensive treatment.
[a] Statistically significant (P<0.05).

modifies an amino acid residue within the highly conserved gelatinase-specific fibronectin type II domain [17], thus affecting MMP-9 activity.

The main findings of this study are: 1) the HH genotype and the H allele of the $(CA)_{14-24}$ polymorphism are associated with hypertension; 2) one MMP-9 haplotype is protective (H3) against the increases in LVMI associated with hypertension, whereas another MMP-9 haplotype (H7) possibly has detrimental effects.

The significant association that we found between the MMP-9 $(CA)_{14-24}$ HH genotype and hypertension is biologically consistent [16] and extends previous clinical reports [24]. However, although our MMP-9 haplotype analysis showed that hypertensive patients have a significantly (P=0.04998) different overall haplotype distribution when compared with normotensive controls, individual between group haplotype comparisons showed no significant differences after correction for multiple comparisons.

Importantly, we found that MMP-9 gene variants may modify LVMI and LV end-diastolic diameter in hypertensive patients. Since LV mass depends on both septal and posterior wall thickness, and LV end-diastolic diameter, it is possible that the observed differences in LVMI associated with these MMP variants result from MMP-9 affecting end-diastolic diameter at a higher extent than LV wall thickness.

Our results are consistent with the idea that increased MMP-9 activity may enhance matrix turnover [25], thus contributing to adverse myocardial remodeling associated with hypertensive heart disease [26]. Indeed, although the H7 haplotype is rare, we found that this haplotype apparently has detrimental effects on LV end-diastolic diameter (P=0.0057) and LVMI (P=0.0015), which may be explained by the fact that this haplotype combines specific MMP-9 variants associated with increased MMP-9 activity [15–17]. Conversely, the H3 haplotype was associated with beneficial effects on LV end-diastolic diameter and LVMI. Because this MMP-9 haplotype is relatively common (frequency>20%), the possible protective effect associated with this haplotype is probably more relevant than the detrimental effect associated with H7 haplotype. Interestingly, when we analyzed female and male patients separately, we found that the protective effect of H3 haplotype against LVMI is present in male patients (β=−19.2524, P=0.0059), but not in female patients (β=−3.8093, P=0.3820), thus suggesting that this effect may be gender dependent. Together, these findings support the notion that MMP-9 haplotypes modify the susceptibility of hypertensive patients to LVH.

The present study has some limitations. Firstly, we studied a relatively small number of patients. However, we found significant associations between MMP-9 gene variants and LV hypertrophy in hypertensive patients. Secondly, the patients included in the present study were under optimized pharmacological treatment. Although our statistical analysis took into consideration this factor, it may have obscured the genetic influence of MMP-9 polymorphisms. In addition, it would not be acceptable not to treat hypertensive patients. Finally, we have not carried out echocardiography studies in healthy controls. While it is possible that MMP-9 haplotypes may affect LV mass variation in healthy subjects, this is probably not the case because there is evidence indicating that MMP-9 polymorphisms or haplotypes do not affect MMP-9 levels in healthy subjects [14,27].

In conclusion, genetic polymorphisms in MMP-9 gene may modify the susceptibility of hypertensive patients to LVH. Further studies are necessary to validate our findings, and to examine whether these polymorphisms affect the incidence of clinical events in hypertensive patients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.cca.2010.08.008.

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ORIGINAL ARTICLE

Common matrix metalloproteinase 2 gene haplotypes may modulate left ventricular remodelling in hypertensive patients

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Matrix metalloproteinases (MMPs) are involved in cardiac remodelling. We examined whether MMP-2 genetic polymorphisms are associated with hypertension and left ventricular (LV) remodelling in hypertensive patients. We studied 160 hypertensive patients and 123 healthy controls. Echocardiography was performed in all patients and the $C^{-1306}T$ (rs243865) and $C^{-735}T$ (rs 2285053) MMP-2 polymorphisms were analysed. Haplo.stats analysis was used to evaluate whether MMP-2 haplotypes are associated with hypertension and with extremes in LV mass index (LVMI). Multiple linear regression analysis was performed to assess whether MMP-2 genotypes or haplotypes affect LVMI and other echocardiography parameters. The $C^{-1306}T$ 'CC' genotype was associated with reduced LVMI and LV end-diastolic diameter (EDD) ($P=0.0365$ and $P=0.0438$, respectively). The haplotype 'C, C' was associated with reduced LVMI and EDD ($P=0.0278$ and $P=0.0322$, respectively). The comparison of upper and lower extremes of the LVMI phenotype showed that the 'C, C' haplotype was more common in the lower LVMI group ($P=0.0060$), whereas the 'T, C' haplotype was more common in the higher quartile of LVMI ($P=0.0187$), and this haplotype was associated with increased risk of higher LVMI values (odds ratio = 3.5121, 95% confidence interval = 1.3193–9.3494). The findings suggest that MMP-2 polymorphisms affect hypertension-induced LV remodelling.

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Introduction

Hypertension challenges health care systems because it affects millions of people and commonly leads to cardiovascular complications. One of the major complications associated with chronic pressure overload is left ventricular hypertrophy (LVH),[1] which may progress to left ventricular (LV) dilatation and cardiac failure, a well-established risk factor for cardiovascular mortality.[2]

Among other mechanisms, mounting evidence indicates that altered activity of a group of structurally related, zinc-dependent enzymes, the matrix metalloproteinases (MMPs) is involved in LVH. Several MMPs are upregulated in cardiac hypertrophy and an imbalance between MMPs and their endogenous inhibitors (tissue inhibitor of metalloproteinases—TIMPs) may underlie the transition of compensated LVH to dilated LVH.[3–7] In addition, experimental evidence suggests that nonspecific inhibition of MMPs, especially MMP-2, ameliorates hypertensive cardiovascular remodelling.[8]

MMPs activities are regulated at different levels including transcription, activation of latent forms of MMPs and inhibition by TIMPs[5,9] In this respect, genetic polymorphisms in *MMP-2* gene may also affect MMP-2 expression or activity, as shown in several disease conditions.[10–12] Although several polymorphisms in the *MMP-2* gene have been described, there is limited information about their functionality. In the present report, we studied two functional polymorphisms in the *MMP-2* gene: the $C^{-1306}T$ SNP (rs243865) and the $C^{-735}T$ (rs 2285053), both in the promoter region of *MMP-2* gene. These polymorphisms affect MMP-2 expression[12,13] and have been associated with several diseases including cancer.[14] and cardiovascular diseases.[10,15–19] However, no previous study has examined whether combinations of *MMP-2* gene polymorphisms within

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haplotypes may affect LV modifications associated with hypertension. This issue is important because the study of MMP-2 genetic variations possibly affecting hypertensive LVH may help to identify subjects at increased cardiovascular risk.

In the present study, we compared MMP-2 allele and genotype distributions in healthy volunteers and hypertensive patients. We then examined MMP-2 haplotypes distributions in hypertensive subjects and assessed whether these haplotypes could influence LV remodelling.

Materials and methods

Subjects

This study was approved by the Human Research Ethics Committee of the University of Campinas, and informed consent was obtained from each participant. This study included 160 hypertensive patients followed up at the Hypertension Unit of the University of Campinas and 123 healthy controls. Clinical data were on the basis of medical history, physical examination and routine analytical tests. Hypertension was defined as systolic blood pressure $\geqslant 140$ mm Hg, or diastolic blood pressure $\geqslant 90$ mm Hg or current antihypertensive medication use. Exclusion criteria were age under 18 years and evidence of moderate or severe cardiac valve disease, hypertrophic cardiomyopathy, previous myocardial infarction, neoplastic disease and suspected secondary hypertension.

Blood pressure was measured using a validated digital oscillometric device (Omron HEM-705CP, Omron Healthcare, Kyoto, Japan) with appropriate cuff sizes. Body mass index (BMI) was calculated as body weight divided by height squared (expressed in kg m^{-2}).

Echocardiography

Echocardiography studies were performed on each subject at rest in the left lateral decubitus position using a Vivid 3 Pro (General Electric, Milwaukee, WI, USA) apparatus equipped with a 2.5-MHz transducer as previously described.[20] LV end-diastolic and end-systolic diameters, interventricular septum thickness, posterior wall thickness and LV mass were measured in accordance with the American Society of Echocardiography guidelines.[21] Relative wall thickness was computed as twice the posterior wall thickness divided by LV end-diastolic diameter (EDD). LV mass index (LVMI) was considered as LV mass/height,$^{2.7}$ and LVH was defined with the use of a cutoff point >51 g m$^{-2.7}$.[22] All the recordings were made by the same physician, who was unaware of other data regarding the subjects. The reproducibility of both acquiring and measuring LV mass was determined in recordings obtained from 10 subjects. Intraobserver LV mass variability was $<8\%$.

Genotyping for the $C^{-1306}T$ and the $C^{-735}T$ polymorphisms in the 5′-flanking region of MMP-2 *gene*

Venous blood samples were collected and genomic DNA was extracted from the cellular component of 1 ml of whole blood and stored at -20 °C until analysed.

Genotypes for the *$C^{-1306}T$ and the $C^{-735}T$ polymorphisms in the 5′-flanking region of MMP-2 gene* were determined by Taqman Allele Discrimination assay (Applied Biosystems, Carlsbad, CA, USA). Probes and primers used for the *$C^{-1306}T$ genotyping* assay were customised as follows: forward 5′-GCCA TTGTCAATGTTCCCTAAAACA-3′, reverse 5′-TGAC TTCTGAGCTGAGACCTGAA-3′ and probes 5′-CAG CACTC[T/C]ACCTCT-3′. TaqMan PCR was performed in a total volume of 12 µl (3 ng of DNA, $1\times$ TaqMan master mix, $1\times$ assay mix) placed in 96-well PCR plates. Fluorescence from PCR amplification was detected using Chromo 4 Detector (Bio-Rad Laboratories, Hercules, CA, USA) and analysed with manufacturer's software. Probes and primers used in MMP-2 $C^{-735}T$ assay were designed by Applied Biosystems (ID: C_26734093-20). TaqMan PCR and fluorescence reading were performed as described above for the $C^{-1306}T$ polymorphism.

Statistical analysis

Differences in genotypes and alleles distributions and deviations from the Hardy–Weinberg equilibrium were assessed using χ^2-tests (Stat-View for Windows; SAS Institute, Cary, NC, USA). The Haplo.stats package (version 1.4.4; http://www.r-project.org) was used to estimate the haplotype frequencies. The function haplo.em computes maximum likelihood estimates of haplotype probabilities using the progressive insertion algorithm, which progressively inserts batches of loci into haplotypes of growing lengths. The function haplo.score was used to compute haplotype-specific score statistics to test for association,[23] with the value of $P<0.05$ considered statistically significant. Only the haplotypes with frequencies $>1\%$ were taken into consideration for the haplotype-specific score analysis. The function haplo.cc was also performed to calculate odds ratio and 95% confidence intervals for each haplotype. The possible haplotypes including genetic variants of two polymorphisms in the *MMP-2* gene studied, $C^{-1306}T$ and $C^{-735}T$ were: H1 (C, C); H2 (C, T); H3 (T, C); H4 (T, T).

Linear regression analysis and non-linear fitting routines were performed to assess univariate relations between variables (software JMP 5.0.1a; SAS Institute). In addition, a bivariate analysis was also used to assess for the potential confounding influence of each covariate on the relation between MMP-2 haplotypes and LVMI. The variables of clinical importance, as identified by the bivariate approach, were then included in the final multiple linear regression models. Septal thickness, posterior

wall thickness, LVMI, relative wall thickness and EDD were considered as dependent variables. MMP-2 haplotypes, gender, BMI, age, pharmacological treatment and systolic blood pressure were considered as independent variables.

Results

Clinical features of studied subjects are shown in Table 1. We found no differences in age, gender, race, cholesterol and triglycerides concentrations between the study groups. However, hypertensive patients had increased arterial pressure and BMI when compared with healthy controls ($P<0.05$; Table 1).

Alleles and genotypes distributions are presented in Table 2. The distribution of genotypes for each polymorphism showed no deviation from Hardy–Weinberg equilibrium. Genotypes and alleles distributions showed no significant differences when healthy and hypertensive groups were compared ($P>0.05$; Table 2). The haplotypes distribution showed no significant differences between healthy and hypertensive groups (global $P=0.9748$, Table 3).

To determine the influence of MMP-2 genotypes or haplotypes on echocardiography parameters, we performed a multiple linear regression analysis for genotypes (Table 4) and another for haplotypes (Table 5), both adjusted for gender, BMI, age, systolic blood pressure and pharmacological treatment.

The systolic blood pressure was significantly and positively associated with septal thickness and LVMI in both models and with posterior wall thickness in haplotypes model. BMI was significantly and positively associated with all parameters in both models. Female gender was associated with reduced posterior wall thickness, LV and septal thickness in both models, whereas age was associated with increased LVMI in haplotype model. Finally, the usage of diuretics was also positively associated with EDD. After adjustment for selected variables, we found that both LV EDD and LVMI were significantly influenced by MMP-2 genotypes and haplotypes (Tables 4 and 5). Although the analysis of genotypes showed that the CC genotype for the $C^{-1306}T$ polymorphism was associated with reduced LV EDD (Estimate (B) $=-2.1171$, $P=0.0438$) and LVMI (B $=-0.0499$, $P=0.0365$), no effect was attributed to any of the $C^{-735}T$ genotypes. Moreover, the analysis of haplotypes showed that the ancestral H1 (C, C) haplotype is associated with reduced EDD (B $=-0.9791$, $P=0.0322$) and LVMI (B $=-0.0228$, $P=0.0278$). No other echocardiography parameters were affected by the studied polymorphisms and haplotypes.

On the basis of these results, we performed a new analysis using Haplostats (Table 6). We compared the upper and lower extreme quartiles of the LVMI phenotype. We found that while the H1 (C, C) haplotype was more common in the lower LVMI group ($P=0.0060$), the H3 (T, C) haplotype was more common in the highest quartile of LVMI ($P=0.0187$). In addition, the H3 haplotype showed

Table 1 Clinical features of healthy and hypertensive subjects

Clinical features	*Healthy controls*	*Hypertensive*	P
N	123	160	—
Gender (male/female)	49/74	64/96	0.9779
Race (white/black)	81/42	119/41	0.1186
Age (years)	56.2 ± 13.0	55.6 ± 9.5	0.6561
BMI (kg m^{-2})	28.1 ± 3.9	31.2 ± 5.8	0.0475[a]
SBP (mmHg)	120 ± 18	152 ± 26	<0.0001[a]
DBP (mmHg)	82 ± 14	88 ± 14	0.0514
Total cholesterol (mg dl^{-1})	185 ± 31	189 ± 42	0.7198
HDL cholesterol (mg dl^{-1})	48 ± 7	51 ± 16	0.0533
LDL cholesterol (mg dl^{-1})	103 ± 28	110 ± 35	0.0705
Triglycerides (mg dl^{-1})	141 ± 52	154 ± 88	0.6862
Glycemia (mg dl^{-1})	107 ± 21	114 ± 50	0.1030
LVMI (g m$^{-2.7}$)	—	75 ± 24	—
Septal thickness (mm)	—	11 ± 1.7	—
Posterior wall thickness (mm)	—	11 ± 1.7	—
Relative wall thickness (mm)	—	0.43 ± 0.07	—
End diastolic diameter (mm)	—	51 ± 6	—
Hypertensive treatment			
Diuretics (%)	—	83.1	—
ACEi or ARB (%)	—	81.9	—
β-blockers (%)	—	51.9	—
Ca^{2+} channel blockers (%)	—	56.9	—

Abbreviations: ACEi, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers; BMI, body mass index; DBP, diastolic blood pressure; LVMI, left ventricular mass index; SBP, systolic blood pressure.
Data are expressed as means ± s.d. or number of subjects.
Student *t*-test or χ^2-test were used to compare groups.
[a]Statistically significant.

Table 2 MMP-2 genotypes and alleles relative frequencies (RF) in healthy control and hypertensive groups

	Healthy control n (RF)	*Hypertensive n (RF)*	P	*Odds ratio*	*95% Confidence interval*
Genotypes					
$C^{-1306}T$					
C, C	88 (0.72)	113 (0.71)	—	1.000	(Reference)
C, T	30 (0.24)	43 (0.27)	0.6915	1.116	0.648–1.922
T, T	5 (0.04)	4 (0.02)	0.4866	0.623	0.162–2.390
$C^{-735}T$					
C, C	99 (0.80)	128 (0.80)	—	1.000	(Reference)
C, T	24 (0.20)	31 (0.19)	0.9974	0.999	0.552–1.810
T, T	0 (0.00)	1 (0.01)	0.3800	2.323	0.094–57.680
Alleles					
$C^{-1306}T$					
C	206 (0.84)	269 (0.84)	—	1.000	(Reference)
T	40 (0.16)	51 (0.16)	0.9175	0.976	0.621–1.535
$C^{-735}T$					
C	222 (0.90)	287 (0.90)	—	1.000	(Reference)
T	24 (0.10)	33 (0.10)	0.8274	1.064	0.611–1.852

Abbreviation: MMPs, matrix metalloproteinases.
Data are expressed as number of subjects (and relative frequencies).
χ^2-tests were used to compare alleles and genotypes distributions.

Table 3 MMP-2 haplotypes in healthy control and hypertensive groups

MMP-2 haplotypes	*Hap-score*	P	*Control (RF)*	*Hypertensive (RF)*	*OR*	*95% Confidence interval*
H 1 C, C	−0.0191	0.9848	0.7446	0.7391	1.0000	(Reference)
H 2 C, T	0.2009	0.8408	0.0928	0.1025	1.0559	0.5901–1.8893
H 3 T, C	−0.1328	0.8943	0.1578	0.1584	0.9766	0.6245–1.5273
H 4 T, T	NA	NA	0.0048	0.0000	NA	NA

Abbreviations: MMPs, matrix metalloproteinases; NA, not available; OR, Odds Ratio; RF, relative frequencies.
Global analysis parameters: global-stat = 0.0512, d.f. = 2, *P*-val = 0.9748.

Table 4 Effects of MMP-2 genotypes on echocardiography parameters in hypertensive patients, after adjusting for selected variables

Source	*Septal thickness (mm)*		*Posterior wall thickness (mm)*		*LVMI ($g\,m^{-2.7}$)*		*Relative wall thickness*		*End-diastolic diameter*	
	Rsquare	*RMSE*	*Rsquare*	*RMSE*	*Rsquare*	*RMSE*	*Rsquare*	*RMSE*	*Rsquare*	*RMSE*
Model	0.1790	1.5774	0.2061	1.5268	0.2451	0.1192	0.1092	0.0724	0.2638	5.2442
	B	*P*	*B*	*P*	*B*	*P*	*B*	*P*	*B*	*P*
Female (vs male)	−0.3108	0.0250[a]	−0.3571	0.0080[a]	−0.0094	0.3657	0.0067	0.2887	−2.5123	<0.0001[a]
Age (years)	0.0007	0.9595	0.0082	0.5431	0.0015	0.1640	0.0002	0.8094	0.0170	0.7134
BMI ($kg\,m^{-2}$)	0.0900	0.0001[a]	0.0989	<0.0001[a]	0.0077	<0.0001[a]	0.0024	0.0223[a]	0.1864	0.0159[a]
ACEi or ARB (y)	0.0452	0.7950	0.0727	0.6657	0.0007	0.9558	0.0024	0.7614	−0.0137	0.9810
Diuretics (y)	0.1810	0.3167	0.1734	0.3215	0.0186	0.1737	−0.0031	0.7081	1.1624	0.0541
β-blockers (y)	−0.0608	0.6449	−0.0526	0.6807	−0.0129	0.1969	0.0001	0.9824	−0.3741	0.3942
CCB (y)	−0.0213	0.8751	0.0444	0.7352	0.0067	0.5118	0.0009	0.8914	−0.0921	0.8380
SBP (mmHg)	0.0113	0.0319[a]	0.0091	0.0720	0.0008	0.0398[a]	0.0004	0.1326	0.0121	0.4870
MMP-2 $C^{-1306}T$ genotypes										
	P = 0.6373		*P* = 0.4534		*P* = 0.0300[a]		*P* = 0.8652		*P* = 0.0434[a]	
	B	*P*	*B*	*P*	*B*	*P*	*B*	*P*	*B*	*P*
C, C	−0.2478	0.4300	−0.3393	0.2647	−0.0499	0.0365[a]	0.0054	0.7089	−2.1171	0.0438[a]
C, T	0.0045	0.9890	−0.0486	0.8766	0.0041	0.8683	−0.0016	0.9148	0.0986	0.9270
T, T	0.2522	0.6548	0.3879	0.4777	0.0459	0.2826	−0.0038	0.8837	2.0185	0.2827
MMP-2 $C^{-735}T$ genotypes										
	P = 0. 0726		*P* = 0.3006		*P* = 0.5321		*P* = 0.0712		*P* = 0.2401	
	B	*P*	*B*	*P*	*B*	*P*	*B*	*P*	*B*	*P*
C, C	−1.0088	0.0791	−0.7886	0.1554	−0.0360	0.4058	−0.0368	0.1624	0.6784	0.7211
C, T	−1.3395	0.0230	−0.8644	0.1278	−0.0120	0.7866	−0.0587	0.0298	2.3669	0.2241
T, T	2.3484	0.0350	1.6530	0.1239	0.0479	0.5667	0.0955	0.0614	−3.0454	0.4079

Abbreviations: ACEi, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers; B, parameter estimates; BMI, body mass index; CCB, Ca^{2+} channel blockers; LVMI, left ventricular mass index; MMPs, matrix metalloproteinases; MAP, mean arterial pressure; Rsquare, 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; y, yes, using that class of drugs.
[a]Statistically significant ($P<0.05$). The final regression model was adjusted for gender, body mass index, age, mean arterial pressure and anti-hypertensive treatment.

a remarkable increased risk of higher LVMI values (odds ratio = 3.5121, 95% confidence interval = 1.3193–9.3494).

Discussion

MMPs are major players in extracellular matrix turnover and myocardial remodelling.[6,7,9] Given the relevance of MMP-2 in these processes, functional genetic polymorphisms of this gene may affect the prevalence of LVH in hypertensive patients. This is the first report to show a protective effect associated with the CC genotype of the $C^{-1306}T$ polymorphism against increases in EDD and LVMI in hypertensive subjects. This finding aligns with a protective effect previously reported after stroke in patients carrying the C allele of this polymorphism.[10] Moreover, we found that the H1 (C, C) haplotype protects against the increases in EDD and LVMI found in hypertensive patients. A more sophisticated analysis comparing the extremes of LVMI phenotype (upper *versus* lower quartiles) confirmed the protective effect associated with this particular haplotype. As LV mass depends on both LV wall thickness and LV EDD, our results suggest that the influence

Table 5 Effects of MMP-2 haplotypes on echocardiography parameters in hypertensive patients, after adjusting for selected variables

Source	*Septal thickness (mm)*		*Posterior wall thickness (mm)*		*LVMI ($g\,m^{-2.7}$)*		*Relative wall thickness*		*End-diastolic diameter*	
	Rsquare	*RMSE*	*Rsquare*	*RMSE*	*Rsquare*	*RMSE*	*Rsquare*	*RMSE*	*Rsquare*	*RMSE*
Model	0.1455	1.5697	0.1881	1.5060	0.2231	0.1180	0.0774	0.0719	0.2377	5.2054
	B	*P*	*B*	*P*	*B*	*P*	*B*	*P*	*B*	*P*
Female (vs male)	−0.3124	0.0011[a]	−0.3662	<0.0001[a]	−0.0116	0.1059	0.0073	0.0942	−2.6060	<0.0001[a]
Age (years)	0.0058	0.5512	0.0119	0.2021	0.0017	0.0230[a]	0.0001	0.4611	0.0157	0.6256
BMI ($kg\,m^{-2}$)	0.0906	<0.0001[a]	0.1010	<0.0001[a]	0.0081	<0.0001[a]	0.0024	0.0014[a]	0.2030	0.0001[a]
ACEi or ARB (y)	−0.0041	0.9730	0.0443	0.7019	0.0009	0.9191	−0.0001	0.9943	0.1128	0.7778
Diuretics (y)	0.1078	0.3860	0.1149	0.3357	0.0156	0.0965	−0.0055	0.3318	1.1649	0.0050[a]
β-blockers (y)	−0.0598	0.5162	−0.0439	0.6190	−0.0107	0.1216	−0.0008	0.8482	−0.2640	0.3874
CCB (y)	−0.0117	0.9001	0.0487	0.5871	0.0073	0.2985	0.0011	0.7940	−0.0968	0.7548
SBP (mmHg)	0.0099	0.0067[a]	0.0081	0.0206[a]	0.0007	0.0073[a]	0.0003	0.0511	0.0108	0.3711
MMP-2 Haplotypes										
	P = 0.6501		*P* = 0.5191		*P* = 0.0372[a]		*P* = 0.7638		*P* = 0.0458[a]	
	B	*P*	*B*	*P*	*B*	*P*	*B*	*P*	*B*	*P*
H1 C, C	−0.0561	0.6832	−0.1217	0.3560	−0.0228	0.0278[a]	0.0046	0.4635	−0.9791	0.0322[a]
H2 C, T	−0.1072	0.5958	−0.0279	0.8855	−0.0014	0.9276	−0.0031	0.7376	−0.0415	0.9506
H3 T, C	0.1632	0.3673	0.1496	0.3890	0.0242	0.0762	−0.0015	0.8852	1.0206	0.0897
H4 T, T	—	—	—	—	—	—	—	—	—	—

Abbreviations: ACEi, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers, BMI, body mass index; B, parameter estimates; CCB, Ca^{2+} channel blockers; LVMI, left ventricular mass index; MAP, mean arterial pressure; MMPs, matrix metalloproteinases; H1 to H4, different MMP-2 haplotypes. H4 haplotype was not observed in hypertensive group; Rsquare, 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; y, yes, using that class of drugs.
[a]Statistically significant ($P<0.05$).
The final regression model was adjusted for gender, body mass index, age, mean arterial pressure and anti-hypertensive treatment.

Table 6 MMP-2 haplotypes in lower ($n=40$) and upper ($n=40$) LVMI groups

MMP-2 haplotypes	*Hap-score*	P	*Lower LVMI (RF)*	*Upper LVMI (RF)*	*OR*	*95% Confidence interval*
H1 C, C	−2.7456	0.0060[a]	0.8375	0.6625	1.0000	(Reference)
H2 C, T	0.9092	0.3633	0.0625	0.1000	2.6024	0.7294–9.2845
H3 T, C	2.3512	0.0187[a]	0.1000	0.2375	3.5121	1.3193–9.3494[a]
H4 T, T	NA	NA	0.0000	0.0000	NA	NA

Abbreviations: MMPs, matrix metalloproteinases; NA, not available; OR, Odds Ratio; RF, relative frequencies.
Global analysis parameters: global-stat = 7.6621, df = 2, *P*-val = 0.0217*.
[a]Statistically significant.

of MMP-2 haplotypes on LVMI was related to modifications in chamber diameter rather than to alterations in wall thickness. To our knowledge, no previous study has examined whether MMP-2 polymorphisms or haplotypes are associated with hypertension, although experimental evidence has implicated MMP-2 in the pathogenesis of hypertension[24–27] and in hypertensive LVH.[8]

Previous studies suggest that genetic polymorphisms modify MMP-2 expression or activity.[10–12] For instance, the $C^{-1306}T$ polymorphism disrupts a Sp1-type promoter site (CCACC box), thus causing a 1.6-fold increase in the promoter activity when the C allele is present as compared with the T allele.[13] In addition, similar mechanisms explain a threefold increase in MMP-2 promoter activity when the C allele of the $C^{-735}T$ polymorphism is present.[12] Consistent with these findings, the MMP-2 haplotype combining both C alleles of both polymorphisms causes remarkable increases in MMP-2 expression.[12] In cardiac hypertrophy, it has been shown that the overall MMP gelatinolytic activity is increased and has crucial roles in the development of LVH and in the progression to decompensated LVH and heart failure.[6,7,28] Although our results suggesting a protective role for a MMP-2 polymorphism and haplotype associated with higher MMP-2 expression may seem paradoxical, little is known about the specific role for each MMP in this process and the possible signalling pathways that depend on them.[29] In this respect, transgenic models have contributed to the understanding of the roles for

MMPs in LVH, and MMP-2 knockout mice present interesting reductions in LVH and its complications in models of heart hypertrophy.[30] These mice have increased TIMP-4 levels in the heart, and TIMP-4 is a major MMPs inhibitor.[31] Whether the reduced LVH seen in these animals is because of lack of MMP-2 activity or to the inhibitory effects of TIMP-4 on various MMPs is not known. In addition, other MMPs, including MMP-9, may be involved in LVH associated with hypertension.[32] It is also relevant to mention that many other actions independent of matrix degradation have been attributed to MMPs, especially MMP-2, and these actions may be very relevant to cardiac remodelling.[29,33,34] Although we do not have a mechanistic explanation for the associations being reported here, it is clear that complex interactions of factors regulate MMPs activities in the heart.

The present study has some limitations. First, we studied a relatively small number of patients. However, we found significant associations between *MMP-2* gene variants or haplotypes and LVH in hypertensive patients. Second, the patients included in the present study were under pharmacological treatment. It is clearly unacceptable not to treat hypertensive patients. However, although our statistical analysis took into consideration this factor, it may have obscured the genetic influence of MMP-2 polymorphisms. Despite these limitations, it is important to consider that the H1 (C, C) and H3 (T, C) haplotypes are common haplotypes (>10% frequency), thus increasing their importance. Finally, we should state that few of our healthy controls may have subclinical hypertension or metabolic syndrome, as some of the clinical features of these subjects may be slightly above normal limits.

In conclusion, genetic polymorphisms in *MMP-2* gene may be risk factors for the development of hypertension-induced LV remodelling. Further studies are necessary to validate our findings, and to examine whether these polymorphisms affect long-term outcomes in hypertensive patients and maybe targeted therapies such as MMPs inhibition before LVH is present.

What is known about this topic

- MMPs are involved in cardiac remodelling associated with different conditions.
- Genetic polymorphisms in the *MMP-2* gene ($C^{-1306}T$ and $C^{-735}T$) affect MMP-2 levels and activity, and may modify disease susceptibility.

What this study adds

- The CC genotype for the MMP-2 $C^{-1306}T$ polymorphism is associated with lower left ventricular mass index and end-diastolic diameter in hypertensive subjects.
- The H1 (C, C) haplotype exerts protective and the H3 (T, C) haplotype exerts detrimental effects on left ventricular mass and diameter in hypertensive subjects.

Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL RESEARCH ARTICLES

Interethnic Differences in the Distribution of Matrix Metalloproteinases Genetic Polymorphisms Are Consistent with Interethnic Differences in Disease Prevalence

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Interethnic differences exist in disease prevalence, especially with regard to cancer and cardiovascular diseases, which involve altered expression or activity of matrix metalloproteinases (MMPs). The hypothesis being tested in this study is that interethnic differences exist between blacks and whites with regard to the distribution of genetic variants of *MMP* polymorphisms and haplotypes. We examined the distribution of polymorphisms of *MMP-2* and *MMP-9* genes in 177 black and 140 white subjects. We studied the following polymorphisms: the $C^{-1306}T$ in the promoter of the *MMP-2* gene, the $C^{-1562}T$ and a microsatellite $-90(CA)_{14-24}$ in the promoter, and the Q279R in exon 6 of the *MMP-9* gene. We have also compared our results with those from Hapmap or Seattle SNPs Projects and estimated the haplotype frequency in these two ethnic groups. The "C" allele for the $C^{-1306}T$ polymorphism was more common in blacks (91.5%) than in whites (80.4%; $p < 0.0001$). The "T" allele for the $C^{-1562}T$ polymorphism was more common in blacks (15.0%) than in whites (8.9%; $p = 0.0279$), as well as the alleles with >21 repeats for the $-90(CA)_{14-24}$ were more common in blacks than in whites (61.9% in blacks and 49.3% in whites; $p = 0.0017$). We found no interethnic differences for the Q279R polymorphism. Moreover, two haplotypes that combine "detrimental" alleles were found at higher frequencies in blacks than in whites (31% vs. 16.4%, respectively; $p < 0.05$). The interethnic differences being reported here replicate those previously found with smaller number of subjects in the Hapmap or Seattle SNPs data and may help explain the higher prevalence of cancer and cardiovascular diseases in blacks compared with whites. Our findings suggest a proportional significance of these polymorphisms in each ethnic group.

Introduction

Matrix metalloproteinases (MMPs) are a family of structurally related, zinc dependent enzymes that degrade several components of the extracellular matrix (Nagase and Woessner, 1999). These enzymes are involved in tissue remodeling and histological alterations in several disease processes (Egeblad and Werb, 2002; Ahmed *et al.*, 2006; Spinale, 2007). Among others, MMP-2 and MMP-9 (gelatinase A and gelatinase B, respectively) play key roles in the pathogenesis of many diseases. In fact, a number of experimental and clinical studies have shown altered levels of MMP-2 or MMP-9 in many disease conditions including hypertensive (Castro *et al.*, 2008, 2010), neurologic (Fernandes *et al.*, 2009; Martins-Oliveira *et al.*, 2009), respiratory (Fortuna *et al.*, 2007; Uzuelli *et al.*, 2008), and metabolic (Martinez *et al.*, 2008b; Belo *et al.*, 2009; Goncalves *et al.*, 2009) disorders; periodontal disease (Marcaccini *et al.*, 2009a, 2009b); and cancer (Coussens and Werb, 1996; Egeblad and Werb, 2002). The role of MMPs in disease processes is becoming progressively clearer with the use of MMPs inhibitors or other drugs that can downregulate MMPs activities and improve disease conditions (Souza-Costa *et al.*, 2005, 2007; Martinez *et al.*, 2006, 2008a, 2008b; Castro *et al.*, 2009).

Most of our knowledge about many disease conditions is derived from studies of Caucasian populations (Forouhi and Sattar, 2006). However, the incidence of disease conditions varies according to ethnicity. For example, African American women are exposed to higher risks of cardiovascular deaths compared with Caucasian Europeans, although this difference may not be present when comparing African descendents from different locations (Forouhi and Sattar, 2006). Moreover, it seems that malignant hypertension and related renal complications are more frequent in blacks than in whites (van den Born *et al.*, 2006). Racial differences have also been found with regard to cancer: white men seem to have fourfold higher incidence of esophageal cancer than African American men (Kubo and Corley, 2004), and white women

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have higher incidence of breast cancer than African American women (Ghafoor *et al.*, 2003). Although many factors including socioeconomic and environmental conditions may underlie these interethnic differences in disease susceptibility, it is now becoming clear that genetic differences among populations may also contribute (Tanus-Santos *et al.*, 2001; Montenegro *et al.*, 2006; Rezende *et al.*, 2007; Muniz *et al.*, 2009). No previous study has examined whether significant differences exist in the distribution of genetic variants of *MMP* polymorphisms and haplotypes when comparing black and white Brazilians.

The hypothesis being tested in this study is that interethnic differences exist between blacks and whites with regard to the distribution of genetic variants of *MMP* polymorphisms and haplotypes. These differences could help explain interethnic differences in disease prevalence. Therefore, we studied one clinically relevant polymorphism in the *MMP-2* gene, a single-nucleotide polymorphism (SNP) in the promoter region ($C^{-1306}T$, rs243865), and three clinically relevant polymorphisms in the *MMP-9* gene: an SNP ($C^{-1562}T$, rs3918242) and a microsatellite ($-90(CA)_{14-24}$, rs2234681) in the promoter of the *MMP-9* gene and an SNP in exon 6, $A^{855}G$ (rs17576). We have also compared our results with those available in relevant databanks (Hapmap or Seattle SNPs Projects) and estimated the haplotype frequency in these two ethnic groups.

Materials and Methods

Subjects

Approval for use of human blood was obtained from the Institutional Review Board at Faculty of Medicine of Ribeirao Preto and at State University of Santa Cruz. One hundred and seventy-seven healthy subjects self reported as black and 140 healthy subjects self reported as white (total $n = 317$; age range: 18–56 years) were recruited to give blood after informed consent was obtained. Black subjects were recruited from Salvador (Bahia, Brazil), whereas white subjects were recruited in Ribeirao Preto (Sao Paulo, Brazil). Venous blood samples were collected, and 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.

Genotyping

$C^{-1306}T$ polymorphism in the 5′-flanking region of *MMP-2* gene. Genotypes for the $C^{-1306}T$ polymorphism in the 5′-flanking region of *MMP-2* were determined by Taqman® Allele Discrimination assay. Probes and primers used in *MMP-2* assay were customized as follows: forward 5′-GCC ATTGTCAATGTTCCCTAAAACA-3′, reverse 5′-TGACTTC TGAGCTGAGACCTGAA-3′, and probes 5′-CAGCACTC[T/C]ACCTCT-3′. TaqMan polymerase chain reaction (PCR) was performed in a total volume of 12 µL (3 ng of dried DNA, 1× TaqMan master mix, 900 nM of each primer, and 200 nM of each probe) placed in 96-well PCR plates. Fluorescence from polymerase chain reaction amplification was detected using Chromo 4 Detector (Bio-Rad Laboratories) and analyzed with manufacturer's software.

$C^{-1562}T$ polymorphism in the 5′-flanking region of *MMP-9* gene. Genotypes for the $C^{-1562}T$ polymorphism in the 5′-flanking region of *MMP-9* were determined by restriction fragment length polymorphism, as described earlier (Demacq *et al.*, 2006). Briefly, products amplified by polymerase chain reaction were digested with *Sph*I (New England Biolabs) overnight at 37°C, producing fragments of 247 and 188 bp (allele T), or an undigested 435-bp product (allele C). Fragments were separated by electrophoresis in 12% polyacrylamide gels and visualized after silver staining.

$-90(CA)_{14-24}$ microsatellite in the 5′-flanking region of *MMP-9* gene. The $-90(CA)_{14-24}$ microsatellite was detected by polymerase chain reaction, as described earlier (Demacq *et al.*, 2009). Amplified products were separated in a 7% polyacrylamide–urea gel and visualized after silver staining. Products were always compared with 10-bp DNA ladder (Invitrogen) and with homozygous samples previously sequenced. Alleles were grouped as "low" (L) when the number of CA repeats was less than 21 and as "high" (H) when the number of CA repeats was 21 or more (Demacq *et al.*, 2008).

Q279R polymorphism in exon 6 of *MMP-9* gene. Genotypes for the *MMP-9* Q279R polymorphism were determined by Taqman Allele Discrimination assay. Probes and primers used in *MMP-9* assay were designed by Applied Biosystems (ID: C_11655953_10). TaqMan polymerase chain reaction and fluorescence reading were performed, as described earlier, for the $C^{-1306}T$ polymorphism.

Estimation of linkage disequilibrium

The Estimating Haplotype (EH) software program (http://linkage.rockefeller.edu/ott/eh.htm; assessed on January 15, 2010) was used to perform a linkage analysis between each pair-wise combination of variants, as previously described (Marroni *et al.*, 2005). Briefly, this program was used to calculate the maximum-likelihood estimate of disequilibrium (D'), which is a standard measure of linkage disequilibrium (LD). The estimated disequilibrium D' values for each pair-wise combination of variants were calculated as $D' = D/D_{max}$, where $D = (h - p)\, q$. Here, p and q are the frequencies for the rarer variants of the two polymorphisms being tested for linkage, such that $p < q \leq 0.5$ and h is the frequency of the haplotype including two specific variants. When $D < 0$, $D_{max} = -p \times q$; when $D > 0$, $D_{max} = p\,(1 - q)$. Thus, D' values can vary from +1 to −1, with a positive D' indicating that the rarer variants are associated, and a negative D' indicating that the rarer variant of one polymorphism is associated with the common variant at the other locus.

Haplotypes were inferred using the Bayesian statistical-based program PHASE version 2.1 (www.stat.washington.edu/stephens/software.html) (Stephens *et al.*, 2001) to estimate the haplotype frequencies in the two ethnic groups. The possible haplotypes including genetic variants of three *MMP-9* polymorphisms studied (C-1562T, $-90(CA)_{14-24}$, and Q279R) were H1 (C, L, Q); H2 (C, L, R); H3 (C, H, Q); H4 (C, H, R); H5 (T, L, Q); H6(T, L, R); H7 (T, H, Q); and H8 (T, H, R).

Statistical analysis

The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium

TABLE 1. GENOTYPE AND ALLELE FREQUENCY IN THE TWO ETHNIC GROUPS STUDIED HERE AND IN OTHER POPULATIONS

		This study		*Other populations*[a]	
		Blacks	*Whites*	*Blacks*	*Whites*
Genotypes					
MMP-2		$n=177$	$n=140$	$n=113$	$n=113$
$C^{-1306}T$	C, C	0.859 (152)	0.643 (90)[b]	0.947 (107)[c]	0.575 (65)
	C, T	0.113 (20)	0.321 (45)[b]	0.053 (6)[c]	0.363 (41)
	T, T	0.028 (5)	0.036 (5)[b]	0 (0)[c]	0.062 (7)
MMP-9		$n=177$	$n=140$	$n=48$	$n=46$
$C^{-1562}T$	C, C	0.706 (125)	0.829 (116)[d]	0.667 (32)	0.739 (34)
	C, T	0.288 (51)	0.164 (23)[d]	0.292 (14)	0.261 (12)
	T, T	0.006 (1)	0.007 (1)[d]	0.042 (2)	0 (0)
		$n=177$	$n=140$		
$(CA)_{14-24}$	L, L	0.209 (37)	0.271 (38)[d]	Not available	
	H, L	0.345 (61)	0.471 (66)[d]		
	H, H	0.446 (79)	0.257 (36)[d]		
		$n=177$	$n=140$	$n=113$	$n=113$
Q279R	Q, Q	0.441 (78)	0.443 (62)	0.389 (44)[c]	0.381 (43)
	Q, R	0.350 (62)	0.421 (59)	0.496 (56)[c]	0.513 (58)
	R, R	0.209 (37)	0.136 (19)	0.115 (13)[c]	0.106 (12)
Alleles					
MMP-2		$n=354$	$n=280$	$n=226$	$n=226$
$C^{-1306}T$	C	0.915 (324)	0.804 (225)[b]	0.973 (220)[c]	0.757 (171)
	T	0.085 (30)	0.196 (55)[b]	0.027 (6)[c]	0.243 (55)
MMP-9		$n=354$	$n=280$	$n=96$	$n=92$
$C^{-1562}T$	C	0.850 (301)	0.911 (255)[d]	0.812 (78)	0.870 (80)
	T	0.150 (53)	0.089 (25)[d]	0.188 (18)	0.130 (12)
		$n=354$	$n=280$		
$(CA)_{14-24}$	L	0.381 (135)	0.507(142)[d]	Not available	
	H	0.619 (219)	0.493 (138)[d]		
		$n=354$	$n=280$	$n=226$	$n=226$
Q279R	Q	0.616 (218)	0.654 (183)	0.637 (144)	0.637 (144)
	R	0.384 (136)	0.346 (97)	0.363(82)	0.363 (82)

n = number of subjects for genotyped subjects (genotype) or number of alleles (allele).
[a]Data obtained from Hapmap database (www.hapmap.org; The International Haplotype Map Project) or Seattle SNPs (http://pga.mbt.washington.edu/); Hapmap black subjects are from Yoruba in Ibadan, Nigeria, and white subjects from Utah with ancestry from northern and western Europe; Seattle SNPs black subjects are African Americans and white subjects are Europeans.
[b]<0.001 comparison between black and whites in our study (chi-squared test).
[c]<0.05 comparison between black groups in our study and in other populations (chi-squared test).
[d]<0.05 comparison between black and whites in our study (chi-squared test).
MMP, matrix metalloproteinases; SNP, single-nucleotide polymorphism.

by using chi-squared tests (StatView for Windows). Differences in the genotype frequency of each polymorphism and in the allele frequency between the two ethnic groups were also assessed with chi-squared tests. A value of $p<0.05$ was considered statistically significant.

Differences in haplotype frequency distributions were further tested using chi-squared tests, and to compare specific haplotypes frequencies in blacks and whites, a value of $p<0.00625$ (0.05/number of haplotypes) was considered significant to correct for the number of comparisons made.

Results

Table 1 shows the frequency of genotypes and alleles in blacks and whites included in this study and in subjects from Hapmap or Seattle SNPs Projects. The distribution of genotypes for each polymorphism showed no deviation from Hardy–Weinberg equilibrium. We found a notable disparity between black and white subjects with regard to the distribution of genotypes and variants for all polymorphisms (Fig. 1 and Table 1; all $p<0.05$), except for the Q279R. The C allele in *MMP-2* $C^{-1306}T$ was more common in blacks (91.5%) than in whites (80.4%; $p<0.0001$). With regard to the *MMP-9* polymorphisms, the T allele for the $C^{-1562}T$ polymorphism was more common in blacks (15.0%) than in whites (8.9%; $p=0.0279$). In addition, the H allele for the $-90(CA)_{14-24}$ polymorphism was also more common in blacks (61.9%) than in whites (49.3%; $p=0.0017$).

Interestingly, although we found slight differences in allele frequency between the data retrieved from Hapmap or Seattle SNPs and our data (see Table 1: small but significant differences for the $C^{-1306}T$ and for the Q279R polymorphisms), the interethnic differences being reported here replicate those previously found with smaller number of subjects in the Hapmap or Seattle SNPs data.

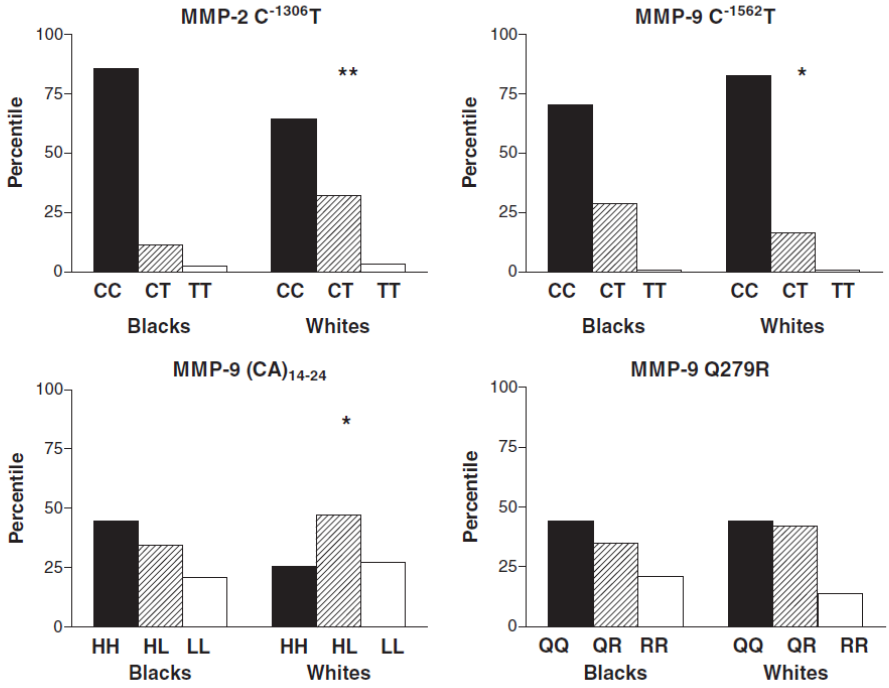

FIG. 1. Percentile distribution of genotypes in blacks and whites. Closed bars, wild-type homozygotes; diagonal, heterozygotes; Open bars, homozygote variants. *$p < 0.05$ compared with the other ethnic groups (chi-squared tests). **$p < 0.001$ vs. the other ethnic group.

Table 2 shows the haplotypes distribution in the two ethnic groups. As anticipated, the most common haplotype for the two ethnic groups combines the wild type alleles for three polymorphisms. After Bonferroni's correction for multiple comparisons, we found that the H1 (C, L, Q) haplotype was more common in whites (48.9%) than in blacks (29.1%; $p < 0.0001$). Conversely, the H2 (C, L, R), H3 (C, H, Q), and H7 (T, H, Q) haplotypes were more frequent in blacks than in whites.

TABLE 2. MATRIX METALLOPROTEINASE-9 HAPLOTYPES IN BLACKS AND WHITES GROUPS

MMP-9 *haplotypes*		*Blacks* n = *354*	*Whites* n = *280*	p
H1	C, L, Q	0.291 (103)	0.489 (137)	<0.0001[a]
H2	C, L, R	0.071 (25)	0.014 (4)	0.0008[a]
H3	C, H, Q	0.268 (95)	0.164 (46)	0.0020[a]
H4	C, H, R	0.220 (78)	0.243 (68)	0.5078
H5	T, L, Q	0.014 (5)	0 (0)	0.0701
H6	T, L, R	0.006 (2)	0.004 (1)	1.0000
H7	T, H, Q	0.042 (15)	0 (0)	0.0002[a]
H8	T, H, R	0.088 (31)	0.086 (24)	1.0000

A value of $p < 0.00625$ (0.05/number of haplotypes) was considered significant to correct for the number of comparisons made in haplotype analysis (Bonferroni's correction). Overall $p < 0.0001$.
[a]Statistically significant.

The linkage analysis between each pair-wise combination showed specific associations between alleles for both ethnic groups. In whites, a positive D' value was found between the less common alleles (all $p < 0.05$; Fig. 2). Conversely, in blacks, we found a positive D' value only for the association between the $(CA)_{14-24}$ and the Q279R ($D' = +0.37$, $p < 0.05$) polymorphisms.

Discussion

Our study shows for the first time a notable interethnic disparity in the distribution of *MMP* genotypes and alleles. The marked interethnic differences that we report here may help to understand, at least in part, how the combination of SNPs and distinct haplotypes may affect the variability in susceptibility to disease conditions when individuals from different ethnic groups are compared.

The functionality of the polymorphisms studied here has been shown in previous studies. The *MMP-2* $C^{-1306}T$ polymorphism disrupts an Sp1-type promoter site (CCACC box), causing a 1.6-fold increase in the promoter activity when C allele is present compared with the T allele (Price *et al.*, 2001). The $C^{-1562}T$ *MMP-9* polymorphism results in loss of a nuclear repressor protein binding site, which decreases

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Disclosure Statement

No competing financial interests exist.

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DISCUSSÃO

Os principais resultados deste estudo foram:

1. Há uma associação do genótipo HH e do alelo H do microssatélite -90 CA $_{(14-24)}$ no promotor da MMP-9 com hipertensão, e portadores deste alelo apresentaram maior risco de serem hipertensos.
2. Haplótipos da MMP-9 podem ter efeitos protetores (H3) ou deletérios (H7) sobre o fenótipo hipertrofia cardíaca
3. Não encontramos uma associação dos genótipos da MMP-2 com hipertensão
4. Haplótipos da MMP-2 podem ser capazes de exercer efeitos protetores (H1) ou deletérios (H3) sobre o fenótipo hipertrofia cardíaca
5. Encontramos uma extensa diferença na distribuição de alelos, genótipos e haplótipos da MMP-9 e MMP-2 entre brancos e negros em nossa população.
6. O alelo H do microssatélite -90 CA $_{(14-24)}$ no promotor da MMP-9, associado com hipertensão, é mais frequente em negros do que em brancos
7. Os haplótipos H3 e H7 da MMP-9 são mais frequentes em negros, sendo que o H7, que era muito raro no estudo de hipertensos alcançou aproximadamente 5% na amostra da população negra.

8. Há uma variabilidade genética maior no gene da MMP-9 nos negros do que nos brancos.

Todos os polimorfismos que estudamos têm evidências *in vitro* de efeitos sobre a expressão e/ou a atividade da MMP-9 ou da MMP-2. O SNP $C^{-1562}T$ do promotor da MMP-9 destrói um sítio de anelamento do tipo AP-1, o qual age reprimindo o gene. Desta forma, o alelo T gera uma maior expressão da MMP-9 (33). O microssatélite -90 $CA_{(14-24)}$ tem alelos que se distribuem mais frequentemente ao redor de 14 repetições (tratadas nesta tese como "L", *low*) e ao redor de 21-24 repetições (tratadas nesta tese como "H", *high*). Estudos *in vitro* mostraram que alelos com repetições em torno de 21(H) tem maior expressão da MMP-9 (34), provavelmente por afetar o distanciamento entre sítios de ligação de fatores de transcrição. O SNP no exon 6 da MMP-9, $A^{855}G$ leva a substituição do aminoácido Glutamina para Arginina dentro de um domínio tipo fibronectina, responsável pelo reconhecimento ao colágeno tipo IV, e ligação no sítio catalítico da enzima. Este domínio é altamente conservado, e quando removido resulta em uma enzima com baixa afinidade pelo colágeno tipo IV (35). Desta forma, podemos imaginar que o alelo que gera a enzima com Glutamina na posição 279 a faz ter alta afinidade pelo colágeno, resultando em atividade plena da enzima. Por outro lado, o alelo que gera a enzima com Arginina resultaria em uma enzima com afinidade reduzida pelo colágeno. Em relação à MMP-2, existem dois SNPs funcionais no promotor do gene, $C^{-1306}T$ e o $C^{-735}T$. Os dois SNPs agem abolindo independentemente dois sítios tipo SP-1, que agem como *enhancers* de expressão da MMP-2. Os alelos C no caso destes dois polimorfismos aumentam a

expressão da MMP-2(36), e o haplótipo unindo estes dois alelos parece ter efeito sinérgico sobre a expressão do gene (37).

Diversos estudos mostraram variações nas concentrações plasmáticas de MMP-9 (42-46), e MMP-2 (42, 46) em hipertensos. Alguns estudos inclusive relacionam essas alterações com maior risco cardiovascular (31, 47), porém níveis plasmáticos de MMPs não necessariamente refletem a expressão e atividade das MMPs no tecido cardíaco (8). Isto ocorre pela produção das enzimas por outras fontes, e pelo fato da liberação de MMPs do interstício para o sangue não necessariamente ser proporcional às concentrações internas ao tecido (8).

A participação das MMPs no processo de hipertrofia cardíaca é clara, porém existem pontos ainda nebulosos, principalmente em relação à sequência de eventos que leva a transição de um coração normal para a hipertrofia concêntrica, e desta para a excêntrica. Sabe-se que tanto em modelos agudos como em modelos crônicos de remodelamento cardíaco, existem diferenças temporais e regionais na expressão entre a MMP-2 e a MMP-9 (28, 48). Estudos usando modelos de estenose de aorta (que geram hipertrofia induzida por sobrecarga de pressão) têm mostrado que enquanto a MMP-2 está aumentada na hipertrofia concêntrica, há uma redução na sua expressão e aumento da inibição via TIMP-2 na progressão para a hipertrofia excêntrica (15, 29). Além disto, foi observado que a transição para hipertrofia excêntrica envolve um aumento da expressão da MMP-9 acompanhada da redução da atividade da MMP-2 (29). Isto dá uma ideia que provavelmente a MMP-2 está envolvida no espessamento das paredes ventriculares observado durante a fase inicial, de hipertrofia concêntrica, enquanto a MMP-9 poderia estar envolvida na dilatação da câmara com subsequente

redução da fração de encurtamento das fibras, observadas na hipertrofia excêntrica. Esta ideia é consistente com resultados dos mesmos modelos de estenose em animais knockout para MMP-2 e MMP-9 (49, 50). Nestes estudos, é possível observar que a hipertrofia é resultado da soma das ações das duas MMPs e possivelmente outros fatores, já que o knockout para as enzimas isoladamente não impede a instalação da hipertrofia. Além disto, é possível notar que o knockout para a MMP-2 tende a reduzir o espessamento das paredes sem afetar o diâmetro diastólico final e fração de encurtamento das fibras. Por outro lado, o knockout da MMP-9 reduz a hipertrofia sem afetar o espessamento das paredes, mas reduzindo a dilatação do ventrículo. Finalmente, é importante encararmos o efeito do knockout da MMP-2 com cuidado, já que no modelo de hipertrofia aparentemente houve um aumento significativo do TIMP-4, principal inibidor de diversas MMPs no coração. Isto significa que possivelmente a inibição de alguma outra MMP (incluindo a MMP-9) via TIMP-4 possa ter sido responsável pelos efeitos benéficos observados nos animais knockout da MMP-2.

Nossos resultados apresentados no primeiro artigo estão de acordo com a ideia de que variantes associadas com aumentos na expressão ou atividade da MMP-9 podem ter efeitos deletérios em fenótipos envolvidos com doenças cardiovasculares, como observado em relação à falência cardíaca (31, 38), aterosclerose (32) e espessamento arterial (51, 52). De fato, observamos uma associação do genótipo “HH” e do alelo “H” do microssatélite -90 CA $_{(14\text{-}24)}$ com hipertensão. O maior risco de hipertensão observado nos portadores desta variante não foi reportado previamente por outros grupos, porém é consistente com a observação de maior risco para aterosclerose (32), doença que envolve

alguns mecanismos em comum com a gênese da hipertensão (por exemplo, disfunção endotelial). A distribuição dos haplótipos entre hipertensos e normotensos foi significativamente diferente (P=0.04988), porém não foi possível discernir diferenças nas distribuições de cada haplótipo entre normotensos e hipertensos, após correção de Bonferroni. Este resultado negativo não impede que os haplótipos da MMP-9 afetem o fenótipo hipertrofia cardíaca, secundária à hipertensão. De fato, a combinação de variantes que causam maior expressão/atividade da enzima (haplótipo H7) estão relacionados a um aumento no diâmetro diastólico final (medida que denota a dilatação ventricular e redução da fração de encurtamento das fibras, característicos da hipertrofia excêntrica), que levou indiretamente a um aumento no índice de massa do ventrículo esquerdo (IMVE) nos pacientes hipertensos. Apesar de este resultado ser interessante, foi encontrado em poucos pacientes, o que significa que uma parcela muito pequena dos efeitos genéticos da hipertrofia pode ser explicada por este haplótipo. De qualquer forma, este haplótipo parece ter maior importância em negros, como abordado adiante. O haplótipo H3, por outro lado, foi associado a efeitos protetores em relação ao diâmetro diastólico final, e uma vez que este haplótipo é relativamente comum (frequência > 20%), podemos imaginar que seu efeito é mais relevante que o atribuído ao haplótipo H7. Este haplótipo parece ter seu efeito mediado pelo gênero, uma vez que na análise de regressão múltipla, ao separarmos os pacientes por sexo, o efeito protetor se mantém apenas no grupo masculino (B= -19.2524, P=0.0059).

No estudo 2, que o genótipo CC do polimorfismo $C^{-1306}T$ e o haplótipo H1, combinando os dois polimorfismos estudados da MMP-2, que levam a maior

expressão de MMP-2, aparentemente exerceram um efeito protetor, reduzindo o diâmetro diastólico final e o IMVE. Este resultado é interessante, pois denota uma possível ação antagônica à ação da MMP-9 em relação à transição da hipertrofia concêntrica para a hipertrofia excêntrica. Isto está de acordo com os resultados discutidos anteriormente em modelos animais, uma vez que há uma inibição da MMP-2 associada à hipertrofia excêntrica (15, 29). É possível imaginar que alelos que levem a maior expressão de MMP-2 possam conferir um efeito benéfico retardando a progressão para a hipertrofia excêntrica. Isto é consistente com efeitos benéficos atribuídos ao genótipo CC do polimorfismo $C^{-1306}T$ em relação à mortalidade cardiovascular (38) e à melhor recuperação pós-infarto em pacientes hipertensos (39). Os resultados da regressão múltipla foram confirmados por uma abordagem alternativa, analisando os extremos do fenótipo de hipertrofia cardíaca: comparamos a distribuição dos haplótipos da MMP-2 no quartil de pacientes com valores maiores de IMVE contra o quartil de pacientes com menores valores de IMVE. Nesta análise, encontramos o mesmo efeito benéfico do haplótipo H1 sobre IMVE, e, adicionalmente, encontramos um efeito deletério do haplótipo H3 sobre IMVE, provavelmente associado a menor expressão da enzima MMP-2 (este efeito deletério também foi observado de maneira não significativa no modelo de regressão múltipla, com valor de P=0,0617). A MMP-2 também é capaz de exercer outras ações independentemente da degradação da matriz, que podem afetar de maneira relevante a hipertrofia cardíaca. Podemos citar entre essas, a clivagem de pro-peptídeos vasoativos (53), e a degradação da cadeia leve de miosina (24, 54). Neste estudo não encontramos nenhuma associação dos genótipos, alelos e haplótipos com hipertensão. Este resultado deve ser visto com cautela, visto a

possibilidade de um erro tipo dois por baixo número amostral (como abordado adiante).

No terceiro estudo avaliamos possíveis diferenças interetnicas na distribuição dos três polimorfismos funcionais da MMP-9 e do polimorfismo da MMP-2 que mostrou ter efeitos sobre a hipertrofia cardíaca isoladamente. Foi encontrada diferença na distribuição de genótipos e alelos entre três dos quatro polimorfismos estudados. Além disto, a distribuição de haplótipos foi diferente entre quatro dos oito possíveis haplótipos. Finalmente, a análise de desequilíbrio de ligação entre os três polimorfismos da MMP-9 mostrou uma variabilidade genética muito maior nos negros do que nos brancos, já que os três alelos raros estavam ligados em brancos, enquanto só foi encontrada uma ligação dos alelos raros do microssatélite e do SNP do exon 6 em negros. Estes fatos em conjunto evidenciam uma diferença importante no background genético da MMP-2 e MMP-9 entre os grupos étnicos, o que pode causar diferenças na magnitude da influência genética sobre os fenótipos envolvendo as MMP-2 e MMP-9 entre os dois grupos. De fato, o haplótipo H7 da MMP-9, associado a efeitos deletérios sobre a hipertrofia cardíaca, que tinha sido observado em uma proporção muito pequena no primeiro estudo com hipertensos alcança aproximadamente 5% na população de negros do terceiro estudo.

Além disto, o alelo H e o genótipo HH do microssatélite -90 $CA_{(14-24)}$, associados com hipertensão no primeiro estudo são mais frequentes em negros. Isto é consistente e talvez possa ajudar a explicar em parte as maiores incidências de hipertensão e complicações cardiovasculares observadas em negros. Também foram observadas maiores frequências de genótipos protetores (CC do SNP C^-

^{1306}T da MMP-2) e haplótipos protetores (H3 da MMP-9) em negros, mostrando que a interação entre diversos polimorfismos, inclusive de diferentes genes, e a interação com o ambiente provavelmente tem papel fundamental na determinação do risco final ao qual os pacientes estão expostos. Apesar das frequências genotípicas dos polimorfismos estudados diferirem sutilmente com as frequências disponíveis nos bancos de dados internacionais do Hapmap e Seattle SNPs, as diferenças observadas em nosso estudo se repetem ao observarmos os dados dos bancos de dados internacionais.

Nossos estudos tiveram limitações. Entre elas, podemos citar o reduzido tamanho amostral, apesar da seleção cuidadosa dos pacientes e do pareamento criterioso dos grupos em relação à idade, gênero e raça. Apesar de não ter sido o foco principal dos artigos 1 e 2, os resultados de associação com hipertensão devem ser vistos com cautela. Calculamos o poder estatístico do nosso estudo utilizando o programa PGA (55), disponível no site http://dceg.cancer.gov/bb/tools/pga. Utilizamos a tabela com os dados brutos das genotipagens para o cálculo do grau efetivo de liberdade. Inserimos em um modelo dominante, a frequência da hipertensão na América latina, em torno de 36% (56); a frequência do alelo mais raro, 11%; o grau de efetivo de liberdade calculado, 2,77; um alfa de 0,05; e a razão entre controles e casos incluídos no estudo de 0,79. Encontramos um poder de 96,54%, considerado adequado, para detectarmos uma razão de chances de 2,0. Infelizmente, a razão de chances que encontramos no primeiro artigo foi de 1,58. Nesse cenário, recalculamos o poder estatístico e encontramos o valor de 64%, abaixo do recomendado (acima de 80%). Isto pode representar um viés em nosso primeiro estudo, porém uma vez

que o poder estatístico é mais relevante para determinarmos verdadeiramente um resultado negativo, acreditamos que é possível (apesar de não termos como provar isto), que o P<0,05 encontrado naquele estudo tenha nos levado a uma conclusão verdadeira quanto à associação do alelo H com hipertensão. Por outro lado, é possível que os outros alelos também tenham associação com a hipertensão levando a um risco relativo menor que 1,7 (menor risco relativo detectável no nosso desenho experimental, com um poder >80%), e a um erro tipo II. Para contornarmos este problema, teríamos que incluir um número maior de indivíduos no grupo controle, preferencialmente 1,5 vezes o número de pacientes. Nessa realidade, o poder da nossa análise teria sido de 82% para detectar uma razão de chances de 1,58. De qualquer forma, estudos analisando um número adequado de indivíduos, considerando o efeito que encontramos, são necessários para validar nossos achados.

O estudo da MMP-2, essencialmente negativo, quanto à associação com hipertensão, apresentou um poder de 95% para detectar uma razão de chances de 2.0 (grau de liberdade efetivo calculado de 2.0). É possível que existam associações gerando riscos relativos abaixo de 1.74 (menor risco relativo detectável em nosso desenho experimental, com um poder >80%), portanto esse dado deve ser visto com cautela. Na figura 2 estão demonstrados gráficos do número de indivíduos contra o poder estatístico em nosso desenho experimental.

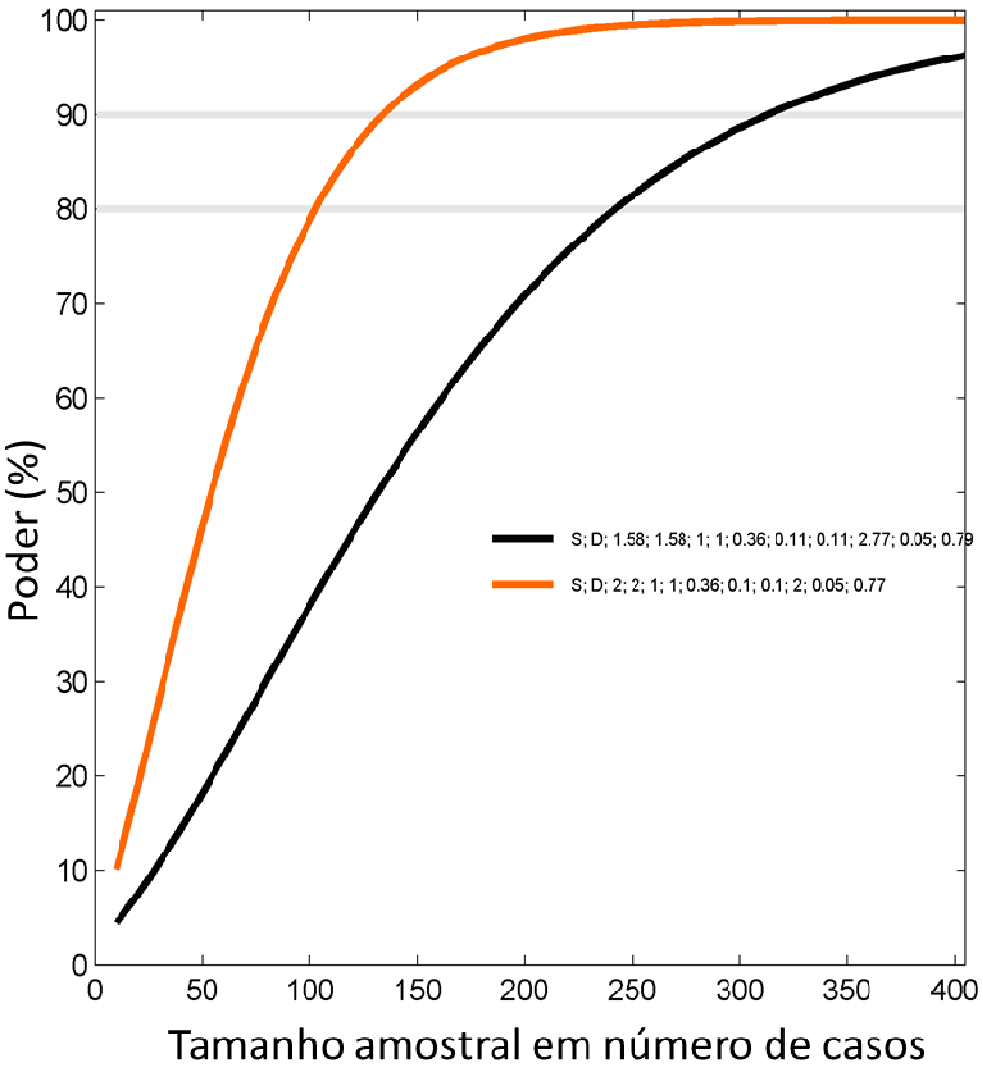

Figura 2. Cálculo do poder estatístico de acordo com o número de amostras. Em preto o estudo 1, e em vermelho o estudo 2.

Outras limitações também afetaram os estudos, como o uso de medicamentos. Apesar da influência de anti-hipertensivos serem consideradas nas análises de regressão linear múltipla, é possível que seus efeitos tenham obscurecido efeitos

genéticos em nosso estudo. Seria claramente antiético retirar a medicação destes pacientes para incluí-los no estudo.

Por fim, devemos também considerar uma limitação inerente de estudos inferenciais, como o nosso: a simples associação de um efeito com um genótipo não atribui uma relação causal direta, portanto nossas conclusões não são definitivas, e devem ser confirmadas em outros estudos.

Nossos resultados devem ser replicados em outras populações, para validação das conclusões de nossos estudos. Além disto, ainda se sabe muito pouco a respeito da interação entre efeitos genéticos, e entre genes e ambiente. Nossos resultados podem contribuir para o estabelecimento de painéis com marcadores de risco e marcadores protetores para doenças complexas, como a hipertensão. Esses painéis, no futuro, devem ser validados quanto à interação entre os diversos marcadores de susceptibilidade, uma vez que os mais diversos efeitos (aditivo, sinérgico, ou anulante) podem aparecer. Essas interações podem compor novos painéis de risco, agora compilando combinações de polimorfismos que geram maior ou menor risco a determinada doença. Por fim, devem ser investigados os fatores ambientais capazes de acionar gatilhos fisiológicos que trazem à tona uma determinada susceptibilidade genética a determinada doença. Esse último cenário comporia um painel definitivo com as interações entre fatores ambientais e uma determinada combinação de diversos polimorfismos. Neste ponto seria possível apontar com maior precisão as diferenças interetnicas em relação ao risco para doenças complexas, e atribuir um risco palpável que a classe médica poderia utilizar como guia para o tratamento de seus pacientes.

CONCLUSÕES

Em conclusão, nossas evidências sugerem que o genótipo HH do microssatélite -90 CA $_{(14-24)}$ da MMP-9 está associado a um maior risco à hipertensão. Além disto, os haplótipos da MMP-9 podem ter efeitos protetores (H3) ou deletérios (H7) no fenótipo de hipertrofia cardíaca. O genótipo CC do SNP $C^{-1306}T$ da MMP-2 é capaz de exercer efeitos benéficos na hipertrofia cardíaca por si só, e os haplótipos H1 e H3 exercem efeitos protetores e deletérios, respectivamente, sobre a hipertrofia cardíaca.

Encontramos uma diferença considerável no background genético entre os grupos de raça branca e negra. O genótipo HH do microssatélite -90 CA $_{(14-24)}$ da MMP-9, associado à hipertensão, é mais frequente em negros. Além disto, o haplótipo H7, que apresentou efeitos deletérios na hipertrofia cardíaca, e é bastante raro em brancos, chega a quase 5% em negros. Estes fatos podem ajudar a explicar em parte o componente genético das diferenças interetnicas na incidência de doenças cardiovasculares.

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ANEXOS

FACULDADE DE CIÊNCIAS MÉDICAS
COMITÊ DE ÉTICA EM PESQUISA

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CEP, 25/10/07.
(Grupo III)

PARECER CEP: N° 760/2007 (Este n° deve ser citado nas correspondências referente a este projeto)
CAAE: 0550.0.146.000-07

I - IDENTIFICAÇÃO:

PROJETO: **"CONSUMO DE SÓDIO EM SUJEITOS HIPERTENSOS: ASSOCIAÇÃO COM ATEROSCLEROSE E VARIANTES GENÉTICAS RELACIONADAS AO SISTEMA NADPH-OXIDADE".**
PESQUISADOR RESPONSÁVEL: Maria Carolina Salmora Ferreira
INSTITUIÇÃO: Hospital das Clínicas / UNICAMP
APRESENTAÇÃO AO CEP: 10/10/2007
APRESENTAR RELATÓRIO EM: 23/10/08 (O formulário encontra-se no *site* acima)

II - OBJETIVOS

Caracterizar e verificar a relação entre genótipo, hipertensão arterial, arteriosclerose, variáveis clínicas e comportamento de consumo de sódio na dieta habitual de pacientes hipertensos, atendidos nos Ambulatórios: de Genética e Cardiologia Molecular e de Hipertensão Arterial do Hospital de Clínicas da Universidade Estadual de Campinas.

III - SUMÁRIO

O estudo propõe a ampliação da amostra de sujeitos e da caracterização clínica e genética. Além disso, será investigado o papel do consumo de sódio como fator independente para o aumento do risco cardiovascular, por meio da medida da espessura de intima média (EIM) de carótidas e da análise do haplótipo envolvendo os polimorfismos C242T e -930 A/G da subunidade p22phox do sistema NADPH oxidase, em sujeitos hipertensos e controles normotensos. O estudo anterior oferece subsídios para a continuidade do projeto pela correlação significativa dos métodos utilizados para quantificar o consumo de sódio com o sódio urinário (r=0,25 p=0,03 -QFASó -Questionário de freqüência Alimentar de alimentos com alto teor de sódio), validando os instrumentos de análise de consumo e devido à correlação significativa entre consumo de sódio e massa ventricular esquerda (r=0,20 p=0,03). A média de consumo diário da população foi de 15,3 gramas para homens e 12,24 gramas para mulheres, mais que o dobro do recomendado para pacientes hipertensos. Além disso, foram analisadas as crenças sobre seguir uma dieta hipossódica e foi observado que quanto maior a percepção de benefícios da adesão à dieta hipossódica, menor a massa ventricular esquerda (r=-0,22 p=0,01) e que quanto maior a percepção de barreiras, maior o consumo de sódio (recordatório de 24 h) (r=0,17 p=0,05), demonstrando a influência das crenças sobre a clínica dos sujeitos. O presente estudo, com a ampliação da avaliação clínica e genética relacionada ao consumo de sódio em hipertensos e controles normotensos, possibilitarão melhor entendimento do papel do consumo elevado de sódio como preditor independente de risco cardiovascular em hipertensos brasileiros.

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TCLE – Termo de Consentimento Livre e Esclarecido

Título do projeto: CONSUMO DE SÓDIO EM SUJEITOS HIPERTENSOS: ASSOCIAÇÃO COM ATEROSCLEROSE E VARIANTES GENÉTICAS RELACIONADAS AO SISTEMA NADPH-OXIDASE.

OBJETIVO DA PESQUISA

Eu entendo que fui convidado a participar de uma pesquisa envolvendo pessoas com hipertensão arterial (pressão alta), que tem como objetivo estudar se o consumo elevado de sal pode influenciar no risco cardiovascular pela sua associação com Aterosclerose e se essa associação pode ser determinada geneticamente.

PROCEDIMENTO

Eu entendo que se concordar em participar desse estudo, terei uma amostra de sangue colhida em jejum (20 ml, mais ou menos 2 seringas). Este sangue será utilizado para pesquisa de genes e para dosagens do nível de açúcar e colesterol.

Sei que o pesquisador fará perguntas sobre meus problemas de saúde, e consultará meu prontuário médico. Ele irá medir meu peso e altura. Não haverá nenhuma alteração nos meus medicamentos.

Entendo que serei também solicitado a realizar um exame de Ecodopplercardiograma, por Médico especializado e treinado, que fará parte de minha rotina de exames do ambulatório onde sou atendido, ficando o resultado do exame disponibilizado em meu prontuário.

RISCO E DESCONFORTO

Estou ciente de que o risco associado à coleta de sangue será mínimo, pois se trata de um exame de sangue tradicional, que será realizado na veia do braço por um profissional treinado e habilitado.

Entendo também que o Ecodopplercardiograma é um exame não invasivo, não oferecendo risco algum e que qualquer diagnóstico associado ao exame será prontamente encaminhado para tratamento no serviço sob a responsabilidade do Prof. Dr. Wilson Nadruz Júnior.

SIGILO E CONFIDENCIALIDADE

Sei que haverá sigilo sobre todos os dados coletados, mantido através da utilização de códigos numéricos para identificação dos participantes.

VANTAGENS

Eu entendo que não obterei nenhuma vantagem direta com a minha participação neste estudo, e que apenas receberei o resultado dos exames realizados rotineiramente no serviço, como dosagens de açúcar e colesterol no sangue, eletrocardiograma e ecocardiograma.

Qualquer dúvida ou informação poderei contatar o Dr. Wilson Nadruz Junior ou a Enfª Maria Carolina Salmora Ferreira pelo telefone: 19-3788-8951

INFORMAÇÃO SOBRE RESULTADO DA PESQUISA

A qualquer momento poderei questionar sobre os resultados da pesquisa e do estudo genético realizado com meu sangue caso seja do meu interesse.

FORNECIMENTO DE INFORMAÇÃO ADICIONAL

Em caso de recurso, dúvidas ou reclamações poderei contatar o Comitê de Ética da Faculdade de Ciências Médicas – UNICAMP, tel (19) 3788-8936.

RECUSA OU DESCONTINUAÇÃO DA PARTICIPAÇÃO

Eu entendo que a minha participação é voluntária; que eu posso me recusar a participar ou retirar meu consentimento; e interromper a minha participação no estudo a qualquer

momento, sem comprometer os cuidados médicos que recebo atualmente ou receberei no futuro.

ARMAZENAMENTO DE MATERIAL BIOLÓGICO

Do sangue coletado extrai-se o DNA para a realização de estudo genético, este DNA pode ser armazenado para uso no estudo atual e em estudos futuros.

( ) Autorizo o armazenamento do meu DNA para estudos

( ) Não autorizo o armazenamento do meu DNA para estudos

Eu confirmo que o (a) Dr. (a) _____ explicou-me o objetivo do estudo, os procedimentos aos quais serei submetido e os riscos e desconforto advindos dessa pesquisa. Eu li e/ou recebi explicação, assim como compreendi esse formulário de consentimento e estou de acordo em participar desse estudo.

_____

Nome e RG do participante

_____ _____

Assinatura do participante ou responsável Data

RESPONSABILIDADE DO PESQUISADOR:

Eu expliquei a _____ o objetivo do estudo, os procedimentos requeridos e os possíveis riscos que poderão advir do estudo, usando o melhor do meu conhecimento. Eu me comprometo a fornecer uma cópia desse formulário de consentimento ao participante ou responsável. Ademais, comprometo-me a submeter à aprovação pelo Comitê de ética da FCM-UNICAMP qualquer novo estudo que se utilize de informações referentes ao presente estudo.

_____No

me e RG da pesquisador

_____ _____

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Observação: o projeto “influência de fatores genéticos relacionados às metaloproteinases da matriz extracelular sobre a susceptibilidade à hipertrofia cardíaca em hipertensos” é um desdobramento do projeto “consumo de sódio em sujeitos hipertensos: associação com aterosclerose e variantes genéticas relacionadas ao sistema NADPH-oxidase” realizado em colaboração com o professor Wilson Nadruz Jr do departamento de clínica médica da FCM/UNICAMP.

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