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Fabricia Junqueira das Neves

FUNÇÃO AUTONÔMICA E REATIVIDADE VASCULAR EM INDIVÍDUOS COM PARENTESCO DE DIABETES TIPO 2 E EM PORTADORES DO POLIMORFISMO 894G>T DA ÓXIDO NÍTRICO SINTASE ENDOTELIAL

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Tese apresentada como requisito para obtenção do título de Doutor ao Programa de Pós-Graduação em Fisiopatologia Clínica e Experimental da Universidade do Estado do Rio de Janeiro.

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Aos meus amados pais, Tony e Inês,
que por uma vida de dedicação e amor
sempre possibilitaram a oportunidade
de realizar sonhos e conquistas.

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todo amor, dedicação e
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realização deste trabalho.

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A ciência será sempre uma busca, jamais
um descobrimento real. É uma viagem,
nunca uma chegada.

Karl Popper

RESUMO

DAS NEVES, Fabricia Junqueira. **Função autonômica e reatividade vascular em indivíduos com parentesco de diabetes tipo 2 e em portadores do polimorfismo 894G>T da óxido nítrico sintase endotelial**, Brasil. 2009. Tese de Doutorado em Fisiopatologia Clínica e Experimental - Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2009.

As doenças cardiovasculares estão entre as principais causas de mortalidade em muitos países. O sistema nervoso autônomo e a função endotelial constituem mecanismos centrais no desenvolvimento e progressão de doenças cardiovasculares. A função autonômica e a reatividade vascular podem estar alteradas em indivíduos com maior risco para doença cardiovascular, como indivíduos com história familiar de primeiro grau de diabetes tipo 2 (HFDM2) e indivíduos com polimorfismo 894G>T da enzima óxido nítrico sintase endotelial (eNOS). Os objetivos dos três artigos apresentados na tese foram: artigo I. Investigar a influência da HFDM2 na modulação autonômica cardíaca em ausência de desordens metabólicas concomitantes; artigo II. Investigar a influência da HFDM2 na reatividade vascular em ausência de desordens metabólicas concomitantes e, artigo III. Investigar a influência do polimorfismo 894G>T no efeito de uma sessão de exercício dinâmico máximo na reatividade vascular. Foram recrutados indivíduos saudáveis com e sem HFDM2 para os artigos I e II. A variabilidade da frequência cardíaca (VFC) foi determinada através da análise espectral de um registro de intervalos RR durante 10 minutos na posição supina (artigo I) e a reatividade vascular durante a hiperemia reativa através da pletismografia de oclusão venosa (artigo II). Para a realização do artigo III, foram recrutados indivíduos saudáveis com e sem o polimorfismo 894G>T da eNOS. O protocolo consistiu na determinação da reatividade vascular basal e durante a hiperemia reativa, o qual eram realizados pré, 10, 60 e 120 minutos após um teste de esforço cardiopulmonar máximo. Os indivíduos com HFDM2 apresentaram maiores valores para variáveis antropométricas e metabólicas e uma menor VFC (artigo I) e reatividade vascular (artigo II) quando comparados com o grupo-controle ($p<0,05$). Em seguida, os grupos foram emparelhados para essas variáveis consideradas capazes de alterar a VFC e a reatividade vascular e nenhuma diferença significativa foi encontrada entre os grupos nos artigos I e II ($p>0,05$). Foi realizada análise de correlação simples, sendo que as variáveis que apresentaram significância estatística foram submetidas à análise de regressão múltipla. Esta identificou colesterol ($P=0,014$) e triglicerídeos ($P=0,014$) como preditores independentes da VFC (modelo $r^2=0,16$; $P<0,001$) e insulina ($P<0,05$) e razão cintura-quadril ($P<0,05$) como preditores independentes da reatividade vascular (modelo $r^2=0,22$; $P=0,006$). No artigo III, não foram observadas diferenças entre os indivíduos com e sem o polimorfismo 894G>T em relação as características antropométricas, metabólicas e hemodinâmicas e medidas de fluxo sanguíneo antes do exercício dinâmico máximo ($P>0,05$). Os indivíduos polimórficos apresentaram menor reatividade vascular independente do tempo (efeito do grupo $P=0,019$) e a análise de post-hoc revelou que os indivíduos polimórficos apresentavam valor menor apenas no momento 120 minutos ($P=0,022$) quando comparados com indivíduos sem o polimorfismo. Estes achados sugerem que indivíduos com HFDM2, em ausência de desordens metabólicas concomitantes, não apresentam alteração da modulação autonômica cardíaca e de reatividade vascular.

Em adendo, indivíduos com polimorfismo 894G>T, têm menor reatividade vascular após um sessão de exercício, denotando a presença de disfunção vascular.

Palavras-chave: Sistema nervoso autônomo. Reatividade vascular. História familiar de diabetes tipo 2. Polimorfismo. Óxido nítrico sintase endotelial.

ABSTRACT

Cardiovascular diseases are among the leading causes of mortality in many countries. The autonomic nervous system and the endothelial function are central mechanisms in the development and progression of cardiovascular diseases. The autonomic function and vascular reactivity may be altered in subjects with higher risk for cardiovascular disease, as subjects with family history of first-degree relatives of type 2 diabetes (FDRs), and subjects with the 894G>T polymorphism of the endothelial nitric oxide synthase (eNOS). The aims of these three papers presented at this thesis were: paper I. To investigate the influence of FDR on cardiac autonomic modulation in the absence of concomitant metabolic disorders; paper II. To investigate the influence of FDR on vascular reactivity in the absence of concomitant metabolic disorders; paper III. To investigate the influence of the 894G>T polymorphism on the effect of a single bout of maximal dynamic exercise on vascular reactivity. Healthy subjects with and without FDRs were recruited for the paper I and II. The heart rate variability (HRV) was determined by spectral analysis of inter-beat intervals recorded during 10 min in the supine position (paper I) and vascular reactivity during the reactive hyperemia by venous occlusion plethysmography (paper II). For the paper III, healthy subjects with and without the 894G>T polymorphism of the eNOS were recruited. The protocol consisted of vascular reactivity assessment at baseline and during reactive hyperemia, which were performed pre, 10, 60 and 120 min after a maximal cardiopulmonary exercise test. The FDR's exhibited higher values for anthropometric and metabolic variables and lower values for HRV (paper I), and vascular reactivity (paper II) when compared to the control subjects ($p<0.05$). After matching the groups for variables, that are known to alter HRV and vascular reactivity, no significant difference was observed between groups in the paper I and II ($p>0.05$). Following single correlation analysis, only the variables with statistical significance were submitted to multiple regression analysis. This identified cholesterol ($P=0.014$) and triglycerides ($P=0.014$) as significant predictors of HRV (model $r^2=0.16$; $p<0.001$), and insulin ($P<0.05$) and waist-to-hip ratio ($P<0.05$) as independent predictors (model $r^2=0.22$; $P=0.006$). There were no differences between the subjects with and without the 894G>T polymorphism concerning anthropometric, metabolic, and hemodynamic characteristics, and blood flow measurements before maximal dynamic exercise ($P>0.05$), in the paper III. The polymorphic subjects presented lower vascular reactivity regardless of time ($P=0.019$ for group main effect), and post-hoc analysis revealed that polymorphic subjects had lower values only at the 120 min measurement ($P=0.022$) when compared with subjects without the polymorphism. These findings suggest that FDRs, in the absence of concomitant metabolic disorders, does not impair cardiac autonomic modulation and vascular reactivity. Furthermore, subjects with the 894G>T polymorphism had lower vascular reactivity after a single bout of exercise, denoting the presence of vascular dysfunction.

Keywords: Autonomic nervous system. Vascular reactivity. Family history of type 2 diabetes. Polymorphism. Endothelial nitric oxide synthase.

LISTA DE ABREVIATURAS E SIGLAS

ARIC	The Atherosclerosis Risk in Communities
BH4	Tetraidrobiopterina
CaM	Calmodulina
CARDIA	Coronary Artery Risk Development in the Young Adults
cGMP	Guanosina monofosfato cíclica
DM2	Diabetes mellitus tipo 2
eNOS	Óxido nítrico sintase endotelial
GTP	Guanosina trifosfato
HFDM2	História familiar de primeiro grau de diabetes tipo 2
LACE	Laboratório de Ciências do Exercício
NADPH	Fosfato de nicotinamida adenina dinucleotídio
NO	Óxido nítrico
VFC	Variabilidade da frequência cardíaca

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1. APRESENTAÇÃO

Esta tese apresenta os resultados das atividades desenvolvidas como parte do doutoramento da autora junto ao Programa de Pós-Graduação em Fisiopatologia Clínica e Experimental da Universidade do Estado do Rio de Janeiro, sob orientação do Prof. Dr. Antonio Claudio Lucas da Nóbrega, no Laboratório de Ciências do Exercício (LACE) da Universidade Federal Fluminense.

O projeto de doutorado consistiu de dois experimentos. O primeiro gerou dois artigos já publicados em periódicos. O segundo experimento gerou, até o presente momento, um artigo que foi submetido para publicação. Vale a pena ressaltar que todos os artigos foram publicados/submetidos à revistas indexadas no PubMed e no Web of Science. O primeiro artigo foi publicado em 2007 no *Diabetic Medicine* (fator de impacto 2,970) e teve como objetivo responder à pergunta se indivíduos sob risco de diabetes - com parentesco de primeiro grau de diabetes tipo 2 (DM2) – apresentam disfunção autonômica como característica hereditária.

O segundo artigo foi publicado no primeiro semestre de 2009 no *Diabetes Care* (fator de impacto 7,851) e teve como objetivo investigar a influência da história familiar de primeiro grau de DM2 (HFDM2) na reatividade vascular, na ausência de desordens metabólicas. Ainda analisando indivíduos que apresentam maior risco para doença cardiovascular, verificamos o impacto da presença do polimorfismo da enzima óxido nítrico sintase endotelial (eNOS) na reatividade vascular em condições basais e após uma sessão de exercício dinâmico máximo. O manuscrito referente a esses resultados foi submetido ao *Journal of Physiology* (fator de impacto 4,580) em agosto de 2009 e encontra-se em fase de análise.

É importante ressaltar ainda, que além dos artigos desenvolvidos e aqui apresentados, durante o período de doutoramento, a autora teve a experiência de realizar o doutorado sanduíche sob supervisão do Prof. Dr. Christian Roberts, professor do *Department of Physiological Science – University of California*, Estados Unidos, com duração de 6 meses (dezembro de 2008 a maio de 2009). Durante este período no exterior, além de participar da execução do projeto de pesquisa que está em andamento, ela foi a responsável por padronizar a técnica de isolamento de células mononucleares, que já está sendo utilizada no LACE com o objetivo de avaliar cultura de células progenitoras endoteliais circulantes.

Desta forma, conforme as normas do Programa de Pós-Graduação em Fisiopatologia Clínica e Experimental da Universidade do Estado do Rio de Janeiro, a presente tese está estruturada em termos de uma introdução geral, apresentação dos três artigos previamente mencionados, discussão, considerações finais e referências bibliográficas.

Na introdução são abordados os aspectos relacionados à fisiopatologia do sistema nervoso autônomo e reatividade vascular em duas populações distintas que apresentam risco aumentado para o desenvolvimento de doenças cardiovasculares, que são: indivíduos com parentesco de primeiro grau de DM2 e indivíduos portadores do polimorfismo 894G>T da eNOS. Os três artigos publicados/submetidos são apresentados com seus respectivos itens: introdução, métodos, resultados, discussão, conclusões e referências bibliográficas. A discussão dos resultados como um todo é apresentada na sessão de discussão respectiva e as considerações finais referem-se igualmente, ao que pode ser comentado com base nos resultados gerais obtidos nos três artigos desenvolvidos.

2. INTRODUÇÃO

As doenças cardiovasculares estão entre as principais causas de morbidade e mortalidade em muitos países (1-3), inclusive no Brasil (4, 5). Independentemente dos fatores causais, o sistema nervoso autônomo (6) e a função endotelial (7) constituem mecanismos centrais no desenvolvimento e progressão de doenças do sistema cardiovascular, e são considerados marcadores precoces de doença (8, 9), assim como preditores independentes de eventos cardiovasculares (10, 11).

2.1. Sistema nervoso autônomo

O sistema nervoso autônomo é responsável pelo controle das funções viscerais ou involuntárias em contra-posição ao sistema somático que inerva a musculatura esquelética. O sistema nervoso autônomo influencia a atividade da maioria dos tecidos e órgãos por meio de dois componentes distintos: o parassimpático (representado principalmente pelo nervo vago) e o simpático. Tanto o parassimpático quanto o simpático atuam na manutenção da estabilidade do ambiente interno do organismo, sendo portanto, fundamentais para a manutenção da homeostasia (6), mas ambos diferem quanto à anatomia, neurotransmissores, receptores e suas ações fisiológicas (12).

Entre as funções exercidas pelo sistema nervoso autônomo está a regulação do sistema cardiovascular, em especial da frequência cardíaca e da pressão arterial. A estimulação do sistema parassimpático promove diminuição do automatismo, da excitabilidade, condutibilidade e da contratilidade do coração. Por outro lado, a atividade simpática tende a estimular a função cardíaca, promovendo efeitos opostos ao parassimpático, atuando no aumento da frequência cardíaca e da excitabilidade elétrica do coração. Desta forma, a regulação rápida e precisa da resposta cardiovascular a modificações ambientais e estímulos fisiológicos como, por exemplo, a realização de exercício físico e situações de estresse emocional é

realizada predominantemente através do balanço entre a atividade do sistema nervoso parassimpático e simpático (12).

Como dito anteriormente, a frequência cardíaca está constantemente submetida a flutuações do tônus autonômico, determinadas pela ativação e/ou inibição parassimpática e simpática em resposta a estímulos diversos (12). Em repouso há um predomínio do tônus vagal e as variações da frequência cardíaca são amplamente dependentes da modulação parassimpática. A resposta do nodo sinusal à atividade nervosa parassimpática é extremamente rápida, ou seja, após um único estímulo, o pico de resposta ao estímulo parassimpático pode ocorrer em 400 milisegundos. Desta forma, o sistema nervoso parassimpático promove um pico de resposta na frequência cardíaca no primeiro ou segundo batimento após a estimulação vagal. Com a cessação do estímulo, a frequência cardíaca retorna rapidamente ao nível anterior. Apesar da cinética de recuperação ser um pouco mais lenta que a de início do estímulo, a frequência cardíaca retorna aos valores iniciais em 5 a 10 segundos após o término do estímulo vagal (12). Em contrapartida, o aumento da atividade no sistema nervoso simpático resulta no aumento da frequência cardíaca e na força de contração do miocárdio. Após o início da estimulação simpática existe um período de latência acima de 5 segundos acompanhado por um aumento progressivo da frequência cardíaca, que alcança um *platô* em 20 a 30 segundos (12).

A modulação autonômica em humanos tem sido tradicionalmente avaliada por meio da mensuração da influência da atividade autonômica sobre órgãos-alvo, como o coração e vasos sanguíneos. O estudo da variabilidade da frequência cardíaca (VFC) é um método capaz de analisar as flutuações batimento a batimento da frequência cardíaca em torno da média que ocorrem durante um determinado período. Uma vantagem na utilização do método é a possibilidade da realização de uma avaliação não invasiva e seletiva da função autonômica, na qual essa variação reflete o efeito da modulação parassimpática e simpática e outros mecanismos de controle fisiológico no sistema cardiovascular (13). Estudos demonstraram que a VFC constitui um importante fator prognóstico para o aparecimento de eventos cardíacos de indivíduos saudáveis (14) e de portadores de cardiopatias (15, 16), o qual a diminuição da VFC está relacionada a um maior índice de morbidade e mortalidade cardiovascular (14, 17).

2.2. Reatividade vascular

A descoberta de Furchgott e Zawadzki, em 1980, de que a vasodilatação em resposta à acetilcolina em preparações de anéis de aorta era dependente da presença de células endoteliais íntegras, revolucionou o estudo da fisiologia cardiovascular (18). Atualmente, o endotélio é considerado um órgão endócrino ativo que, em resposta a estímulos humorais, neurais e mecânicos sintetiza e libera substâncias que participam do processo de modulação do tônus vascular, regulação da coagulação, trombólise, remodelamento vascular e resposta inflamatória (19).

Alguns estímulos como a pressão que o sangue exerce sobre a parede vascular, também conhecida como tensão de cisalhamento ou *shear stress,* e modificações no ambiente químico local como, por exemplo, a presença de hipóxia tecidual, promovem a liberação de substâncias vasodilatadoras pelo endotélio, especialmente o óxido nítrico (NO) (20, 21). O NO é sintetizado a partir do aminoácido L-arginina por ação da eNOS e pela presença de diversos co-fatores, como tetraidrobiopterina (BH4), calmodulina (CaM) e NADPH. No músculo liso adjacente, o NO estimula a guanilato ciclase solúvel, resultando no aumento da conversão da guanosina trifosfato (GTP) em guanosina monofosfato cíclica (cGMP), a qual diminui as concentrações de cálcio intracelular e promove o relaxamento da musculatura lisa vascular (22). Além do NO, outras substâncias vasodilatadoras secretadas pelo endotélio, como o fator hiperpolarizante derivado do endotélio e prostaciclinas, e substâncias vasoconstritoras, como endotelina, angiotensina II e tromboxano A_2 promovem a manutenção do tônus vascular. Vale a pena ressaltar que, além do equilíbrio entre substâncias vasodilatadoras e vasoconstritoras, a reatividade vascular é também dependente de fatores miogênicos e do sistema nervoso autônomo (23).

Agressões químicas, mecânicas ou metabólicas ao vaso podem levar à disfunção endotelial e, consequentemente, ao comprometimento da reatividade vascular. A resposta vascular a estes agentes agressores envolve a interação de diversos grupos celulares como monócitos, linfócitos T, plaquetas e células musculares lisas vasculares, dando início a uma série de eventos que culminam com a formação da placa aterosclerótica (19). Sabe-se hoje que o endotélio tem participação central na patogênese da aterosclerose, deflagrando uma resposta

inflamatória que é a responsável pela formação e instabilização da placa aterosclerótica, com influência direta no curso clínico de doenças cardiovasculares, como hipertensão arterial (24) e insuficiência cardíaca (25).

Atualmente, diferentes métodos são utilizados na investigação da função endotelial, abrangendo desde a biologia celular e molecular até a pesquisa clínica aplicada a seres humanos. Estes métodos ganharam importância não apenas por possibilitar a investigação da fisiopatologia da disfunção endotelial, mas também por representar um marcador de risco precoce para eventos cardiovasculares (26-28). Dentre os métodos de avaliação não-invasiva do fluxo de sangue periférico está a pletismografia de oclusão venosa, que é uma técnica capaz de quantificar variações de volume em um determinado segmento corporal em situações como repouso e hiperemia reativa. A hiperemia reativa é utilizada para medir a reatividade vascular como um indicativo de função endotelial, e consiste em ocluir temporariamente a circulação utilizando-se um manguito inflado a uma pressão supra-sistólica (29). O fluxo sanguíneo aumentado logo após a liberação da oclusão exige uma resposta vasodilatadora, que é causada por metabólitos liberados localmente, que foram produzidos pelo tecido isquêmico, e pelo aumento da produção de óxido nítrico pelo endotélio, devido ao grande estresse de cisalhamento provocado pela manobra (30).

2.3. Parentesco de primeiro grau de diabetes mellitus tipo 2

O diabetes mellitus é um conjunto de alterações metabólicas que resulta em uma ausência (diabetes tipo 1) ou diminuição significativa na secreção de insulina pelas células β-pancreáticas e/ou na diminuição da ação celular da insulina (DM2). O DM2 é denominado uma doença multifatorial, pois depende não só de influências ambientais como também de múltiplos fatores genéticos. Dentre os fatores de risco para o desenvolvimento do DM2 estão a obesidade, o sedentarismo e o histórico familiar de DM2.

Indivíduos com HFDM2 apresentam alto risco de desenvolver desordens metabólicas incluindo a própria DM2, como demonstrado por estudos transversais (31) e estudos coorte (32, 33). Dados do *Framingham Offspring Study*, no qual os

filhos da população original do estudo foram acompanhados durante 20 anos, demonstram que o risco para desenvolver DM2 foi 3,5 vezes maior quando o pai ou a mãe era diabético e, 6 vezes maior quando o pai e a mãe eram diabéticos em comparação a indivíduos sem história familiar de DM2 (32). Dados mais recentes demonstram que os fatores genéticos, além de fatores comportamentais relacionados com o ambiente familiar, aumentam o risco de desenvolvimento de DM2 (34). Recentemente, um estudo que investigou 392.935 variações em genes - polimorfismos de nucleotídeo único, conhecidos como SNP - confirmou um *locus* e identificou outros quatro novos *loci* que contribuem significativamente para o risco de desenvolvimento de DM2 (35).

Já está bem definido na literatura que indivíduos com DM2 apresentam comprometimento da função autonômica (36) e da reatividade vascular (37-39), além de um risco de três a quatro vezes maior para eventos cardiovasculares (40, 41) quando comparados com indivíduos sem DM2. Estudos prévios têm sugerido também que indivíduos com HFDM2 apresentam maior risco para disfunção autonômica (42, 43) e endotelial (44, 45). Porém, esses estudos não realizaram o controle para variáveis intervenientes como obesidade (46, 47), resistência insulínica (48, 49), intolerância à glicose (50, 51), dislipidemia (52, 53) e elevação de proteína C-reativa (54, 55), dentre outros, que podem influenciar o sistema nervoso autônomo (48, 56) e a reatividade vascular (57) e que, sabidamente, estão alteradas em indivíduos que apresentam HFDM2 (58). Portanto, a afirmação de que indivíduos com HFDM2 apresentam disfunção autonômica e endotelial per se, como uma característica herdada, originou-se de estudos com viéses metodológicos que impedem tal conclusão.

Considerando que não está claro na literatura se o comprometimento do sistema nervoso autônomo e da reatividade vascular, observada nos indivíduos que apresentam HFDM2, é uma característica primária ou a consequência de desordens metabólicas comumente observadas nesses indivíduos, os objetivos dos estudos, que integram esta tese e foram publicados no *Diabetic Medicine* e *Diabetes Care*, foram investigar a influência da história familiar de primeiro grau de DM2 na variabilidade da frequência cardíaca e reatividade vascular na ausência de desordens metabólicas. A informação sobre a influência ou não do parentesco de DM2 como disfunção primária permite identificar se o comprometimento do sistema nervoso autônomo e reatividade vascular pode ser ou não considerado um problema

determinado geneticamente com consequências médico sociais e com impacto sobre medidas preventivas.

2.4. Polimorfismo 894G>T da óxido nítrico sintase endotelial

Com o avanço do seqüenciamento do DNA humano, tem sido possível caracterizar variações na sequência do genoma que estão associadas à variabilidade inter-individual (59) nas respostas e adaptações a estímulos fisiológicos (60), assim como, sua influência no processo de desenvolvimento e progressão de doenças cardiovasculares (61). Neste contexto, estudos envolvendo polimorfismos do gene da eNOS têm despertado grande interesse da comunidade científica, visto que a produção adequada de NO pela eNOS é fundamental para a homeostasia cardiovascular (62), principalmente devido à regulação do tônus vascular (18).

O gene da eNOS está localizado no *locus* 7q35-36, contendo 26 exons totalizando 21kb onde já foram identificados alguns polimorfismos. Dentre estes, o localizado no exon 7 (894G>T), é caracterizado pela troca do nucleotídio contendo a base nitrogenada guanina pela timina na posição 894 ($G^{894 \rightarrow}T$) e, consequentemente, substituição do aminoácido glutamato pelo aspartato na posição 298, levando a uma alteração na estrutura primária da proteína. Esse polimorfismo tem sido associado com alterações de mecanismos moleculares, como atividade reduzida da eNOS (63) e modificação na localização da enzima nas cavéolas das células endoteliais, o que pode ocasionar a diminuição da resposta dependente do estresse de cisalhamento, assim como, o comprometimento do ciclo regulatório da eNOS (64).

Embora controverso, estudos mostram que as alterações moleculares provocadas pelo polimorfismo 894G>T podem contribuir para o processo de desenvolvimento e progressão da disfunção endotelial, levando a menor produção de NO (64-66) e uma redução na reatividade vascular (66-69). Por sua vez, quando avaliada a influência da presença do polimorfismo 894G>T em desfechos clínicos, observa-se a sua associação a doenças cardiovasculares de modo geral, tais como coronariopatia (70, 71), infarto do miocárdio (61, 67), hipertensão (72) e acidente

cerebrovascular isquêmico (73). Porém, estudos semelhantes não têm identificado associação entre este polimorfismo e doença cardiovascular (74-76) e/ou têm sugerido a relevância de outros polimorfismos em detrimento do 894G>T (77-79).

É importante ressaltar que as associações entre o polimorfismo 894G>T e desfechos clínicos explicam apenas uma parte da etiologia complexa e multifatorial das doenças cardiovasculares. Além disso, a interpretação dos resultados deve ser realizada levando-se em consideração possíveis limitações e características dos estudos, como por exemplo, a população estudada, o tamanho amostral e as respostas a estímulos fisiológicos que são capazes de provocar perturbações da homeostase como a realização de exercício físico dinâmico (80).

A realização de exercício dinâmico promove vasodilatação nos músculos em movimento com respostas cardiovasculares integradas que incluem aumento do débito cardíaco e da pressão arterial, e redução do fluxo sanguíneo para a circulação esplâncnica e dos músculos em repouso. Após o término do exercício, essas respostas fisiológicas podem levar alguns minutos ou horas para que haja o retorno aos valores basais (81). Apesar de alguns estudos mostrarem que o NO não apresenta papel relevante como mediador da vasodilatação dos vasos musculares durante o exercício (82), sua participação após a realização do exercício parece ser importante (83).

Desta forma, considerando que o papel do NO como um vasodilatador está aumentado após a realização de exercício e que o polimorfismo 894G>T parece ter um papel de redução da liberação de NO em resposta ao estresse de cisalhamento (64), ainda não está claro na literatura qual a influência do polimorfismo 894G>T no efeito de uma única sessão de exercício dinâmico na reatividade vascular. Assim, o objetivo principal do terceiro estudo que integra parte da presente tese, foi investigar a influência do polimorfismo 894G>T no efeito de uma única sessão de exercício dinâmico máximo na reatividade vascular de indivíduos sadios.

3. ARTIGO I: PRESERVED HEART RATE VARIABILITY IN FIRST-DEGREE RELATIVES OF SUBJECTS WITH TYPE 2 DIABETES MELLITUS WITHOUT METABOLIC DISORDERS

4. ARTIGO II: IMPAIRED VASCULAR REACTIVITY IN HEALTHY FIRST-DEGREE RELATIVES OF SUBJECTS WITH TYPE 2 DIABETES IS RELATED TO METABOLIC FACTORS

5. ARTIGO III: EFFECT OF A SINGLE BOUT OF DYNAMIC EXERCISE ON VASCULAR REACTIVITY OF SUBJECTS WITH THE 894G>T POLYMORPHISM OF THE ENDOTHELIAL NITRIC OXIDE SYNTHASE

Effect of a single bout of dynamic exercise on vascular reactivity of subjects with the 894G>T polymorphism of the endothelial nitric oxide synthase

Running title: eNOS Polymorphism and exercise

Key word: exercise, plethysmography, polymorphism, nitric oxide

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ABSTRACT

Considering that the role of nitric oxide as a vasodilator is increased after exercise and that the 894G>T polymorphism of the endothelial nitric oxide synthase seems to reduce the nitric oxide release in response to shear stress, the aim of the present study was to investigate the influence of the 894G>T polymorphism on the effect of a single bout of maximal dynamic exercise on vascular reactivity. We studied 110 healthy volunteers (wild type group: 45.5% and polymorphic group: 54.5%). The protocol consisted of vascular reactivity assessment at baseline and during reactive hyperemia (RH), which were performed pre, 10, 60 and 120 min after a maximal cardiopulmonary exercise test. Vascular reactivity was assessed by venous occlusion plethysmography. Genomic DNA was extracted from blood samples to determine the 894G>T polymorphism. There were no differences between the wild type and polymorphic groups concerning anthropometric, metabolic, and hemodynamic characteristics. Blood flow measurements before maximal dynamic exercise were similar between the wild type and the polymorphic groups. The polymorphic group presented lower vascular reactivity regardless of time (P=0.019 for group main effect), and post-hoc analysis revealed that polymorphic subjects had lower values than wild type only at the 120 min measurement (P=0.022). Concerning within group analysis, vascular reactivity increased at 10 min after exercise (P=0.029) returning to baseline at 120 min (P=0.005) in the polymorphic group. In conclusion, subjects with the 894G>T polymorphism had lower vascular reactivity after a single bout of exercise, denoting the presence of vascular dysfunction.

Abbreviations BMI, body mass index; CRP, C-reactive protein; DPB, diastolic blood pressure; eNOS, endothelial nitric oxide synthase; HDL, high density lipoprotein; HOMA-IR, homeostasis model assessment; HR, heart rate; LDL, low density lipoprotein; L-NMMA, *N*G-monomethyl-L-arginine; MAP, mean arterial pressure; NO, nitric oxide; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RH, reactive hyperemia; SBP, systolic blood pressure; VO2peak, peak oxygen uptake.

INTRODUCTION

Vascular tone is under the direct influence of endothelial function via the release of chemical mediators such as nitric oxide (NO), which relaxes the vascular smooth muscle cells causing vasodilation (Hibi *et al.*, 1998; Miyamoto *et al.*, 1998; Lamblin *et al.*, 2005). Different studies have shown the important contribution of NO to physiological vascular responses in humans. For example, the prompt increase in blood flow after a period of ischemia (reactive hyperemia - RH) is known to result from the interaction of several factors, including NO release, since the magnitude of flow is reduced by approximately 25% when NO production is inhibited (Tagawa *et al.*, 1994; Engelke *et al.*, 1996).

Dynamic exercise causes metabolic vasodilation in working muscles along with complex integrative cardiovascular responses, including increased cardiac output and blood pressure and reduced blood flow to splanchnic circulation and resting muscles. These physiological responses take several minutes or hours to return to baseline values after exercise has ceased as described by Nobrega's review (da Nobrega, 2005). Although the role of NO as a mediator of vasodilation of muscle vessels during exercise seems to be of minor importance (Tschakovsky & Joyner, 2008), vascular reactivity is amplified after exercise (Agewall *et al.*, 1999; Bousquet-Santos *et al.*, 2005), a mechanism that seems to depend on NO release since post-exercise hyperemic volume was reduced by 50% when NO synthase is blocked by intra-arterial infusion of *N*G-monomethyl-L-arginine (L-NMMA) (Gordon *et al.*, 2002).

It is well known that vascular reactivity measured by RH presents great inter-individual variability, even among healthy subjects with the same age, gender, aerobic power, and other phenotypic characteristics (Brook *et al.*, 2005; Ishibashi *et al.*, 2006). Therefore, it is conceivable that genetic factors would be responsible for part of the inter-subject variability. Considering that NO is an important mediator of vascular reactivity (Hibi *et al.*, 1998; Miyamoto *et al.*, 1998), polymorphisms of the gene coding for the enzyme responsible for its production are potential candidates to explain part of the inter-individual variability on vascular reactivity.

NO, that specifically regulates vascular tone is synthesized by the endothelial isoform of nitric oxide synthase (eNOS), which converts L-arginine to L-citruline involving several cofactors (Andrew & Mayer, 1999). The eNOS gene is located at

chromosome 7q35-36 and contains 26 exons that span 21 kb (Marsden *et al.*, 1993). Since the characterization of the eNOS gene in the mid-1990s, many related polymorphisms have been identified, and several specific allelic variations of the eNOS gene have been implicated as potential links to cardiovascular diseases (Rossi *et al.*, 2003a; Casas *et al.*, 2004). A common variation of eNOS, that alters the primary structure of the protein, is the 894G>T or Glu298Asp variant located at exon 7. This polymorphism corresponds to a substitution of guanine to thymine at position 894, and leads to a substitution of glutamate to aspartate at amino acid position 298. The 894G>T polymorphism has been associated with reduced eNOS activity (Persu *et al.*, 2002) and altered localization of eNOS at caveolar leading to diminished response to shear stress and impaired coordination of the enzyme regulatory cycle (Joshi *et al.*, 2007), which altogether seem to decrease NO production from the endothelium (Veldman *et al.*, 2002). Albeit controversial, the functional consequences of this eNOS dysregulation may include a reduction of basal vascular reactivity (Schneider *et al.*, 2000; Veldman *et al.*, 2002; Rossi *et al.*, 2003b). Nevertheless, the systemic effect of dynamic exercise on vascular reactivity in subjects with the 894G>T polymorphism of eNOS has never been investigated.

Considering that the role of NO as a vasodilator is increased after exercise (Gordon *et al.*, 2002) and that the 894G>T polymorphism of the eNOS seems to reduce the NO release in response to shear stress (Joshi *et al.*, 2007), the aim of the present study was to investigate the influence of the 894G>T polymorphism on the effect of a single bout of maximal dynamic exercise on vascular reactivity. The working hypothesis was that exercise would disclose differences in vascular reactivity between wild-type and polymorphic subjects that may not be as evident at baseline.

METHODS

Sample

A total of 110 healthy volunteers (23% men; age 32±9 years) were recruited through advertisements in the University and in local newspapers. The eligibility for taking part in the study was verified through a clinical history assessment, physical examination, blood pressure measurement taken on two different days, biochemical blood analyses, resting electrocardiogram and maximal cardiopulmonary exercise testing. All the subjects fulfilled the following criteria: age between 18 and 49 years, women had regular menstrual cycles, absence of any diagnosed disease and no recent infections, body mass index (BMI) between 18.5 and 29.9 $kg.m^{-2}$, systolic blood pressure (SBP) < 140 mmHg and/or diastolic blood pressure (DBP) < 90 mmHg, glycemia < 5.6 $mmol.L^{-1}$, total cholesterol < 6.21 $mmol.L^{-1}$, low density lipoprotein (LDL) < 4.14 $mmol.L^{-1}$, triglycerides < 2.26 $mmol.L^{-1}$, non-smoker, not using medications with exception of oral contraceptives, normal resting and exercise electrocardiogram, and sedentary (not engaged in exercise activities lasting 30 minutes or more, 3 times per week during the last three months). The study procedures were approved by the Ethics Committee of Antonio Pedro University Hospital, and written informed consent was obtained from all participants prior to the experimental procedures.

Experimental Protocol

After the initial clinical and laboratory screening, experiments were conducted in the morning, an hour after a standardized light breakfast. Women were evaluated from the 1^{st} until the 12^{th} day after the onset of menstruation. Participants did not drink alcohol or caffeinated beverages and did not perform intense physical activity for at least 24 h before the experimental visit. In addition, they slept normally the night prior to the measurements. For the experiment, subjects were placed in a supine position in a quiet air-conditioned room (~24^{o}C). After instrumentation they rested quietly for 10 min. The experimental protocol consisted of blood pressure and vascular reactivity assessment at baseline and during RH, which were performed pre, 10, 60 and 120 min after a maximal cardiopulmonary exercise test. Forearm vascular

reactivity was assessed by venous occlusion plethysmography on the right arm and blood pressure was measured on the left arm through the auscultatory method.

Biochemical blood analyses

Plasma glucose was measured by enzymatic in vitro test, and serum insulin was determined using an electrochemiluminescense assay. The homeostasis model assessment (HOMA-IR), an index of insulin resistance, was calculated (Matthews *et al.*, 1985). Cholesterol and subfractions (LDL and HDL), triglycerides, and C-reactive protein were determined by the dry chemistry method.

Genotyping

A blood sample (4mL) was collected in 4-mL EDTA tubes for genotyping analysis. Genomic DNA was isolated from white blood cells using the Salting-out method, and the eNOS 894G>T polymorphism was determined through polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP). A 248-bp DNA fragment containing the polymorphic site was amplified by PCR using forward primer 5'-AAG GCA GGA GAC AGT GGA TGG A-3' and reverse primer 5'-CCC AGT CAA TCC CTT TGG TGC TCA-3'. The PCR conditions were, initial denaturation step at 94^{o}C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 1 minute and a final extension step at 72^{o}C for 7 minutes. The amplified product was digested with 3U restriction enzyme *Ban*II. DNA fragments were separated by electrophoresis on 2% agarose gel and visualized by use of ethidium bromide and UV light. The results were confirmed by at least 2 independent investigators who were unaware of the DNA origin. The Figure 1 is the genotyping of some volunteers.

Venous Occlusion Plethysmography

Subjects were placed in the supine position with the right arm supported in a comfortable position elevated approximately 5 cm above the level of the heart. Two cuffs were used, one (8 cm width) was placed around the right wrist, and another (10 cm width) was placed around the right upper arm, which was attached to a rapid

cuff inflator (EC6, Hokanson, Bellevue, WA, USA). A mercury-in-silastic strain gauge (Hokanson, Bellevue, WA, USA) was placed at the widest girth of the right forearm. The diameter of the strain gauge was one or two cm lower than the widest girth of the forearm. Blood flow was measured at baseline and post-ischemia. The release of arm circulation after ischemia leads to a large vasodilation, and has been widely used to assess vascular reactivity.

Baseline Blood Flow. The wrist cuff was inflated to 220 mmHg, starting 1 min before the onset of blood flow measurement, to isolate the vascular circulation of the hand and was kept inflated throughout blood flow measurement (Burggraaf *et al*., 2000). At the beginning of all measurements the evaluator generated a standard calibration pulse of 1 mV. Blood flow was measured by rapidly inflating the cuff placed around the right upper arm to 50 mmHg (in less than 0.5 s), maintaining this pressure for 10 s, and then rapidly deflating it to 0 mmHg and maintaining this pressure for 10 s, thus completing a 20-s cycle. Six cycles were performed to determine baseline blood flow.

Reactive Hyperemia (RH). After the baseline blood flow assessment, the cuff placed around the right upper arm was inflated to 200 mmHg for 5 min in order to occlude forearm circulation, thus provoking an ischemic stimulus. The wrist cuff was inflated to 220 mmHg once again at the 4^{th} min, and at the end of the 5^{th} min the arm cuff was deflated, a standard calibration pulse of 1 mV was generated, and 10 s after deflation the blood flow was measured for 3 min following the protocol previously described, i.e. the upper arm cuff was kept inflated for 10 s at 50 mmHg and then deflated for 10 s at 0 mmHg.

The plethysmographic signal was transmitted to a computer for off-line analysis using an A/D conversion board that sampled the signal at a rate of 1 MHz (National Instruments, Co., Austin, TX, USA) for off-line analysis. Two researchers analyzed the data and the reproducibility of blood flow analysis in our laboratory was high (intraclass correlation coefficient of 0.98 and 0.99, for baseline and RH, respectively).

Blood pressure was measured on the left arm by the auscultatory method, using a calibrated mercury sphygmomanometer with an appropriate cuff size, once at the beginning of the baseline blood flow measurement, and once at the beginning of RH. Mean blood pressure (MBP) was used to calculate the vascular conductance (blood flow/MBP). The area under the vascular conductance curve, both at baseline

and during RH, was calculated as a flow-time index to provide values of total forearm vascular conductance in each situation, and the percentage increase of the vascular conductance during the RH above the previous baseline value was considered as the vascular reactivity measure.

Maximal cardiopulmonary exercise test

The maximal cardiopulmonary exercise test was performed on a treadmill (Inbramed, Porto Alegre, RS, Brazil). The test consisted of a 3 min warm-up at 3 km/h and 0% grade, followed by a ramp protocol with a linear increase of speed and grade every minute, and a 5 min recovery at 4 km/h and 0% grade. The ramp protocol was individualized according to the predicted maximal exercise capacity to reach volitional fatigue at approximately 10 min. The subjects were verbally encouraged to exercise until exhaustion. Ventilation, oxygen uptake and carbon dioxide output were measured with each breath (CPX Ultima Gas Exchange System, Medgraphics, St Paul, MN, USA). Electrocardiograms were monitored through 12 leads (Welch Allyn CardioPerfect™ Workstation, Welch Allyn, Skaneateles Falls, NY, USA), blood pressure was measured every 2 min through the auscultatory method and perceived exertion was assessed every minute through the Borg 0-10 scale. Breath-by-breath ventilation and expired gases were averaged to 20 s to identify the ventilatory threshold and the peak oxygen consumption. Ventilatory threshold was considered the oxygen uptake corresponding to the increase in ventilatory equivalent of oxygen without an increase in the ventilatory equivalent of carbon dioxide. Peak oxygen uptake (VO2peak) was considered the highest value of oxygen uptake during exercise.

Statistical Analysis

The Shapiro-Wilk test was used to verify the variables distribution. Groups' characteristics were compared using Student´s t-tests, Mann-Whitney test, or Chi-square when appropriate. Vascular conductance data were log transformed to normalize its distribution. A two way ANOVA, followed by the Fisher post-hoc, was used to compare the vascular conductance at moments pre, and 10, 60 and 120 min post exercise between the wild type (GG genotype) and the polymorphic (GT/ TT

genotypes) groups. Statistical significance was considered as $P < 0.05$ for two tailed comparisons. All analyses were performed with the software STATISTICA (version 8, StatSoft, Inc., Tulsa, OK, USA).

RESULTS

Fifty subjects (45.5%) had the homozygous wild-type genotype (GG), 50 (45.5%) had the heterozygote polymorphic genotype (GT) and 10 (9%) had the homozygous polymorphic genotype (TT). The observed genotype frequencies were in accordance with the Hardy-Weinberg equilibrium (P = 0.95). Data were analyzed by pooling the results from GT and TT groups in order to investigate the impact of the presence of the polymorphic allele.

As shown in Table 1, there were no differences between the wild type and the polymorphic groups in terms of gender, age, weight, BMI, peak oxygen uptake, maximal heart rate, biochemical blood analysis, and resting blood pressure. In addition, blood flow measurements before maximal dynamic exercise were similar between the wild type and the polymorphic groups (Table 2).

Vascular reactivity before and after exercise is shown in Figure 2. Maximal exercise increased vascular reactivity in the group as a whole (upper panel). When analyzed separately (lower panel), polymorphic group presented lower vascular reactivity regardless of time (P = 0.019 for group main effect), and post-hoc analysis revealed that polymorphic subjects had lower values than wild type only at the 120 min measurement (P = 0.022). Concerning within group analysis, vascular reactivity increased at 10 min after exercise (P = 0.029) returning to baseline at 120 min (P = 0.005) in the polymorphic group.

DISCUSSION

The purpose of the present study was to investigate the influence of the 894G>T polymorphism of the eNOS gene on the changes seen in vascular activity after a single bout of maximal dynamic exercise in healthy young subjects. The results support the hypothesis that exercise would disclose a difference in vascular reactivity between wild type and the polymorphic subjects that was not as evident before exertion. This effect, i.e. lower vascular reactivity in polymorphic subjects, became clear 120 min after maximal exercise was performed.

RH is the increase in blood flow caused by metabolic vasodilation in response to ischemia induced by vascular occlusion. Increased blood flow causes augmented blood velocity in conduit arteries and thus increases shear stress, which is responsible for the larger arterial diameter and amplification of the hyperemic response. The increased shear stress is a major stimulus for the local release of vasodilatory endothelial factors. Although it is generally accepted that NO plays a role as a mediator during RH, the magnitude of its participation is controversial (Tagawa *et al.*, 1994; Engelke *et al.*, 1996; Agewall *et al.*, 2002). In addition, NO does not seem to be obligatory for the increase in muscle blood flow during exercise (McAllister *et al.*, 2008; Tschakovsky & Joyner, 2008). Nevertheless, NO release is an important mechanism producing RH after exercise. Agewall *et al*. 2002 evaluated the diameter of the brachial artery in healthy men under RH preceded by either a handgrip exercise during NaCl or L-NMMA infusion or no exercise at all. Post-occlusion vasodilation was significantly larger after exercise compared to occlusion only measures, and it was lower after ischemic exercise during intra-arterial infusion of L-NMMA compared to infusion of NaCl (Agewall *et al.*, 2002). Gordon *et al*. have shown that about half of the increase in blood flow during RH, measured immediately after cessation of dynamic exercise, is due to NO release, highlighting that the post-contraction muscular environment boosts the role of NO as a mediator for increasing blood flow (Gordon *et al.*, 2002). It is worth noting that vascular reactivity increases after exercise in vascular beds not directly involved with the contractions (Bousquet-Santos *et al.*, 2005; Baynard *et al.*, 2007), suggesting that a systemic mechanism is operating, a concept supported by the results of the present study.

The 894G>T polymorphism leads to a change in the primary structure of eNOS and has been associated with reduced enzymatic activity (Persu *et al.*, 2002)

and altered localization of eNOS at caveolar (Joshi *et al.*, 2007). When endothelial cells carrying this polymorphism are submitted to shear stress, their NO release is lower than cells without the polymorphism, suggesting impaired capacity to respond to the stimulus (Joshi *et al.*, 2007). Since this function is not completely abolished, the presence of the 894G>T polymorphism of eNOS may cause differences in vascular reactivity that may not be as evident at basal conditions, but could be revealed by physiological challenges, such as maximal exercise. In the present study subjects with the 894G>T polymorphism presented lower vascular reactivity throughout the experiment (ANOVA group effect), but measurements before exercise were not particularly different. Whether subjects with this polymorphism have diminished basal vascular reactivity is a controversial issue. For example, Rassol *et al*. investigated the influence of the 894G>T polymorphism on skin microvascular reactivity to an ischemic stimulus, and found no significant difference between wild-type and polymorphic subjects (Rasool *et al.*, 2009). In addition, Kathiresan *et al*. investigated the influence of various single nucleotide polymorphisms, either isolated or as haplotypes, on brachial artery flow-mediated dilation and hyperemic flow velocity, and found no association between eNOS sequence variants and endothelial function (Kathiresan *et al.*, 2005). Employing an invasive approach, Schneider *et al*. could not find any differences in basal forearm blood flow in response to intra-arterial infusion of acetylcholine or L-NMMA between healthy volunteers with or without the 894G>T polymorphism (Schneider *et al.*, 2000). On the other hand, Veldman *et al*. showed a reduced response of blood flow during the infusion of L-NMMA in subjects with the 894G>T polymorphism, suggesting that their basal NO production is reduced as compared to wild type subjects (Veldman *et al.*, 2002). Godfrey and co-authors observed that carrying the T allele was associated with blunted endothelial-dependent vasodilation in healthy subjects (Godfrey *et al.*, 2007), while the T allele was the only significant and independent predictor of flow-mediated brachial artery dilation and carotid intima-media thickness by multivariate regression analysis (Paradossi *et al.*, 2004). Taken together, these results suggest that if the presence of the 894G>T polymorphism has any influence on basal vascular reactivity it is modest at best, as it seems to be the case for any individual eNOS polymorphism (Metzger *et al.*, 2005; Metzger *et al.*, 2007). However, considering that endothelial cells with this polymorphism have limited capacity to release NO when submitted to shear stress (Joshi *et al.*, 2007), the present study was designed to investigate whether the

increase in vascular reactivity to a non-exercising limb would be different in subjects with and without this genetic variation.

To the best of our knowledge, only one previous study has investigated the influence of the 894G>T polymorphism on vascular reactivity during exercise. Dias *et al*. showed that subjects homozygous for the polymorphism had attenuated muscle vasodilation in the non-exercising limb in response to a handgrip exercise, although at baseline there was no difference between wild type and the polymorphic subjects. In the present study, a different mode of exercise was applied – dynamic contraction of large muscle groups. In addition, vascular reactivity was measured after exercise was ceased, not during the effort. Nevertheless, taken together, these two studies suggest that individuals with 894G>T polymorphism of eNOS have comparable vascular reactivity to wild-type subjects, but reduced capacity to vasodilate resting muscle vessels in response to exercise. Whether this polymorphism blunts the physiological increase in blood flow to the working muscles remains uncertain.

The results of the present study should be interpreted in light of the following limitations. Although our subject population consisted of three different genotypes - homozygous wild-type (GG), heterozygous (GT), and homozygous mutant (TT) - only two groups were considered for data analysis, the wild-type group (GG) and the polymorphic group (GT/TT), because of the small frequency of the TT genotype. This grouping strategy is a standard approach in similar experiments, but precludes analysis to determine whether different responses would occur between subjects with GT and TT genotype. Another limitation was the absence of direct measures of the nitric oxide bioavailability, such as nitrite/nitrate dosage on plasma, or of the eNOS activity such as pharmacological blockade of eNOS. Despite the potential role of other mediators in regulating changes in vascular reactivity after exercise, RH is believed to depend on endothelium-derived NO (Kooijman *et al.*, 2008). In addition, clinical and laboratory variables were similar between polymorphic and wild-type subjects, the only known difference being the presence/absence of the altered gene coding for eNOS along with altered vascular reactivity after exercise.

In conclusion, the present study showed that exercise disclosed a difference in vascular reactivity between wild type and the polymorphic subjects that was not evident before exertion.

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DISCLOSURE

No conflict of interests.

AUTHOR CONTRIBUTIONS

All authors have contributed to all of the following:

1. conception and design, or analysis and interpretation of data,
2. drafting the article or revising it critically for important intellectual content,
3. final approval of the version to be published.

All the experiments were done at Fluminense Federal University, Niterói, RJ, Brazil.

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Table 1. Anthropometric, metabolic, and hemodynamic characteristics for the wild type and the polymorphic groups.

Variables	Groups		*P* value
	Wild type	Polymorphic	
n (male%)	50 (24%)	60 (22%)	0.82
Age (years)	32.6 ± 1.3	31.7 ± 1.1	0.58
Weight (kg)	66.9 ± 1.6	67.4 ± 1.3	0.80
BMI ($kg.m^{-2}$)	24.2 ± 0.5	24.8 ± 0.4	0.32
Waist circumference (cm)	76.8 ± 1.3	77.5 ± 1.2	0.69
Fasting glucose ($mmol.L^{-1}$)	4.69 ± 0.07	4.68 ± 0.06	0.96
Fasting insulin ($pmol.L^{-1}$)	40.59 ± 3.83	42.22 ± 3.72	0.76
HOMA-IR	1.44 ± 0.1	1.5 ± 0.2	0.74
Cholesterol ($mmol.L^{-1}$)	4.54 ± 0.10	4.57 ± 0.12	0.83
HDL ($mmol.L^{-1}$)	1.39 ± 0.04	1.44 ± 0.04	0.47
LDL ($mmol.L^{-1}$)	2.69 ± 0.09	2.70 ± 0.10	0.93
Triglyceride ($mmol.L^{-1}$)	1.00 ± 0.06	1.01 ± 0.06	0.90
CRP ($mg.L^{-1}$)	0.40 ± 0.04	0.53 ± 0.07	0.14
SBP (mm Hg)	114 ± 1	113 ± 1	0.36
DBP (mm Hg)	74 ± 1	72 ± 1	0.14
MAP (mm Hg)	88 ± 1	85 ± 1	0.16
VO_{2peak} ($mL.kg^{-1}.min^{-1}$)	33.4 ± 1	31.5 ± 1	0.18
HR_{max} (beats/min)	183 ± 2	182 ± 2	0.46

Data are mean±SEM.

BMI indicates body mass index; HOMA-IR, homeostasis model assessment; HDL, high density lipoprotein; LDL, low density protein; CRP, C-reactive protein; SBP, systolic blood pressure; DPB, diastolic blood pressure; MAP, mean arterial pressure; VO2peak, peak oxygen uptake; HR, heart rate.

Table 2. Vascular conductance at baseline and during reactive hyperemia, and vascular reactivity before a single bout of maximal dynamic exercise in wild type and polymorphic groups.

Variables	Groups		*P* value
	Wild type	Polymorphic	
Basal blood flow conductance (a.u.)	215.05 ± 10.48	246.02 ± 13.28	0.18
RH blood flow conductance (a.u.)	1021.93 ± 50.97	1046.45 ± 53.85	0.94
Vascular reactivity (a.u.)	802.40 ± 47.08	826.97 ± 49.42	0.19

Data are mean±SEM.

RH indicates reactive hyperemia; a.u., arbitrary units.

Figure 1. The genotyping of some volunteers.

1. GG
2. GT
3. GG
4. GT
5. GT
6. GT
7. GT
8. GG
9. GG
10. GG
11. GG
12. GT
13. GT
14. GG
15. TT
16. GG
17. GG
18. GG
19. negative control
20. MARC 100bp

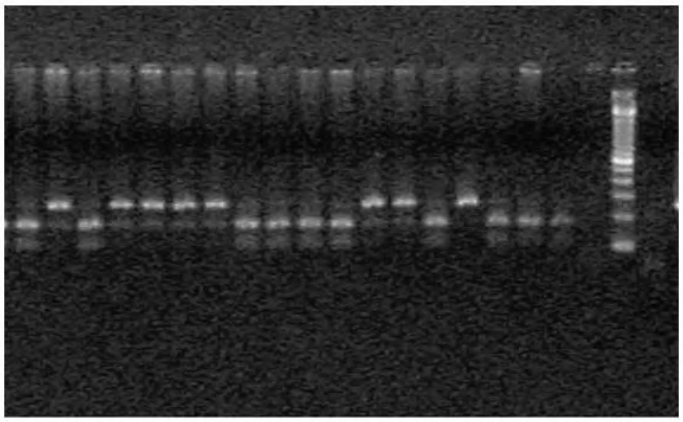
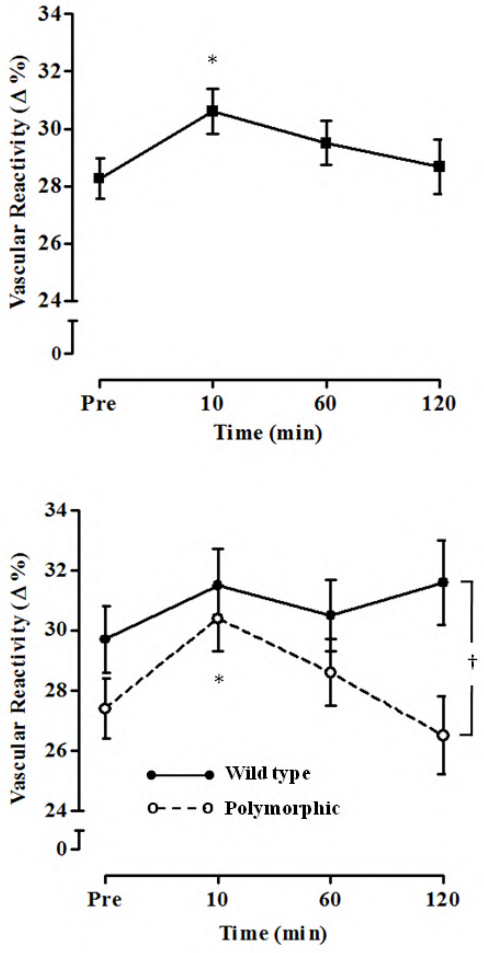


Figure 2. Vascular reactivity before and at intervals after a single bout of maximal dynamic exercise in the whole group of subjects (n=110; upper panel) and separately for wild type (n=50) and polymorphic subjects (n=60; lower panel).
Values are mean±SEM. Upper panel: * *P* = 0.015 vs. pre maximal dynamic exercise; lower panel: * *P* = 0.005 vs. pre maximal dynamic exercise in the polymorphic group; † *P* = 0.019 for main group effect.



6. DISCUSSÃO

Os artigos publicados no *Diabetic Medicine* (2008) e *Diabetes Care* (2009) abordaram a influência da HFDM2 na função autonômica e reatividade vascular, respectivamente. Em ambos os estudos, foram identificados valores aumentados para variáveis metabólicas nos indivíduos com HFDM2 quando comparado com o grupo controle, assim como observado em estudos prévios (45, 58, 84-87). Considerando que esses fatores de risco reduzem a função autonômica (46, 48, 50, 52) e a reatividade vascular (47, 49, 51, 53), é de se esperar que ambos estejam diminuídos nos parentes quando comparados aos indivíduos-controle. Análises subsequentes levaram ao resultado destes estudos, o qual, na ausência de alterações metabólicas, a função autonômica e reatividade vascular apresentavam valores similares entre indivíduos com HFDM2 e aqueles sem qualquer história de DM2.

Estudos prévios mostraram que a HFDM2 está associada com a redução da variabilidade da frequência cardíaca, que é considerado um fator prognóstico para o aparecimento de eventos cardíacos (16). Iellamo e colaboradores (43) sugeriram que indivíduos com HFDM2 apresentavam aumento da modulação simpática periférica como característica primária, mas sabendo que esses indivíduos apresentavam altos níveis de PCR e hemoglobina glicosilada, que são fatores conhecidos por alterarem a função autonômica se comparados com indivíduos-controle (50, 54), é provável que essa redução na modulação autonômica cardiovascular tenha sido provocada por inflamação sistêmica e disglicemia. O mesmo foi observado em outras publicações, nas quais os autores relataram índices reduzidos de modulação autonômica em indivíduos com HFDM2 acompanhados de alterações metabólicas (42, 88). Vale salientar que os níveis dessas variáveis metabólicas estão frequentemente alterados em parentes de diabéticos e são conhecidos por reduzirem a função autonômica (52, 56). Portanto, não se pode excluir a possibilidade de que outros fatores ligados a desordens metabólicas expliquem a redução da VFC observada nos indivíduos que apresentam HFDM2.

Frontoni e colaboradores (89) analisaram a interação entre a função autonômica e resistência insulínica, onde indivíduos com HFDM2 insulinodependentes apresentavam uma modulação exacerbada do sistema nervoso

simpático. Porém, esse resultado foi observado apenas nos parentes que apresentavam resistência insulínica identificada pelo *clamp* hiperinsulinêmico euglicêmico, e não nos parentes de DM2 que eram sensíveis à insulina, fornecendo resultados similares ao estudo atual.

Embora seja evidente que a HFDM2 não seja um fator de risco independente que prejudique a função autonômica, é importante que a associação entre a função autonômica e o desenvolvimento de DM2 não seja negligenciada. O estudo *The Atherosclerosis Risk in Communities (ARIC)* acompanhou mais de 8.000 indivíduos durante aproximadamente oito anos, e concluiu que a frequência cardíaca de repouso e a VFC podem estar associadas com o desenvolvimento de DM2 em indivíduos saudáveis, independentemente de outros fatores como idade, raça, gênero, atividade física e IMC (90). Outro índice que representa a modulação autonômica cardíaca, a frequência cardíaca de recuperação após o término de um teste de exercício máximo em esteira rolante, também atuou como um fator preditivo para a ocorrência de DM2 no estudo *Coronary Artery Risk Development in the Young Adults (CARDIA)*. Entre indivíduos com baixa condição aeróbica e prevalência semelhante de HFDM2, o risco de desenvolver DM2 foi 3,4 vezes maior, um efeito que persistiu mesmo após ajustes para insulina basal (91). Portanto, é provável que a redução na função autonômica e a presença de HFDM2 sejam fatores de risco independentes para DM2, operando através de mecanismos como sedentarismo, desordens metabólicas e obesidade, que são fatores de risco para DM (33) e estão correlacionados com a função autonômica (52, 54, 56).

A literatura científica vem investigando também a influência do parentesco de primeiro grau de DM2 na reatividade vascular, que é considerada um marcador precoce do processo aterosclerótico (18). Quando avaliada a reatividade vascular desta mesma população, ou seja, indivíduos que apresentavam HFDM2, os resultados foram similares aos encontrados em relação ao sistema nervoso autônomo, e foram publicados no *Diabetes Care* em 2008.

O primeiro estudo avaliando a reatividade vascular de indivíduos com HFDM2 foi publicado por Caballero e colaboradores (86). Os autores concluíram que os participantes com HFDM2 apresentavam reatividade vascular reduzida quando comparado com indivíduos sem parentesco. Entretanto, como os parentes apresentavam maiores níveis séricos de endotelina e moléculas de adesão, os quais estão sabidamente envolvidos na disfunção endotelial (92, 93), não é possível

afirmar se a menor reatividade vascular observada nos parentes foi devido às alterações séricas ou está relacionada ao fator hereditário. Balletshofer e colaboradores (85) encontraram vasodilatação reduzida apenas nos indivíduos com HFDM2 que apresentavam resistência insulínica, sugerindo que as desordens metabólicas e não os fatores genéticos, eram os mecanismos envolvidos diretamente na menor reatividade vascular. Mesmo após esse elegante estudo, outras publicações têm sugerido que o comprometimento da reatividade vascular de indivíduos com HFDM2 era devido à susceptibilidade genética, desconsiderando a influência de alterações metabólicas (43, 45, 84, 87, 94, 95), mais comuns em parentes de DM2 e sabidamente fatores causais para disfunção endotelial (96-98).

Nossos resultados sobre a reatividade vascular de indivíduos com HFDM2 estão de acordo com os encontrados por Balloshofer e colaboradores (85), nos quais a redução da reatividade vascular estava associada à resistência insulínica nos parentes. O presente estudo está de acordo com a idéia de comprometimento da reatividade vascular dos indivíduos com HFDM2, mas essa alteração funcional precoce parece ser causada por variáveis como resistência insulínica, inflamação subclínica, valores elevados de glicose e lipídios, os quais são mais comuns em indivíduos com HFDM2. Desta forma, apesar da hipótese de “susceptibilidade hereditária primária” como causadora do comprometimento da reatividade vascular em indivíduos com HFDM2, um mecanismo metabólico subjacente parece estar envolvido em todos os artigos publicados até o momento.

Os resultados dos estudos que investigaram a função autonômica e a reatividade vascular de indivíduos que apresentavam HFDM2, e que foram publicados no *Diabetic Medicine* e *Diabetes Care*, respectivamente, devem ser interpretados mediante a presença de algumas limitações. Primeiramente, o padrão-ouro para a determinação da homeostasia glicose-insulina é o *clamp* hiperinsulinêmico euglicêmico que não foi realizado. Todavia, a falta de sensibilidade metodológica para detectar a resistência insulínica seria mais relevante como explicação potencial se indivíduos com e sem qualquer história familiar de DM2 apresentassem diferentes índices de VFC. Os índices de VFC obtidos em repouso podem não refletir a função autonômica em outras condições fisiológicas que causam ativação simpática e inibição vagal, como o exercício físico. Diversos métodos têm sido utilizados para avaliar a reatividade vascular em humanos, e a resposta intra-arterial à infusão de substâncias vasoativas pode ser considerada o

método padrão. Entretanto, o método invasivo demanda maior tempo e riscos intrínsecos aos participantes devido ao acesso arterial. O método não invasivo para avaliação do fluxo sanguíneo do antebraço em resposta à hiperemia reativa tanto pelo ultra-som (99) quanto pela pletismografia de oclusão venosa (100), têm sido validados para avaliação da reatividade vascular.

Como os distúrbios metabólicos são conhecidos por prejudicar o sistema nervoso autônomo e a reatividade vascular, e estes constituem mecanismos centrais no desenvolvimento e progressão de doenças do sistema cardiovascular, os resultados de ambos os artigos podem ter implicações importantes para a identificação de populações que podem se beneficiar de modificações precoces de estilo de vida.

Os resultados submetidos ao *Journal of Physiology* também abordam a questão relacionada à reatividade vascular, mas com foco em uma outra população de risco para doença cardiovascular, ou seja, aqueles que apresentam o polimorfismo 894G>T da eNOS. Assim, o terceiro estudo investigou a influência deste polimorfismo nas modificações da reatividade vascular após uma sessão de exercício dinâmico em jovens saudáveis. Os resultados confirmam a hipótese de que o exercício pode expor uma diferença na reatividade vascular que não era evidente antes do esforço entre indivíduos com e sem o polimorfismo 894G>T. A menor reatividade vascular nos polimórficos foi observada 120 minutos após a realização do exercício.

O NO parece ter um papel importante na hiperemia reativa após a realização de exercício físico, quando a contração muscular, consequente ao exercício, pode promover o aumento da produção do NO (83, 101), mesmo em leitos vasculares não envolvidos diretamente com o esforço (102, 103). As respostas fisiológicas ao exercício podem estar prejudicadas na presença do polimorfismo 894G>T, já que este promove mudanças na estrutura primária da eNOS, redução na atividade enzimática (63) e alteração na localização da eNOS na cavéola (64). Isso pode levar a diminuição da liberação de NO e consequentemente da resposta dependente do estresse de cisalhamento (64). Considerando que o NO participa da hiperemia reativa após o esforço físico e que o polimorfismo 894G>T pode levar à redução de liberação de NO, nosso estudo identificou uma menor reatividade vascular nos indivíduos com o polimorfismo 894G>T quando comparados com aqueles que não apresentavam o mesmo polimorfismo (efeito do grupo obtido por ANOVA). Porém,

particularmente no momento anterior ao exercício, essa diferença não foi observada, sugerindo que a função do NO como mediador no maior fluxo sanguíneo pode não estar totalmente anulada na presença do polimorfismo 894G>T e que as diferenças da reatividade vascular que podem não ser evidentes em condição basal, poderiam ser identificadas após manobras fisiológicas como a realização de exercício. Isso demonstra o comprometimento da capacidade de resposta a estímulos pelos indivíduos com polimorfismo 894G>T.

Ainda não está claro na literatura quais são os efeitos da presença do polimorfismo 894G>T em relação à reatividade vascular basal. Alguns artigos mostram que a presença deste polimorfismo não interfere na reatividade vascular basal de indivíduos saudáveis (69, 75, 76), enquanto outros observaram uma menor vasodilatação, o que sugere que a produção basal de NO pode estar reduzida nos polimórficos quando comparado a indivíduos não polimórficos (66, 104, 105). Em conjunto, esses estudos sugerem que, se a presença do polimorfismo 894G>T promove alguma influência na reatividade vascular basal, essa influência parece ser pequena (106, 107). Entretanto, considerando que as células endoteliais que apresentam o polimorfismo 894G>T têm uma capacidade limitada de liberar NO quando submetidas ao estresse de cisalhamento (64), o presente estudo foi delineado para investigar se o aumento na reatividade vascular em um membro não exercitado poderia ser diferente entre sujeitos com e sem o polimorfismo 894G>T. Desta forma, identificamos que a presença do polimorfismo 894G>T não interfere na reatividade vascular basal, mas reduz a capacidade de vasodilatação de leitos vasculares em repouso na resposta ao exercício.

Esses resultados devem ser interpretados mediante a presença de algumas limitações. Embora os participantes apresentassem os três genótipos, ou seja, homozigoto *wild type* (GG), heterozigoto (GT) e homozigoto polimórfico (TT), os participantes foram alocados em dois grupos: ausência (indivíduos GG) ou presença (indivíduos GT/TT) do alelo T. Essa classificação foi feita pela baixa frequência do genótipo TT (7,8%). Essa abordagem é usada com regularidade na literatura científica, mas não permite avaliar se existem diferenças entre os indivíduos GT e TT. Uma outra questão que deve ser levada em consideração é a ausência de medidas para avaliar a biodisponibilidade de NO ou a atividade da eNOS. Apesar de outros mediadores também participarem da regulação da reatividade vascular após a realização de exercício, estudos mostram que a hiperemia reativa é dependente

do NO derivado do endotélio (108). Além disso, a única diferença observada entre os indivíduos avaliados no atual estudo era a presença ou ausência do alelo polimórfico, já que as variáveis antropométricas, bioquímicas e hemodinâmicas eram similares entre os grupos.

Em conclusão, o presente estudo mostrou que o exercício pode expor uma diferença na reatividade vascular que não era evidente antes do esforço entre indivíduos com e sem o polimorfismo 894G>T.

7. CONSIDERAÇÕES FINAIS

Como considerações finais em relação aos três artigos apresentados, podemos destacar que o conhecimento da disfunção autonômica e da alteração da reatividade vascular em indivíduos que apresentam risco aumentado para o desenvolvimento de doenças cardiovasculares podem orientar abordagens preventivas e terapêuticas mais eficazes na promoção da saúde. Uma consequência prática dos nossos achados é ratificar o conceito de que é possível reduzir o risco de disfunção autonômica e endotelial, uma vez que tais problemas não são herdados per se, mas ocorrem como consequência das alterações metabólicas. Por outro lado, é possível que portadores de determinados polimorfismos relacionados a óxido nítrico sintase endotelial apresentem menor reatividade vascular contribuindo para um maior risco de eventos cardiovasculares. Existe a expectativa de que os resultados aqui apresentados poderão subsidiar novas pesquisas nos campos da fisiologia e fisiopatologia cardiovascular, contribuído para o desenvolvimento de medidas preventivas mais eficazes e seguras.

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Short Report

Preserved heart rate variability in first-degree relatives of subjects with Type 2 diabetes mellitus without metabolic disorders

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Abstract

Aims To investigate the influence of a family history of Type 2 diabetes mellitus (T2DM) on resting heart rate variability in the absence of concomitant metabolic disorders.

Methods We studied 55 first-degree relatives (FDRs) of subjects with T2DM and 36 control subjects without any known family history of diabetes. FDRs were recruited from a University Hospital out-patient diabetes clinic. The protocol included: oral glucose tolerance test (30, 60, 90 and 120 min after ingestion of 75 g glucose) blood glucose, plasma insulin, cholesterol and subfractions, triglycerides, leptin and C-reactive protein. Heart rate variability (HRV) at rest was determined by spectral analysis of interbeat intervals recorded during 10 min in the supine position.

Results HRV was lower in FDRs compared with control subjects ($P < 0.05$). Multiple regression analysis identified cholesterol ($P = 0.014$) and triglycerides ($P = 0.014$) as significant independent predictors (model $r = 0.40$; $P < 0.001$) of HRV. Since FDRs had higher values for anthropometric and metabolic variables known to alter HRV, we performed an ANCOVA adjusted for cholesterol and triglycerides and also another analysis in which the groups were comparable for anthropometric and metabolic characteristics. Comparison of FDRs and comparable control subjects revealed no significant difference in HRV ($P > 0.05$).

Conclusions A family history of T2DM, in the absence of concomitant metabolic disorders, does not impair heart rate variability.

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Keywords Type 2 diabetes, heart rate monitoring, family studies

Abbreviations BMI, body mass index; CRP, C-reactive protein; FDR, first-degree relative; HF, high frequency; HOMA, homeostasis model assessment; HRV, heart rate variability; LDL, low-density lipoprotein; LF, low frequency; SDNN, standard deviation of normal-to-normal beats; T2DM, Type 2 diabetes mellitus; TP, total power

Introduction

First-degree relatives (FDRs) of subjects with Type 2 diabetes mellitus (T2DM) are at higher risk for development of metabolic disorders including T2DM, as shown by both cross-sectional [1] and cohort studies [2,3]. Along with behavioural factors linked to the familial environment, it is becoming clear that at least part of the increased risk of FDRs is inherited. A recent genome-wide association study has confirmed one locus and identified four other novel loci that account for a substantial portion of the risk for the development of T2DM [4].

Patients with T2DM display dysfunctional autonomic modulation of the cardiovascular system [5], as evidenced by decreased heart rate variability (HRV), a well known risk factor for cardiac events and sudden death [6]. Considering that genetic factors are involved in the development of T2DM [4], FDRs are at higher risk for the development of the disease [2,3] and it has been proposed that being a FDR of a patient

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with T2DM is a risk *per se* for autonomic dysfunction [7,8]. However, FDRs may have abnormal metabolic variables such as blood insulin, glucose and lipids [9], which are known to impair autonomic function [10–12]. Accordingly, the aim of this study was to investigate the influence of a family history of T2DM on resting HRV in the absence of concomitant metabolic disorders.

Patients and methods

FDRs ($n = 55$; 78% women; age 35 ± 8 years) were compared with a group of control subjects of similar sex and age ($n = 36$; 72% women; age 33 ± 9 years) without a family history of diabetes. The sample size was estimated as 17 subjects for each group based on an $\alpha = 0.05$, power = 0.8, a mean difference between means = 10% and a standard deviation within groups = 10%, for one-tailed comparisons. FDRs were identified by asking hospital diabetes clinic patients whether their family members had diabetes; only one volunteer from each family was included. Before inclusion in the study, a physician took a clinical history, performed a physical examination and resting electrocardiography, and interpreted the biochemical analyses. Exclusion criteria were: second-degree relatives of subjects with T2DM, the presence of diabetes and/or cardiovascular diseases, tobacco use, regular physical activity and use of medications. The study was approved by the institutional Ethics Committee and informed consent was obtained from all participants. Participants did not drink alcohol or caffeine-containing beverages and did not perform intense physical activity for 24 h before the study days. HRV analysis was performed in women during the follicular phase of the menstrual cycle. A standard oral glucose tolerance test was performed after a 12-h fast. Samples for blood glucose and plasma insulin were drawn at 0, 30, 60, 90 and 120 min after 75 g oral glucose. The area under the glucose curve (AUC) was calculated. The homeostasis model assessment (HOMA) value, an index of insulin resistance, was calculated. Cholesterol and subfractions [low-density lipoprotein (LDL) and high-density lipoprotein], triglycerides, leptin and C-reactive protein (CRP) were determined. On a separate day, the RR intervals (intervals between adjacent QRS complexes, equivalent to heart period) were measured and later analysed, according to the standard recommendations [13]. Briefly, the subjects were examined in the morning, after 8 h of fasting. After resting in the supine position for 20 min in a quiet air-conditioned room (~24°C), RR intervals were recorded for 10 min in the supine position (Polar Vantage NV, Electro Oy, Kempele, Finland). The recorded RR intervals were filtered (Matlab 6.0; Mathworks Inc., Natick, MA, USA) and analysed both in the time domain by standard statistics [variable: standard deviation of normal-to-normal beats (SDNN)] and in the frequency domain [Fast Fourier Transformation with Welch's method; variables: total power (TP), low frequency (LF), high frequency (HF), low frequency/high frequency ratio (LF/HF)]. Skewed variables were log-transformed, and differences between groups were compared by Student's *t*-test, the Mann-Whitney test or χ^2 test, when appropriate. Association between variables was determined using Pearson's or Spearman's rank correlation and variables with $P < 0.20$ in these bivariate correlations were included in multiple regression models, using the stepwise forward method. Data are presented as mean ± SD or median ± interquartile range, when appropriate. Statistical significance was considered as $P < 0.05$ for one-tailed comparisons, as the literature indicates that FDRs have anthropometric, metabolic and autonomic abnormalities. All analyses were performed using SPSS (version 15.0; SPSS Inc., Chicago, IL, USA).

Results

Body mass index (BMI), diastolic blood pressure, glucose, HOMA-IR, cholesterol, LDL and leptin were higher ($P < 0.05$), whereas HRV variables (SDNN, TP, LF) were lower ($P < 0.05$) in FDRs than in the control group (Table 1). TP was inversely correlated with glucose (entire cohort, $r = -0.25$, $P = 0.009$; FDRs, $r = -0.19$, $P = 0.082$; control, $r = 0.28$, $P = 0.051$), AUC-glucose (entire cohort, $r = -0.23$, $P = 0.014$; FDRs, $r = -0.29$, $P = 0.016$; control, $r = -0.03$, $P = 0.428$), cholesterol (entire sample, $r = -0.32$, $P = 0.001$; FDRs, $r = -0.26$, $P = 0.029$; control, $r = -0.33$, $P = 0.025$), LDL (entire sample, $r = -0.20$, $P = 0.029$; FDRs, $r = -0.14$, $P = 0.149$; control, $r = -0.16$, $P = 0.172$) and triglycerides (entire cohort, $r = -0.32$, $P = 0.001$; FDRs, $r = -0.31$, $P = 0.010$; control, $r = -0.30$, $P = 0.039$) (Fig. 1). With multiple regression analysis, using TP as the dependent variable, only cholesterol ($P = 0.014$) and triglycerides ($P = 0.014$) were identified as significant predictors (model $r^2 = 0.16$; $P < 0.001$). Since FDRs had higher values for several variables known to alter HRV, the data were re-analysed using an ANCOVA adjusted for variables that were different between groups and were significant predictors of HRV (BMI, fasting glucose, AUC-glucose, fasting insulin, HOMA, cholesterol, LDL, triglyceride, leptin, CRP). This analysis yielded no difference between groups in any of the HRV indices ($P > 0.05$). These results were confirmed by another analysis, in which subjects exhibiting the following criteria were excluded from both groups in order to make them comparable: BMI ≥ 30 kg/m^2, fasting glucose ≥ 5.5 mmol/l, HOMA-IR ≥ 2.5, cholesterol ≥ 6.2 mmol/l, LDL ≥ 4.2 mmol/l, triglycerides ≥ 2.3 mmol/l and/or CRP ≥ 1.0 mg/l. Thereafter, when the groups were compared (FDRs group $n = 22$; control group $n = 31$), no difference was found for any of the HRV indices ($P > 0.05$), denoting comparable autonomic function.

Discussion

The main finding of the present study is that resting HRV was similar between FDRs of subjects with T2DM and control subjects in the absence of glucose intolerance, and differences in BMI, blood lipids, leptin and CRP. Since dysglycaemia and altered blood lipids were more common in FDRs, as also found in previous studies [9,14], and considering that these factors reduce HRV [10–12], it is not surprising that HRV was diminished in FDRs when compared with control subjects when the groups were compared without considering metabolic disorders.

Previous studies aimed to investigate whether a family history of T2DM is associated with reduced HRV, which is

Table 1 Anthropometric, metabolic and heart rate variability data for first-degree relatives of patients with Type 2 diabetes mellitus and control subjects

Variable	FDRs	Control	*P*-value
n (female%)	55 (78%)	36 (72%)	0.516
Age (years)	35.4 ± 8.4	33.3 ± 8.5	0.119
Weight (kg)	72.7 ± 12.7	68.9 ± 13.1	0.084
BMI (kg/m^2)	26.5 ± 4.0	24.6 ± 3.7	0.013
SBP (mmHg)	124 ± 14	73 ± 12	0.059
DBP (mmHg)	119 ± 11	69 ± 9	0.038
Fasting glucose (mmol/l)	5.0 ± 0.5	4.8 ± 0.3	0.009
AUC-glucose (mmol/(min.l)$^{-1}$)	770 ± 148	695 ± 130	0.012
Fasting insulin (pmol/l)*	41 ± 26	31 ± 27	0.045
AUC-insulin (pmol/(min.l)$^{-1}$)*	35 667 ± 22 641	30 537 ± 19 906	0.101
HOMA-IR*	1.51 ± 0.95	1.11 ± 0.90	0.023
Cholesterol (mmol/l)	5.0 ± 0.9	4.5 ± 0.7	0.002
HDL (mmol/l)	1.4 ± 0.4	1.4 ± 0.3	0.391
LDL (mmol/l)	3.1 ± 0.8	2.7 ± 0.6	0.002
Triglyceride (mmol/l)*	1.0 ± 0.5	0.8 ± 0.5	0.053
Leptin (ng/ml)*	21 ± 19	14 ± 10	0.057
CRP (mg/l)*	0.28 ± 0.25	0.20 ± 0.33	0.210
SDNN (ms)*	42.0 ± 19.0	49.0 ± 17.0	0.025
TP (ms^2)*	1787 ± 1765	2376 ± 1697	0.024
LF (ms^2)*	349 ± 476	571 ± 629	0.006
HF (ms^2)*	417 ± 597	517 ± 1084	0.126
LF/HF*	0.87 ± 0.88	0.99 ± 1.05	0.174

Data are mean ± SD or (*) median ± interquartile range.
FDR, First-degree relative;
BMI, body mass index; SBP, systolic blood pressure; DPB, diastolic blood pressure; AUC, area under curve; HOMA, homeostasis model assessment; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; SDNN, standard deviation of normal RR interval; TP, total power; LF, low-frequency power; HF, high-frequency power; LF/HF, low frequency/high frequency ratio.

considered to be a non-invasive estimate of autonomic modulation [7,8] and which is an independent predictor for sudden death in the general population [6]. For example, Lellamo *et al.* [8] have suggested that FDRs have enhanced efferent sympathetic outflow as a primary characteristic, but since FDRs had higher levels of CRP and glycated haemoglobin, which are known to change autonomic function [11,15], it is likely that changes in cardiovascular autonomic modulation were triggered by systemic inflammation and dysglycaemia. The study by Forientini *et al.* [7] has shown that in offspring of subjects with T2DM, reduced HRV was independent of insulin resistance, but they did not report whether lipids or leptin levels, which could influence HRV [10,12], were different between FDRs and control subjects. These relevant comparative data are also lacking in the publication by De Angelis *et al.* [16]. Therefore, one cannot exclude the possibility that other factors linked to metabolic disorders explain the reduced HRV. In this case, alterations in lipid metabolism and the leptin pathway may actually precede the development of autonomic dysfunction. The role of leptin as a pathophysiological mechanism involved in the metabolic syndrome and its consequences has been emphasized recently [17]. Several other publications have reported indices of autonomic modulation in FDRs, supporting the concept that the presence of dysglycaemia might be involved in the development of autonomic dysfunction [15,18–21].

In the present study, HRV was lower in FDRs compared with control subjects, but FDRs also had higher values for BMI, fasting glucose, cholesterol, LDL, HOMA-IR and leptin. In addition, significant negative correlations between HRV and blood glucose, cholesterol, LDL and triglycerides were detected. However, only cholesterol and triglycerides were independently correlated with HRV, for both within-group and combined analyses. When the groups were compared again after adjustment for potential confounding variables, no HRV differences were detected. One previous study focusing on the interaction between autonomic function and insulin resistance has provided similar results. Frontoni *et al.* [22] have shown that offspring of insulin-resistant patients with diabetes had a diminished LF/HF ratio fall during the night as well as an increase in this same variable during a hyperinsulinaemic glucose clamp, suggesting sympathetic overactivity in these subjects. These results were not present in insulin-sensitive FDRs, identified by being in the lower tertile for the rate of glucose infusion during the steady state of the hyperinsulinaemic clamp studies.

The results of the present study should be interpreted in light of some limitations. Although our sample was relatively small, the study was adequately powered for the analyses presented. The young age of our subjects and our selection criteria may have excluded some subjects with more severely impaired HRV. We were unable to use the gold standard for determining

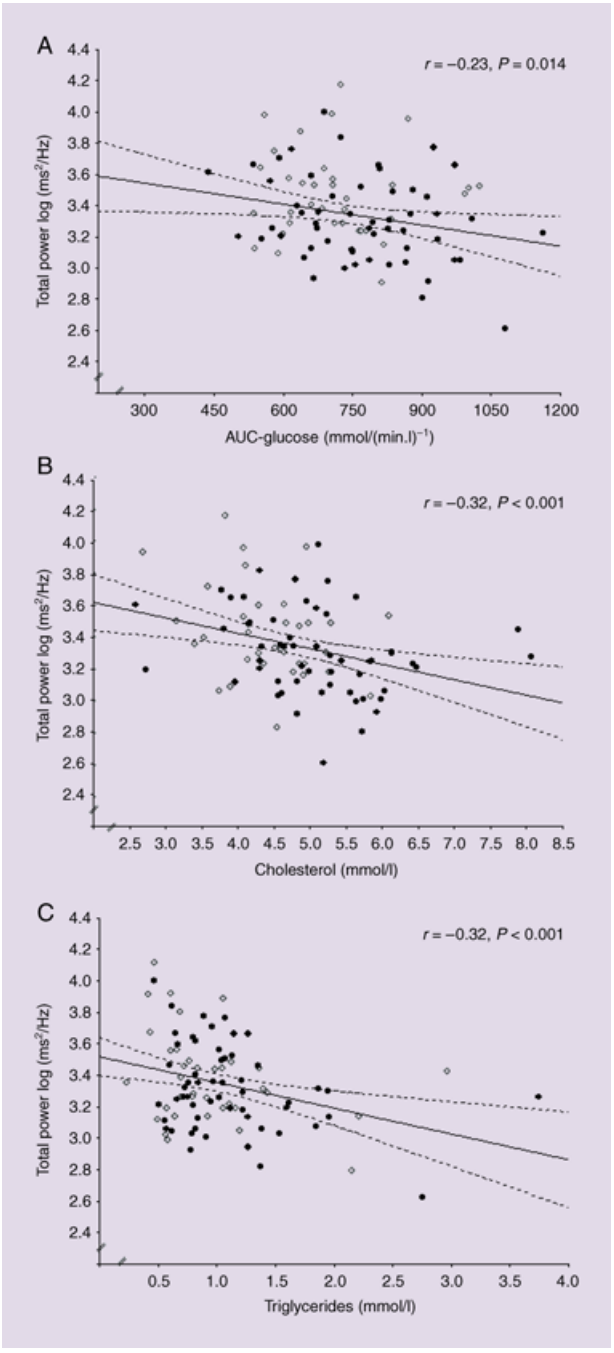

FIGURE 1 Pearson correlation between total power log-transformed and: (A) area under curve of glucose (AUC-glucose), (B) cholesterol, and (C) triglycerides. First-degree relatives ($n = 55$; closed circle) and control subjects ($n = 36$; open circle); r and P-values are for combined groups.

glucose-insulin homeostasis, the hyperinsulinaemic glucose clamp. Nevertheless, the lack of methodological sensitivity to detect insulin resistance would have been more relevant as a potential explanation if FDRs and control subjects had different indices of HRV. Indices of HRV obtained at rest may not reflect autonomic function in other physiological conditions that cause sympathetic activation and vagal withdrawal, such as exercise.

In conclusion, the present findings suggest that a family history of T2DM, in the absence of concomitant metabolic disorders, does not impair resting HRV.

Competing interests

None to declare.

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OBSERVATIONS

Impaired Vascular Reactivity in Healthy First-Degree Relatives of Subjects With Type 2 Diabetes Is Related to Metabolic Factors

Several studies have suggested that vascular changes precede the development of metabolic disorders in first-degree relatives of subjects with type 2 diabetes, indicating that being a first-degree relative of a subject with type 2 diabetes is a risk per se for vascular dysfunction (1–3). However, these studies have failed to control for relevant metabolic and inflammatory variables that are usually altered in first-degree relatives of subjects with type 2 diabetes and are known to impair vascular reactivity (4).

To investigate the hypothesis that vascular reactivity in first-degree relatives of subjects with type 2 diabetes without metabolic disorders is similar to that in a control group without history of type 2 diabetes, 42 first-degree relatives of subjects with type 2 diabetes (79% women; mean ± SD age 33 ± 9 years) and 45 age- and sex-matched control subjects (78% women; age 34 ± 9 years) were recruited. Vascular reactivity was assessed by reactive hyperemia measured by forearm venous occlusion plethysmography.

Although all values for blood analysis were within normal limits, insulin resistance measured by homeostasis model assessment and plasma glucose levels were higher in the first-degree relatives of subjects with type 2 diabetes than in the control group ($P < 0.05$). Fasting insulin, cholesterol, LDL cholesterol, and leptin showed a trend to be higher in the first-degree relatives of subjects with type 2 diabetes than in the control group. The first-degree relatives of subjects with type 2 diabetes exhibited, at basal conditions, similar forearm blood flow and forearm vascular conductance compared with those in the control group. During reactive hyperemia, forearm blood flow was similar between the groups, but vascular conductance was lower in the first-degree relatives of subjects with type 2 diabetes than in the control group (31.32 ± 8.36 arbitrary units [AU] vs. 34.26 ± 6.72 AU, respectively; $P = 0.037$).

Multiple regression analysis using conductance during reactive hyperemia as the dependent variable yielded only fasting insulin and waist-to-hip ratio as independent predictors (model $r^2 = 0.22$; $P = 0.006$). Because the first-degree relatives of subjects with type 2 diabetes had higher values for several variables known to alter vascular reactivity, the data were reanalyzed using an ANCOVA adjusted for variables that were different between the groups. This analysis yielded no differences between the groups in any of the forearm blood flow measurements ($P = 0.161$). These results were confirmed by another analysis, in which subjects exhibiting insulin resistance and/or inflammation were excluded from both groups in order to match them. This approach revealed no differences for any of the forearm blood flow variables ($P > 0.05$), indicating comparable vascular function between the groups when subjects with metabolic or inflammatory changes are excluded.

In the present study, higher values of metabolic variables were more common in the first-degree relatives of subjects with type 2 diabetes, as found in previous publications (1–3,5), and considering that these factors reduce vascular reactivity (4), it is not surprising that vascular reactivity was diminished in the first-degree relatives of subjects with type 2 diabetes initially studied. The subsequent analysis leads to the main finding of the present study that in the absence of difference of metabolic variables, vascular reactivity in the first-degree relatives of subjects with type 2 diabetes is similar to that in the control group.

Because metabolic disorders are known to impair vascular reactivity and these alterations are an early step in the atherosclerotic process, the present study may have important implications for identifying populations that can derive substantial benefits from early lifestyle modifications. In the absence of metabolic disorders, vascular reactivity in the first-degree relatives of subjects with type 2 diabetes is similar to that in the control group. Therefore, family history of type 2 diabetes seems to be not a risk factor per se for vascular reactivity but, rather, a consequence of metabolic disorders that are more common in these subjects.

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