UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

TESE DE DOUTORADO

ASPECTOS RELACIONADOS A PRESSÃO ARTERIAL E COMPLICAÇÕES CRÔNICAS MICRO- E MACRO VASCULARES EM PACIENTES COM DIABETES MELLITUS TIPO 2

BRUNO SCHMIDT DELLAMEA

Orientador: Prof. Dr. Luis Henrique Canani

Co-orientador: Profª Drª Cristiane Bauermann Leitão

Porto Alegre, julho de 2015

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A apresentação desta tese é exigência do Programa de Pós-graduação em Ciências Médicas : Endocrinologia, Universidade Federal do Rio Grande do Sul, para obtenção do título de Doutor.

Porto Alegre, Brasil
2015

CIP - Catalogação na Publicação

Schmidt Dellaméa, Bruno
ASPECTOS RELACIONADOS A PRESSÃO ARTERIAL E COMPLICAÇÕES CRÔNICAS MICRO- E MACRO VASCULARES EM PACIENTES COM DIABETES MELLITUS TIPO 2 / Bruno Schmidt Dellaméa. -- 2015.
88 f.

Orientador: Luis Henrique Canani.
Coorientador: Cristiane Bauermann Leitão.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Porto Alegre, BR-RS, 2015.

1. diabetes mellitus tipo 2. 2. hipertensão arterial sistêmica. 3. complicações crônicas. 4. doenças cardiovasculares. 5. genética. I. Canani, Luis Henrique, orient. II. Bauermann Leitão, Cristiane, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

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Alguns homens vêem as coisas como são, e dizem 'Por quê?' Eu sonho com as coisas que nunca foram e digo 'Por que não?'.

George Bernard Shaw

DEDICATÓRIA

À minha família
Tudo só aconteceu por causa de vocês

À minha noiva
Pelo amor e apoio incondicional

AGRADECIMENTOS

Ao meu orientador Prof Dr Luis Henrique Canani, pelo apoio na conclusão desta trajetória, pela disponibilidade de tempo, dedicação, compartilhamento de conhecimento, além de muita compreensão e muita paciência ao longo desta caminhada; mas além de tudo por ser um grande exemplo e modelo de médico e professor;

À Profa Dra Cristiane Bauermann Leitão, por toda a ajuda disponibilizada durante a realização deste projeto; partilhando o conhecimento e me ensinando como fazer pesquisas;

Ao Prof. Luiz Alberto Pereira, por quem fui contagiado pelo interesse na área da endocrinologia, que desde então tem sido a minha vida;

Aos professores de pós-graduação, pesquisadores do Serviço de Endocrinologia e colegas de pós-graduação; que me ajudaram, cada um ao seu modo, ao longo de toda essa caminhada;

A banca examinadora, por aceitarem o convite e disponibilizarem tempo para me ajudarem nesse processo;

Aos colegas de profissão, tantos amigos, que me ajudam a crescer diariamente através de discussões de caso e atualizações, em especial a Tiago Schuch, colega com o qual dividi a docência por longos anos de muito aprendizado e discussões;

Ao alunos que tive na ULBRA, uma experiência incrível onde eu aprendia tanto quanto ensinava;

Aos amigos que sempre estiveram juntos no tempo bom e ruim, e me acompanham pela vida;

Aos meus familiares, tudo que sou e alcancei foi devido ao esforço, carinho e sacrifício de vocês, e vou ser eternamente grato por isso;

A minha noiva, pela parceria, pela alegria, pelo apoio, e pelo amor que cresce a cada dia

A todos um muito obrigado.

SUMÁRIO

ABREVIATURAS E SIGLAS

A1c – hemoglobina glicada

ABPM – ambulatory blood pressure measurement

ADMA – dimetilarginina assimétrica / assimetric dimethylarginine

AER – Albumin excretion rate

AGE – produtos avançados de glicação

Akt – protein kinase B

AVC – acidente vascular cerebral

BT4 - tetrahidrobiopterina

BP – blood pressure

CAN – cardiac autonomic neuropathy

CKD – chronic kidney disease

CV- cardiovascular

DM – diabetes mellitus

DN – diabetic nephropathy

eNOS – óxido nítrico sintase endotelial / endothelial Oxide Nitric Synthase

EO – estresse oxidativo

ERO – espécie reativa de oxigênio

ESRD – end-stage renal disease

EUA – excreção urinária de albumina

GFR – glomerular filtration rate

HAS – hipertensão arterial sistêmica

HWE – Hardy-Weinberg equilibrium

IAM – infarto agudo do miocárdio

iNOS – inducible nitric oxide synthase

MAPA – monitorização ambulatorial da pressão arterial

NAC – neuropatia autonômica cardíaca

ND – nefropatia diabética

nNOS – neuronal nitric oxide synthase

NO – óxido nítrico / nitric oxide

NOS – nitric oxide synthase

NOSs – nitric oxide synthases

PA – pressão arterial

PAI-1 – inibidor do ativador do plasminogênio –1 (plasminogen activator inhibitor – 1)

PAS – pressão arterial sistólica

PI3K – phosphatidylisinotide 3-kinases

PP – pressão de pulso

PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-analysis

RAGE – receptor do produto avançado de glicação

RD – Retinopatia diabética

RI – Resistência a insulina

SNP – single nucleotide polymorphism

UCP-2 – uncoupling protein 2

UAER – urinary albumin excretion rate

VEGF – vascular endothelial growth factor

VE – ventrículo esquerdo

QUADROS, TABELAS E FIGURAS

RESUMO

A prevalência de diabetes mellitus (DM) tem aumentado progressivamente no mundo. O controle da glicemia é a base do tratamento do DM, sendo importante para a prevenção das complicações crônicas. No entanto, pode ser insuficiente em alguns casos, visto que controle glicêmico intensivo falhou em mostrar evidências na redução da progressão da doença macrovascular, trazendo a necessidade de uma avaliação complementar além da glicemia.

A hipertensão arterial sistêmica tem sido considerada um dos principais fatores relacionado a complicações crônicas do DM. Fatores pressóricos não identificados na verificação da pressão arterial no consultório podem explicar parte destes achados, como hipertensão do avental branco, hipertensão mascarada, alteração cicardiana da variabilidade da pressão arterial e avaliação do descenso noturno. A ausência do descenso noturno da pressão arterial tem sido associado a nefropatia e neuropatia diabética.

O endotélio promove o tônus vasomotor e regula a inflamação e coagulabilidade. Quanto maior o tempo de diabetes, maior a disfunção endotelial secundário ao estresse oxidativo.

Neste contexto, resolvemos avaliar a ausência do descenso noturno da pressão arteiral e o gene candidato óxido nítrico sintase endotelial como fatores de risco para complicações do DM macrovasculares e microvasculares, respectivamente.

Em um coorte do ambulatório de endocrinologia do Hospital de Clínicas de Porto Alegre com 361 pacientes, avaliamos a ausência de descenso noturno como fator de risco cardiovascular. Os pacientes não apresentavam histórico prévio de doença cardiovascular e realizaram a monitorização ambulatorial da pressão arterial basal sem medicamentos anti-hipertensivo, sendo avaliados posteriormente desfechos cardiorrenais. No acompanhamento, após 54±39 meses, 297 pacientes foram reavaliados, dos quais 72% mostraram ausência do descenso noturno da

pressão arterial na avaliação basal. Estes apresentaram 32% mais risco de apresentar desfechos que os pacientes com descenso normal da pressão arteiral. Após uma revisão sistemática e meta-análise, analisamos nossos dados em conjunto com outros estudos, e demonstramos um aumento de 1,7 vezes no risco de desfechos micro- e macrovasculares em pacientes com ausência do descenso da pressão arterial quando comparados a pacientes com descenso.

Afim de avaliar o papel da disfunção endotelial no desenvolvimento das complicações crônicas do DM, estudamos a relação do óxido nítrico com a nefropatia do DM (ND). O óxido nítrico é fundamental para a atuação normal do endotélio, onde é produzido pela óxido nítrico sintase endotelial. Este gene tem sido considerado um gene candidato para a ND, e alguns polimorfismos foram estudados quanto a progressão da ND, como G894T, 4 b/a, T786C; porém, os resultados apresentados na literatura são contraditórios. Portanto, conduzimos uma revisão sistemática e meta-análise sobre os polimorfismos citados, considerando todos os modelos genéticos, quanto a associação com a ND, que demonstrou resultados estatisticamente significativos com os polimorfismos 4 b/a e T786C, e concluímos que o gene candidato estudado pode contribuir para o desenvolvimento de ND.

Desta maneira, a utilização da monitorização ambulatorial da pressão arterial acrescenta dados que não obtidos pela medida da pressão arterial na consulta médica. A constatação da ausência do descenso noturno confere ao paciente com DM tipo 2 aumento de risco do desenvolvimento de complicações micro- e macrovasculares. Por outro lado, o estresse oxidativo está relacionado a disfunção endotelial, por alterações no óxido nítrico, e polimorfismos no gene candidato óxido nítrico sintase endotelial podem estar relacionados ao desenvolvimento de ND. O desenvolvimento das complicações crônicas do DM tipo 2 então estão intimamente ligados ao continuum resistência insulínica, estresse oxidativo, hipertensão arterial e disfunção endotelial.

APRESENTAÇÃO

Este trabalho consiste na tese de doutorado "**ASPECTOS RELACIONADOS A PRESSÃO ARTERIAL E COMPLICAÇÕES CRÔNICAS MICRO- E MACRO VASCULARES EM PACIENTES COM DIABETES MELLITUS TIPO 2**", apresentada ao Programa de Pós-Graduação em Ciências Médicas da Universidade Federal do Rio Grande do Sul, em 17 de julho de 2015. O trabalho será apresentado em 4 partes, descritas a seguir:

1. Introdução

2. Capítulo 1

3. Capítulo 2

4. Conclusões e considerações finais

INTRODUÇÃO

1. DIABETES MELLITUS

A prevalência de diabetes mellitus (DM) no Brasil é de aproximadamente 11,6 milhões da população adulta, ou seja 8,6% [1], sendo que o DM tipo 2 (DM2) é responsável por mais de 90% dos casos, o DM tipo 1 por 5-10 % e o restante é secundário a outras causas específicas [2].

O manejo adequado da homeostase glicêmica é um dos fatores principais no controle do DM2, principalmente para prevenção secundária ou terciária de complicações crônicas [3]. DM2 é a principal causa de cegueira por retinopatia diabética (RD), nefropatia diabética (ND) levando a insuficiência renal e amputações não traumáticas de membros inferiores, além de ser um dos principais responsáveis pelo desenvolvimento de doença cardiovascular (CV). [4]. O tratamento adequado do DM2 é um grande desafio [5, 6], devido à dificuldade na aderência ao tratamento (farmacológico e mudanças de estilo de vida) e à inércia clínica prevalente [7, 8]. Assim, 1 em cada 20 pacientes com DM2 com hemoglobina glicada (A1c) >8% apresenta uma complicação microvascular em 5 anos [9].

Há um aumento do gradiente de risco conforme a elevação progressiva da glicemia, onde cada 1% de aumento na A1c aumenta o risco relativo para doença CV em 1,18% [10]. Apesar do controle glicêmico intensivo ter demonstrado redução de eventos microvasculares [3, 11], não houve redução de eventos macrovasculares em pacientes com DM2 submetidos a tratamento intensivo [12-14]. Atualmente, o controle glicêmico ideal deve ser feito numa abordagem individualizada, conforme condições clínicas do paciente, mantendo de um modo geral a A1c <6,5-7,0% [15].

A evolução de alguns pacientes para complicações microvasculares e macrovasculares apesar de controle glicêmico razoável traz a necessidade da

avaliação de outros fatores que poderiam contribuir para isso. Conceitos como memória metabólica [16, 17], variabilidade glicêmica [18, 19] e desdiferenciação da célula-beta [20, 21] contribuem em parte para o melhor entendimento entre controle glicêmico e risco CV [22-25], mas ainda são insuficientes para total entendimento deste fenômeno.

Além do hiperglicemia *per si*, o DM2 se associa a outros fatores de riscos [26-28], como hipertensão arterial sistêmica (HAS), obesidade visceral, dislipidemia [26, 29], assim como hiperuricemia [30-32] e aumento da hipercoagulabilidade, com aumento do fibrinogênio e redução do inibidor do ativador do plasminogênio (PAI-1)[33-35]. Além destes, os pacientes com DM2 ainda podem apresentar outros fatores de risco clássicos como dislipidemia, tabagismo, aumento da albuminúria, sedentarismo e hiperhomocisteinemia [36]. A obesidade parece ter um papel determinante, que representa um estado inflamatório crônico [37, 38], onde a lipotoxicidade e consequente depósito extra-adipocitário de lipídios levam a inflamação, disfunção endotelial (DE) e aterotrombose [39-41]. A alteração da produção de citocinas pelo tecido adiposo branco, predominantemente pró-inflamatórias [42-44], associado a redução das miocinas anti-inflamatórias, secundário ao sedentarismo [45, 46], estimulam sinergicamente o aumento da inflamação e o consequente aumento do risco CV [47].

A avaliação genética tem sido explorada para tentar identificar outros fatores que possam explicar a presença de complicações crônicas em pacientes sem fatores de risco conhecidos. Dentro deste contexto, muitos genes candidatos tem sido avaliados como possível causa do desenvolvimento de complicações crônicas, como os genes TCF7L2 [48], UCP1-3 [49, 50], eNOs [51], entre outros, com o objetivo de ampliar o entendimento dos fatores de risco para o desenvolvimento das complicações do DM.

2. **HIPERTENSÃO ARTERIAL**

Dentre os fatores de risco citados acima, a HAS tem sido considerada o principal fator relacionado a complicações crônicas do DM. Aproximadamente 40% dos pacientes com DM2 são hipertensos ao diagnóstico [52]. O risco para doença CV está aumentado em 4 vezes em pacientes com DM2 e HAS quando comparados a pacientes sem ambas patologias [53], e com tratamento adequado, cada redução de 10 mmHg na pressão arterial sistólica (PAS) acarreta uma redução de 12% no risco de qualquer complicação relacionada ao DM [54]. O controle intensivo da HAS reduz a incidência de acidente vascular cerebral (AVC), mas não de mortalidade geral ou infarto agudo do miocárdio (IAM) [55]. Hiperinsulinemia, expansão do volume e aumento da rigidez arterial [56-58] contribuem para a HAS no DM2, e mesmo na fase de pré-diabetes já se observa maior incidência de HAS ou aumento da pressão de pulso (PP) [59].

Fatores pressóricos não identificados pela medida da pressão arterial (PA) no consultório podem explicar parte destes achados, onde muitos pacientes presumidamente normotensos no consultório podem apresentar aumento do risco CV [60-62]. A medida da PA nas 24 horas, através da monitorização ambulatorial da PA (MAPA), apresenta melhor correlação com desfechos do que a simples medida da PA no consultório [63-65] em pacientes hipertensos. Nos pacientes com DM2, a PA na MAPA está associada com maior excreção urinária de albumina (EUA) em pacientes normoalbuminúricos [66, 67]. Outros parâmetros avaliados pela MAPA também parecem ser de particular importância em pacientes com DM2. A HAS mascarada (PA normal no consultório e alta na MAPA) está associada a maiores níveis de EUA e da espessura do ventrículo esquerdo (VE) em comparação com os valores encontrados em pacientes normotensos verdadeiros [61]. Da mesma maneira, a HAS do avental branco (PA alta no consultório e normal na MAPA) confere maior risco de desenvolvimento de macroalbuminúria e RD [68]. Os pacientes com DM2 apresentam alterações no padrão de variação da PA nas 24

horas, mesmo antes do diagnóstico de HAS, quando comparados a indivíduos sem DM, tanto normo- como hipertensos [69]. Alterações no ritmo circadiano da PA em outros horários do dia, por exemplo aumento da PA vespertina, aumenta o risco de complicações microvasculares no DM2 [70].

Os indivíduos normotensos apresentam uma variação normal da PA nas 24 horas, caracterizada pela diminuição da PA durante o sono e aumento após o despertar. Esta queda dos níveis pressóricos é chamada de descenso noturno da PA e é expressa pela diferença absoluta entre a média da pressão noturna e diurna ou por uma relação das médias da pressão da noite/dia. A ausência do descenso noturno tem sido descrito como associado a ND e neuropatia diabética [71, 72] entretanto, as evidências são fracas, realizadas através de pequenos estudos de associação. A ausência de descenso noturno pode ser considerada uma manifestação da neuropatia autonômica cardíaca (NAC) [73, 74]. A NAC tem uma prevalência de 22% no DM2 [75], podendo levar a hipertrofia de VE e disfunção diastólica, que pode evoluir para insuficiência cardíaca [76, 77]. Este fato associado a DE e inflamação leva a uma maior resistência a ação da insulina (RI), que leva a um ciclo vicioso de piora glicêmica e efeito deletério sobre o miocárdio [78], com maior disfunção diastólica nos pacientes com pior controle glicêmico [79]. Estas fatores pode levar a uma redução no pré-condicionamento cardíaco [80-82] e isquemia ou IAM silencioso, aumentando o risco cardiovascular.

O aumento do estresse oxidativo (EO) é um fator patogênico chave na HAS no DM2 [83], e espécies reativas do oxigênio (ERO) podem levar a DE, redução da bioavaliabilidade do óxido nítrico (NO) e vasolidatação mediada pelo NO reduzida [84].

Como mencionado, há provavelmente fatores genéticos envolvidos na patogênese das complicações crônicas. Neste sentido, os genes que regulam as rotas da PA ou da fisiologia renal podem ser candidatos ao desenvolvimento das complicações da ND [85].

3. DISFUNÇÃO ENDOTELIAL

O endotélio promove o tônus vasomotor, regula a transferência de células e nutrientes, mantém a fluidez do sangue, regula o balanço local de mediadores inflamatórios e anti-inflamatórios, assim com atividade coagulante e anti-coagulante [86, 87]. A insulina é um regulador da ativação do eNOS e produção NO, e a RI pode atenuar este processo, suprimindo a secreção normal de NO [88].

Entre os mecanismos envolvidos na doença CV em pacientes com DM, estão a DE, cujo grau de disfunção está relacionada ao tempo de DM [89]; a ativação plaquetária [90]; anormalidades de coagulação, com aumento do fibrinogênio plasmático, atividade fibrinolítica reduzida e aumento da PAI-1 [91] . A DE ocorre primariamente pela perda de bioatividade do NO [92], e entre as vias de lesão, o acúmulo de produtos avançados de glicação (AGE) tem recebido um atenção principal, sendo relacionado a múltiplas patologias vasculares [93, 94] assim como o receptor do AGE (RAGE) [95].

Alto EO e inflamação levam ao metabolismo anormal do NO, referente a biodisponibilidade, uso/resposta, produção, liberação e degradação, produzindo um efeito global de vasoconstrição, caracterizado pela geração de ERO [96], podendo estar relacionados a citocinas [97, 98]. Além disso, a endotelina também contribui para a resposta vasoconstritora exagerada em artérias ateroescleróticas [99], aumentando o risco CV. Pacientes com ausência do descenso noturno mostram maior EO comparado a pacientes com descenso normal [100], com aumento de marcadores de DE, como a dimetiarginina assimétrica (ADMA) [101]. ADMA é um inibidor do NO sintase endotelial (eNOS), e sua elevação tem sido associado a doença arterial coronariana (DAC) [102]. Nesse contexto de EO, a produção de LDL oxidada está aumentada, assim como outros marcadores além da ADMA, como a

tetrahidrobiopterina (BH4), fatores estes implicados na ateroesclerose no DM2 [103, 104].

4. COMPLICAÇÕES CRÔNICAS

No contexto dos itens explanados acima, resolvemos avaliar a ausência do descenso noturno da PA e o gene candidato eNOS no desenvolvimento de complicações micro- e macrovasculares do DM.

O capítulo 1 avalia a ausência de descenso noturno, e consiste em dois artigos. O primeiro é um artigo original, onde foram reavaliados 361 pacientes em atendimento no Hospital de Clínicas de Porto Alegre, onde foi visto a ocorrência de desfechos micro-macrovasculares e sua associação com a ausência do descenso noturno da PA. O segundo artigo é uma revisão sistemática com meta-análise, onde foram analisados em conjunto os artigos longitudinais disponíveis na literatura, que somados ao nosso artigo original, demonstraram que a ausência de descenso noturno da PA é fator de risco para o desenvolvimento de desfechos micro- e macrovasculares do DM.

O capítulo 2 avalia polimorfismos do gene candidato eNOS, e consiste em dois artigos. O primeiro é um artigo de revisão, onde é avaliado o sistema eNOS e seus efeitos na fisiologia renal e patofisiologia no DM2. O segundo artigo é uma revisão sistemática e meta-análise de estudos genéticos, onde é realizado um avaliação de associação genética entre polimorfismos do gene eNOS , sem modelo pré-definido, que evidenciou uma razão de risco entre polimorfismos especificados e ND.

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Non-dipping Diastolic Blood Pressure and Adverse Outcomes in Patients with type 2 Diabetes Mellitus.

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Abstract

Introduction: Twenty-four-hour ambulatory blood pressure (BP) monitoring (ABPM) defines better the cardiovascular risk than ordinary office BP measurements. Patients without the normal nighttime drop in BP are named non-dippers, lately associated with complications in patients with diabetes mellitus (DM). The aim of this study was to evaluate prospectively the non-dipper pattern as a predictor of cardiovascular events.

Methods: A prospective cohort study was performed with 361 type 2 DM patients without cardiovascular disease, evaluated between 1994 and 2008 and re-evaluated between 2011 and 2012. ABPM was performed at baseline, as well as clinical and laboratorial data collection. The primary outcome analyzed was the combination of acute myocardial infarction, ischemic heart disease, stroke, lower limbs non-traumatic amputation, need for lower limbs revascularization, and/or chronic kidney disease with/without need of hemodialysis.

Results: Non-dipping pattern was observed in 261 (72%) patients for systolic BP, in 177 (49%) patients for diastolic BP, and in 267 (74%) when considered any of them. After a mean follow-up of 54±39 months, 297 patients were re-evaluated (82% of the original cohort). Fifty-four (18%) patients presented 66 outcomes. Diastolic non-dipping pattern was associated in survival analysis with shorter time-to-outcome ($p = 0.01$).

Discussion: A diastolic non-dipping pattern was associated with a shorter period of events free in subjects with type 2 DM. Patients with non-dipping BP had 32% more risk than dippers to present an outcome. These associations remained significant even after adjustment for possible confounding factors, including daytime systolic BP.

Introduction

Hypertension and type 2 diabetes mellitus (DM) frequently coexist, and this combination has additive effects on the development diabetes micro- and macrovascular complications [1].

Twenty-four-hour ambulatory blood pressure (BP) monitoring (ABPM) defines better the cardiovascular risk in both hypertensive subjects [2] and general population [3] than ordinary office BP measurements. The better performance of this test probably is associated with the possibility of evaluating a broader spectrum of BP parameters such as 24-h, daytime and nighttime BP means and loads, and the presence of white-coat and masked hypertension, otherwise not documented by the office BP evaluation [4]. Each of these variables has been associated with higher cardiovascular risk in non-diabetic patients [5, 6] and with chronic complications in both type 1 and type 2 DM patients [7-10].

Other ambulatory BP parameters have been studied in patients with DM and special attention has been given to the blunted nocturnal physiological drop of BP. BP varies along the 24-h according to a circadian rhythm, which is characterized by a morning rise, daytime plateau and a nocturnal decrease of at least 10% of the daytime mean BP levels. Patients without the normal nighttime drop in BP are named non-dippers, and the non-dipping phenomena has been associated with increased glomerular filtration rate (GFR) [11] as well as with higher incidence of microalbuminuria [12-14], diabetic retinopathy (DR) [13, 15] and macrovascular complications [13] in DM patients.

However, this issue is still in debate [8]. In most studies non-dipper patients had also elevated 24-h ABPM means [13, 14, 16], and the cause-effect relation between non-dipping phenomena and outcomes remains controversial, because there are few data from prospective studies [17-19]. Additionally, dipper pattern definition sometimes was based on systolic BP and other times it was based also in diastolic BP nocturnal drop [18, 19].

Therefore, the aim of this study was to evaluate if the non-dipping BP pattern is an independent predictor of cardiovascular and renal complications in patients with type 2 DM, and to determinate which component of BP (systolic or diastolic) is a better predictor of adverse outcomes.

Methods

A prospective cohort study was performed with 361 type 2 DM patients without prior cardiovascular events who regularly attended the DM outpatient clinic at Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil. The inclusion criterion was the diagnosis of type 2 DM (>30 years of age at onset of DM, no previous episode of ketoacidosis or documented ketonuria, no need for insulin in the first 5 years of diagnosis). The exclusion criterion was the presence of previous cardiovascular disease. Subjects were evaluated twice: first at baseline, in the period between 1994 and 2008; and then between 2011 and 2012, to identify outcomes. The ethics committee of the Hospital approved the study protocol. Written informed consent was obtained from all patients.

Baseline assessments

Patients underwent an interview and clinical examination to record demographic and anthropometric data. BP evaluations were performed one week after withdrawal of all medications with an antihypertensive effect. Office BP was measured twice, with a two-minute interval, with a mercury sphygmomanometer using the left arm and with the patient in a sitting position, after a 5-min rest, on the same day as the ABPM, and the mean of these two measurements was considered for analysis. Hypertension was defined by an office BP measurement of ≥140/90 mm Hg on two occasions or use of antihypertensive medications. ABPM was obtained by oscillometry (Spacelabs 90207 serial nos. 207/024751 and 207/038016 with calibration certification), with a 15-min interval in the daytime and 20-min interval in the nighttime period. ABPM was performed on an ordinary workday, and patients

were advised to maintain their usual daily activities. Sleep time was recorded as the period between the time when the patient went to bed and the time when the patient woke up the next morning. The means of 24-h, daytime, and nighttime systolic and diastolic BP were recorded, as well as systolic and diastolic BP loads (percentage of 24-h and daytime BP ≥140/90 mmHg and nighttime BP ≥120/80 mmHg). The dipping pattern was based on the ratio between nighttime and daytime BP (nighttime BP/daytime BP). A ratio ≤0.9 was considered normal (dipping pattern) and a ratio >0.9 was considered abnormal (non-dipping pattern). Patients were classified as dippers or non-dippers for systolic BP, diastolic BP, and systolic or diastolic BP.

DR evaluation was performed by an experienced ophthalmologist after mydriasis, and patients were classified as with or and without DR. Albuminuria evaluation was performed by measurement of urinary albumin excretion rate (UAER) in 24-h urine samples, and patients were classified based on two out of three mesurements in: normoalbuminuric (UAER <20 µg/min), microalbuminuric (UAER 20 - 199 µg/min), or macroalbuminuric (UAER ≥200 µg/min).

Previous cardiovascular disease was identified by history [20] or by routine cardiovascular evaluation (EKG, ergometric test or myocardial cintigraphy, as clinically indicated).

Outcome evaluation during follow-up

The development of predefined outcomes was evaluated by revision of patient's charts or by direct contact by telephone. The outcome analyzed was the combination of acute myocardial infarction, ischemic heart disease (documented by EKG, ergometric test, myocardial cintigraphy, or coronary arteriography) stroke, lower limbs non-traumatic amputation, need for lower limbs revascularization, and/or chronic kidney disease (eGFR <60 ml/min). If a patient had more than one event, time in months for the first event was used for analysis.

Laboratory methods

UAER was measured by immunoturbidimetry (Microalb; Ames-Bayer, Tarrytown, NY; intra- and interassay coefficients variation of 4.5 and 11.0%, respectively). HbA1C was measured by a high-performance liquid chromatography system (normal range 4–6%; Merck-Hitachi 9100). Fasting plasma glucose was measured by the glucose-peroxidase colorimetric enzymatic method (Biodiagnóstica). Creatinine was measured by the Jaffé method and the lipid profile by a colorimetric method.

Statistical analyses

Continuous variables are presented as means ± standard deviation (SD) or median (interquartile interval), and categorical variable as absolute (number) and relative frequency (percentage). Student *t* test was used to compare continuous variables. Variables with non-normal distribution were log transformed. Chi-square test was used to compare categorical variables. Kaplan-Meier curves (Log-Rank test) were used to assess the risk of development of the primary endpoint, and Cox's regression analysis was employed to adjust the results to possible confounding factors found on univariate analysis or based on their biological relevance. $P < 0.05$ (two-tailed) was considered significant. All analyses were performed using the statistical package SPSS (version 18.0; SPSS, Chicago, IL)

Results

Baseline characteristics

A total of 361 patients with type 2 DM (male: n = 195, 54%; white: n = 272, 75%) were evaluated at baseline. Mean age was 56 ± 9.2 years, and mean duration of DM was 10.1 ± 6.7 years. Fifty-eight patients (16%) were current smokers, the mean BMI was 28.6 ± 4.5 kg/m², and mean HbA1c was 8.19% ± 2.26. Hundred

nineteen (33%) patients had DR and 123 (34%) had increased UAER (85 micro- and 38 macroalbuminuria).

Regarding BP values, mean office BP was 141/84 mmHg, and 188 (52%) patients had arterial hypertension. During ABPM, mean 24-h BP was 132/78 mmHg. Daytime BP was 135/81 mmHg and nighttime BP was 127/73 mmHg. Non-dipping pattern was observed in 261 (72%) patients for systolic BP, in 177 (49%) patients for diastolic BP, and in 267 (74%) when considering the presence of non-dipping patterns for systolic and/or diastolic BP.

Follow-up results

After a mean follow-up of 54 ± 37 months (median: 48 months; interquartile range: 24-70 months), 297 patients were re-evaluated, comprising 82% of the original cohort. From these patients, 219 (73%), 149 (50%) and 223 (75%) presented non-dipping pattern at baseline for systolic BP, diastolic BP and systolic and/or diastolic BP combined, respectively. Fifty-four (18%) patients had 66 outcomes (chronic kidney disease [n=7]; hemodialysis [n=7]; ischemic heart disease [n=17], acute myocardial infarction [n=9]; stroke [n=10]; amputation/vascular graft [n=4]), and 12 (4.0%) patients died during the follow-up.

Sixty-four patients were lost from follow-up. At baseline, 36 patients had dipping and 28 non-dipping pattern, considering DBP. This group did not differ from the re-evaluated group regarding mean age, sex, DM duration, nephropathy, retinopathy, BMI, waist circunference or lipid profile (all P values >0.05).

Systolic and/or diastolic BP non-dipping

Patients with a non-dipping pattern at baseline based on systolic and/or diastolic BP had 50 events (17.6%), while those with dipping pattern at had 16 events (14.7%), leading to an incidence rate of 47 e 36 events by 1000 persons/year, respectively, which resulted in a relative risk of 1.32 (95% IC 0.72-2.47) for the combined

outcome. Mortality for all causes was similar between groups (non-dippers: 9 vs. dippers: 3; p = 0.99). The time-to-outcome was similar when patients were classified based on the combination of systolic and/or diastolic BP [126 months (95% CI 111-140 months) vs. 147 months (95% CI 131-162 months), p = 0.38].

Systolic BP non-dipping

Patients with a non-dipping pattern at baseline for systolic BP had 49 events (22.3%), while those with dipping pattern at baseline had 17 events (21.8%), leading to an incidence rate of 41 e 37 events by 1000 persons/year, respectively, which resulted in a relative risk of 1.11 (95% IC 0.66-1.86) for the combined outcome. Mortality for all causes was similar between groups (non-dippers: 9 vs. dippers: 3; p = 0.83).

The time-to-outcome was similar when patients were classified based on systolic BP [dipping: 126 months (95% CI 112-141 months) vs. non-dipping: 145 months (95% CI 129-161 months), P = 0.42].

Diastolic BP non-dipping

Patients with a non-dipping pattern at baseline for diastolic BP had 40 events (26.0%), while those with dipping pattern had 26 events (17.6%), leading to an incidence rate of 51 e 30 events by 1000 persons/year, respectively, which resulted in a relative risk of 1.42 (95% IC 0.98-2.05) for the combined outcome. Mortality for all causes was similar between groups (non-dippers: 6 vs. dippers: 6; p = 0.94).

When the diastolic non-dipping pattern was evaluated, it was associated with shorter time-to-outcome [non-dipping: 115 months (95%CI 101-128 months) vs. dipping: 150 months (CI 95% 130 -170 months, P = 0.02) (Figure 1).

Since differences in outcomes were observed only for non-dipping diastolic BP, we evaluated baseline characteristics of the included patients based on the diastolic dipping pattern in order to find out possible confounding factors that could explain the adverse outcomes present by this group. Table 1 depicts baseline patients clinical and laboratory characteristics based in the diastolic dipping pattern.

Non-dipping subjects were older and had higher serum creatinine values than dippers. Regarding other BP parameters, diastolic non-dipper patients showed no differences in office BP compared to dippers, but had higher 24-h and nighttime systolic, diastolic and mean BP, and higher diurnal systolic BP (Table 2).

A Cox-regression analysis was conducted with time-to-outcome as the dependent variable and diastolic non-dipping BP, age, serum creatinine, and daytime systolic BP as the independent ones. The diastolic non-dipping pattern group remained associated with shorter time-to-outcome compare to dipping pattern group, even after the adjustments (RR 1.91, CI 95% 1.03 – 3.56; P = 0.04) (Figure 2).

Discussion

In this sample of type 2 DM patients, the diastolic non-dipping pattern was associated with a higher risk for cardiovascular and renal outcomes. These associations remained significant even after adjustment for possible confounding factors, including daytime systolic BP, age and renal status at baseline.

Hypertension is a complex disorder that is commonly seen in patients with DM [21], probably associated do insulin resistant and resultant hyperinsulinemia [22]. BP normally varies over twenty-four hours, being in average 10-20% lower at night than during the day [23, 24]. Approximately 25% of individuals with essential arterial hypertension have a non-dipping BP profile [25], which increases with age [26]. Non-dippers have increased sympathetic nervous system and decreased parasympathetic nervous system activity through the night [27, 28], and this autonomic deregulation is an independent predictor of cardiovascular events [29].

The use of ABPM in patients with DM allowed the study of several BP parameters, as the absence of nocturnal dipping status; BP variability and identification of masked hypertension [30]. ABPM adds to clinic and self-measurement BP as the optimal clinical procedure [31-33]. There is evidence

suggesting that nighttime BP is superior to daytime pressure in predicting adverse outcomes [34-37]. Isolated nocturnal hypertension can be better characterized with ABPM [33].

Non dipping pattern is common in patients with DM and may reach a prevalence over 30% [38, 39]. This could explained by several factors, as obstructive sleep apnea, orthostatic hypotension/autonomic neuropathy, diabetic kidney disease or heart failure [40].

We previously reported an association of DR and nocturnal blood pressure values [8], as well masked hypertension with albuminuria and echocardiographic abnormalities [7]. White coat hypertension [9] and afternoon blood pressure increase [41] are also associated with microvascular complications, remarking the importance of this exam as a tool for a better prediction of diabetic complications.

In some transversal studies the presence of non-dipping pattern was associated with a higher prevalence of cardiovascular events [42-44]. Considering prospective studies, Nakano [17] and Carmona [18] showed that non-dippers at baseline had a higher incidence of cardiovascular events during follow-up. However, although the non-dipper pattern was associated with events in Nakano et al. study [17], other parameters, such as 24-h pulse pressure and nighttime systolic BP, were also related to outcomes, but with greater magnitude than the non-dipper pattern. Similar observation was made by Eguchi et al. [19], which demonstrated that the nighttime BP values were more important to predict cardiovascular events than dipping pattern. These three prospective studies evaluated 754 patients with type 2 DM, with median follow-ups of 28, 86, and 50 months. In all three studies the non-dipping pattern was defined on the systolic BP only.

As a novelty of our study, we analyzed the dipping pattern considering each BP one alone (systolic and diastolic) or their combination, and differences in results were observed among them. The decision to evaluated SBP, DBP, or a combination of SBP and DBP is relevant, since the impact of night-day ratio may differ [45-47].

In the present study, there was no association between non-dipping pattern based on systolic BP or combined systolic and diastolic BP and the combined cardio-renal outcome. However, non-dipping BP based on diastolic BP alone was associated with early development of adverse outcomes. This observation suggests that the BP parameter used for definition of dipping is relevant. The strength of the present study is its prospective design and the evaluation of the effect of different BP parameters to define the study groups. The major limitation is the sample size. This let us with a limited number of events; therefore we were unable to analyze the data by each event individually. To overcome this limitation a larger, prospective study needs to be performed.

Conclusions

The absence of diastolic BP dipping pattern is associated with earlier CV and renal events in patients with type 2 DM. This was not observed using the dipper definition based on systolic BP. Therefore, the BP parameter used to define the status of dipper or non-dipper is relevant. However, longer and larger follow-up studies should be performed to address this question.

Conflicts of interest

There is no conflict of interests.

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Table 1 – Baseline clinical and laboratory characteristics based on diastolic pressure dipping pattern

	Dippers (n = 148)	Non-dippers (n = 149)	P
Age (years)	55.7 ± 9.2	58.3±9.2	0.015
Diabetes Mellitus Duration (years)	9.6 ± 6.8	11.1±6.7	0.059
Male - n (%)	77 (52)	85 (57)	0.385
White - n (%)	118 (80)	104 (70)	0.126
Smoking - n (%)	79 (53)	80 (53)	0.897
Body Mass Index (kg/m^2)	28.6±4.2	28.9±4.8	0.515
Waist (cm)	97.2±9.7	99.3±10.9	0.102
Nephropathy – n (%)	45 (30)	51 (34)	0.158
Retinopathy – n (%)	46 (31)	56 (37)	0.123
Glucose (mg/dl)	158±46	140±42	0.140
HbA1c (%)	7.97±2.2	8.19±2.1	0.427
Creatinine (mg/dl)	0.84±0.19	0.91±0.26	0.014
Total cholesterol (mg/dl)	195±45	196±47	0.868
HDL (mg/dl)	45±10	47±11	0.346
LDL (mg/dl)	118 ±39	116±36	0.687
Triglycerides (mg/dl)	125 (101)	141 (118)	0.552

Data are means ± SD or n (%); triglycerides = median (interquartile range)

Table 2 – Baseline blood pressure values based on diastolic pressure dipping pattern

	Dippers (n = 148)	Non-dippers (n = 149)	P
Office blood pressure			
Systolic	139±20	143±21	0.108
Diastolic	84±12	85±12	0.595
24-h blood pressure			
Systolic	128±13	136±19	<0.001
Diastolic	77±9	80±10	0.006
Mean	94±10	98±14	0.003
Daytime blood pressure			
Systolic	133±13	137±18	0.030
Diastolic	81±9	81±10	0.851
Mean	99±10	100±12	0.260
Nighttime blood pressure			
Systolic	119±12	136±18	<0.001
Diastolic	67±9	78±10	<0.001
Mean	85±9	98±12	<0.001
Pulse Pressure			
24 h pulse pressure	52.35±9.4	57.19±13.1	<0.001
Daytime pulse pressure	52.44±9.9	56.44±13.1	0.001
Nighttime pulse pressure	51.21±9.1	58.08±13.4	<0.001

Data are means ± SD.

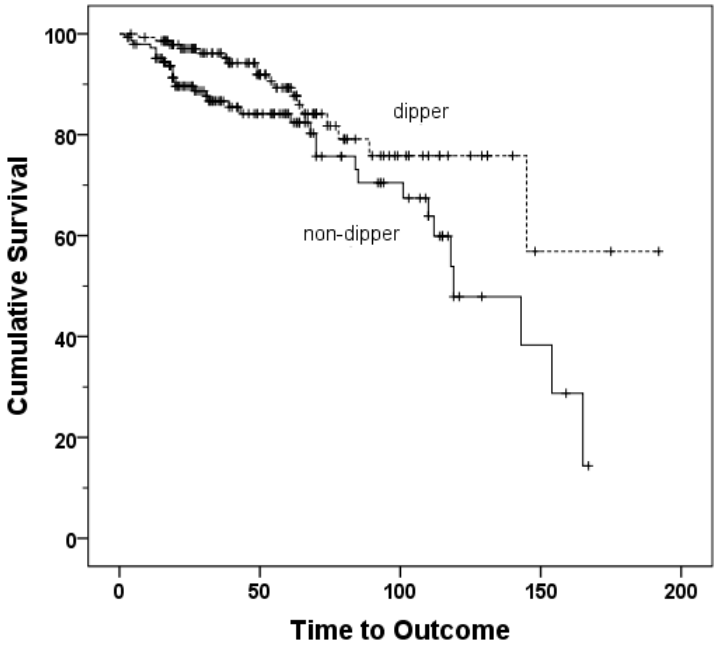

Figure 1 – Survival analyzes for diastolic blood pressure (Kaplan-Meyer). Time measured in months, and cumulative survival in percentual.

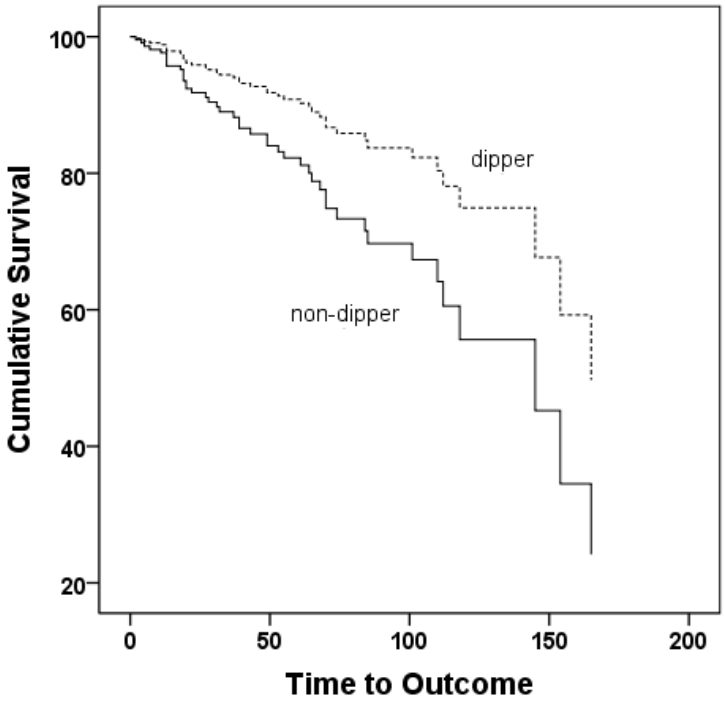

Figure 2 – Survival analyzes for diastolic blood pressure adjusted for age, creatinine, and daytime systolic pressure (Cox Regression). Time measured in months, and cumulative survival in percentual.

Non-dipping blood pressure pattern and micro- and macrovascular outcomes in type 2 diabetes mellitus: a systematic review and meta-analysis of longitudinal studies

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Abstract

Introduction –Non-dipping blood pressure (BP) pattern in ambulatory BP monitoring (ABPM) have been associated with increased diabetes micro- and macrovascular complications. However, there are few longitudinal studies addressing this issue. The aim of this study was to evaluate the association of micro- and macrovascular events and the dipper pattern in patients with type 2 diabetes mellitus (DM), through a systematic review and meta-analysis of longitudinal studies.

Methods – Pubmed database was searched from inception to May 31st, 2015, using index terms: "Diabetes Mellitus, Type 2" AND "Ambulatory Blood Pressure Monitoring" OR "Ambulatory Blood Pressure Measurement". Longitudinal observational studies evaluating the association of BP dipping pattern with micro- and macrovascular DM complications were included in the systematic review and meta-analysis.

Results – Three hundred seventy-eight studies were identified. A total of 4 studies fulfilled the eligibly criteria and were included in the meta-analysis. A higher incidence of micro- and macrovascular events was observed among non-dipper subjects (fixed effect: RR = 1.67, CI 95% 1.14-2.45, P = 0.009) when compared to those with a dipping pattern.

Discussion – Non-dipping type 2 DM patients have a 1.7 fold increase in the risk of micro- and macrovascular DM complications compared to dipping subjects.

Introduction

Type 2 diabetes mellitus (DM) is a chronic disease, frequently associated with other comorbidities, mostly derived from metabolic disarrangements. Hypertension is one of the most commonly associated diseases, which adds risk for the development of chronic diabetic complications [1]. Nearly 40% of type 2 DM patients are hypertensive when diagnosed [2], and with appropriate hypertension treatment each 10 mmHg decrease in systolic blood pressure (BP) reduces about 12% the risk of any diabetic complication [3].

Twenty-four-hour ambulatory BP monitoring (ABPM) is an useful tool to evaluate cardiovascular risk in DM patients [4-7], as its values are better predictors of cardiovascular events when compared to ordinary office BP, even in patients apparently not hypertensive [8]. ABPM provides the possibility to evaluate a broader spectrum of BP parameters such as 24-h, daytime and nighttime BP means and loads, presence of white-coat and masked hypertension, evaluation of dipping pattern, pulse pressure and even estimating arterial stiffness [9]. These variables have been associated with higher cardiovascular risk profile in non-diabetic patients [10-12] and in both type 1 and type 2 DM patients [13-18].

BP varies along the 24-h according to a circadian rhythm, which is characterized by a morning rise, daytime *plateau* and a nocturnal decrease of at least 10% of the daytime mean BP levels. Individuals without the normal decrease in nocturnal BP are named non-dippers, and the non-dipping phenomena has been associated with several complications in DM patients, such as increased glomerular filtration rate [19], higher incidence of albuminuria [20-22], diabetic retinopathy [21, 23] and macrovascular complications [21]. However, the cause-effect relation between non-dipping phenomena and outcomes remains controversial, because there are few studies addressing this association in a longitudinal design [14, 24-27].

Therefore, the aim of this study was to evaluate the risk of micro- and macrovascular DM complications according to the dipping pattern of BP thought a systematic review and meta-analysis of longitudinal studies in type 2 DM patients

Methods

The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement was used in this report [28].

Selection criteria and search strategy

Longitudinal studies that evaluated ABPM patterns in patients with type 2 DM were searched. Studies that included dipping pattern evaluation and had micro- and macrovascular outcomes reported were eligible. The combined outcome included non-fatal acute myocardial infarction, non-fatal stroke, occlusive arterial disease, fatal myocardial infarction, fatal stroke, cardiac sudden death, end-stage renal disease and severe retinopathy (proliferative disease requiring laser therapy).

No publication language, publication date, or publication status restrictions were imposed. All studies published up to May 31st 2015 were identified by searching Medline (1966-Present) electronic database. The following index terms were used: Diabetes Mellitus, Type 2 AND “Ambulatory Blood Pressure Monitoring” OR “Ambulatory Blood Pressure Measurement".

Study selection and data extraction

Eligibility assessment was made by title and abstracts review and in doubtful cases by full article review. This was performed independently in a standardized manner by three investigators (BSD, MD, RN). Disagreements between reviewers were resolved by consensus.

Two independently investigators extracted the data (BSD and MD). Disagreements were resolved by a third author (LHC). In the case of duplicate publications, the first manuscript published was included in the analysis. Information was extracted from each individual study based on: (1) characteristics of study participants (including age and gender), (2) time of follow-up; (3) number of patients lost.

We included a non-published study from our group, that evaluated the presence of non-dipping pattern and its association with micro- and macrovascular combined outcomes in 361 type 2 DM patients. At baseline, mean age was 56 ± 9 and 54% were male. Patients had no previous history of cardiovascular disease, and ABPM at baseline

was measured without any antihypertensive medication for at least one week. ABPM was obtained by oscillometry (Spacelabs 90207 serial nos. 207/024751 and 207/038016 with calibration certification), with a 15-min interval in the daytime and 20-min interval in the nighttime period. Dipping pattern was defined when nighttime fall in systolic BP of 10% or more was observed. Two hundred nine-seven patients were followed for an average of 54 ± 37 months (range 2 – 192 months). Outcomes were defined as non-fatal acute myocardial infarction, non-fatal stroke, occlusive arterial disease, fatal myocardial infarction, fatal stroke, and end-stage renal disease. Thirty-six patients had events.

Quality assessment

To ascertain the quality of each eligible study, two investigators (BSD and MD) assessed independently the presence of major biases in study design and analysis. In addition, an evaluation of the strategies utilized to minimize the risks of bias or confusion in each study was performed. We utilized the Newcastle Ottawa scale to evaluate studies quality. It consists of nine possible points from eight questions: 4

questions about selection of the cases and controls, with one possible point for each; 1 question about comparability with two possible points for it; 3 questions about outcome, with one possible point for each. (http://www.ohri.ca/programs/clinical_epidemiology/nosgen.pdf).

Statistical analysis

The magnitude of effect was evaluated by relative risk (RR), calculated using random and fixed effect models. Heterogeneity was tested by Cochran's Q test and inconsistency with I^2. If P_Q <0.10 or I^2 >50% then heterogeneity was considered statistically significant. The risk of publication bias was evaluated using funnel plot graphics and Egger's test. Data were analyzed using Stata/SE 11.2 (http://www.stata.com).

Results

Three hundred seventy-eight studies were identified, and 366 were excluded based on review of titles and abstracts. Thirteen articles were eligible and had the full text evaluated. Eight studies were excluded due to lack of information required. A total of 3 studies fulfilled the eligible criteria and were included in the meta-analysis. We added a non-published study from our group to the meta-analysis (Figure 1).

The 4 studies included provided information on 1054 patients, of which 631 subjects had a non-dipping and 423 a dipping BP pattern. One hundred forty three subjects had cardiovascular

outcomes. In all studies dipping pattern was defined when the nighttime fall in systolic BP was 10% or more.

Main clinical characteristics of individual studies are described in Table 1. Carmona et al. [25] evaluated prognostic markers for type 2 DM, most of them on antihypertensive and antidiabetic medications. The population had high-risk profile for cardiovascular outcome, since 60% had dyslipidemia and 39% already had vascular events. Nakano et al. [24] evaluated ABPM patterns, as systolic BP, diastolic BP, mean 24 hour BP, pulse pressure and dipping status, and the ABPM at baseline was performed without any antihypertensive medication. Patients who developed outcomes had longer diabetes duration, were older and had lower glomerular filtration rate. Eguchi et al. [29] evaluated ABPM in comparison to office BP in asymptomatic patients.

The meta-analysis results are depicted on Figure 2. Non-dipper BP pattern was associated with an increased incidence of micro- and macrovascular events during the follow-up (fixed effect: RR = 1.67, CI 95% 1.14-2.45, P = 0.009). Random effect model was performed, and demonstrated similar data (data not shown). No heterogeneity was detected (I^2 = 0%, P_Q= 0.43).

No publication bias was observed in funnel plot or Egger test (p = 0.178) (**Figure 3)**. The studies presented at Newcastle-Ottawa scale result between 7-9, meaning they have good quality, not requiring sensitive analysis.

Discussion

In this systematic review with meta-analysis of longitudinal studies, non-dipping pattern of BP was associated with a 1.7 fold increase in the risk of micro- and macrovascular DM complications.

Except for Carmona study, which presented marginal statistic effect, the other studies showed no statistically significance when analyzed separately, probably because individual studies didn't had enough power to detect differences due to a small number of patients, and when meta-analyzed together the effect came out.

Non-dipping BP pattern is considered a manifestation of autonomic failure, presenting in type 2 DM patients with cardiac autonomic neuropathy (CAN) [30]. This is also associated left ventricular hypertrophy and diastolic dysfunction that can evolve to heart failure [31, 32]. There is evidence that in these patients bedtime anti-hypertensive treatment could be beneficial [33].

ABPM should be considered in the routine evaluation of cardiovascular risk in patients with type 2 DM, since the non-dipping pattern is associated with vascular outcomes. Besides the dipper pattern, some other parameters, such as pulse pressure, arterial stiffness, white-coat hypertension and masked hypertension could also be estimated at the same time [34]. We have previously demonstrate that the presence of white-coat effect [15], the late afternoon increase BP [35] and the presence of masked hypertension [13] were associated with vascular complications in patients with DM. In order to prevent the onset and progression of diabetic complications we need to look beyond glycemic control, and ABPM might be a useful tool to help accomplishing that.

However, we must acknowledge some limitations. Dipping patterns were defined in all studies only by systolic blood pressure, not considering the presence of only diastolic BP non-dipping pattern. Some authors suggested that we should consider diastolic BP in addition, since it represents an indirect measure of peripheral vascular resistance [36]. Another limitation is the cardiovascular outcome definition. All studies included fatal and non fatal stroke and acute myocardial infarction, as well sudden death. Carmona e Nakano included end-stage renal disease

and severe retinopathy as cardiovascular outcome, while Eguchi have excluded these features. In our study for instance, a composite outcome was defined as fatal stroke and acute myocardial infarction, non-fatal stroke and acute myocardial infarction, severe peripheral arterial disease, and ESRD development.

Conclusions

In conclusion, this study shows an association between non-dipping pattern and micro- and macrovascular DM complications. These results are original and help to understand the role of ABPM patterns as risk factors.

Conflicts of interest

There are no conflict of interests.

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Table 1: Studies Characteristics

First author	Year	n	Loss from follow-up (n)	Completed Follow-up (%)	Follow-up time (months)	Range of follow-up (months)	Mean Age (years)	Male (%)	Non-dipping (%)	Events (n)	Newcastle-Ottawa
Carmona et al.	2004	97	5	94.84	28.0 ± 5	15-43	63 ± 8	54	72,8	28	7
Nakano et al.	2004	392	28	92.85	86.0 ± 46	2-168	55 ± 14	63	75,3	50	8
Eguchi et al.	2008	301	0	100	50 ± 23	1-116	70.4 ± 10	38	48,2	29	9
Dellamea et al.	2015	361	64	82.27	54.0 ± 37	2-192	56 ± 9	54	50,2	36	8

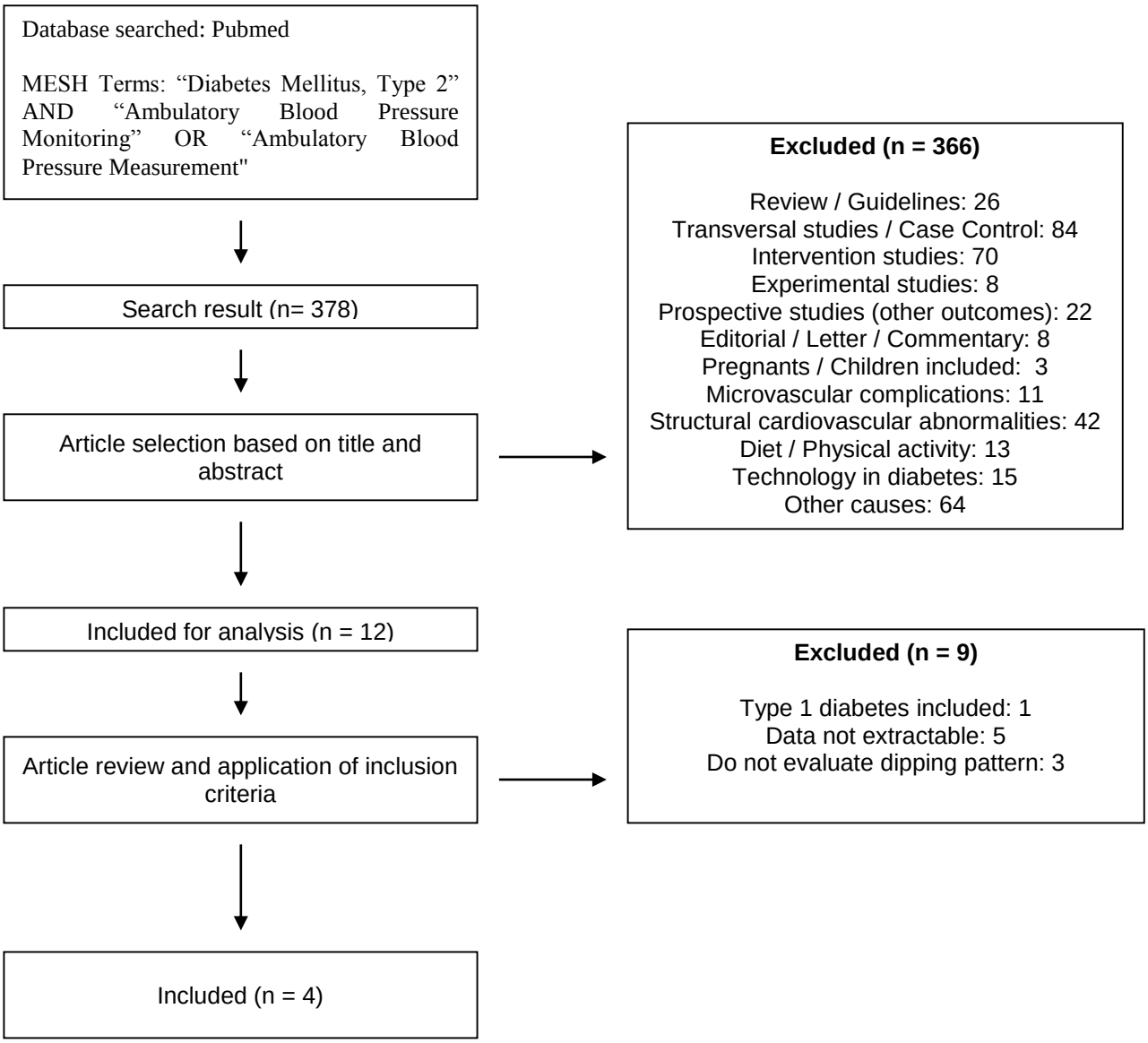

Figure 1: Search algorism.

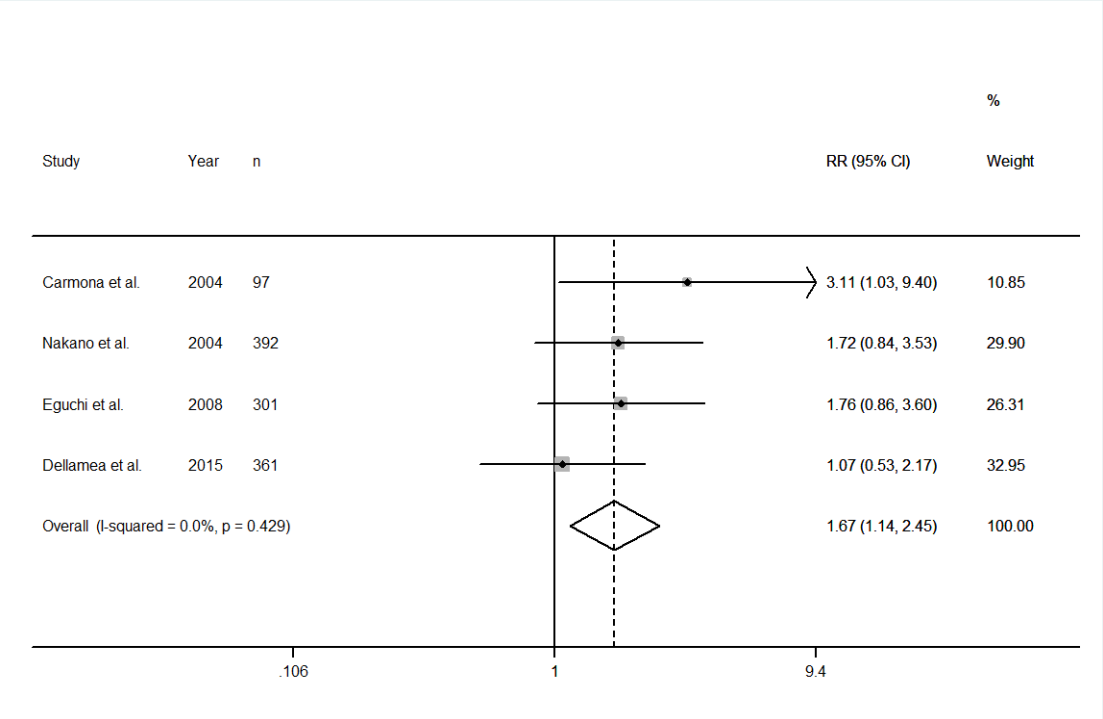

Figure 2: Micro- and macrovascular outcomes between non-dippers and dippers. Deviation to the right side denotes a higher risk for non-dippers.

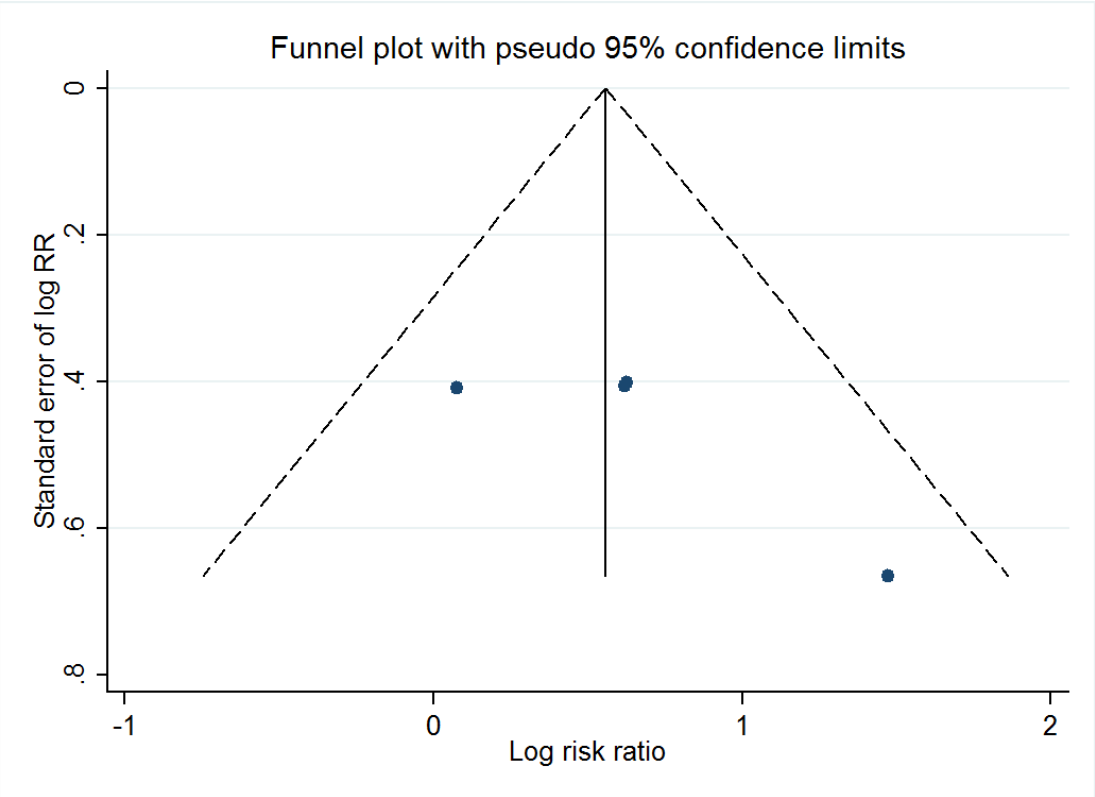

Figure 3: Funnel plot

Title: Nitric Oxide System and Diabetic Nephropathy

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Abstract

About 30% of patients with type 2 diabetes mellitus develop clinically overt nephropathy. Hyperglycemia is necessary, but not sufficient, to cause the renal damage that leads to kidney failure. Diabetic nephropathy (DN) is a multifactorial disorder that results from interaction between environmental and genetic factors.

Nitric oxide (NO) is a short-lived gaseous lipophilic molecule produced in almost all tissues, and it has three distinct genes that encode three NO synthase isoforms (NOS): neuronal (nNOS), inducible (iNOS) and endothelial (eNOS).

The correct function of the endothelium depends on NO, participating in hemostasis control, vascular tone regulation, proliferation of vascular smooth muscle cells and blood pressure homeostasis, among other features. In the kidney, NO plays many different roles, including control of renal and glomerular hemodynamics. The net effect of NO in the kidney is to promote natriuresis and diuresis, along with renal adaptation to dietary salt intake.

The eNOS gene has been considered a potential candidate gene for DN susceptibility. Three polymorphisms have been extensively researched: G894T missense mutation (rs1799983), a 27-bp repeat in intron 4, and the T786C single nucleotide polymorphism (SNP) in the promoter (rs2070744). However, the potential link between eNOS gene variants and the induction and progression of DN yielded contradictory results in the literature.

In conclusion, despite the discrepant results of many studies, the eNOS gene remains a good candidate gene for DN.

Keywords: *diabetes, diabetic nephropathy, polymorphism, eNOS, NOS-3, G894T, 4b/a, T786C.*

1 - Introduction

About 30% of patients with type 2 diabetes mellitus develop clinically overt nephropathy [1]. Thus it appears that in humans hyperglycemia is necessary, but not sufficient, to cause the renal damage that leads to kidney failure. The risk is not linearly correlated to the duration of diabetes, with a decline after an initial progressive incidence, likely due to exhaustion of the subgroup of susceptible subjects [2, 3].

Diabetic nephropathy (DN) is a multifactorial disorder that results from interaction between environmental and genetic factors. DN is histologically defined by thickening of the glomerular basement membrane, increased fractional mesangial volume, and podocyte abnormalities [2]. Hyperglycemia, hypertension and proteinuria are the main insults that cause structural abnormalities in a diabetic kidney [1, 4-6].

The earliest known manifestation of diabetic kidney disease is the presence of small amounts of albumin in the urine, known as microalbuminuria. So far it is still unknown who will evolve to end-stage renal disease, and the genetic factor is a matter for debate and further study [7].

2 – Nitric Oxide System Characterization

Nitric oxide (NO) is a short-lived gaseous lipophilic molecule produced in almost all tissues and organs, a free radical exerting a variety of biological actions under both physiological and pathological conditions. NO is a paracrine mediator formed from its precursor L-arginine by a family of NO synthases (NOSs) with stoichiometric production of L-citruline. The NO system consists of three distinct NO synthase (NOS) isoforms, encoded by three distinct genes, including neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2)

and endothelial (eNOS or NOS-3). The gene encoding eNOS is located on chromosome 7 (7q35-q36) and contains 26 exons with an entire length of 21 kb[8-10].

3 – Nitric Oxide System Physiology

eNOS expression is regulated by transcription (changes in the rate of eNOS gene transcription), stabilization (alterations in eNOS mRNA stability), and phosphorylation [11]. The presence of these consensus sites is consistent with evidence showing that levels of eNOS transcripts are elevated by sheer stress, exercise and hypoxia [12]. Regulation of eNOS transcription by estrogens is still a matter of debate. Both lipopolysaccharide and tumor necrosis factor-α decrease eNOS gene expression by reducing the stability of eNOS-mRNAs. The constitutively expressed eNOS-mRNA is about 4052 nucleotides long and has a half-life of 10–35 h. Therefore, synthesis of the encoded proteins is likely to persist long after gene expression has been repressed [12]. Also, the activity of eNOS and the production of NO are diminished in senescent human endothelial cells [13].

The endothelium is a fundamental layer in the arterial wall both for the local regulation of flow to critical organs and for the protection of the vascular system from atherogenic insults. The correct function depends on the NO generation rate [14]. NO participates in regulatory functions including control of hemostasis, fibrinolysis, platelet and leukocyte interactions with the arterial wall, vascular tone regulation, vascular smooth muscle cell proliferation and blood pressure homeostasis. Disturbances in NO bioavailability have been found to cause endothelial dysfunction, leading to increased susceptibility to atherosclerotic lesion progression, hypertension, hypercholesterolemia, diabetes mellitus, thrombosis and stroke [15, 16].

Insulin increases NO production, leading to vasodilatation and increased blood perfusion, and it also has anti-apoptotic and pro-survival effects on the ischemic/reperfused

heart. Impairment of the phosphatidylinositide 3-kinases (PI3K) – protein kinase B (AKT) – eNOS – NO pathway as a manifestation of insulin resistance contributes to endothelial dysfunction, predisposing the endothelium to hyper-inflammatory and thrombotic states, while endothelin-1 expression and mitogenic effects are not affected [17]. Exercise, diet, cardiovascular drugs and insulin sensitizers, such as angiotensin-converting enzyme inhibitors, angiotensin receptor inhibitors, and the PPARg agonists, modulate both metabolic and cardiovascular effects of insulin simultaneously by regulating PI3K-AKT-eNOS signaling [18].

Data from animal models have suggested that eNOS null mice show a phenotype that resembles the human metabolic syndrome phenotype [19]. The oxidative effects of NO may play a role in insulin resistance and type 2 diabetes [20]. Two studies performed in a Spanish population found a positive association between e*NOS* polymorphisms and metabolic syndrome [21, 22]. Another study, in an Italian population found an association with eNOS polymorphisms and insulin resistance [23]. Additionally, a positive association between G*894T* polymorphism and metabolic syndrome has been shown in Chinese and Japanese populations [24, 25]. An association between G894TeNOS gene polymorphism and features of the metabolic syndrome was demonstrated in a southern Brazilian population, assuming a recessive model of inheritance [26].

4 – Nitric Oxide System and Kidney

NO must be considered in the pathogenesis of DN, since it plays numerous physiological roles in the kidney, including control of renal and glomerular hemodynamics, by interfering at multiple and physiologically critical steps of nephron function. NO dilates both the afferent and the efferent arteriole; it may augment the glomerular filtration rate (GFR) and influence renal sodium handling along various tubule segments from the thick

ascending limb to the distal tubule and the collecting duct [27]. NO is also responsible for mediation of pressure natriuresis, maintenance of medullary perfusion, blunting of tubuloglomerular reabsorption, and modulation of renal sympathetic nerve activity [27, 28]. The net effect of NO on the kidney is to promote natriuresis and diuresis, along with renal adaptation do dietary salt intake [29, 30].

High levels of nNOs are expressed in macula density and in minor intensity in specialized neurons within renal arteries of the hilus, arcuate and interlobular arteries. eNOs is strongly expressed in renal vascular endothelium, although tubular expression of eNOS also occurs. iNOS is weakly expressed in the kidney [27]. Changes in NOS gene expression do not always correlate well with measures of actual NO synthesis, because synthesis of NO by nNOS and eNOS is highly dependent on both adequate substrate and co-factor availability [31]. Changes in requirements for NO synthesis in the kidney often occur very fast, so regulation of total NOS expression seems not to play an important role, and it makes it more difficult to study molecular events in the regulation of NOS in the kidney [27, 31, 32].

The overall production of NO is decreased in chronic kidney disease (CKD), which contributes to cardiovascular events and further progression of kidney damage. There are many likely causes of NO deficiency in CKD, such as limitations on substrate (L-Arginine) availability, increased circulating levels of endogenous NOS inhibitors, in particular asymmetric dimethylarginine (ADMA). Reduced renal cortex abundance of the nNOS protein correlates with injury while increasing nNOS abundance may provide a compensatory, protective response [33].

In CKD, ongoing endothelial damage in the capillary system of the renal medulla and accompanying vascular rarefaction are thought to be central processes toward progressive kidney damage [34]. Reduced NO synthesis by endothelial cells due to accumulation of inhibitors of the eNOS, such as ADMA, has been pointed out as the cause of accelerating

progression. Also, erythropoietin may have vasculoprotective effects on renal endothelium, which may be critically dependent on the activation of eNOS[35].

5 - Nitric Oxide System and Diabetic Nephropathy

Recently, endothelial dysfunction has been common in subjects with DN, and is considered the central pathophysiologic denominator for all cardiovascular complications of diabetes. In animal models of CKD and arteriosclerosis, blocking endothelial NO leads to an increase in microvascular disease, known to impair renal autoregulation [36].

Endothelial dysfunction has also been shown to lead to an uncoupling of the vascular endothelial growth factor (VEGF)-nitric oxide axis resulting in enhanced proinflammatory and proliferative effects of VEGF [36, 37]. VEGF is increased in glomeruli and tubules in response to hyperglycemia. Whereas most studies have suggested that VEGF may be beneficial in non-diabetic renal disease, there is increasing evidence that VEGF may have a deleterious role in diabetic nephropathy. Endothelial dysfunction with NO deficiency may result in a loss of negative regulatory activity in the VEGF pathway. Consequently, VEGF may cause excessive endothelial cell proliferation, pathological macrophage infiltration, and overactivation of vascular smooth muscle cell, causing vascular injury [38-39].

Endothelin-1 is inhibited by vasodilators like NO and prostacyclins. Endothelin-1 acts in the kidney rising vascular resistance, which leads to reduction in blood flux, glomerular filtration rate and inhibition of salt and water reabsorption. It also causes glomerular cellular proliferation and accumulation of extracellular matrix [17].

NOS could be involved in the development of chronic diabetes complications through others pathways, such as uncoupling protein 2 (UCP2). UCP2 is expressed in several tissues, and protects against oxidative stress, in the regulation of insulin secretion by beta cells, and in fatty acid metabolism. Moreover, UCP2 preserves endothelial function through increasing

NO bioavailability secondary to the inhibition of ROS production in the endothelium [40]. ~~UCP2 has a protection role against oxidative stress, with a positive association between *UCP2* gene polymorphisms and the occurrence of chronic complications of diabetes [41].~~ UCP2 has a potential role for inflammation and apoptosis regulation. These functions have major implications for cardiovascular and cerebrovascular chronic complications of diabetes. In fact, some UCP2 polymorphisms have been associated with the presence of diabetic chronic complications [41].

The metabolic abnormalities of diabetes cause mitochondrial superoxide overproduction in endothelial cells of both large and small vessels, enhancing five major pathways to diabetic complications: polyol pathway flux, activation of protein kinase C isoforms, overactivity of the hexosamine pathway, increased formation of advanced glycation end products and increased expression of the receptor for advanced glycation end products [38].

A broad spectrum of findings and issues has been amassed concerning the pathophysiology of the renal NO system in diabetes. Severe diabetes with profound insulinopenia can be viewed as a state of generalized NO deficiency. Available evidence suggests that diabetes triggers mechanisms that at the same time enhance and suppress NO bioavailability in the kidney [20, 43]. It has been hypothesized that during the early phases of nephropathy, the balance between these two opposing forces is shifted toward increased NO [20, 44, 45]. This plays a role in the development of characteristic hemodynamic changes and may contribute to consequent structural alterations in glomeruli. The enhanced NO production may contribute to hyperfiltration and microalbuminuria that characterizes early diabetic nephropathy [43]. Both eNOS and nNO synthase can contribute to the altered NO production, particularly the first. As the duration of exposure to the diabetic milieu increases, factors that suppress NO bioavailability eventually prevail [20, 45]. Increasing accumulations

of advanced glycation end products may be one of the culprits in this process, leading to severe proteinuria, declining renal function, and hypertension [43]. In addition, this balance is continuously modified by actual metabolic control and the degree of insulinopenia[45].

Progression of early stages of DN to end-stage kidney disease is manifested by the gradual, inexorable scarring of the renal glomerulus followed by a similar fibrosing process in the tubulointerstitial region. Diabetic glomerular fibrosis is caused by accumulation of extracellular matrix proteins in the mesangial interstitial space resulting in fibrosis manifested by either diffuse or nodular changes. The use of daidzein (caveolin inhibitor), hemin (hemoxygenase activator) or NO substrate in rats significantly decreases the renal cortical collagen content as compared to diabetic rats, presenting significant improvement in BUN, serum creatinine, proteinuria, urinary output, kidney weight/ body weight, renal cortical collagen content and nitrite/nitrate levels [46-47]. Although there is some controversy, most reports suggest higher levels of NO production early in diabetes but reduced levels in progressive DN [48].

The eNOS gene has been considerate as a potential candidate gene to DN susceptibility. Over the last few years, several polymorphisms of the eNOS gene have been identified, and their association with various diseases has been explored. Three polymorphisms have been extensively subject of research in efforts to identify genetic predisposition to chronic diabetes mellitus microvascular complications. These polymorphisms of interest in DN are the G missense mutation (rs1799983), a 27-bp repeat in intron 4, and the T786C single nucleotide polymorphism (SNP) in the promoter (rs2070744) [49-53]. However, not all studies support this association [54-56]. A recent meta-analysis [57], which analyzed these polymorphisms in the progression of DN, showed that G894T is significantly associated with DN, mainly in the allele contrast genetic model. However, in this meta-analysis patients with DN were compared to healthy subjects used as controls.

Therefore, one cannot be sure if the polymorphisms were associated with DN or diabetes mellitus itself. Another meta-analysis [58] diabetic patients without nephropathy (controls) to diabetic patients with nephropathy (cases), and these polymorphisms were associated with increased risk for DN, supporting the involvement of the eNOS gene in the pathogenesis of DN.

The potential link between eNOS gene variants and the induction and progression of DN yielded contradictory results, exemplified by the association of the cited polymorphisms with ESRD and DN by some [49, 51-54, 59-69], but not by all studies [54-56,70]. G894T was linked to increased risk of macroalbuminuria and progression from microalbuminuria to macroalbuminuria, with declining glomerular filtration rate as serum creatinine value rises progressively, culminating in ESRD [66-67], independent of other risk factors.

These polymorphisms seem to change eNOS expression and to be associated with different levels of eNOS that make these associations clinically plausible. Intron 4 of eNOS contains a variable number of 27-pb consensus sequence repeats with the *b* allele having five repeats and the *a* allele having four repeats. A single nucleotide polymorphism affecting transcription of the eNOS promoter T786C reduces its activity to less than the half [71]. Plasma concentrations of NO metabolites are reduced in carriers of the "a" allele in intron 4 (intron with four repeats). The T786C SNP is strongly linked to the intron 4 polymorphism and functional studies reveal that the T786C mutation reduces eNOS gene promoter activity [50].

In conclusion, NOS seems to be involved in the development and progression of DN. Despite the discrepant results of many studies, the eNOS gene is also a good candidate gene for DN.

List of abreviations

ADMA –Dimethylarginine

AKT - Protein kinase B

CKD - Chronic kidney disease

DN - Diabetic nephropathy

eNOS - Endothelial nitric oxide synthase

GFR - Glomerular filtration rate

iNOS - Inducible nitric oxide synthase

nNOS - Neuronal nitric oxide synthase

NO - Nitric oxide

NOS - Nitric oxide synthase

NOSs - Nitric oxide synthases

NOS-1 - Neuronal nitric oxide

NOS-2 - Induciblenitric oxide

NOS-3 - Endothelialnitric oxide

PI3K - phosphatidylinositide 3-kinases

UCP2 - Uncouplingprotein 2

VEGF - Vascular endothelial growth factor

Competing interests

The author(s) declare that they have no competing interests

Authors' contributions

BSD reviewed the subject, searched for studies in databases, selected articles to include in the review and wrote the manuscript. CBL participated in the search and selection of articles and helped to draft the manuscript. RF helped in selection and helped to draft the manuscript. LHC conceived of the study, participated in coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Title: Endothelial nitric oxide synthase gene polymorphisms and risk of diabetic nephropathy:

a systematic review and meta-analysis

Short running title: eNOS polymorphysm and diabetic nephropathy risk

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Abstract

Background: Endothelial nitric oxide synthase gene (eNOS-3) polymorphisms have been associated with diabetic nephropathy (DN), however some studies do not confirm this association. Therefore, we conducted a systematic review and meta-analysis, including studies with diabetic patients with nephropathy (cases) and diabetic patients without nephropathy (controls) that evaluated at least one of the three polymorphisms of interest. All studies published up to December 31st, 2012 were identified through electronic databases. Gene-disease association was measured using odds ratio estimation based on the following genetic contrast/models: (1) allele contrast; (2) additive; (3) recessive; (4) dominant, and (4) codominant. The analyzed polymorphisms in eNOS-3 gene were 4b/4a, T-786C, and G986T. **Results:** Twenty-two studies were eligible for meta-analysis (4b/a: 15 studies, T-786C: 5 studies, and G984T: 12 studies). Considering 4b/a polymorphism, an association with DN was observed for all genetic models: allele contrast (OR=1.14, CI:1.04-1.25); additive (OR=1.77, CI:1.37-2.28); recessive (OR=1.77, CI:1.38-2,27); dominant (OR=1.12, CI:1.01-1.24), with the exception for co-dominance model. As well, T-786C polymorphism showed association with all models, with exception for co-dominance model: allele contrast (OR= 1.22, CI: 1.07-1.39), additive (OR = 1.52, CI: 1.18-1.97), recessive (OR = 1.50, CI: 1.16-1.93), and dominant (OR = 1.11, CI: 1.01-1.23). For the G894T polymorphism, an association with DN was observed in allelic contrast (OR = 1.12, CI: 1.03-1.25) and co-dominance models (OR= 1.13, CI: 1.04-1.37). **Discussion:** In the present study, there was association of DN with eNOS 4b/a and T-786C polymorphism, which held in all genetic models tested, except for co-dominance model. G894T polymorphism was associated with DN only in allele contrast and in co-dominance model. This data suggested that the eNOS gene could play a role in the development of DN.

Introduction

Nitric oxide (NO) is a short-lived gaseous lipophilic molecule produced in almost all tissues and organs[1-2]. It is a free radical that exerts a variety of biological actions under both physiological and pathological conditions[3]. NO is formed from its precursor L-arginine by a family of NO synthases (NOSs). NOS system consists of three distinct isoforms, encoded by three distinct genes, including neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial (eNOS or NOS-3). The gene encoding eNOS is located on chromosome 7 (7q35-q36) and contains 26 exons, with an entire length of 21 kb[3-4]

NO has numerous functions in the kidney, including control of renal and glomerular hemodynamics, by interfering at multiple pathological and physiologically critical steps of nephron function. NO dilates both the afferent and the efferent arteriole, augmenting the glomerular filtration rate (GFR) and influencing renal sodium handling [5]. NO also mediates pressure natriuresis, maintenance of medullary perfusion, decrease of tubuloglomerular reabsorption, and modulation of renal sympathetic nerve activity [6]. The net effect of NO in the kidney is to promote natriuresis and diuresis, along with renal adaptation to dietary salt intake [7-8].

eNOS gene has been considered a potential candidate gene to diabetic nephropathy (DN) susceptibility. Since 1998, several polymorphisms of the eNOS gene have been identified, and their association with various diseases has been explored. Three polymorphisms have been the subject of research in relation to DN, however the results are highly variable. The polymorphisms potentially associated with DN are a 27-bp repeat in intron 4 (VNTR), the T-786C single nucleotide polymorphism (SNP) in the promoter region (rs2070744), and G894T missense mutation in exon 7 (rs1799983) [9]. Some of these

polymorphisms are associated with reduction of either eNOS activity (-786C in the promoter area) or plasma concentrations of NO (four repeats in intron 4) [2].

However, the potential association of eNOS gene variants with the induction and progression of DN remains controversial. Some authors found a higher frequency of eNOS polymorphisms in patients with end-stage renal disease (ESRD) and DN [10-17], but not all studies reported this association [18-20].

The objective of the present study was to evaluate if eNOS gene polymorphisms are associated with DN through a systematic review of the literature and a meta-analysis.

Results and discussion

Three-hundred and nine studies were identified, and 281 were excluded based on review of titles and abstracts (70 animal experimental studies, 17 pharmacological studies, 86 without adequate cases or controls, 58 without the genes or polymorphisms of interest, 3 review articles, 5 meta-analysis, 35 studies with multiple publications of the same data presented with different titles, 7 no accesses to original data even after contacting authors). Twenty-eight articles were eligible and had the full text evaluated. Six studies were excluded due to lack of information regarding genotypic distribution. A total of 22 studies fulfilled the eligible criteria and were included for the meta-analysis (Figure 1).

Clinical characteristics of individual studies are described in Table 1. Regarding quality assessment, the phenotype definitions as cases or controls were appropriated, but none of the studies included information if genotyping was performed by personnel blinded to clinical status. Of the 22 studies included, 15 provided 4054/3405 cases/controls for 4b/a; 5 provided 1436/1286 cases/controls for T-786C; and 12 provided 3316/2765 cases/controls for G894T. The allelic frequency of 4b, T-786, and G894 in cases/controls was 6647/5702, 1863/1795, and 4691/4017 respectively (Table 2).

Hardy-Weinberg equilibrium (HWE) was assessed using exact test and P-value < 0.05 were considered significant. Only 4 studies (1 study for T-786C; 2 for G984T; and 1 for 4b/a) with controls were not in HWE (Table 2). These studies were subjected to a sensitive analysis, and their exclusion did not show significant difference on OR.

For the 4b/a polymorphism, an association with DN in all genetic models, except for co-dominance, was observed: allele contrast (OR= 1.15, CI(95%): 1.05-1.25, P_Q <0.01, I^2 = 66%); additive (OR= 1.52, CI(95%): 1.18-1.97, P_Q <0.01, I^2 = 62%); recessive (OR= 1.50, CI(95%): 1.16-1.93, P_Q <0.01, I^2 = 64%); and dominant (OR= 1.11, CI(95%): 1.01-1.23, P_Q= 0.01, I^2 = 49%). Similarly, for the T-786C polymorphism the association with DN was found with all models, with exception for co-dominance model: allele contrast (OR= 1.22, CI (95%): 1.07-1.39, P_Q= 0.59, I^2 = 0%), additive (OR = 1.52, CI (95%): 1.18-1.97, P_Q<0.01, I^2 = 62%), recessive (OR = 1.50, CI (95%): 1.16-1.93, P_Q <0.01, I^2 = 64%) and dominant (OR = 1.11, CI (95%): 1.01-1.23, P_Q <0.01, I^2 = 49%). The G894T polymorphism showed association with DN in allelic contrast (OR = 1.12, CI (95%): 1.03-1.25, P_Q <0.01, I^2 = 75%) and co-dominance model (OR= 1.13, CI (95%): 1.04-1.37, P_Q= 0.01, I^2 = 60%). (Table 3 and Figure 2). A random model analysis was performed confirming the fixed model results.

Publication bias was observed for the majority of the polymorphisms evaluated and are presented as a funnel plot for 4b/a polymorphsism. (Figure 3). In order to identify non published data, we performed manual search for abstracts in some of the major scientific meetings in the field in the last seven years. We estimated the effect of these potential publication biases using trim and fill method and no major differences were observed from the original results.

Since some studies included only subjects of specific ethnicities or with type 1 or type 2 DM, we performed a sensitive analysis stratifying the studies according to these

characteristics. Considering 4b/a polymorphism, there was an association in White and East Asian populations in allele contrast, additive and recessive models; only for Whites in the dominant model; and none for the co-dominant model. For T-786C variant, no association was shown for Whites in allele contrast analysis or in any other genetic model, but in African populations the polymorphism was associated with DN in allele contrast, dominance and co-dominance models. Considering G894T polymorphism, in African populations the association was observed for all genetic models, with the exception of co-dominance model. There were insufficient studies to perform a meta-analysis for G894T in South Asians and West Asians.

According to the type of diabetes mellitus (DM), for 4b/a polymorphism an association was observed in additive and recessive models for both type 1 and type 2 diabetes, and only for type 1 in allele contrast and dominant models. There was no association with any type of DM in co-dominant model for 4b/a variant. For T-786C, no association in any genetic model was found in type 2 diabetes. There was insufficient data for this analysis in type 1 DM. Likewise, for G894T variant there was an association only in the allele contrast model with type 2 diabetes (Table 3).

We compared the ORs of our meta-analysis with the results from a previous meta-analysis that used non-diabetic patients as controls [21]. The results were similar and no statistical differences in the ORs of the two studies were observed in all genetic models analyzed (data not shown).

Conclusions

In the present study, the most robust association of DN was with eNOS 4b/a and T-786C polymorphism that held in all genetic models tested, except for co-dominance model. G894T polymorphism was associated with DN only in allele contrast and in co-dominance

model. 4b/a polymorphism association with DN was confirmed in all ethnic groups evaluated and for all types of diabetes. The subgroup analysis of the T-786C variant should be viewed with caution, since it was limited due to the small number of studies.

Analyzing genetic model is important, considering the difference between them. Each individual genotype is formed by two alleles (for example G and T for G984T polymorphism), and the risk of every genotype depends on the number of variant allele copies carried, where one of which is thought to be associated with a disease (e.g., T), association studies will collect information on the numbers of diseased and disease-free subjects with each of the three genotypes (GG, GT, and TT). So we used the allele contrast, which compares the number of alleles G with the number of alleles G; the additive model, which contrasts extreme homozygotes, comparing the genotype GG with the genotype TT; in recessive model two copies of T allele are essential to modify the risk, combining the GG and GT genotypes and comparing with TT; the dominant model, which heterozygous GT and homozygous TT genotypes have the similar risk as a single copy of T is sufficient to alter the risk, then compares GG with combined GT and TT genotypes; and the codominance model, commonly used genetic model, where each genotype gives a diverse and non additive risk. which combines the GG and TT genotypes and compares with GT. So OR in each particular genetic model gives us different interpretations about the risk of the polymorphisms.

These results are original and help to understand the role of these polymorphisms in the development of DN. However, it was not possible to exclude a publication bias of negative studies. Therefore, the exact effect could be smaller. As discussed before, other explanations, besides classic risk factors, are needed for understanding the progression of a diabetic patient from normoalbuminuria to macroalbuminuria, and a polymorphism identification of a specific gene would propitiate the development of a new therapy aimed directly to it.

In contrast to a recent meta-analysis performed by Zintzaras et al. [21], which analyzed the same polymorphisms in the progression of DN, our analysis compared diabetic patients with DN (cases) with diabetic patients without DN (controls). In Zintzaras' study, healthy subjects were used as controls, mixed with patients with DN. When the controls are defined as non-diabetic subjects, the observed association could reflect a genetic predisposition for individuals to develop "diabetic nephropathy". The obtained results could reflect a mixture of a susceptibility to diabetes per se and to nephropathy, which cannot be discriminated. In this regard, to serve non-diabetic individuals as controls seem rationale to estimate a risk of diabetic nephropathy. However, from clinical points of view, most of medical staff would be interested in risks for nephropathy among individuals with diabetes, as in the case with hyperglycemia, rather than combined risks for developing diabetes and for nephropathy thereafter. That is why diabetic individuals showing no or little nephropathy despite a term of duration have been widely investigated as controls, in most of the previous studies. So, our work and Zintzaras are derived from different standing points: a clinical aspect and a bio-mathematic research.

In this sense, we considered that the optimal control group when studding a DM complication is a diabetic patient without the complication and with disease duration long enough the permit a genetic predisposition to become clinically detected in the presence of hyperglycemia. Moreover, the disease duration must be comparable between cases and controls. Most included studies fulfilled the two pre-requisitions. As can be seen in Table 1, the DM duration is similar between cases and controls in each study and the majority has more than 10 years of DM, reflecting that authors from original studies probably took this important issue in consideration.

Despite the different control used by Zintzaras, they found 92 articles, being 20 included for meta-analysis; that provided 1942/1461 cases/controls for G894T, 2663/2232;

cases/controls for 4b/a, and 857/845 cases/controls for T-786C. That was similar to ours that had 22 studies included, but provided about one third more cases/controls. The OR observed in their analyzes showed significance in allelic contrast model for G894 polymorphism, recessive and additive model for 4b/a polymorphism, and allelic contrast model for T-786C, all observed in our study; but our analyze showed association in more genetic models than that, like codominant model for G894T; allele contrast and dominant model for 4b/a; recessive, dominant and additive model for T786C. Furthermore, we compared our ORs with those reported by Zintzaras et al. and no statistical differences were found. With that said, our study reinforce the findings from Zintzaras.

DN development predisposition has not been fully explained, since glycemic control and environmental factors, as well as traditional risk factors, do not accurately predict the occurrence of this diabetic complication in all patients. With this in mind, studies have been trying to resolve this question using genetic approaches. Many candidate genes have been explored in this context, and eNOS polymorphisms have been implicated in the susceptibility to glomerular disease, by mechanisms yet unknown [15]. However, there is no consensus on the role of these polymorphisms in modulation of risk for DN, since the available literature demonstrates mixed results and most of the studies have a small sample. In this scenario, the recommended approach to help investigators in understanding the effect of each polymorphism in DN development is a systematic review and meta-analysis. Our data ~~indicates~~ suggest an ~~positive~~ association between eNOS polymorphisms and DN. Assuming a recessive model, the relative risk, attributable risk and population attributable risk for the 4a variant ranges are, respectively, 1.20; 0.11; and 0.09.

The present paper has some limitations. The inclusion of studies evaluating patients with DM in several stages of DN, ranging from microalbuminuria to chronic renal insufficiency in kidney replacement therapy, could bias the results due to clinical

heterogeneity of cases. Some studies did not present the data separated by DN stages. Furthermore, inclusion criteria in the reviewed studies utilized different methods and cutoffs to define microalbuminuria or macroalbuminuria. Although all clinically validated [22], these aspects made impossible to evaluate the effect of each polymorphism in the stages of DN in this meta-analysis. Finally, the polymorphisms true effects could be overestimated in the present study, since there is some indication of publication bias.

In conclusion, this study shows an association between DN and polymorphisms in eNOS gene. This effect is very consistent for the 4b and T-786 polymorphism.

Methods

The Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement was used in this report [23-24].

Selection criteria and search strategy

Case-control studies that had diabetic patients with DN as cases and diabetic patients without nephropathy as controls, as well as that evaluated at least one of the three polymorphisms of interest (4b/4a, T-786C, G986T) were considered eligible. Only studies in humans and using validated genotyping methods were considered. No publication language, publication date, or publication status restrictions were imposed. All studies published up until December 31st, 2012 were identified by searching electronic databases: Medline (1966-Present), EMBASE (1980-Present), LILACS and Cochrane Library.

Abstracts presented at scientific events held by: The American Diabetes Association (ADA); The European Association for the Study of Diabetes (EASD); The National Kidney Association (NKA); and The American Society of Nephrology (ASN) were searched over the last seven years. The authors were contacted for more details in the case of abstracts with missing information.

The following index terms were used: ("Nitric Oxide Synthase Type III" OR "NOS3 protein, human") AND ("Databases, Genetic" OR "Genetic Predisposition to Disease" OR "Genetic Phenomena" OR "Genetic Processes" OR "Genetic Markers" OR "Genetic Variation" OR "Polymorphism, Genetic" OR "Genetic Research" OR "Genetic Determinism" OR "Genes" OR "Genetics" OR "Mutation" OR "Genetics, Medical" OR "DNA") AND ("Proteinuria" OR "Albuminuria" OR "Kidney Failure" OR "Kidney Failure, Chronic" OR "Kidney Diseases" OR "Diabetic Nephropathies").

Study selection and data extraction

Eligibility assessment was made by title and abstracts review and in doubtful cases by full article review. This was performed independently in a standardized manner by two investigators (BSD and CBL). Disagreements between reviewers were resolved by consensus.

Two investigators extracted the data, one independent to another (BSD and LCFP). Disagreements were resolved by a third author (LHC). For articles with missing information, (n=3) the authors were contacted for further information, but none responded. In the case of duplicate publications, the first manuscript published was included in the analysis. Information was extracted from each individual study based on: (1) characteristics of study participants (including age, gender, type of diabetes, diabetes duration, nephrologic status, and ethnicity) [25], (2) case and control definition; (3) genetic data (including allelic distribution and genotypic frequency).

Quality assessment

To ascertain the validity of each eligible case-control study, two investigators (BSD and LCFP) worked independently during the initial search and after worked together to determine the adequacy of studies selection. It was assessed if the same exclusion criteria for cases and controls were used; if cases were easily differentiated from controls; if analysis of

studied polymorphisms were conducted in a standard, valid, and reliable way, if major biases were identified and considered in design and analysis; and how good the study was to minimize the risks of bias or confusion. Hardy-Weinberg equilibrium assessment among the control group within each polymorphism in all studies was checked by exact test using an online HWE calculator (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

Statistical analysis

Gene-disease association was measured using odds ratio estimation based on the following genetic contrast/models: (1) allele contrast; (2) additive model; (3) recessive model; (4) dominant model and (4) co-dominant model [26-27]. Heterogeneity was tested by chi-squared test, Cochran's Q, and inconsistency with I^2. If P_Q <0.10, then heterogeneity was considered statistically significant. Odds ratio was calculated using fixed-effect models (Mantel-Haenszel), and random models when heterogeneity was observed. Multiple comparisons were not made because meta-analysis of genetic association studies is considered an exploratory study, without a prespecified key hypothesis [28-29].

The risk of publication bias was evaluated using funnel plot graphics [30].

Sensibility tests were made concerning to ethnia and type of diabetes.

Data were analyzed using Data were analyzed using Stata/SE 11.2 (http://www.stata.com).

We compared the ORs of our meta-analysis with the results from a previous one [21] that used non-diabetic patients as controls using the differences of OR and 95%CI (WinPepi version 11.3).

List of Abreviations:

ADA: The American Diabetes Association

ASN: The American Society of Nephrology

DM: Diabetes Mellitus

DN: Diabetic Nephropathy

EASD: The European Association for the Study of Diabetes

eNOS: Endothelial Nitric Oxide Synthase

ESRD: End Stage Renal Disease

GFR: glomerular filtration rate

iNOS: Inducible Nitric Oxide Synthase

NKA: The National Kidney Association

nNOS: Neuronal Nitric Oxide Synthase

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

PRISMA: The Preferred Reporting Items for Systematic Reviews and Meta-Analysis

SNP: single nucleotide polymorphism

Authors contributions: BSD participated in design, selection of included articles, data collection, statistical analysis and wrote the manuscript; LCFP participated in data collection; CBL participated in selection of included articles and wrote the manuscript; KGS data collection and wrote the manuscript, LHSC wrote the manuscript.

Conflict of interests: None to declare.

Funding: LHC and CBL received a scholarship from *Conselho Nacional de Desenvolvimento Cinetífico e Tecnológico (CNPq).*

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Table 1 – Baseline studies characteristics.

Author	Year	Polymorphism	Type of DM	Ethnicity	Cases/Controls (n)	Criteria	Male/Female (%)	Age	DM duration (years)
Ahluwalia et al. [31]	2008	G894T, 4a/b, T-786C	2	East Asians	Case (195)	Overt proteinuria	35/65	60.0 ± 6.15	16.5 ± 6.3
					Control (255)	Normoalbuminuria	41/59	60.5 ± 5.7	15.6 ± 5.2
Bessa et al. [32]	2011	G894T	2	African	Case (40)	Albuminuria > 30 mg/24h	21/19	58.8 ± 12.5	19.4 ± 4.2
					Control (40)	Albuminuria < 30 mg/24h	17/23	55.4 ± 8.8	15.3 ± 3.7
Cai et al. [33]	1998	G894T	2	Whites	Case (116)	Microalbuminuria	NA	NA	NA
					Control (284)	Normoalbuminuria	NA	NA	NA
Degen et al. [34]	2001	4a/b	1and 2	Whites	Case (207)	AER >30 mg/24h	NA	NA	>10 yrs
					Control (418)	AER <30 mg/24h	NA	NA	>10 yrs
Ezzidi et al. [10]	2008	G894T , 4a/b, T-786C	2	African	Case (515)	AER >30 mg/24h	46/54	59.6 ± 10.8	13.5 ± 6.3
					Control (402)	AER <30 mg/24h	42/58	59.1 ± 11.2	11.5 ± 6.2
Fujita et al. [35]	2000	4a/b	2	East Asians	Case (102)	AER >200 mcg/min	60/40	61.0 ± 21.0	NA
					Control (65)	AER <20 mcg/min	46/54	62.0 ± 10.0	NA
Ksiasek et al.[13]	2003	4a/b	2	Whites	Case (178)	With DN	48/52	57.9 ± 8.2	8.7 ± 3.1
					Control (232)	Without DN	51/49	58.3 ± 6.8	8.0 ± 2.6
Lin et al. [36]	2002	4a/b	2	East Asians	Case (80)	With DN	NA	NA	NA
					Control (48)	Normoalbuminuria	NA	NA	NA

Mollsten et al. [37]	2006	G894T, 4a/b	1	Whites	Case (955)	AER >20 mcg/min	58/42	40.3 ± 10.0	28 (5-65)
					Control (555)	AER <20 mcg/min + DM duration >20 yrs	41/59	42.2 ± 10.2	28 (20-57)
Mollsten et al. [18]	2009	G894T	1	Whites	Case (458)	AER >300 mg/24h	39/61	42.0 ± 10.4	27 (7-65)
					Control (319)	AER <30 mg/24h	55/45	43.7 ± 11.0	23 (15-63)
Neuguebauer et al. [14]	2000	4a/b	2	East Asians	Case 1 (104)	AER 20-200 mg/g Cr	53/47	59.0 ± 11.1	13.8 ± 5.1
					Case 2 (39)	AER >200 mg/g Cr	74/26	59.0 ± 8.6	15.2 ± 4.5
					Control (82)	AER <20 mg/g Cr	65/35	56.0 ± 8.6	13.3 ± 4.5
Rahimi et al. [38]	2012	G894T	2	West Asians	Case 1 (68)	Albumin to creatinin ratio >300 mg/g	33/35	57.1 ± 8.7	11.1 ±6.4
					Case 2 (72)	Albumin to creatinin ratio 30-299 mg/g	23/46	55.3 ± 8.6	8.6 ± 5.2
					Control (72)	Albumin to creatinin ratio <30 mg/g	23/49	54.4 ± 7.9	7.7 ± 5.4
Rippin et al. [39]	2003	4a/b	1	Whites	Case (464)	Overt proteinuria	NA	NA	NA
					Control (396)	Normoalbuminuria	NA	NA	NA
Santos et al. [41]	2009	G894T, 4a/b, T-786C	2	Whites	Case (376)	AER >20 mcg/min or >17 mg/dl	57/43	60.4 ± 9.7	15.0 ± 9.1
					Control (268)	AER <20 mcg/min or <17 mg/dl	37/63	62.0 ± 9.4	16.7 ± 6.8
Shestakova et al. [16]	2006	4a/b	1	Whites	Case (63)	AER >300 mg/24h	47/53	25.7 ± 6.4	12.6 ± 2.8
					Control (66)	AER <30 mg/24h	37/63	40.8 ± 10.2	26.8 ± 6.9
Shimizu et al. [40]	2002	4a/b	2	East Asian	Case 1 (107)	Overt proteinuria	70/30	63.1 ± 10.6	15.5 ± 11.0
					Case 2 (124)	Overt proteinuria + Cr >1.5 mg/dl	75/25	65.1 ± 8.8	19.8 ± 7.8

Shin Shin et al. [41]	2004	G894T	2	East Asians	Control (203)	Normoalbuminuria >DM >10 yrs	65/35	63.7 ± 8.8	18.6 ± 7.8
					Case 1 (35)	Microalbuminuria	46/54	62.9 ± 10.9	16 (12-20)
					Case 2 (83)	Overt proteinuria	46/54	58.8 ± 9.7	16 (11-20)
					Control (59)	Normoalbuminuric	25/75	61.6 ± 11.7	12 (10-16)
Shoukry et al. [42]	2012	G894T, 4a/b, T-786C	2	African	Case	Albumin to creatinin ratio >300 mg/g	108/92	55.3 ± 5.8	14.5 ± 4.3
					Control	Albumin to creatinin ratio <30 mg/g	116/84	54.6 ± 5.2	13.8 ± 3.2
Tamemoto et al. [43]	2008	G894T	NA	East Asians	Case (124)	Microalbuminuria	NA	NA	NA
					Control (211)	Normoalbuminuria	NA	NA	NA
Taniwaki et al. [20]	2001	4a/b	2	East Asians	Case 1 (44)	Microalbuminuria	59/41	60.5 ± 8.5	10.9 ± 7.4
					Case 2 (22)	Overt proteinuria	68/32	59.0 ± 10.5	12.8 ± 6.5
					Case 3 (20)	Overt proteinuria + Cr >1.5 mg/dl	50/50	64.2 ± 7.8	19.1 ± 9.7
					Control (69)	Normoalbuminuria	59/41	60.1 ± 9.8	7.4 ± 4.5
Tiwari et al. [19]	2009	G894T	2	South Asians	Case 1 (90)	DM >2 yrs + Cr >2 mg/dl from N India	87/13	53.6 ± 11.0	9.6 ± 6.8
					Case 2 (106)	DM >2 yrs + Cr >2 mg/dl from S India	76/24	55.9 ± 11.5	14.0 ± 6.4
					Control 1 (75)	DM >10 yrs + Cr <2 mg/dl from N India	53/47	61.0 ± 8.9	15.4 ± 8.1
					Control 2 (149)	DM >10 yrs + Cr <2 mg/dl from S India	68/32	60.5 ± 11.4	15.5 ± 6.91
Zanchi et al. [17]	2000	4a/b, T-786C	1	Whites	Case 1 (74)	AER >200 mcg/mg	42/58	35.5 ± 7.3	24.9 ± 9.0
					Case 2 (78)	AER >200 mcg/mg + Cr >1.5 mg/dl	49/51	35.7 ± 6.5	24.5 ± 6.8
					Control (195)	AER <20 mcg/mg + DM >15 yrs	52/48	36.5 ± 7.6	23.7 ± 6.3

Where:AER : albumin excretion rate; DM: diabetes mellitus; DN: diabetic nephropathy; NA: not available; Cr: creatinine

Table 2 – Polymorphisms distribution. HWE (Hardy-Weinberg equilibrium).

Author	**Distribution of the T-786C polymorphism**						**HWE**
	Cases			**Controls**			**p value**
	TT	**TC**	**CC**	**TT**	**TC**	**CC**	
Ahluwalia et al. 2008	121	62	12	165	87	3	0.020
Ezzidi et al. 2008	261	215	34	224	139	32	0.115
Santos et al. 2011	140	160	76	93	104	44	0.138
Shoukry et al. 2012	57	89	54	84	83	33	0.129
Zanchi et al. 2000	57	65	30	75	100	20	0.123
	Distribution of the G894T polymorphism						**HWE**
	Cases			**Controls**			**p value**
	GG	**GT**	**TT**	**GG**	**GT**	**TT**	
Ahluwalia et al. 2008	82	81	32	125	105	25	0.658
Bessa et al. 2011	10	18	12	17	19	4	1.000
Cai et al. 1998	65	44	7	148	109	27	0.310
Ezzidi et al. 2008	185	247	81	165	195	41	0.151
Mollsten et al. 2006	492	365	89	268	232	51	0.919
Mollsten et al. 2009	293	133	32	182	121	16	0.540
Rahimi et al. 2012	68	45	13	39	17	7	**0.038**
Santos et al. 2011	176	166	32	118	95	22	0.640
Shin Shin et al. 2004	95	23	0	52	7	0	1.000
Shoukry et al. 2012	66	94	40	99	77	24	0.140
Tamemoto et al. 2008	104	18	2	181	27	3	0.117
Tiwari et al. 2009	82	21	3	91	43	13	**0.035**
	Distribution of the 4b/4a polymorphism						**HWE**
	Cases			**Controls**			**p value**
	bb	**ba**	**aa**	**bb**	**ba**	**aa**	
Ahluwalia et al. 2008	146	28	21	189	61	5	1.000

Degen et al. 2001	229	94	4	297	105	9	1.000
Ezzidi et al. 2008	314	162	29	234	143	21	1.000
Fujita et al. 2000	81	21	0	55	10	0	1.000
Ksiasek et al. 2003	105	58	15	147	66	19	**0.007**
Lin et al. 2002	115	21	1	41	6	1	0.271
Mollsten et al. 2006	656	248	39	389	145	19	0.220
Neugebauer et al. 2000	101	26	6	71	10	1	0.351
Rippin et al. 2003	344	108	12	297	90	9	0.519
Santos et al. 2011	237	99	11	168	59	5	1.000
Shestakova et al. 2006	14	48	1	34	31	1	0.052
Shimizu et al. 2002	180	44	6	156	44	3	1.000
Shoukry et al. 2012	124	64	12	131	60	9	0.502
Taniwaki et al. 2001	63	21	2	50	19	0	0.340
Zanchi et al. 2000.	80	27	37	144	47	4	1.000

Table 3 - Meta-analysis in all genetic models with all patients and subgroup analysis, in fixed-model analysis, presenting heterogeneity (P_Q and I^2).

	Population	Studies	OR	IC (95%)	P	P_Q	I^2 (%)
4b/a							
Allele	All	15	1.15	1.05-1,25	<0.01	<0.01	66
contrast	African	2	0,98	0.81-1.18	0.88	0,25	22
	East Asians	6	1.21	0.97-1.50.8	0.08	0.29	18
	Whites	7	1.20	1.07-1.34	<0.01	<0.01	80
	Type 1	5	1.17	1.02-1.34	0.02	0.07	54
	Type 2	10	1.12	0.99-1.27	0.07	0.28	18
Additive	All	15	1.52	1.18-1.97	<0.01	<0.01	62
	African	2	1,13	0,69-1,81	0.62	0.56	0
	East Asians	6	3.25	1.58-6.68	<0.01	0.31	16
	Whites	7	1.49	1.06-2.08	0.01	<0.01	74
	Type 1	5	2.21	1.50-3.25	<0.01	<0.01	81
	Type 2	11	1.36	0.98-1.88	0.06	0.08	41
Recessive	All	15	1.50	1.16-1.93	<0.01	<0.01	64
	Africans	2	1.13	0.69-1.83	0,61	0.83	0
	East Asians	6	3.44	1.68-7.05	<0.01	0.28	21
	Whites	7	1.43	1.03-1.99	0.03	<0.01	75
	Type 1	5	2.19	1.49-3.21	<0.01	<0.01	81
	Type 2	11	1.49	1.07-2.07	0.02	0.08	42
Dominant	All	15	1.11	1.01-1.23	0.03	0,01	49
	African	2	0.94	0.75-1.18	0.64	0.24	27
	East Asians	6	1.04	0.81-1.34	0.71	0.44	0
	Whites	7	1.20	1.05-1.36	<0.01	<0.01	67
	Type 1	5	1.22	1.04-1.43	0.01	<0.01	78
	Type 2	11	1.05	0.92-1.20	0.44	0.62	0

Codominant	All	15	0.98	0.88-1.09	0.81	0,02	46
	African	2	1.09	0.87-1.38	0.42	0.29	7
	East Asians	6	1.17	0.90-1.55	0.22	0.14	38
	Whites	7	0.90	0.79-1.04	0.16	0.04	54
	Type 1	5	0.94	0.80-1.11	0.46	0.01	68
	Type 2	11	1.01	0.88-1.17	0.80	0.19	26
T-786C							
Allele	All	5	1.28	1.14-1.44	<0.01	0.25	24
contrast	African	2	1.44	1.21-1.71	<0.01	0.26	19
	Whites	2	1.13	0.94-1.36	0.19	0.44	0
	Type2	4	1.29	1.13-1.46	<0.01	0.15	42
Additive	All	5	1.48	1.14-1.92	<0,01	0.01	67
	African	2	1.43	0.98-2.09	0.05	0.01	84
	Whites	2	1.36	0.93-1.98	0.10	0.18	42
	Type2	4	1.40	1.06-1.86	0.01	<0.01	73
Recessive	All	5	1.38	1,09-1.76	<0,01	0.01	68
	African	2	1.24	0.88-1.76	0.21	0.01	81
	Whites	2	1.39	0.98-1.95	0.06	0.09	0
	Type2	4	1.27	0.98-1.65	0.06	0.01	72
Dominant	All	5	1.21	1,04-1.42	0.01	0.29	18
	African	2	1.39	1.11-1.73	<0.01	0.13	54
	Whites	2	1.05	0.81-1.37	0.70	0.95	0
	Type2	4	1.24	1.05-1.47	<0.01	0.22	31
Codominant	All	5	0.95	0.81-1.11	0.53	0.12	45
	African	2	0.78	0.62-0.98	0.03	0.48	0
	Whites	2	1.15	0.89-1.49	0.28	0.24	25
	Type2	3	0.90	0.75-1.06	0.20	0.131	15

G986T							
Allele	All	12	1.12	1.03-1.21	<0.01	<0.01	75
contrast	African	3	1.63	1.39-1.91	<0.01	0.61	0
	East Asian	3	1.33	1.05-1.70	0.01	0.74	0
	Whites	4	0.93	0.84-1.04	0.20	0.67	0
	Type 1	2	0.92	0.80-1.04	0.18	0.18	0
	Type 2	9	1.27	1.15-1.42	<0.01	<0.01	72
Additive	All	12	1.19	0.99-1.43	0.05	<0.01	63
	African	3	2.01	1.50-2.94	<0.01	0.27	22
	East Asian	3	1.85	1,05-3.25	0.03	0.59	0
	Whites	4	0.86	0.67-1.10	0.23	0.69	0
	Type 1	2	0.87	0.65-1.16	0.34	0.44	0
	Type 2	9	1.47	1.16-1.86	<0.01	<0.01	63
Recessive	All	12	1.16	0.97-1.38	0.09	0.02	52
	Africa	3	1.80	1.31-2.46	<0.01	0.43	0
	East Asian	3	1.73	1.01-2.96	0.04	0.63	0
	Whites	4	0.88	0.69-1.11	0.29	0.62	0
	Type 1	2	0.91	0.69-1.20	0.49	0.31	0
	Type 2	9	1.36	1.08-1.70	<0.01	0.03	53
Dominant	All	12	0.99	0.89-1.11	0.92	0.07	45
	African	3	1.46	1.17-1.82	<0.01	0.11	54
	East Asian	3	1.32	0.98-1.79	0.06	0.73	0
	Whites	4	0.93	0.80-1.07	0.31	0.59	0
	Type 1	2	0.89	0.75-1.06	0.19	0.74	0
	Type 2	9	1.19	0.92-1.26	0.35	0.04	57
Codominant	All	12	1.03	1.04-1.37	0.01	0.01	60
	African	3	0.92	0.74-1.14	0.45	0.29	18

East Asian	3	0.89	0.65-1.21	0.48	0.52	0
Whites	4	1.02	0.89-1.18	0.69	0.44	0
Type 1	2	1.08	0.91-1.29	0.35	0.32	0
Type 2	9	0.94	0.82-1.08	0.41	0.34	11

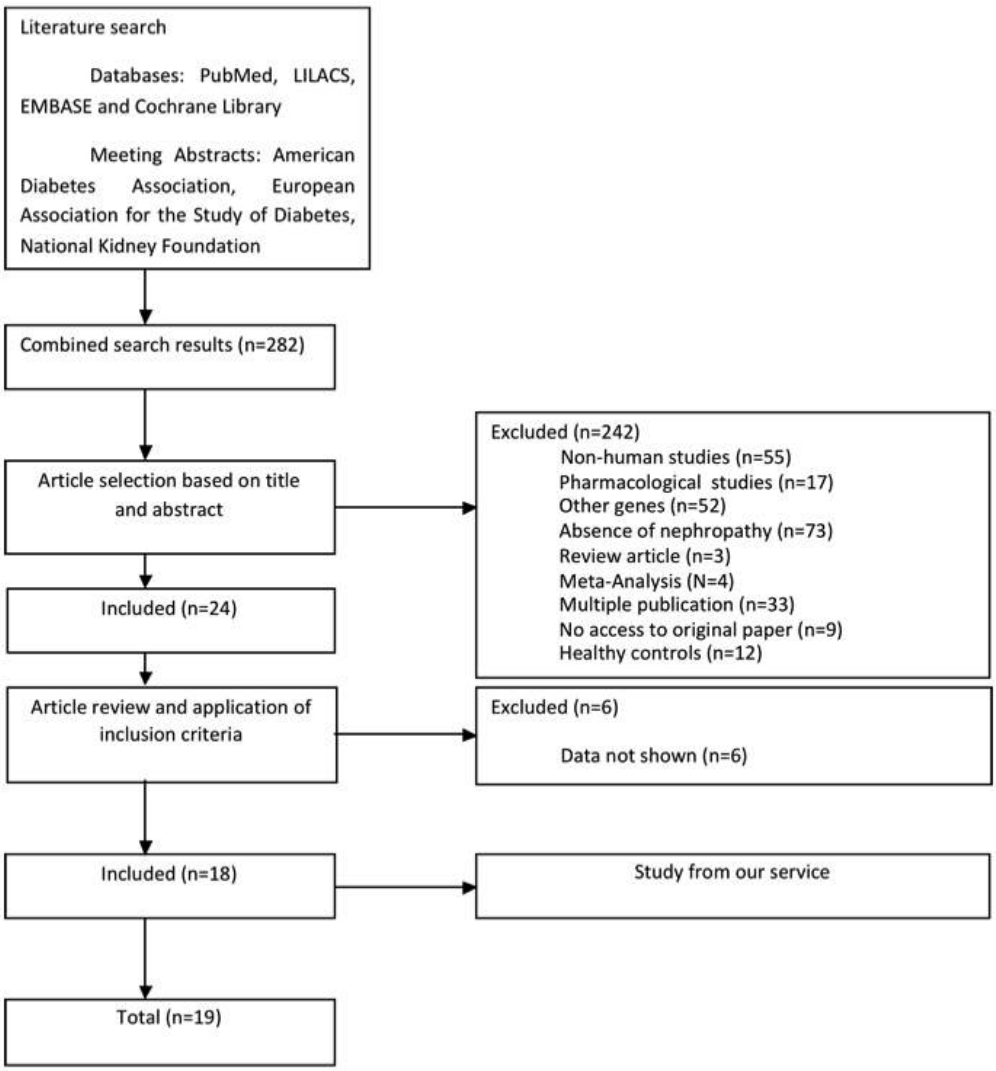

Figure 1 – Search strategy

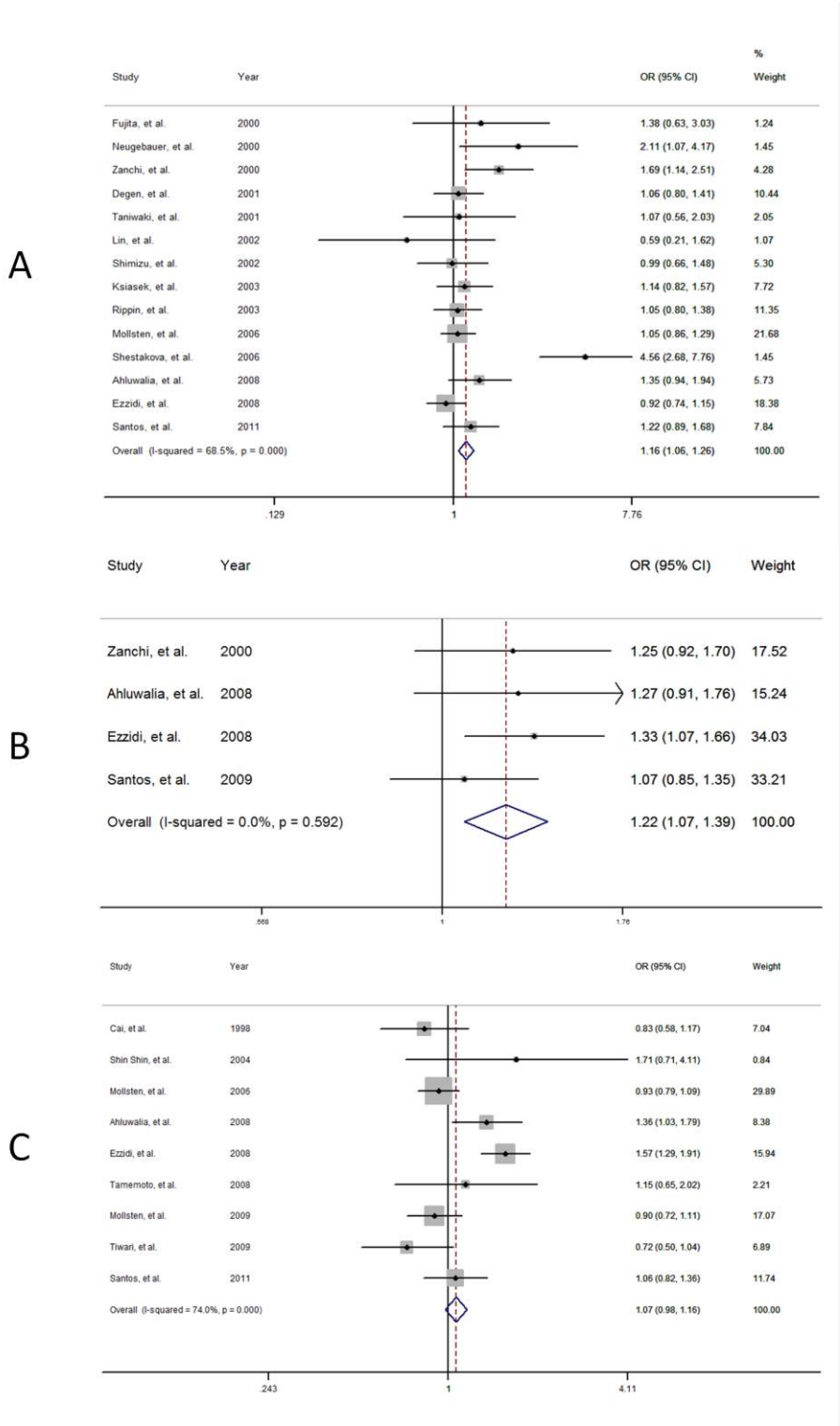

Figure 2. – Forest plot for contrast allele model for (A) 4b/a polymorphism; (B) T-786C polymorphism; and (C) G894T polymorphism.

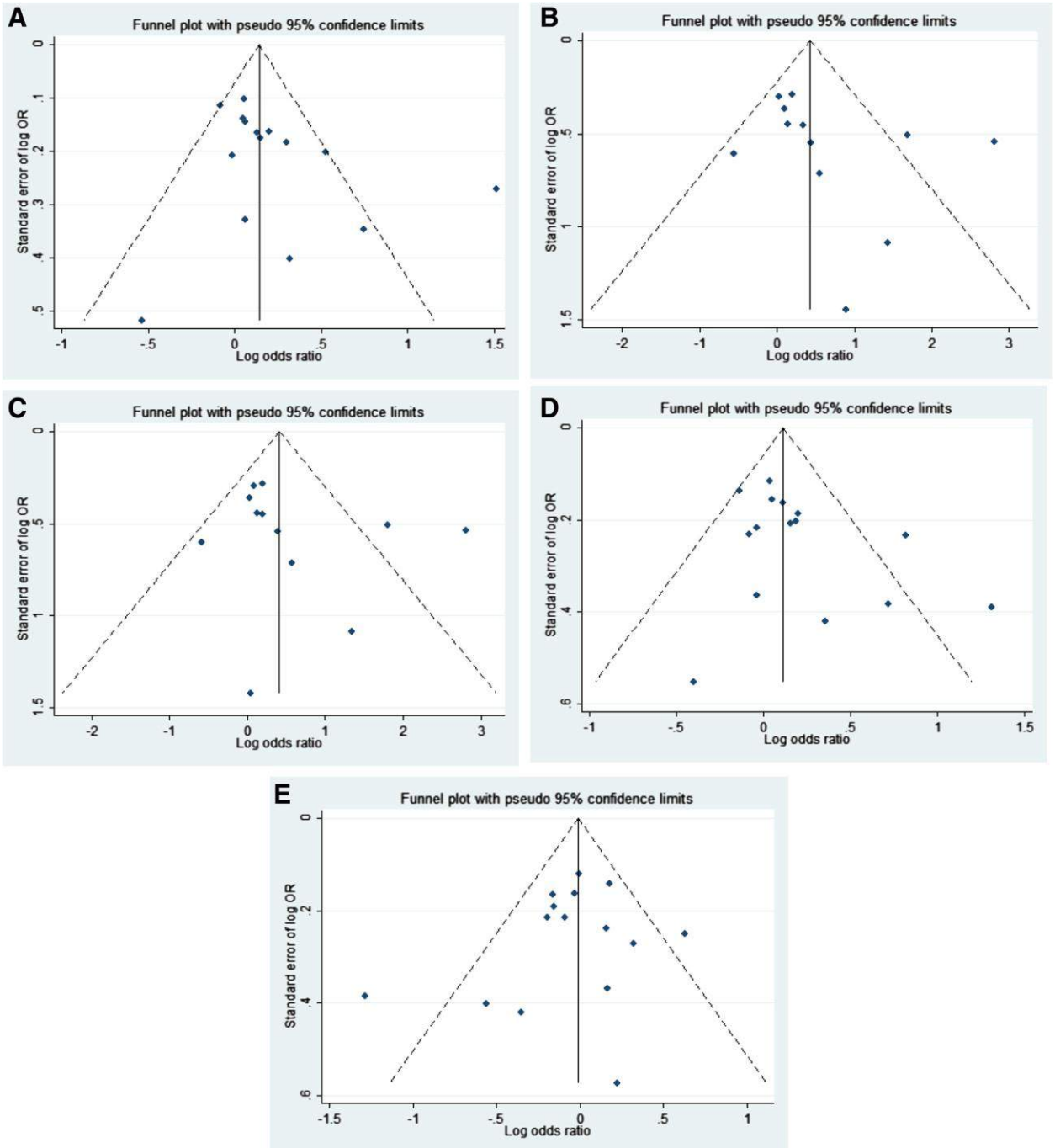

Figure 3 – Funnel plot for 4b/a polymorphism: (A) allele contrast; (B) additive; (C) recessive; (D) dominant; (E), codominant.

CONCLUSÕES

A HAS é um dos maiores contribuintes para a ocorrência de complicações crônicas do DM2. A utilização da MAPA nos acrescenta dados que não conseguimos obter apenas através da simples verificação da PA no consultório. A constatação da ausência do descenso noturno confere ao paciente com DM2 um fator de risco CV adicional, que deve ser acrescentado ao atendimento global do paciente com DM2, na tentativa de controlar todos os fatores de risco presentes.

A ausência do descenso noturno pode associar-se a NAC, aumento do VE e insuficiência cardíaca. EO parece ser a pedra fundamental no desenvolvimento destas patologias, sendo visto mais nos pacientes com ausência de descenso que em pacientes com variabilidade da pressão arterial normal. O EO está relacionado a DE, principalmente relacionado a alteração quantitativa e qualitativa do NO, e polimorfismos no gene candidato eNOS podem estar relacionados ao desenvolvimento dessa complicação.

O desenvolvimento das complicações crônicas do DM2 então estão intimamente ligados ao continuum resistência insulínica, EO, HAS e DE. Observamos este fenômeno tanto na avaliação macrovascular, como apresentado no capítulo 1 da presente tese, onde a ausência do descenso noturno levou a maiores complicações; assim como na avaliação microvascular, como apresentado no capítulo 2, onde polimorfismos do gene candidato eNOS foram associados a nefropatia diabética.

CONSIDERAÇOES FINAIS

DM é uma doença crônica, multi-sistêmica, que deve ser tratada agressivamente para controle de fatores de risco e diminuição da incidência de complicações crônicas micro e macrovasculares.

Cada vez mais se amplia o conhecimento sobre o controle glicêmico, com adição de novas descobertas e conceitos. Conhecimentos além da glicemia vão sendo ampliados a vários sistemas, como o envolvimento de órgão endócrinos “novos”, como o tecido adiposo, músculo esquelético, hormônios gastrointestinais, endotélio e microbiota intestinal; assim como uma maior compreensão sobre órgãos que antes eram apenas alvos de complicações, mas que agora podem ser contribuintes para complicações crônicas, como rim e coração.

DM2 é uma doença multifatorial, que além dos fatores clínicos necessita de uma avaliação genética complementar, já existindo atualmente vários genes candidatos a serem objetos de estudos.

Quanto cada parte da equação contribui para a prevenção é incerto, mas quanto mais fatores de risco forem estudados e descobertos, estaremos diminuindo a quantidade de pacientes que progridem para complicações crônicas, apesar de todo esforço para controle glicêmico e não-glicêmico já existentes na prática clínica.